Chemical Forays of Fungal Metabolites

A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

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Author’s Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

A portion of the work presented in chapter two: collection of mushrooms MMSP01, MMSP02, MMSP03 and MMSP10 (section 2.3) was carried out under the leadership of Mr Stewart Wossa and Mr John Nema. The initial solvent extraction of these mushrooms (section 2.3) was performed within the Science Department, University of Goroka. The NMR spectra for compounds 2.1 and 2.2 presented in the Appendix were run by Dr Edwin Castillo who also isolated these compounds from a PNG fungus with the local name Fulaga dive.

A portion of the work presented in chapter six: the collection of a Papua New Guinea (PNG) mushroom with the local name Kula avara (section 6.1.2) was carried out under the leadership of Mr Stewart Wossa and Mr John Nema. The initial solvent extraction of this mushroom (section 6.1.2) was performed within the Science Department, University of Goroka.

With these exceptions, all of the research described was carried out within the Research School of Chemistry at The Australian National University during the period of July 2013 and March 2018, under the supervision of Associate Professor Russell Allan Barrow. No part of this thesis has been previously submitted for any other degree.

Li Liao
21th June 2018
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Abstract

This thesis contains six chapters and the research presented within focuses on three parts. The first part (chapter one to three) was the isolation of antibacterial and antioxidant mushroom metabolites, and the synthesis towards analogues of discovered natural products. The following part (chapter four) was to assess antioxidant activities of synthetic compounds and investigate their SAR (structure-activity relationship), by use of a developed DPPH assay. The last part (chapter five to six) was the discovery of an abiotic halogenation reaction among a class of fungal metabolites, azaphilones. A series of their synthetic analogues were applied as model compounds to understand the mechanism of the discovered unusual and facile non-enzymatic nucleophilic halogenation reaction.

Chapter one is a review of antioxidants in mushrooms. The components with antioxidant activities discovered from mushrooms were summarised by their chemical structures and representative samples were listed. In addition, the potential mechanisms of those antioxidants were described in this chapter.

In chapter two, extracts of 20 mushrooms from PNG (Papua New Guinea) were screened against both Gram-positive and Gram-negative bacteria, six of them were chosen as chemical survey candidates for their strong antibacterial activities. Extracts of 37 PNG mushrooms were screened for their antioxidant using a modified DPPH assay, 14 of them were chosen as chemical survey candidates based on their antioxidant capacities. In chemical examination guided by bioactivity, five natural products including two novel furan fatty acids (2.1-2.2) and three known compounds, i.e. grifolin (2.3), grifolic acid (2.4) and grifolic acid methyl ether (2.5), were isolated from four selected mushroom samples.

Chapter three described the synthetic efforts towards analogues of three isolated antioxidant fungal metabolites (2.3-2.5). Inspired by 15 synthetic targets, a short and versatile general synthetic route was established to deliver 40 compounds possessing structures related to the natural products. Based on these structures, a compound library was established for antioxidant assessment in the following SAR research. The synthetic work resulted in the preparation of 33 novel structures.
In chapter four, various antioxidant assays and their working mechanisms were reviewed, as well as their corresponding advantages and drawbacks. A developed DPPH assay was optimised to offer an accurate and reproducible antioxidant evaluation for SAR research in the achieved compound library. The results indicated that a structure with an aromatic ring to be allylated with a carbon chain in addition to the presence of phenols, is required to show an antioxidant activity, and the increased length of a carbon chain (more prenyl units) enhances the antioxidant activity.

Chapter five focused on naturally occurring organohalogen compounds with biotic origins. The representative halogenated natural products with a variety of bioactivities were categorised following a chemical structural classification as well as a bioactive classification, to showcase both their structural diversity and bioactive variety. The biosynthetic mechanisms of halogenated natural products were reviewed in this chapter.

Chapter six detailed the investigation of a novel halogenation reaction that was discovered to be occurring with some azaphilone fungal metabolites, for example the conversion of (+)-deschlorosclerotiorin (6.2) into (+)-sclerotiorin (6.1). Various non-halogenated model compounds with an azaphilone core structure were created to shed light on the authenticity of numerous halogenated azaphilones reported as natural products. The ensuing synthetic work resulted in an efficient general procedure producing the required targets. The following investigation of the halogenation revealed a novel, facile, non-enzymatic nucleophilic reaction and its proposed mechanism is discussed. The synthetic efforts resulted in the creation of 22 novel structures.
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Glossary

A.C.S. The chemical meets the specifications of American Chemical Society
AR Analytical reagent
EC\textsubscript{50} Half maximal effective concentration
EI Electron ionisation
ESI Electrospray ionisation
GalNAc \(N\)-Acetylgalactosamine
Gal Galactose
HMBC Heteronuclear multiple bond correlation
HR High resolution
HSQC Heteronuclear single quantum correlation
IC\textsubscript{50} Half maximal effective concentration
ManNAc \(N\)-Acetylmannosamine
MS Mass spectrum
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR Nuclear magnetic resonance
PNG Papua New Guinea
RSC Research School of Chemistry
SAR Structure-activity relationship
TIC Total ion chromatogram
TLC Thin layer chromatography
Chapter 1: Antioxidants in Mushrooms

1.1 Introduction

The term “mushroom” has had various specific meanings in different regions and at different times. While generally speaking, mushroom refers to all larger fungi, so “a mushroom is a macrofungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (under ground) and large enough to be seen with the naked eye and to be picked by hand.”1-4 The typical characteristics of a mushroom are an umbrella shape with a cap (pileus), a stem (stipe), sometimes additionally with a ring (annulus) or a cup (volva).2-4

Since their first existence 300 million years ago, mushrooms have evolved to kingdom status with a vast number of species. Current knowledge indicates around 15,000 mushrooms have been described, however, that is only 10% of the 150,000 estimated total mushroom species. Mushrooms are ecologically categorised into three groups: saprophytes, parasites, and mycorrhiza, the first category compromises the dominant grouping.2,4,5

Mushrooms can also be classified into four types: edible, medicinal, poisonous and miscellaneous mushrooms based on their usages.2 Mushrooms have been collected and used for dietary and medicinal purposes by humans since the prehistoric era, numerous records of mushroom can be found in all the civilizations over the world, namely Europe, Africa, Asia and America, such as Romans, Greek, Egyptians, Indian, Chinese and Mayan. While thousands of years of historical use are documented, mushroom have only been cultivated for hundreds of years, with Auricularia auricula being first farmed in China on wood logs around A.D. 600.2 Naturally, knowledge of mushrooms has accumulated from generation to generation, thus mushrooms are widely applied in ethnologically diverse diets and medicines, such as Shiitake (Lentinula edodes) and Ringzhi (Ganoderma lucidum).6-20 In many cases, mushrooms are served as both foods and medicines. Poisonous mushrooms are generally divided into five classes: Amanita-type poisoning, Muscarine-type poisoning, psychotropic (hallucinogenic) poisoning, Coprinus and poisoning from external sources. Some of those mushrooms are used for religious ceremonies or used as recreational drugs.2
Chapter 1: Antioxidants in Mushrooms

As traditionally used foods and medicines, many known mushrooms have been intensively studied. Abundant research shows mushrooms are rich in proteins and amino acids, fat, vitamins, carbohydrate, fibre, pigments, minerals and nucleic acids. These components among others have contributed to mushrooms’ promising bioactivities, such as antioxidant, anti-inflammatory, antimicrobial, antiviral, antitumor, antidiabetic, anti-hypercholesterolemia, antihypertensive, hepatoprotective, immunomodulatory, cardiovascular and brain protective effects.1-4,6-13,21-25 Among those health benefits, antioxidant value is often highlighted and regarded as the most important function, so antioxidant bioactive components and their mechanisms are attracting increasing attention from scientists.4,6,8,10,12,13,22-25 Since many synthetic antioxidants are found to exhibit dose-dependent toxicity restricting their use, mushrooms and their extracts, with antioxidant property, are regarded as an important source of natural antioxidants, with promising applications in the nutrition, cosmetic and pharmaceutical industries.4,6,12,13,22,24,25

In Australia, mushrooms play a very important role in agriculture, generating the second most profitable crop after potatoes in the fresh vegetable market.15 Many Australasian mushrooms display significant health benefits including antioxidant effects, thus numerous research groups including our group contribute to research on antioxidant mushrooms.15,26,27

This chapter gives a brief introduction to antioxidant components in mushrooms and their mechanisms action. For more detail and other bioactivities, several literature sources are available and readers can refer to these comprehensive reviews.2,3,22,24,28

1.2 Antioxidant Components in Mushrooms

1.2.1 Introduction

Based on Nawar’s definition, antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching $\mathrm{O}_2^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localised $\mathrm{O}_2$ concentrations.29,30 However, reactive oxygen species (ROS) are the major reactive
species (RS) in aerobic cells, others are reactive nitrogen species (RNS), reactive carbon species (RCS) and reactive sulphur species (RSS).\textsuperscript{22,24} Thus antioxidants in a broader sense are compounds which can inhibit or reduce the generations of RS or scavenge the existing RS. It is necessary to note that, recent research has found that in many cases, antioxidants play reversible roles depending on concentrations. They act as antioxidants in low concentration while they work as prooxidants in high concentration.\textsuperscript{24,25,31} So the public should be aware that an antioxidant may be a two-edged sword; in some circumstances, a simple overdose of antioxidants will bring negative effects. For example, ascorbic acid, vitamin E and vitamin A are widely recognised antioxidants, but lots of evidence show long-term frequent intakes of these individual compounds can increase the risk of diseases caused by oxidative stress.\textsuperscript{24} In this chapter, any compound, as long as it displays antioxidative effects, regardless of its possible reversible effect, will be described as “antioxidant”.

In an organism, there is an equilibrium between free radical production and antioxidant defences to maintain the normal metabolism and keep this process in order. Free radicals can either be produced endogenously, as in energy production in mitochondrial or inflammatory reactions caused by diseases or drugs, or produced exogenously as through the impacts of radiation.\textsuperscript{22,24} Overproduction of free radicals or weaker antioxidant defences results in \( \approx 70\% \) of chronic diseases, in that moment intake of antioxidants to rebalance the equilibrium can play a significant role in reducing oxidative damages.\textsuperscript{22}

Synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) have been confirmed to possess many side effects which has led to the use of synthetic antioxidants to be more restricted, while natural antioxidants are more widely applied.\textsuperscript{22,24} For example, dairy foods are a rich source of natural antioxidants.\textsuperscript{30} Besides the unique textures and flavours, the antioxidant benefit is an important reason for the broad acceptance and increasing consumption of mushrooms.

### 1.2.2 Chemical Structures and Mechanisms

Antioxidants in mushrooms are comprised of various chemical structures. In this chapter these compounds are classified into four categories; polyphenols, polysaccharides, vitamins and minerals.
1.2.2.1 Polyphenols

Polyphenols are the most common antioxidants in the diet and are well represented in mushrooms. Polyphenols can be sub-classified into phenolic compounds and flavonoids.

1.2.2.1.1 Phenolic Compounds

Phenolic compounds are simply aromatic hydroxylated compounds, possessing one or more aromatic rings with one or more hydroxy groups. The main phenolic compounds found in mushrooms are phenolic acids. Phenolic acids are represented by two major groups, namely hydroxybenzoic acids and hydroxycinnamic acids (Figure 1-1), which can be derived from the non-phenolic molecules benzoic acid (1.1) and cinnamic acid (1.2), respectively. Benzoic acid (1.1) has been discovered from the Korean mushrooms Sparassis crispa and Agaricus blazei. Protocatechuic acid (1.3) was isolated from various mushrooms, like the Finnish mushroom Agaricus bisporus, the Indian mushroom Termitomyces heimii, the Portuguese mushroom Lepista nuda, and the Korean mushroom Pleurotus ostreatus. Gallic acid (1.4) has also been found in mushrooms Termitomyces heimii and Pleurotus ostreatus. Cinnamic acid (1.2), caffeic acid (1.5), p-coumaric acid (1.6) and ferulic acid (1.7) are very common in mushrooms, Termitomyces heimii was reported to contain all these phenolic compounds. Termitomyces heimii is one of the most intensively researched mushroom species, and it possesses the highest diversity phenolic compounds.

![Figure 1-1 Antioxidant Phenolic Compounds in Mushrooms](image)

The antioxidant activity of phenolic acids is mainly due to phenolic hydrogens. Firstly, the number of hydroxy groups affects antioxidant effects; more hydroxy groups generally means better activity. For instance, protocatechuic acid (1.3) is a weaker antioxidant than gallic acid (1.4) due to possessing fewer hydroxy groups. Similarly
caffeic acid (1.5) possesses better antioxidant activity than both p-coumaric acid (1.6) and ferulic acid (1.7). Intramolecular hydrogen bonds can enhance antioxidant activity. An intramolecular hydrogen bond can be formed between 3-CH$_3$O and 4-OH, which enables ferulic acid (1.7) to have better antioxidant activity than p-coumaric acid (1.6). Nevertheless, the acid proton contributes little on antioxidant activity, some reports suggest the allylic group may enhance the stability of phenolic radical.$^{22}$

The main mechanism of phenolic compounds exerting antioxidant effects is to interrupt the autoxidative chain reaction (Scheme 1-1).$^{22}$

\[
\begin{align*}
\text{RH} + \text{RO}_2^\cdot & \quad \rightarrow \quad \text{R}^\cdot + \text{ROOH} \\
\text{RH} + \text{RO}_2^\cdot + \text{ArOH} & \quad \rightarrow \quad \text{RH} + \text{ROOH} + \text{ArO}^\cdot
\end{align*}
\]

\textbf{Scheme 1-1 Mechanism of Phenolic Compounds Interrupting the Chain Reaction}

The electron in the generated phenolic radical ArO$^\cdot$ can be delocalised by the aromatic ring, which becomes a less active free radical and slowly reacts with substrate RH while RO$_2^\cdot$ can rapidly be scavenged by a phenolic compound ArOH, so it can protect RH from oxidative damage of active free radical RO$_2^\cdot$. It is clear that the antioxidant capacity of ArOH mainly depends on the stability of the generated radical ArO$^\cdot$ which well explains the aforementioned involvement of phenolic hydrogens in antioxidant activity.$^{22}$ Apart from direct reaction with free radicals, phenolic compounds currently are also regarded to either promote endogenous antioxidant capacity or stimulate synthesis of endogenous antioxidants, or they are involved in cell signalling pathways to modify the redox status of the cell or modulate certain molecules activities which can repair damage caused by free radicals, in order to conduct their antioxidant effects.$^{22,24}$

\subsection{1.2.2.1.2 Flavonoids}

The basic structure of flavonoid is a C6-C3-C6 skeleton, which is a flavan nucleus consisting of two benzene rings (A and B) joined by an oxygen-containing pyran ring (C) (Figure 1-2).
The various classes of flavonoids derive from the level of oxidation of the C ring of the basic 4-oxoflavonoid (2-phenyl-benz-γ-pyron) nucleus. The six common subclasses of flavonoids (1.8-1.20) in mushrooms are flavonols, flavones, flavanols, flavanones, isoflavones and anthocyanidins (Figure 1-3), however, only one example of an anthocyanidin in mushrooms has been determined even though anthocyanidins are frequently regarded as an antioxidant component in mushrooms and other foods.20,22,24,33-39 Flavonols quercetin (1.8), rutin (1.9), kaempferol (1.10) and myricetin (1.11) were isolated from Portuguese Suillus luteus, Indian Pleurotus ostreatus, Korean Sparassis crispa and Korean Ganoderma lucidum mushrooms, respectively.22

**Figure 1-3 Structures of Flavonoids in Mushrooms**
As phenolic compounds, flavonoids can directly exert antioxidant effects by interrupting the chain reaction, following the same mechanism shown in Scheme 1-1, which is regarded as the primary mechanism. Additionally, flavonoids can chelate trace metal ions involved in RS formation or inhibit enzymes involved in RS production or regenerate membrane-bound antioxidants, which also result in antioxidant effects.\textsuperscript{22,24}

1.2.2.2 Polysaccharides

Polysaccharides are biomolecules which are comprised of simple sugars in different ratios showing diversity in their structure and composition.\textsuperscript{10} Polysaccharides, as well as polysaccharide-protein complexes, are a class of antioxidants found in mushrooms.\textsuperscript{24} For instance, \textit{Cordyceps sinensis} (Dong Chong Xia Cao) is a high value Traditional Chinese Medicine, while several polysaccharides, like poly-\textit{N}-acetylhexosamine (1.21) (Figure 1-4), \textit{Cereus sinensis} polysaccharide-1 (CSP-1) and capsular polysaccharide-1 (CPS-1) have exhibited significant antioxidant activity.\textsuperscript{10,40} The structure elucidation of polysaccharides is challenging, as they frequently produce complicated, poorly dispersed nuclear magnetic resonance (NMR) spectra. As a result only a few polysaccharides have had their structures confirmed. Among polysaccharides, $\beta$-glucans are recognised as the major active components while $\alpha$-glucans are eukaryotic nutrient components and are easily degraded by mammalian enzymes.\textsuperscript{10}

\[ [-4,\beta-D-ManNAc-(1\rightarrow 3),\beta-D-GalNAc-1\rightarrow]_n \]

\[ m=1-2, \ n=9-13, \ MW=6 \text{ kDa} \]

\textit{poly-N-acetylhexosamine (1.21)}

\textbf{Figure 1-4 Structure of poly-\textit{N}-acetylhexosamine}

The antioxidant capacity of polysaccharides is attributed to their ability to directly scavenge RS, following a similar mechanism to that exhibited in Scheme 1-1 involving hydrogen atom transfer reactions involving polysaccharides abundant hydroxy group. Also, polysaccharides are thought to chelate trace metal ions involved in RS generation, or to modulate enzymes’ activities, either enhancing enzymes using RS as substrates or inhibiting enzymes forming RS.\textsuperscript{10,24}
1.2.2.3 Vitamins

1.2.2.3.1 Vitamin C

Vitamin C, also known as L-ascorbic acid (1.22) (Figure 1-5), is an essential nutrient for a number of animals, including humans, that are incapable of its de novo synthesis and must therefore acquire it from dietary uptake. Vitamin C is recognised as an antioxidant and is abundant in foods. As one of the simplest vitamins, it has been detected in diverse mushrooms including chanterelles (Cantharellus cibarius), wood ears (Auricularia mesenterica), maitakes (Grifola frondosa) and peppery milk-caps (Lactarius piperatus). While the content varies in different mushrooms, in some mushrooms vitamin C content is much higher than in some fruits and vegetables which are recommended as a good natural source of vitamin C.22,24

\[
\text{Figure 1-5 Structure of Vitamin C}
\]

Vitamin C is a powerful RS scavenger, it far more effectively inhibits lipid peroxidation triggered by a lipid peroxyl radical (LOO•) initiator than other plasma constituents, like vitamin E. Vitamin C can protect biomembranes from being damaged by LOO•, where the basic mechanism is recognised to involve direct trapping of LOO• before they initiate lipid peroxidation.24 Apart from direct reaction with RS, the primary mechanism of vitamin C antioxidant function is to recycle vitamin E (tocopherols) via a synergistic procedure (also mentioned for vitamin E; section 1.2.2.3.2), which is shown below (Scheme 1-2). That is, vitamin E firstly reacts with a RS to generate a vitamin E radical, which is rapidly trapped by vitamin C, the vitamin C radical (semidehydroascorbate) formed is reduced by nicotinamide adenine dinucleotide (NADH).22,24

\[
\begin{align*}
\text{Vitamin C} + \text{Vitamin E} & \rightarrow \text{Vitamin C}^\cdot + \text{Vitamin E}^\dagger \\
\text{Vitamin C}^\cdot + \text{NADH} & \rightarrow \text{Vitamin C}^\dagger + \text{NAD}^\cdot
\end{align*}
\]

\[
\text{Scheme 1-2 Primary Mechanism of Vitamin C Antioxidant Effect}
\]
1.2.2.3.2 Vitamin E

Like vitamin C, vitamin E is another well recognised antioxidant which also exists abundantly in foods such as grains and nuts, vegetables and fruits. Essentially, “vitamin E” is a family of chemicals which share a common structure with a chromanol head and isoprenic side chain.\textsuperscript{22,24} Based on the structures, vitamin E can be divided into two classifications tocopherols and tocotrienols, which both have four types $\alpha, \beta, \gamma, \delta$ (Figure 1-6). Therefore vitamin E does not refer to one single molecules, but is instead comprised of eight structures (1.23-1.30). Numerous references have confirmed that diverse mushrooms contain vitamin E, however, the detected vitamin E content is only limited to tocopherols, so far no tocotrienols have been detected in mushrooms.\textsuperscript{22,24} For instance, $\alpha$-tocopherol (1.23) was found in \textit{Auricularia mesenterica} and \textit{Grifola frondosa}, $\beta$-tocopherol (1.24) was found in horse mushrooms (\textit{Agaricus arvensis}) and porcini mushrooms (\textit{Boletus edulis}), $\gamma$-tocopherol (1.25) was found in reishi mushrooms (\textit{Ganoderma lucidum}) and shitakes (\textit{Lentinula edodes}), $\delta$-tocopherol (1.26) was found in lion’s mane (\textit{Hericium erinaceus}) and the white beech mushroom (\textit{Hypsizygus marmoreus}).\textsuperscript{22} From these eight forms of vitamin E, $\alpha$-tocopherol (1.23) is regarded as the most active one, nevertheless, other vitamin forms are recently emphasised in terms of their pro-health functions.\textsuperscript{22,24}

![Figure 1-6 Chemical Structures of Vitamin E](image-url)
The main biological role of vitamin E is to protect cell membranes against oxidative damage. The general mechanism is described below (Scheme 1-3). After lipids react with the initial RS, a lipid radical $L^\cdot$ is generated, which can react with oxygen to form a peroxyl radical $LOO^\cdot$ with high activities and toxicities, however, $LOO^\cdot$ can be rapidly scavenged by tocopherol to form neutral hydroperoxide lipids $LOOH$ and a poorly active tocopherol radical. Then the tocopherol radical, as mentioned above (section 1.2.2.3.1), can be neutralised by vitamin C and consequently tocopherol is recycled through this synergistic procedure (Scheme 1-2).22,24 The interactions between vitamin E and vitamin C suggests the concentration of each antioxidant alone may not be adequate to effectively protect cells, therefore ingestion of vitamin E and vitamin C together may be helpful in order to improve the antioxidant effect.22

\[
\begin{align*}
LH + \text{Oxidant initiator} & \rightarrow L^\cdot \\
L^\cdot + O_2 & \rightarrow LOO^\cdot \\
LOO^\cdot + \text{Tocopherol} & \rightarrow LOOH + \text{Tocopherol}^\cdot
\end{align*}
\]

**Scheme 1-3 General Mechanism of Vitamin E Antioxidant Effect**

1.2.2.3.3 **Vitamin A and Carotenoids**

Carotenoids are a class of chemicals possessing a polyisoprenoid skeleton with 40 carbons. Within that common structure, a conjugated double bond chain forms the central part of the molecule which in some instances forms a bilateral symmetry (e.g. 1.31) located around the *trans* disubstituted central double bond (Figure 1-7).22,24 Carotenoids can be synthesised by plants and microorganisms, not by animals. They are the most distributed pigments in nature, and dozens of carotenoids are found in foods such as fruits and vegetables. The predominant carotenoids, greater than 90%, in the human diet are represented by $\beta$-carotene (1.31), $\alpha$-carotene (1.32), lycopene (1.33), $\beta$-cryptoxanthin (1.34) and lutein (1.35) (Figure 1-7). Among them, $\beta$-carotene, $\alpha$-carotene and $\beta$-cryptoxanthin are able to function as provitamin A and play important biological roles as vitamin A dietary sources. Vitamin A represents a group of unsaturated nutritional compounds with biological significance including its activity as an antioxidant. Apart from the carotenoids, vitamin A exists as other major forms such as retinol (1.36) and retinal (1.37) (Figure 1-7) in animals.41 Many carotenoids have been discovered in mushrooms species, particularly, $\beta$-carotene (1.31) widely distributed in
various species like peppery milk-caps (*Lactarius piperatus*), the bell morel (*Verpa conica*), *Agaricus romagnesii* and the white beech mushroom (*Hypsizygus marmoreus*).\(^{22,24}\)

![Diagram of carotenoids and vitamin A](image)

**Figure 1-7 Structures of Major Carotenoids and Vitamin A**

The antioxidant property of carotenoids has been the focus of a lot of recent research, as carotenoids are believed to directly react with RS and function as a chain-breaking antioxidant in a lipid environment to generate inactive and short-lived carotenoid radicals (Scheme 1-4).\(^{22,24}\) The peroxyl radical \(\text{LOO}^+\) is produced after the lipid radical reacts with oxygen, and is very harmful to cells, can be attacked by a carotenoid and consequently forms an inactive product to minimise the oxidative damage. The extensive conjugated double bonds enable carotenoids to react with the peroxyl radical...
LOO'. As such the conjugated double bonds are regarded as essential for antioxidant function. Since carotenoid radicals can be stabilised by virtue of the delocalization of the unpaired electron over the conjugated polyene and this delocalization also allows additional reactions to take place at many sites on the radical, reactivity of the scavenging lipid radical depends on the length of the conjugated polyene chain and the characteristics of the end groups.\textsuperscript{22,24}

\[
\begin{align*}
LH + \text{Oxidant initiator} & \rightarrow L^- \\
L^- + O_2 & \rightarrow \text{LOO}^-
\end{align*}
\]

Scheme 1-4 Proposed Mechanism of Carotenoids Antioxidant Activities

\textbf{1.2.2.3.4 Vitamin D}

Vitamin D (Figure 1-8) is a group of fat-soluble secosteroids which are responsible for enhancing intestinal absorption of calcium (Ca), iron (Fe), magnesium (Mg), phosphorus (P) and zinc (Zn), so vitamin D is vital for bond function. It is also has other biological effects like increase immune system and muscle functions.\textsuperscript{24} The most important compounds of the vitamin D family in humans are vitamin D\textsubscript{2} (1.38), or ergocalciferol and vitamin D\textsubscript{3} (1.39), or cholecalciferol (Figure 1-8). However, both vitamin D\textsubscript{2} and D\textsubscript{3} are biologically inactive and must be metabolised in the liver to generate 25-hydroxyvitamin D (1.40) and further metabolised in the kidney to produce the biological active form 1,25-dihydroxyvitamin D (1.41) (Figure 1-8).\textsuperscript{24} Vitamin D is sourced from food, generally, vitamin D\textsubscript{3} has animal origins while vitamin D\textsubscript{2} is predominantly from mushrooms and yeast.\textsuperscript{24} For instance, white and brown button mushrooms (\textit{Agaricus bisporus}) are a major dietary source of vitamin D\textsubscript{2} but it is also found in edible chanterelle mushrooms \textit{Cantharellus cibarius} and \textit{Cantharellus tubaeformis}.\textsuperscript{42,43} Recently vitamin D, including D\textsubscript{2}, D\textsubscript{3} and their bioactive metabolite 1,25-dihydroxyvitamin D was recognised as a powerful antioxidant,\textsuperscript{24,44-50} but the mechanism of vitamin D antioxidant activity remains unclear.
Vitamin D antioxidant property is proposed to associate with its conjugated double bonds system, which is similar to vitamin A, in addition, vitamin D is regarded to stabilise membrane with reducing membrane fluidity to inhibit iron-dependent liposomal lipid peroxidation.44

1.2.2.4 Ergothioneine

Ergothioneine (ET) (1.42), is a naturally occurring amino acid and is a thiourea derivative of histidine (Figure 1-9). In humans, ET is obtained exclusively through dietary intake. It is widely distributed in various species of edible mushrooms including Agaricus bisporus, Lentinus edodes, Grifola frondosa, and the oyster mushrooms Pleurotus ostreatus and Pleurotus eryngii. ET has also been used as an antioxidant in food storage and preservation.24,51-56
Chapter 1: Antioxidants in Mushrooms

Figure 1-9 Structure of Ergothioneine

A deficiency of ET is associated with increased mitochondrial deoxyribonucleic acid (DNA) damage and protein oxidation. High mitochondrial concentration suggests that ET is able to protect mitochondrial components against oxidative damage from $O_2^-$ produced within mitochondria.\textsuperscript{24} The detail of how ET actually works as an antioxidant is not known.

1.2.2.5 Minerals

Minerals are another important aspect of components attributed to mushroom’s antioxidant effects. Mushrooms are able to accumulate trace elements and deliver them to our bodies to achieve bio-accumulative effects through the diet.\textsuperscript{57,58} Wild mushrooms are reported to possess a stronger capacity to accumulate minerals than those produced in monocultural practices.\textsuperscript{24}

Many dietary trace elements like selenium, zinc, copper, manganese and iron are cofactors of enzymes, which scavenge RS or eliminate oxidative damage caused by RS.\textsuperscript{22,24} Peroxidases are a large family of enzymes that use hydrogen peroxide or organic hydroperoxides as substrates. Se is essential for the biosynthesis of glutathione peroxidases (GPx), and it can reduce lipid hydroperoxides to their corresponding alcohols and reduce free hydrogen peroxide to water. Selenoprotein-P has a similar function to GPx in terms of minimising cellular oxidative harm. Those two enzymes are the major Se compounds secreted in blood, so they play a vital role in endogenous enzymatic antioxidant defences.\textsuperscript{22,24} Heme peroxidases are another important type of peroxidases with metal chelated to enzymes. They utilise hydrogen peroxide as the electron acceptor to catalyse a number of oxidative reactions to reduce the cytotoxic effects. Mushrooms are also capable of accumulating heavy metals, such as cadmium (Cd) and lead (Pb).\textsuperscript{24}
1.3 Conclusions

Around 70% of chronic diseases are associated with imbalance between free radical production and antioxidant defences, so research on antioxidant reagents and their corresponding mechanisms play a significant role in the prevention and therapy of such diseases. Functional foods with antioxidant properties have been receiving increasing attention and are perceived to be of high value for this reason. As the traditionally used food and medicines in various regions of the world, mushrooms represent a readily available resource for the discovery of antioxidants for academia and the pharmaceutical industry. Inspired by their tremendous biological diversity and their promising bioactivities, our group has an ongoing interest in mushrooms and one aspect of this program focuses on a chemical survey and bioactivity assessment of mushrooms traditionally used by communities in the Eastern Highland province of Papua New Guinea.

1.4 References

(3) Cheung, P. C. K.; Editor *Mushrooms As Functional Foods*; John Wiley & Sons, Inc., **2008**.


(41) Gilbert, C. *Community Eye Health* 2013, 26, 65.
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(54) Cremades, O.; Diaz-Herrero, M. M.; Carbonero-Aguilar, P.; Gutierrez-Gil, J. F.; Fontiveros, E.; Bautista, J. Food Biosci. 2015, 10, 42.


Chapter 2: Chemical Forays of Mushrooms Traditionally Used in Papua New Guinea

2.1 Introduction

Natural products and their derivatives are major sources of drug discovery, and they greatly benefit the pharmaceutical industry,\(^1\) with the wealth of fungi-derived drugs demonstrating the importance of investigation of fungal metabolites.\(^2,3\) Not only in ancient but also contemporary practice, macrofungi (mushrooms) are used worldwide for dietary or medicinal purposes, examples include Oyster mushrooms and Reishi.\(^4\) The usage of mushrooms in traditional medicines, such as Chinese, Indian and European, dates from thousands of years ago and the validation of medicinal mushrooms and their various bioactivities have been confirmed by modern researchers.\(^5-12\) Nevertheless, out of an estimated total 150,000 species of mushrooms, only 10%, or approximately 14,000 species have been described in the literature.\(^13,14\) Such surprisingly limited knowledge of mushrooms illustrates lead compounds from mushrooms could be a solution of economical and sustainable attraction to challenges in current drug discovery.

Papua New Guinea (PNG) is located to the north of Australia. It has very close ties with Australia with regard to economy, culture or ecology. PNG is one of the world’s biologically diverse regions, especially rich in fungi (2390 species), with a large amount still undescribed.\(^4,15\) Indigenous tribes of PNG widely and traditionally use mushrooms as food and/or medicine and abundant firsthand knowledge has been accumulated (Figure 2-1). However, such knowledge is poorly documented. Due to the developing economic levels and living conditions associated with poor public service and infrastructure in many areas of PNG, residents do not have ready easy access to established healthcare, and as a result functional foods and medicinal mushrooms play a crucial role in the wellbeing and health of a community. Thus the investigation of traditionally used mushrooms is very useful for both PNG and the broader scientific communities. Searching for bioactive compounds from mushrooms with demonstrated efficacy and inherent low toxicity, coupled with traditional knowledge, appears an obvious advantage over random screening.\(^16\) Additionally, the PNG community will benefit from the feedback of this investigation. Firstly, traditional culture and knowledge will be preserved and extended. The local community is able to use those mushrooms
more safely and efficiently. In the general case, validated medicinal mushrooms seem more useful than distant hospitals and unavailable drugs. Also, mushrooms confirmed to have functions such as antioxidant, antihyperglycemic and antihypertensive activities can be applied as functional food or cosmetic ingredients, and the local economy could benefit from production of those mushrooms. Therefore, it is necessary and worthy to conduct some chemical studies of mushrooms traditionally used in PNG.

![Figure 2-1 Images of PNG and Local Traditionally Used Mushrooms](image)

**Figure 2-1 Images of PNG and Local Traditionally Used Mushrooms**

1 Photo comes from google map  
3 Photos are courtesy of colleague Dr Edwin M Castillo Martinez

Bacterial infections are major causes of illness and death in PNG. As reviewed in chapter one, oxidative damage is associated with a number of diseases including cancer, diabetes and Alzheimer’s disease.¹⁶⁻²⁰ Thus, chemical survey of mushrooms in my PhD program will focus on the chemicals possessing antibacterial and antioxidant activities. By means of collaboration with local communities in PNG, our group profiled native mushrooms and their common usages and corresponding traditional knowledge. These mushrooms were collected and their ethanol extracts were sent back to Australia and stored for further investigation.
2.2 Screenings of Mushroom Extracts

In order to rapidly identify bioactive candidates from mushroom extracts for chemical survey, crude screenings were conducted, including antibacterial and antioxidant assays.

2.2.1 Antibacterial Screening

Ethanol extracts of 20 mushroom samples (MMSP01-MMSP20) were subjected to antibacterial screening against bacteria *Staphylococcus epidermidis* (Gram-positive) and *Escherichia coli* (Gram-negative) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 2-2). The evaluation of their antibacterial activities was used to choose candidate mushroom samples for further study.

*Figure 2-2 Crude Antibacterial Screening of Mushroom Extracts by MTT Assay*

MTT is a tetrazolium dye, it gives a yellowish colour in solution. MTT can be reduced by dehydrogenases and reducing agents present in metabolically active cells, yielding a water insoluble violet-blue formazan (Figure 2-3).\(^{21,22}\) The minimum inhibitory concentration (MIC) of a test sample can be visually determined by solution colour after adding MTT to the incubated bacterial inoculum containing the testing sample. A yellow colour indicated bacterial inhibition while a blue colour indicates bacterial growth. Also,
the amount of formazan generated from MTT has been widely recognised as the indicator of cell activity, it is directly proportional to the number of living bacteria. Since MTT and its reduced product formazan have the greatest absorption difference around 580 nm wavelength (Figure 2-4), the antibacterial activity of a test sample can be quantified by measuring an individual well’s absorbance. Based on inhibitory ratio of testing samples, its half maximum inhibitory concentration (IC$_{50}$) can be calculated.

As a result of testing (Table 2-1), mushroom extracts MMSP 01, 02, 03, 05, 06, 10, 15, 18 show obvious antibacterial capacities against *Escherichia coli*. Mushroom extracts MMSP 01, 02, 03, 04, 07, 08, 10, 12, 15, 16, 17, 18 show obvious antibacterial capacities.
Chapter 2: Chemical Forays of Mushrooms Traditionally Used in Papua New Guinea

against *Staphylococcus epidermidis*. However, attempts to measure antibacterial capacity of mushroom extracts based on absorbance often encountered severe interference from either extracts’ background absorbance, or absorbance of complexes from reactions between extracts and MTT. So 6 mushroom samples MMSP 01, 02, 03, 10, 15, 18 were chosen as chemical survey candidates for their strong antibacterial activities both against Gram-positive and Gram-negative bacteria.

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC (mg/mL)</th>
<th>E. coli</th>
<th>S. epidermidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin sulfate</td>
<td>0.00008</td>
<td>0.00008</td>
<td></td>
</tr>
<tr>
<td>MMSP01</td>
<td>37.6</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>MMSP02</td>
<td>10.2</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>MMSP03</td>
<td>3.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>MMSP04</td>
<td>—</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>MMSP05</td>
<td>37.6</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MMSP06</td>
<td>10.2</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MMSP07</td>
<td>—</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td>MMSP08</td>
<td>—</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>MMSP09</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MMSP10</td>
<td>5.1</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>MMSP11</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MMSP12</td>
<td>—</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>MMSP13</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MMSP14</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MMSP15</td>
<td>28.3</td>
<td>3.5</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 Antioxidant Screening

Ethanol extracts of 37 mushroom samples* (MMSP03-MMSP20, KMSP1-KMSP5 and KUMSP2-25) were subjected to antioxidant screening via DPPH assay (Figure 2-5). The evaluation of their antioxidant activities was used to choose candidate mushroom samples for further investigation.

* Two collections of mushrooms were received from PNG. When the second collection was received the focus of my work had changed to be solely on antioxidants.
radicals. Based on the DPPH scavenging ratio, its half maximum effective concentration (EC₅₀) can be determined.

As a result (Table 2-2), 14 mushroom samples MMSP03, MMSP06, MMSP07, MMSP08, MMSP15, MMSP16, MMSP19, MMSP20, KMSP3, KMSP5, KUMSP3, KUMSP14, KUMSP15 and KUMSP19 were regarded to be good antioxidants (EC₅₀ < 0.50 mg/mL), they were chosen as chemical survey candidates. However, absorbance data were also found to be interfered with through absorbance of either an extract’s background or the generated complex’s absorbance from interacting with DPPH.

**Table 2-2 Antioxidant Screening Result of Mushroom Extracts**

<table>
<thead>
<tr>
<th>Samples</th>
<th>EC₅₀ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-ascorbic acid</td>
<td>0.005</td>
</tr>
<tr>
<td>MMSP03</td>
<td>&lt; 0.45</td>
</tr>
<tr>
<td>MMSP04</td>
<td>0.69</td>
</tr>
<tr>
<td>MMSP05</td>
<td>1.05</td>
</tr>
<tr>
<td>MMSP06</td>
<td>&lt; 0.37</td>
</tr>
<tr>
<td>MMSP07</td>
<td>0.46</td>
</tr>
<tr>
<td>MMSP08</td>
<td>&lt; 0.61</td>
</tr>
<tr>
<td>MMSP09</td>
<td>0.87</td>
</tr>
</tbody>
</table>
2.3 Bioactivities Guided Isolation of Mushroom Extracts

Among the selected candidates, 4 mushroom extracts with code numbers MMSP01, MMSP02, MMSP03 and MMSP10 were selected for chemical examination, guided by antibacterial screening using paper disc assay or antioxidant screening using the DPPH assay.

The MTT assay has been already reported to afford false results due to its susceptibility and many interferences.\textsuperscript{24,25} On account of the significant interference to absorbance values found in the current MTT antibacterial assay, Kirby-Bauer paper disc diffusion
assay offers the complementary antibacterial assessment.\textsuperscript{26} As a rapid procedure, the paper disc assay was used to determine antibacterial activities of the mushroom extracts subjected to chemical survey and to track the activity in partitioned fractions’ antibacterial capacity. In the paper disc assay, the bacterium \textit{Escherichia coli} was cultivated on agar in a petri plate, paper discs soaked with samples were laid on the surface of agar plate. After incubation, an inhibition zone around a paper disc will be observed if a sample has antibacterial activity (Figure 2-7). The size of the inhibition zone is proportional to the strength of the sample’s antibacterial capacity and indicative of its ability to diffuse through the aqueous agar medium.

\textbf{Figure 2-7 Antibacterial Paper Disc Assay Guided in Chemical Survey}\textsuperscript{†}

\section*{2.3.1 Chemical Survey of Mushroom MMSP01 Extract}

This mushroom (Figure 2-8) (local name is Nagisavoi sma in PNG) was collected from Eastern Highland province. This specimen has not been classified according to Linnaean taxonomy. Recollection in the remote PNG highlands is required to allow extraction of DNA and this was not achieved in the timeframe of this study. A voucher sample is kept in the University of Goroka and the assignment of taxonomy is to be completed when experts travel to PNG. This mushroom is found growing on soil under the trunks of fallen trees.

\textsuperscript{†} A-E refer to PE, DCM, EtOAc, n-BuOH and aqueous fractions respectively.
The crude ethanol extract was subjected to a modified Kupchan solvent partition and divided into five fractions, specifically, petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAc), n-Butanol (n-BuOH) and aqueous fractions (Figure 2-9). PE, DCM, EtOAc fractions exhibited significant antibacterial activities, and thin layer chromatography (TLC) indicated the same components were predominant in these fractions. Two compounds with very similar retention factors (Rf) values and behaviour on TLC were isolated from these fractions in the following purification. $^1$H NMR spectra of these two compounds were also very similar, especially two proton resonances, $\delta$ 5.92, 1H, d, $J= 3.1$ Hz and $\delta$ 6.00, 1H, d, $J= 3.1$ Hz; and $\delta$ 5.92, 1H, d, $J= 3.2$ Hz and $\delta$ 6.15, 1H, d, $J= 3.2$ Hz respectively which suggested the presence of a 2,5-dialkylated furan structure in these two compounds. The two different olefinic proton resonances, $\delta$ 6.05, 1H, dt, $J= 15.9$, 6.7 Hz and $\delta$ 6.13, 1H, dt, $J= 15.9$, 1.0 Hz; and $\delta$ 5.40, 1H, dt, $J= 11.7$, 7.3 Hz and $\delta$ 6.28, 1H, dt, $J= 11.7$, 1.7 Hz respectively, indicated they were diastereomers, the former structure had a trans configuration while the later had a cis configuration. Based on heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) and MS analysis, two furan fatty acid isomers ($E$)-9-(5-pentylfuran-2-yl) non-8-enoic acid (2.1) and ($Z$)-9-(5-pentylfuran-2-yl) non-8-enoic acid...
(2.2) (Figure 2-10) were elucidated as the structures, which represented previously unreported compounds simultaneously isolated by group colleague Dr Edwin M Castillo Martinez from a PNG mushroom with local name Fulaga dive, identified as a new *Amanita* sp. Compounds 2.1 and 2.2 represent novel fatty acids.

![Figure 2-9 Modified Kupchan Solvent Partition](image)

*(E)-9-(5-pentylfuran-2-yl) non-8-enoic acid (2.1)*

*(Z)-9-(5-pentylfuran-2-yl) non-8-enoic acid (2.2)*

![Figure 2-10 Structures of Isolated Mushroom Metabolites Furan Fatty Acids](image)

### 2.3.2 Chemical Survey of Mushroom MMSP02 Extract

This mushroom (Figure 2-11) (local name is Fella Yaluwasa sma, which translates to bracket fungus) was collected from the Eastern Highland province and is used traditionally, to treat swollen body parts, by the Lohu tribe in Ungga-bena district. This
specimen has not been classified according to Linnaean taxonomy. Voucher samples are kept in the University of Goroka. This mushroom grows on stems and bark of trees of the genus *Castanopsis*.

![Mushroom MMSP02 (Fella Yaluwasma)](image)

**Figure 2-11 Mushroom MMSP02 (Fella Yaluwasma sma)**

Following the same modified Kupchan solvent partition protocol used previously, this mushroom ethanol extract was also divided into five fractions, with DCM, EtOAc, \( n \)-BuOH fractions displaying significant antibacterial activities. Further purification was conducted on these fractions but unfortunately no pure compound was isolated due to a lack of material.

### 2.3.3 Chemical Survey of Mushroom MMSP03 Extract

Mushroom MMSP03 (Figure 2-12) (local name is Fella Nagisavoi in PNG) was collected from the Eastern Highland province and traditionally used as food by the Lohu tribe in Ungga-bena district (section 2 in Figure 2-1). This specimen has not been classified according to Linnaean taxonomy. A voucher sample is kept in the University of Goroka and is awaiting assessment by taxonomists. Recollection of a fresh sample is planned to provide DNA for molecular identification. This mushroom grows on soil and under tree trunks.
Using the modified Kupchan partition protocol, this mushroom’s ethanol extract was divided into five fractions. The PE, DCM and EtOAc fractions showed significant antioxidant activities (Figure 2-13 and Table 2-3). All three fractions were found to contain the same major components based on TLC and NMR spectra. Subsequently, three natural compounds with very similar Rf values and behaviour on TLC were isolated from the DCM fraction. These isolated compounds possessed very similar $^1$H NMR spectra including four singlet CH$_3$ signals from $\delta$ 1.56 to $\delta$ 1.82, two broad olefinic proton resonances around $\delta$ 5.08, and a triplet olefinic proton resonance around $\delta$ 5.20 with the coupling constant around 7.0 Hz, which suggests the presence of a farnesyl structure. In addition, a singlet CH$_3$ signal with the chemical shift over $\delta$ 2.00 and singlet aromatic proton resonance around $\delta$ 6.25 indicated the presence of a multi-substituted phenyl structure. The isolated natural products were identified as known structures grifolin (2.3), grifolic acid (2.4) and grifolic acid methyl ether (2.5) (Figure 2-14) after the analysis of 2D NMR spectra and MS. Grifolin (2.3) had also been isolated by a group colleague, Dr Edwin M Castillo Martinez from a PNG mushroom with local name Igura hivi (Albatrellus sp. Gray).28
Figure 2-13 Antioxidant Screening of MMSP03 Fractions by DPPH Assay

Table 2-3 Antioxidant Screening of MMSP03 Fractions by DPPH Assay

<table>
<thead>
<tr>
<th></th>
<th>PE</th>
<th>DCM</th>
<th>EtOAc</th>
<th>n-BuOH</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (mg/mL)</td>
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Figure 2-14 Structures of Isolated Mushroom Metabolites Grifolin and Its Derivatives

grifolin (2.3)
grifolic acid (2.4)
grifolic acid methyl ether (2.5)
2.3.4 Chemical Survey of Mushroom MMSP10 Extract

This mushroom (Figure 2-15) (local name is Yalluwasa sma in PNG) was collected from the Eastern Highland province. This specimen has not been classified according to Linnaean taxonomy. A voucher sample is kept in the University of Goroka. This mushroom grows on fallen, dead logs.

![Figure 2-15 Mushroom MMSP10](image)

The ethanol extract of the mushroom was divided into five fractions following the modified Kupchan solvent partition protocol employed previously. However, no further isolation was conducted because no fraction displayed activity in antibacterial assay. This suggests either a false positive result of antibacterial screening via MTT assay or more than likely decomposition of the active component.

2.4 Discussion and Conclusions

20 Mushroom samples were screened for antibacterial activity against bacteria *Staphylococcus epidermidis* (Gram-positive) and *Escherichia coli* (ATCC 25922) (Gram-negative) via MTT assay. Six of these mushroom extracts (see Table 2-1) were chosen as chemical survey candidates for their strong antibacterial activities both against Gram-positive and Gram-negative bacteria.
37 Mushroom samples were screened for their antioxidant activities using the modified DPPH assay. Out of them, 14 mushroom samples were chosen as chemical survey candidates based on their antioxidant capacities (see Table 2-2).

Among these selected candidates, investigations of four PNG mushroom extracts were carried out resulting in the isolation and identification of five natural products. The two isolated compounds (E)-9-(5-pentylfuran-2-yl) non-8-enoic acid (2.1) and (Z)-9-(5-pentylfuran-2-yl) non-8-enoic acid (2.2), isolated from mushroom MMSP01 extract, represented previously unreported compounds and are new fatty acids. They show strong antibacterial activities against Staphylococcus aureus with IC₅₀ 29.63 μg/mL and 20.01 μg/mL, respectively. Grifolin (2.3), grifolic acid (2.4) and grifolic acid methyl ether (2.5) were isolated from the edible mushroom MMSP03 extract. These meroterpenoid natural products have been identified previously and evidence of their antioxidant properties have been demonstrated by many references.

Crude bioactivity screenings of mushroom extracts, MTT antibacterial assays in particular, were found to be interfered with by either extracts’ background absorbance, or complexes’ absorbance yielded from reactions between extracts and essential working reagents (MTT). It is worthy to note that previous researchers have found that antioxidants present in crude extracts and pure compounds can react with MTT and also generate the reduced formazan product. So the false positive antibacterial outcome of mushroom MMSP10 may be the result from such interference. The susceptibility of MTT to possibly afford false negative or positive results will also influence the results’ accuracy. Because of the severe interference in MTT assay, paper disc assay was applied in the following mushroom extracts’ chemical survey. Due to the complicated components in crude extracts, their interactions with the essential working reagents, either MTT or DPPH, are inexplicable and the subsequent influence on absorbance is indelible. The influence of an extract’s background absorbance can be eliminated by adding a blank group to the 96-well plate. Despite this the bioassay results of mushroom extracts give an initial assessment and offer leads to the discovery of bioactive compounds. The bioassay guided isolation also enables a more efficient discovery of bioactive natural products. The MTT and DPPH based assays are promising methods for the assessment of a pure compound’s bioactivity.
2.5 Experimental

2.5.1 Equipment and Chemical Reagents

2.5.1.1 Bioassays

Westinghouse WFM0900 bar freezer was set at -30 °C to store frozen bacterial strains. Thermoline I60G incubator was set at 37 °C for incubation and set 120 revolutions per minute (RPM) for agitation. Labconco Logic A2 biological safety cabinet was used to afford sterile operating conditions. Tomy ES-315 vertical autoclave was used to autoclave experimental materials. BioTek Epoch microplate spectrophotometer was used to read the absorbance of 96-well plates. Shimadzu UVmini-1240 UV-Vis spectrophotometer was used to measure the absorbance (optical density) of the bacterial cultures. Eppendorf Research® series pipettes were used to transfer solutions. Ratek OM6 orbital mixer was used to blend suspensions. Hanna HI1290 pH meter was used to adjust culture medium pH.

Culture media, Mueller Hinton broth (MHB) and Mueller Hinton agar (MHA), were supplied by Bacto Laboratories Pty Ltd, Mount Pritchard, NSW 2170, Australia and prepared as per the manufacturer’s instruction unless otherwise noted. The pH of the medium was adjusted to 7.3 ± 0.1 with ultrapure water before autoclaving. MHA plates were prepared following a previously reported method. Plastic tissue culture tubes, plastic petri dishes, clear flat bottom 96-well culture plates, MTT and L-ascorbic acid were supplied by Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC 3179, Australia. DPPH, Kanamycin sulfate, methanol and dimethyl sulfoxide (DMSO) were supplied by Sigma Aldrich Australia Pty Ltd, Castle Hill, NSW 2154, Australia. Paper discs with 6 mm diameter were cut from filter paper supplied by Whatman International Pty Ltd, Maidstone, ME14 2LE United Kingdom. All standard drugs were BioReagent grade, all chemicals and solvents were AR or A.C.S. grade, unless otherwise stated.

2.5.1.2 Chemical Survey

All nuclear magnetic resonance spectra (NMR) were recorded on a Bruker Biospin GmbH spectrometer at 300 K with a 5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0258 probe. \(^1\)H and \(^{13}\)C NMR experiments were undertaken at 400 MHz and 100 MHz respectively, unless otherwise indicated. \(^1\)H and \(^{13}\)C signals were described by chemical shift δ (integration, multiplicity, coupling constant \(J\) (Hz), assignment) using ppm. The
multiplicities were expressed by the abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Assignments are supported by 2D NMR data.

Low resolution electron ionisation mass spectra (LREIMS) were recorded on a Finnigan Polaris Q ion trap mass spectrometer using ionisation energy at 70 eV. LREIMS were described by the Mass/charge ratios (m/z) followed the format (value, the relative abundance), molecular ions were symbolised as M while fragment ions weren’t specified unless otherwise stated. The relative abundance is shown as percentage of the base peak intensity. High resolution electron ionisation mass spectra (HREIMS) were recorded on a Waters VG autospec premier spectrometer using ionisation energy at 70 eV. HREIMS were described by (m/z) followed the format (ion actual value, ion calculation value). Low resolution electrospray mass spectra (LRESIMS) were recorded on a Waters Micromass ZMD single quadrupole mass spectrometer using ionisation electric field at 3500 V, source temperature 100 °C, desolvation temperature at 150 °C, coupling to a Waters Alliance 2995 HPLC system. LRESIMS were described followed the same format as LREIMS. High resolution electrospray mass spectra (HRESIMS) were recorded on a Waters LCT Premier mass spectrometer using ionisation electric field at 2500 V, source temperature 100 °C, desolvation temperature at 150 °C, coupling to a Waters Alliance 2995 HPLC system. HRESIMS are described following the same format as for HREIMS. All MS used positive ionisation mode unless otherwise indicated.

Gas chromatography-mass spectra (GC-MS) experiments were recorded using an Agilent 7890A GC and 5975C MS detector. The instrument was equipped with a HP-5 column [(cross-linked 5 % phenyl methyl silicone), 30 m x 0.25 mm x 0.25 μm film thickness] and flow split 2:1 using Helium as carrier gas (1 mL/min) by using positive ion detection. Oven temperature program started at 40 °C for 1 min, increasing 10 °C/min up to 280 °C, holding that temperature for 3 min. Inlet temperature was 250 °C unless otherwise indicated. In GC-MS, MS were described followed the same format as LREIMS.

Semi-preparative HPLC was performed on an Agilent 1100 HPLC system, using a Phenomenex Luna 5μ Silica column (250 x 10.00 mm).

Fluorescent active thin layer chromatography (TLC) plates (silica gel 60 F254) and silica gel (230-400 mesh) for flash chromatography were supplied by Merck Millipore Pty Ltd, Bayswater, VIC 3153, Australia. Sephadex LH-20 was supplied by GE Healthcare Pty Ltd, Parramatta, NSW 2150, Australia. Other fine chemicals and solvents were supplied by
Chapter 2: Chemical Forays of Mushrooms Traditionally Used in Papua New Guinea

Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC 3179, Australia. HPLC grade solvents were only used for HPLC operation, other chemicals and solvents were AR or ACS grade, unless otherwise stated. Petroleum ether has its boiling range 60-80 °C unless otherwise stated.

2.5.2 General Procedure of Antibacterial Screening

2.5.2.1 Preparation of Test Bacteria

*Escherichia coli* (ATCC 25922, β-lactamase negative) and *Staphylococcus epidermidis* (clinical isolate) were used for antibacterial assay. *E. coli* was supplied by our collaborators from Macquarie University, Department of Chemistry and Biomolecular Sciences, NSW 2109. *S. epidermidis* was obtained from the Department of Microbiology, Canberra Hospital, Garran, ACT 2605. Bacterial strains were sub-cultured in liquid media as described below and stored at -30 °C in glycerol/water matrix.

A small portion of the glycerol/water matrix containing the bacteria was scraped and mixed thoroughly into 10 mL sterile Mueller Hinton broth (MHB) in a sterile tissue culture tube. After incubation overnight at 37 °C, approximately 5 μL of the bacterial suspension was transferred into a sterile MHA plate, spread evenly and incubated at 37 °C overnight. A single colony was selected from the incubated MHA plate and transferred into 10 mL of sterile MHB in a sterile tissue culture tube. After incubation at 37 °C overnight, the optical density (OD) at 600 nm (0.08-0.1) of the bacterial suspension was adjusted to achieve a 0.5 McFarland Standard, then diluted 1:100 in sterile MHB, the diluted suspension was used as bacterial inoculum for the assays.

2.5.2.2 Preparation of Test Samples

Mushroom extracts were concentrated to dryness, 20-30 mg of which were dissolved in 50 μL fresh distilled DMSO, then added to 450 μL sterile MHB and mixed thoroughly to produce test samples.

2.5.2.3 Preparation of Positive Controls

Antibiotic kanamycin sulfate was used as positive control for *E. coli* and *S. epidermidis*. Kanamycin sulfate (2.5 mg) was dissolved in 5mL sterile MHB, then 50 μL of this solution was mixed with 50 μL distilled DMSO and 400 μL sterile MHB to produce the positive control solution (50 μg/mL).
2.5.2.4 MTT Assay

This procedure represents a modification based on previously reported methods.28,37-39 Kanamycin sulfate (80 μL), sample (80 μL) and sterile MHB (80 μL), representing the negative control and blank, were dispensed into wells in the first row (A1-A12) of a 96 well plate (Figure 2-16). Subsequently, 40 μL was removed from each well of the first row and added into the next well in the same column (B1-B12), mixed with 40 μL sterile MHB. This operation was repeated to serially dilute the positive control (kanamycin sulfate) and the samples in duplicate through to the final row (H1-H12). Column 11 (A11-H11) was used as negative control. Column 12 (A12-H12) was used as blank group, containing only MHB. Finally, to each well except those in the blank group (column 12) was added 150 μL bacterial inoculum to make total volume 190 μL. To the wells in the blank group was added 150 μL sterile MHB. The 96-well plate was incubated for 20 h at 37 °C. After incubation, 10 μL MTT methanol solution (5 mg/mL) was added to each well in the plate. Absorbance of each well was measured at 600 nm wavelength after the 96-well plate was incubated at 37 °C for 1 h. The average value of blank group’s absorbance was applied for autozero, the original absorbance of each well was subtracted and the processed value was recorded. The average value of negative controls absorbance was used as A negative control. In positive control, the average absorbance value of two wells in the first row was used as A positive control. In each test sample, absorbance was taken as the average value of two wells with same concentration.
The following equation (Equation 2-1) is used to calculate test sample inhibitory ratio. Since all test samples were crude mushroom extracts, some of which were highly coloured, and/or contained metabolites that could react with MTT, some absorbance values had interference around 600 nm and consequently produced unreliable results of inhibitory ratio. In order to confirm antibacterial activity of mushroom extracts to guide chemical examination, the calculation of inhibitory ratio was used with caution, complemented with visual observations of the MIC.

Equation 2-1 Antibacterial Inhibitory Ratio

\[
\% \text{ inhibition} = 100 \times \left( \frac{A_{\text{negative control}} - A_{\text{test sample}}}{A_{\text{negative control}} - A_{\text{positive control}}} \right)
\]

2.5.3 General Procedure of Antioxidant Screening

2.5.3.1 Preparation of DPPH Testing Solution

DPPH (99 mg) was dissolved in 200 mL ethanol, then 4 mL of the solution was diluted with ethanol to 50 mL, which was sealed in an amber bottle covered by aluminium foil and used as the DPPH testing solution (100 μM). The entire operation was conducted under low light conditions (laboratory lighting off).
2.5.3.2 Preparation of Test Samples
Mushroom extracts were concentrated to dryness, 20-30 mg of which were dissolved in 250 μL distilled DMSO to produce test samples.

2.5.3.3 Preparation of Positive Controls
L-ascorbic acid is a widely-recognised antioxidant, it was used as positive control for scavenging of the DPPH radical. 177 mg L-ascorbic acid was dissolved in 100mL distilled DMSO to produce positive control solution (10 mM).

2.5.3.4 DPPH Assay
This procedure represents a modification based on previously reported methods. L-ascorbic acid solution (20 μL), test sample solution (20 μL) and distilled DMSO (20 μL), representing the negative control and blank, were dispensed into wells in the first row (A1-A12) of a 96 well plate (see Figure 2-16). Subsequently, 10 μL was removed from each well of the first row and added into the next well in the same column (B1-B12), and mixed with 10 μL of distilled DMSO. This operation was repeated to serially dilute the positive control (L-ascorbic acid) and the samples in duplicate through to the final row (H1-H12). Column 11 (A11-H11) was used as negative control. Column 12 (A12-H12) was used as blank group, containing only DMSO. Finally, to each well except those in the blank group (column 12) was added 190 μL DPPH solution to make total volume 200 μL. To the wells in the blank group was added 190 μL ethanol. The absorbance of each well was measured at 515 nm wavelength after the 96-well plate was incubated at 37 °C for 0.5 h. The whole operation was conducted under dark conditions. The average value of blank group’s absorbance was applied for autozero, the original absorbance of each well was subtracted and the processed value was recorded. The average values of negative controls absorbance were used as A negative control. In positive control, the average absorbance value of two wells in the first row was used as A positive control. In each test sample, absorbance was taken as the average value of two wells with same concentration.

Equation (Equation 2-1) was used to calculate test sample DPPH radical scavenging activity. Subsequently, various test sample concentrations (mg/mL) and their corresponding radical scavenging activities were analysed by Origin (OriginLab, Northampton, MA) software, followed by sigmoidal fit using category “growth/sigmoidal”, function “dose/response”. EC50 value of each test sample were determined.
2.5.4 General Procedure of Antibacterial Paper Disc Assay

2.5.4.1 Preparation of Agar Petri Plate

*Escherichia coli* (ATCC 25922, β-lactamase negative) was the test bacterium used to track activity. The preparation of a bacterial suspension (OD$_{600} = 0.5$ McFarland Standard) followed the same method described in the MTT assay above (Section 5.2). A suspension of bacteria (1 mL) was transferred to a sterile MHA petri plate, spread evenly and incubated at 37 °C for 12 h. After that, the incubated petri plates with a thin and even layer of bacterial colonies were selected as test petri plates.

2.5.4.2 Preparation of Test Samples

Various extracts partitioned fractions were concentrated to dryness, then the same mass (around 5 mg) solid materials were dissolved in 1 mL methanol or other soluble solvents to produce various fraction solutions.

2.5.4.3 Paper Disc Assay

The procedure was slightly modified based on previously reported method.$^{26,43}$ Sterile paper discs with 6 mm diameter were soaked into these prepared test sample solutions, allowed to dry, then gently pasted on the surface of test petri plate. After incubation at 37 °C for 20 h, inhibition zone were measured as diameters of the annulus surrounding the disc.

2.5.5 Characterisation of Natural Products Isolated

The crude ethanol extract of mushroom MMSP01 was concentrated to dryness, to return 3.4 g of material. This was suspended in methanol (80 mL), extracted with petroleum ether (260 mL), the methanol layer was concentrated to dryness again and suspended in water (80 mL), then extracted with dichloromethane (260 mL), ethyl acetate (260 mL) and n-butanol (260 mL) respectively. All partitioned fractions were concentrated to dryness to return the following masses: PE (782 mg), DCM (618 mg), EtOAc (66 mg), n-BuOH (316 mg) and aqueous (1414 mg). The DCM fraction was subjected to flash column chromatography on silica using 70% EtOAc in petroleum ether as eluent. Fractions possessing antibiotic activity were further purified by semi-preparative HPLC using 15% EtOAc in petroleum ether as eluent to give two pure compounds, namely *(E)-9-(5-pentylfuran-2-yl) non-8-enoic acid* (2.1) as a pale yellow
solid (11 mg) and (Z)-9-(5-pentylfuran-2-yl) non-8-enoic acid (2.2) as a pale yellow solid (8 mg).

\[
\text{(E)-9\''-(5-pentylfuran-2-yl) non-8\''-enoic acid (2.1).} \quad 1^H \text{NMR (400 MHz, CDCl}_3\text{):} \delta 0.90 (3H, t, J = 6.9 Hz, 5\'-H), 1.31-1.68 (14H, m, 2', 3', 4', 3'', 4'', 5'', 6''-H), 2.12-2.18 (2H, m, 7''-H), 2.35 (2H, t, J = 3.1 Hz, 4-H), 6.00 (1H, d, J = 3.1 Hz, 3-H), 6.05 (1H, dt, J = 15.9, 1.0 Hz, 9''-H). 13C NMR (100 MHz, CDCl}_3\text{):} \delta 14.2 (5'-C), 22.6 (4'-C), 24.8 (3''-C), 27.9 (2'-C), 28.3 (1'-C), 28.9 (5''-C), 29.1 (4''-C), 29.3 (6''-C), 31.6 (3'-C), 32.8 (7''-C), 34.1 (2''-C), 106.3 (4-C), 106.9 (3-C), 119.0 (9''-C), 128.4 (8''-C), 151.7 (2-C), 155.8 (5-C), 179.5 (1''-C). EIMS: m/z [M]+ 292 (74), 235 (22), 177 (100), 151 (21), 121 (26), 107 (84), 95 (24), 55 (26). HREIMS: m/z 292.2033 (calculated for C_{18}H_{28}O_3 292.2038).
\]

\[
(Z)-9\''-(5-pentylfuran-2-yl) non-8\''-enoic acid (2.2). \quad 1^H \text{NMR (800 MHz, C}_6\text{D}_6\text{):} \delta 0.83 (3H, t, J = 7.1 Hz, 5\'-H), 1.11-1.59 (14H, m, 2', 3', 4', 3'', 4'', 5'', 6''-H), 2.04 (2H, t, J = 7.5 Hz, 2''-H), 2.46-2.51 (4H, m, 1', 7''-H), 5.40 (1H, dt, J = 11.7, 7.3 Hz, 8''-H), 5.92 (1H, d, J = 3.2 Hz, 4-H), 6.15 (1H, d, J = 3.2 Hz, 3-H), 6.28 (1H, dt, J = 11.7, 1.7 Hz, 9''-H). 13C NMR (200 MHz, C}_6\text{D}_6\text{):} \delta 14.2 (5'-C), 22.8 (4'-C), 24.9 (3''-C), 28.1 (1'-C), 28.5 (2'-C), 29.2 (4''-C), 29.3 (5''-C), 29.7 (6'',7''-C), 31.7 (3'-C), 33.9 (2''-C), 106.9 (4-C), 110.3 (3-C), 118.2 (9''-C), 129.7 (8''-C), 152.4 (2-C), 155.8 (5-C), 178.8 (1''-C). EIMS: m/z [M]+ 292 (74), 235 (22), 177 (100), 151 (21), 121 (26), 107 (84), 95 (24), 55 (26). HREIMS: m/z 292.2033 (calculated for C_{18}H_{28}O_3 292.2038).
\]

The crude ethanol extract of mushroom MMSP03 was concentrated to dryness to return 3.3 g of material. This was suspended in methanol (80 mL), extracted with petroleum ether (260 mL), the methanol layer was concentrated to dryness and suspended in water (80 mL), then extracted with dichloromethane (260 mL), ethyl acetate (260 mL) and n-butanol (260 mL) respectively. All partitioned fractions were concentrated to dryness to return; PE (1222 mg), DCM (162 mg), EtOAc (105 mg), n-BuOH (315 mg) and aqueous (1616 mg). The DCM fraction was purified by semi-preparative HPLC using 12% EtOAc in petroleum ether as eluent to give three pure compounds (Figure 2-17). The peak eluting with the shortest retention time (t=17.5 min) was concentrated to give grifolin (2.3) as
a colourless solid (1 mg). The peak eluting with the intermediate retention time (t=20.1 min) was concentrated to give grifolic acid methyl ether (2.5) as a colourless solid (2 mg). The peak eluting with the longest retention time (t=29.7 min) was concentrated to give grifolic acid (2.4) as a colourless solid (6 mg).

Figure 2-17 HPLC Chromatogram of Mushroom MMSP03 Isolation

grifolin (2.3). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.59 (3H, s, 14’-H), 1.60 (3H, s, 13’-H), 1.68 (3H, s, 12’-H), 1.82 (3H, s, 15’-H), 1.87-2.10 (8H, m, 4’, 5’, 8’, 9’-H), 2.21 (3H, s, 7-H), 3.39 (2H, d, $J$= 7.1 Hz, 1’-H), 5.09 (2H, brs, 6’, 10’-H), 5.27 (1H, t, $J$= 7.1 Hz, 2’-H), 6.24 (2H, s, 4, 6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 16.2 (15’-C), 16.4 (14’-C), 17.8 (13’-C), 21.2 (7-C), 22.3 (1’-C), 25.8 (12’-C), 26.5 (5’-C), 26.8 (9’-C), 39.8 (4’, 8’-C), 109.2 (4, 6-C), 110.6 (2-C), 121.8 (2’-C), 123.7 (6’-C), 124.5 (10’-C), 131.4 (11’-C), 135.7 (7’-C), 137.6 (5-C), 139.0 (3’-C), 154.5 (3-C), 154.9 (1-C). It has the same Rf value and behaviour on TLC as the standard reference compound.

grifolic acid (2.4). $^1$H NMR (400 MHz, CDCl$_3$):

$\delta$ 1.59 (6H, s, 13’, 14’-H), 1.67 (3H, s, 12’-H), 1.82 (3H, s, 15’-H), 1.94-2.12 (8H, m, 4’, 5’, 8’, 9’-H), 2.53 (3H, s, 7-H), 3.43 (2H, d, $J$= 7.1 Hz, 1’-H), 5.08 (2H, brs, 6’, 10’-H), 5.27 (1H, t, $J$= 7.1 Hz, 2’-H), 6.26 (1H, s, 5-H), 11.89 (1H, s, COOH-H). $^{13}$C NMR (100 MHz, CDCl$_3$):
\[ \delta 16.2 (15'-C), 16.4 (14'-C), 17.8 (13'-C), 21.2 (7-C), 22.2 (1'-C), 25.8 (12'-C), 26.5 (5'-C), 26.8 (9'-C), 39.8 (4', 8'-C), 103.9 (1-C), 111.6 (3-C), 111.9 (5-C), 121.4 (2'-C), 123.7 (6'-C), 124.5 (10'-C), 131.4 (11'-C), 135.8 (7'-C), 139.3 (3'-C), 142.4 (6-C), 160.5 (4-C), 163.7 (2-C), 175.1 (COOH-C). \] It has the same Rf value and behaviour on TLC as the standard reference compound.

grifolic acid methyl ether (2.5). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta 1.56 (3H, s, 14'-H), 1.58 (3H, s, 13'-H), 1.67 (3H, s, 12'-H), 1.77 (3H, s, 15'-H), 1.92-2.06 (8H, m, 4', 5', 8', 9'-H), 2.58 (3H, s, 7-H), 3.33 (2H, d, \( J = 6.9 \) Hz, 1'-H), 3.86 (3H, s, OMe-H), 5.07 (2H, brs, 6', 10'-H), 5.19 (1H, t, \( J = 7.0 \) Hz, 2'-H), 6.31 (1H, s, 5-H), 11.63 (1H, s, COOH-H). EIMS: \( m/z \) [M]+ 386 (10), 368 (5), 342 (11), 335 (15), 334 (100), 329 (13), 299 (20). It has the same Rf value and behaviour on TLC as the standard reference compound.

### 2.6 References

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2.8 Appendix NMR Spectra of Natural Products

Figure 2-18 $^1$H NMR Spectrum of Furan Fatty Acid (2.1) (400 MHz, CDCl$_3$)

Figure 2-19 $^{13}$C NMR Spectrum of Furan Fatty Acid (2.1) (100 MHz, CDCl$_3$)

Figure 2-20 $^1$H NMR Spectrum of Furan Fatty Acid (2.2) (800 MHz, C$_6$D$_6$)

Figure 2-21 $^{13}$C NMR Spectrum of Furan Fatty Acid (2.2) (100 MHz, C$_6$D$_6$)

Figure 2-22 $^1$H NMR Spectrum of Grifolin (2.3) (400 MHz, CDCl$_3$)

Figure 2-23 $^1$H NMR Spectrum of Grifolic Acid (2.4) (400 MHz, CDCl$_3$)

Figure 2-24 $^1$H NMR Spectrum of Grifolic Acid Methyl Ether (2.5) (400 MHz, CDCl$_3$)
Figure 2-18 $^1$H NMR Spectrum of Furan Fatty Acid (2.1) (400 MHz, CDCl$_3$)
Figure 2-19 $^{13}$C NMR Spectrum of Furan Fatty Acid (2.1) (100 MHz, CDCl$_3$)
Figure 2-20 $^1$H NMR Spectrum of Furan Fatty Acid (2.2) (800 MHz, $C_6D_6$)
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Figure 2-21 ¹³C NMR Spectrum of Furan Fatty Acid (2.2) (100 MHz, C₆D₆)
Figure 2-22 $^1$H NMR Spectrum of Grifolin (2.3) (400 MHz, CDCl$_3$)
Figure 2-23 $^1$H NMR Spectrum of Grifolic Acid (2.4) (400 MHz, CDCl$_3$)
Figure 2-24 $^1$H NMR Spectrum of Grifolic Acid Methyl Ether (2.5) (400 MHz, CDCl$_3$)
Chapter 3: Synthesis of Grifolin Analogues

3.1 Introduction

The antioxidant activities of grifolin (2.3), grifolic acid (2.4) and their derivatives have been widely reported and our research has also confirmed grifolin’s antioxidant activity.1-8 As mentioned in the first chapter, the application of synthetic antioxidants is more restricted with increased regulations for their toxicities and side-effects, while natural antioxidants are becoming more and more popular for health authorities and the public for the perceived wellbeing they offer. These natural antioxidants have attracted the attention of organic chemists in academia and pharmaceutical industry, with many approaches applied to the synthesis of grifolin and analogues.2,6,9-19 However, the common challenges in organic synthesis, high cost, high step count and low yields limited their applications in industrial production. The application of biosynthesis offers a considerable and promising solution to produce natural antioxidants in an environmentally friendly and sustainable manner.20,21 Mushrooms, producing natural antioxidants as metabolites, such as grifolin, are able to be utilised for harvesting functional foods or to produce semi-synthetic antioxidants. Research correlated to these objectives is ongoing within our group with studies focussed on the traditional use of mushrooms in PNG.22 PNG has abundant mushroom resources afforded by its climate and geography, providing the local communities exceptional advantages to boost their mushroom production, with concomitant economic benefits.

Grifolin and its derivatives comprise two portions (Figure 3-1); an aromatic core like orcinol or orsellinic acid and an aliphatic side chain of varying length such as prenyl, geranyl or farnesyl. Following the principles of biosynthesis, the aromatic core originates from the polyketide pathway, while the aliphatic side chain most likely comes from the isoprene pathway, then they are assembled together to generate meroterpene metabolites. In order to yield grifolin and its derivatives, it is practicable to add components with these two classes of chemical structures to mushroom culture medium in anticipation of them being incorporated to increase metabolite yield.
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Figure 3-1 Some Known Grifolin Derivatives

The literature reports contradictory results of antioxidant activities among grifolin and its derivatives, and different opinions were put forward regarding their structure-activity relationships (SAR), specifically the impacts of the position and the length of an aliphatic side chain, and the number of free phenols, on the effect of antioxidant capacity.\textsuperscript{1,4,6,23}

We embarked upon a study to systematically investigate grifolin and its derivatives’ SAR for the application of aberrant biosynthesis. In this direction, 15 synthetic targets (3.1-3.15) were designed (Figure 3-2), 10 of which have not been previously synthetically...
achieved. The targeted compounds belong to two different substitution patterns based around grifolin and neogrifolin architectures (see Figure 3-1), with different numbers of free phenol. The investigation of relationship between these structures and their antioxidant activities should prove useful to identify optimal precursors for cost-efficient antioxidant production through aberrant biosynthesis.

\[
\begin{align*}
\text{Target 1 (3.1)} & \quad \text{Target 2 (3.2)} & \quad \text{Target 3 (3.3)} \\
\text{Target 4 (3.4)} & \quad \text{Target 5 (3.5)} & \quad \text{Target 6 (3.6)} \\
\text{Target 7 (3.7)} & \quad \text{Target 8 (3.8)} & \quad \text{Target 9 (3.9)} \\
\text{Target 10 (3.10)} & \quad \text{Target 11 (3.11)} & \quad \text{Target 12 (3.12)} \\
\text{Target 13 (3.13)} & \quad \text{Target 14 (3.14)} & \quad \text{Target 15 (3.15)}
\end{align*}
\]

\textit{Figure 3-2 Synthetic Targets for SAR Investigation}

### 3.2 Synthesis

#### 3.2.1 Synthetic Efforts

##### 3.2.1.1 Suzuki Coupling

Retrosynthetic analysis indicates disconnections that would allow the target framework to be assembled through Suzuki coupling of an appropriately substituted boronic acid and an allyl halide. Investigation of this route (Scheme 3-1) began with commercially available orcinol. After dimethylation under standard Williamson conditions, the generated ether (3.16) was then regioselectively brominated by \textit{N}-bromosuccinimide (NBS), providing bromide (3.17) in 51% yield as the only isolable product.\textsuperscript{24} With this
bromide in hand, the next synthetic step was to generate a boronic acid which can be utilised for Suzuki coupling with a prenyl halide.\textsuperscript{25,26} It was reasoned the aryl bromide (3.17) could be employed in a lithium halogen exchange using \textit{n}-butyl lithium as the reagent and subsequently reacted with triisopropyl borate to furnish the corresponding boronic acid after work-up. However, all efforts were unsuccessful with a hydroxy substituted product 2,4-dimethoxy-6-methylphenol (3.18). When phenylboronic acid was used to test the effectiveness of the Suzuki coupling with prenyl bromide, the desired compound (3.19) was only ever isolated in 6\% yield.\textsuperscript{27} Based on these results, this synthetic pathway was abandoned.

\begin{center}
\includegraphics[width=\textwidth]{Scheme_3-1}
\end{center}

\textit{Scheme 3-1 Halogen-Metal Exchange and Suzuki Coupling Synthetic Attempt}

\textbf{3.2.1.2 Friedel-Crafts Acylation and Ketone Reduction}

Friedel-Crafts acylation using acid anhydrides to acylate aromatic rings had been successfully utilised by members of our group to produce related compounds so exploratory work was conducted in attempts to exploit this chemistry. This route (Scheme 3-2) began with commercially available 3-methylcrotonic acid, after dehydration to afford the corresponding anhydride (3.20) which was used to acylate the compound 3.16, generating three acylation products (3.21-3.23) in a ratio of 50:16:39. In order to furnish the prenyl chain, these products required the selective reduction of the carbonyl moiety in the presence of an α,β-unsaturated ketone. Various conditions, looking for selective 1,2-addition at the carbonyl while suppressing 1,4-addition which reduces the carbon-carbon double bond were explored.\textsuperscript{28-30} Unfortunately all efforts proved to be unsuccessful, with carbon-carbon double bond being more susceptible
than the carbonyl double bond. For instance, one of the acylation products 3.21 was subjected to a multitude of reducing conditions (Scheme 3-2). Compound 3.21 underwent a retro Friedel-Crafts reaction resulting in a deacylation product 3.16 using triethylsilane (TES) in a large excess of trifluoroacetic acid (TFA). Using increased equivalents of the silane reducing reagent compound 3.21 underwent a similar deacylation albeit along with concomitant double bond reduction (7%), resulting from 1,4-addition to produce 3.24. Sodium borohydride in methanol solution was found too weak to reduce the carbonyl double bond returning starting material. When Luche reduction conditions were applied, 1,4-addition resulting in product 3.24 was generated in 4%. When compound 3.21 was treated with the reducing reagent, diisobutylaluminium hydride (DIBAH), only starting material was recovered. Finally, reduction of the carbonyl was achieved using TES along with PdCl₂ catalyst, however this was secondary to the initial 1,4-addition returning a mixture of 3.24 and the compound 3.25 possessing a fully reduced sidechain. Based on these observations, this synthetic route was abandoned.
Scheme 3-2 Friedel-Crafts Acylation and Ketone Reduction Synthetic Attempt
3.2.1.3 Claisen Rearrangement and Wittig Reaction

In order to avoid the problematic reduction of the carbonyl moiety encountered above the Claisen rearrangement was considered as a route to access the desired architecture. Theoretically two regioisomers can be generated after a Claisen rearrangement, which can be used to access both the neogrifolin and grifolin substitution patterns (3.28 and 3.30 respectively, Scheme 3-3). The allyl resorcinol derivatives thus formed can then undergo ozonolysis to yield a phenylacetaldehyde, which is ready for Wittig olefination to furnish the required prenyl substituted compound. Execution of this pathway (Scheme 3-3) started from commercially available orcinol, which was monomethylated under Williamson conditions to return the desired monomethy ether (3.26) in 31% yield. The isolated product 3.26 was subject to a second etherification with an allyl bromide, generating the 3-allyloxy-5-methoxy toluene (3.27) in excellent yield, which was submitted to the Claisen rearrangement under standard conditions. Upon workup and chromatography three products were obtained, two of them were the expected allyl resorcinol regioisomers (3.28, 19% and 3.30, 34%) in addition to a dihydrobenzofuran (3.29, 16%) the result of a 5-exo-trig ring closure from compound 3.28. Curiously the equivalent dihydrobenzofuran from cyclisation of the other regioisomer (3.30) was not observed. In order to gain a substrate for Wittig olefination, compound 3.28 was subjected to ozonolysis, working up with dimethyl sulfide to produce the phenylacetaldehyde 3.31, supported by the appearance of a characteristic aldehyde resonance in the $^1$H NMR spectrum at 9.77 ppm as a sticky yellow oil. However, this ozonolysis reaction was found difficult to control with the reaction not generating any product if ozone were streamed for less than 3 min while the product was completely decomposed if reacted with streaming ozone for more than 10 min. The maximum yield of the phenylacetaldehyde recovered was an unacceptable 21%. Compound 3.28 was then methylated to generate product 3.32 and ozonolysis carried out under the same reaction conditions, consistently affording the methanol hemiacetal 3.33 in in low yield (28%). The possible reason for the refractory reactivity was that electron donating groups such as methoxy and hydroxy enriched the electron density of the aromatic ring rendering it vulnerable to ozonolysis, resulting in decomposition. It was reasonable to believe that compound 3.31 with the same electron donating groups would present similar challenges during its ozonolysis, so this approach was discarded.
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3.2.2 General Synthetic Procedure

As previously described, the Claisen rearrangement of the allyl ether derived from Williamson etherification of orcinol afforded two regioisomeric products representing the precursors for grifolin and neogrifolin type targets. We considered that cross metathesis with the terminal olefin would furnish the desired prenyl group, and upon implementing this strategy the designed targets were achieved (Scheme 3-4).

Scheme 3-4 Retrosynthetic Strategy

Scheme 3-3 Claisen Rearrangement and Wittig Reaction Synthetic Attempt
The synthetic pathway was instigated by protecting the phenol groups in the Claisen products of \textit{3.34} as \textit{tert}-butyldimethylsilyl (TBS) ethers and cross metathesis employing a second generation Grubbs catalyst,\textsuperscript{33,34} then TBS deprotection generated the required final products. However, the application of mild acid conditions during deprotection resulted in low yields and it was found that such protection and deprotection of phenols was entirely unnecessary allowing cross metathesis to be conducted directly, without protecting the phenol in advance. So the modified method shortened the number of synthetic steps, saved reagents and gave excellent yields of a stable product.

What follows describes the general synthetic route. It is divided into two categories, which start from two different commercially available materials orcinol and orsellinic acid in order to target grifolin/neogrifolin and grifolic/neogrifolic acid analogues, respectively.

3.2.2.1 Grifolin and Neogrifolin Analogues

The syntheses of targets 1 to 7 (\textit{3.1-3.7}, Figure 3-2) were achieved following the chemistry described in Scheme 3-5. The commercial product orcinol was alkylated employing one equivalent of allylic bromide under Williamson conditions to generate a mono-ether \textit{3.34}. In previous synthetic attempts, the neat material was heated at 220 °C for prolonged periods (2 h) in order for them to undergo Claisen rearrangements, producing two regioisomers (\textit{3.28} and \textit{3.30}) in addition to a heterocyclic product (\textit{3.29}) (Scheme 3-3) along with significant decomposed material. In contrast to this traditional method, a microwave mediated Claisen rearrangement in \textit{N,N}-diethyl aniline was found to produce the same three products but with shortened reaction duration and without thermal decomposition improving yields.

The neogrifolin type product (\textit{3.35}) was the major product isolated, supported by the appearance of an AB spin system in the aromatic region of the \textsuperscript{1}H NMR spectrum (\(\delta\) 6.22, 1H, d, \(J\) = 2.3 Hz; \(\delta\) 6.28, 1H, d, \(J\) = 2.3 Hz) representing a yield of 53%. In addition 5\% of its 5-exo-trig ring closure product (\textit{3.36}) was isolated. The yield of the grifolin type product (\textit{3.37}) was 31\%, as indicated by the symmetrical A\textsubscript{2} spin system of the aromatic protons in the \textsuperscript{1}H NMR spectrum (\(\delta\) 6.26, 2H, s). With a ratio of 58:31 (approx. 2:1) these data suggest that the Claisen rearrangement had no discernible regiochemical preference. In order to form the target compound, the 2-allyl resorcinol (\textit{3.37}) was
directly subjected to the olefin cross metathesis reaction with 2-methyl-2-butene using the second generation Grubbs catalyst, giving an excellent yield of the desired prenylated resorcinol 3.1. Product formation was supported by two singlet CH$_3$ signals δ 1.64, 3H, s; δ 1.74, 3H, s and a triplet olefinic proton resonance, δ 5.21, 1H, t, $J$= 7.1 Hz. In order to diversify the library of compounds, thereby allowing us to explore the relationship between free hydroxy groups and antioxidant activity, the resorcinol (3.1) was alkylated using 1.3 equiv. of dimethyl sulfate to afford, after separation, 26% of monomethylated product 3.2 and 51% of dimethylated product 3.3. These compounds were easily recognised by the appearance of singlet methoxyl resonances at 3.74 ppm integrating for three hydrogens and 3.76 ppm integrating for six hydrogens for compounds 3.2 and 3.3 respectively.

Following the same method, compound 3.4 was generated from compound 3.35 after the cross metathesis. The product was once again readily identified as possessing the 1,3,4,5-tetrasubstituted pattern around the aromatic ring, consistent with neogrigolin type compounds, by the appearance of two aromatic hydrogen resonances in the $^1$H NMR spectrum (δ 6.11, 1H, d, $J$= 2.1 Hz; δ 6.13, 1H, d, $J$= 2.1 Hz). Due to the unsymmetrical nature of the starting resorcinol (3.4) the subsequent methylation reaction returned three compounds in the form of 2 monomethylated derivatives (3.5 and 3.6) and the dimethylated derivative 3.7. These monomethylated derivatives (3.5 and 3.6) were easily recognised by the correlations in HMBC spectrum. In compound 3.6, the singlet methoxyl signal at 3.73 ppm was associated with a carbon signal at 159.6 ppm (C-1) which was also associated with an allylic methylene resonance at 3.22 ppm. Despite the stability of the longer chain farnesylated natural product, the synthetic targets (3.1-3.7) were found to be sensitive to acid, decomposing rapidly even in chloroform solution.
Scheme 3-5 Synthetic Route to Grifolin and Neogrifolin Analogues
3.2.2.2 Grifolic Acid and Neogrigenic Acid Analogue

In order to achieve targets 3.8-3.15, the synthetic route needed to start from the commercially available compound orsellinic acid. The carboxylic acid was firstly esterified to form a methyl ester 3.38, followed by Williamson reaction with allyl bromide to generate the ether 3.39. Subjecting this material to our established microwave mediated conditions for Claisen rearrangement resulted in the formation of 3.40 (86%) without its regioisomer (Scheme 3-6). Since the hydroxy group ortho to the carbonyl is able to form a 6-centred hydrogen bond, the electrons are unevenly distributed between A and B, which makes the electron density at A higher than B. The resulting Claisen rearrangement can occur in a concerted intramolecular process whereas the alternative Claisen rearrangement requires an intermolecular deprotonation event, which may explain the exclusive product 3.40 (Scheme 3-7). We reasoned that elimination of the hydrogen bonding capacity can redistribute the electron density enabling formation of the expected two regioisomers. The Claisen rearrangement was conducted again after the ortho hydroxy group in compound 3.39 was protected as the t-butyldimethylsilyl ether 3.41, returning two regioisomers 3.42 and 3.43 with yields of 63% and 29% respectively (Scheme 3-6). The compound 3.43, possessing the substitution pattern consistent with grifolic acid was subjected to the conditions for cross metathesis furnishing the desired prenylated material 3.44 as supported by two singlet CH₃ signals δ 1.64, 3H, s; δ 1.70, 3H, s and a triplet olefinic proton resonance, δ 5.13, 1H, t, J= 6.3 Hz. Deprotection of the silyl ether with tetrabutylammonium fluoride proceeded as expected to afford the 2,4-dihydroxybenzoate ester 3.45. We envisaged that the ester (3.45) would readily be hydrolysed to return the target acid 3.8, however all efforts to effect this transformation resulted in decomposition returning complex mixtures (Scheme 3-6).
Scheme 3-6 Synthetic Attempt to Grifolic Acid and Neogrifolic Acid Analogues
Turning our attention to the compound 3.42 with the substitution pattern consistent with neogrifolic acid, which represented the major compound obtained from the Claisen rearrangement of the allyl ether (3.41), we implemented the cross metathesis and deprotection to return compounds 3.46 and 3.47 respectively (Scheme 3-6). As for 3.45 the 2,4-dihydroxybenzoate (3.47) was also unstable under all conditions trialed and the desired acid 3.12 was not generated. However, whereas previous efforts resulted in complex mixtures the acid catalysed hydrolysis of 3.47 returned high yields of a cyclised dihydropyran product 3.48. The formation of this heterocycle was supported by two singlet CH$_3$ signals $\delta$ 1.29, 6H, s and two triplet methylene proton resonances $\delta$ 1.82, 2H, t, $J$ = 6.9 Hz; $\delta$ 2.61, 2H, t, $J$ = 6.9 Hz (Scheme 3-6). A review of the relevant literature confirmed that a free hydroxy placed at the para position to the carboxyl group resulted in a decarboxylation product under basic conditions.$^{35-39}$ The only reported solution to hydrolyse such esters was to stir it in concentrated sulfuric acid for 20 h.$^{40,41}$ While we considered these conditions harsh, exploration of this pathway was considered using the 2,4-dihydroxybenzoates 3.40 and 3.49 which were much less acid sensitive than compounds 3.45 and 3.47. Unsurprisingly both compounds also underwent decomposition under these conditions returning small amounts of heterocyclic products, identified as the dihydrobenzofurans 3.50, 3.51 and 3.52, the structure of which were supported by analysis of the 2D NMR data (Scheme 3-8). Formation of a dihydrofuran
here as opposed to the previously observed formation of the dihydropyran above merely reflects the stability of the intermediate carbocation in the allyl versus prenyl precursors.

![Chemical structures and reactions]

**Scheme 3-8 Sulfuric Acid Catalysed Ester Hydrolysis**

So far there has not been a synthetic method reporting the synthesis of target *para* hydroxy benzoic acids, targets 8, 9, 12 and 13. Given the instability of esters 3.45 and 3.47 to hydrolysis under either basic or acidic condition resulting in either decarboxylation or an increased propensity of the prenyl group toward cyclization to form a dihydropyran, these target *para* hydroxy benzoic acids were removed from consideration in the SAR profile. So synthetic targets 8, 9, 12 and 13 were abandoned.

The simpler *p*-methoxy esters 3.53 and 3.55, which were the methylation products of allylated compounds 3.49 and 3.40 respectively, were successfully hydrolysed to their corresponding acids 3.54 and 3.56 under basic conditions (Scheme 3-9) supporting the hypothesis that a *p*-hydroxy group was necessary for decarboxylation. Nevertheless, while the allylated materials are moderately stable to acid the corresponding prenylated targets 10, 11, 14 and 15 would still be susceptible due to their prenyl structures, affording the stable tertiary carbocation upon protonation and resulting in decomposition (cyclisation). Thus targets 10, 11, 14 and 15 were abandoned. The alternative targets were the corresponding methyl esters conferring a greater stability by reducing the possibility of self acid catalysis. So compounds 3.57, 3.58 and 3.59 were formed from methylation of 3.45 and 3.47 (Scheme 3-10).
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Scheme 3-9 Hydrolysis of the Methylated Esters under Basic Conditions

Scheme 3-10 Synthesis of Alternative Targets

In addition, several side-products 3.60-3.63 (Figure 3-3) were isolated during the preparation of synthetic targets, they were added to the compound library to assess their antioxidant activities as well.

Figure 3-3 Isolated Side Products during Synthetic Targets Preparation

3.3 Conclusions

Grifolin and neogrigolin analogues 3.1-3.7 were successfully prepared, while grifolic acid and neogrigolic acid analogues 3.8-3.15 were abandoned because of their instability. Synthesis of the alternatives targets, esters 3.45, 3.47, 3.57, 3.58 and 3.59 were
achieved. A general synthetic route to those designed targets was worked out. Based on this synthetic route, 28 other compounds possessing the similar associated structures to synthetic targets were prepared. All these structures built up a compound library, which are ready for bioassay to investigate SAR, they have two main regiochemistry patterns. Within the same regiochemistry pattern, these structures have different numbers of free phenol. The assessment of these structures’ antioxidant activities is useful to elucidate how the number of prenyl units and their regiochemistry affect antioxidant activity as well as the number of free phenol groups.

The original designed targets belongs to two different chemical patterns, namely grifolin and neogrifolin pattern, which are two regioisomers. So the investigation of a versatile synthetic pathway to those targets is very important, a reaction generating relatively even different regioisomers at one step is a promising candidate to constitute the versatile synthetic pathway. The Claisen rearrangement generating two regioisomer products gives a considerable solution. Our methodology investigation discovered that compared with the traditional protocol, the microwave aid method offers a higher yield and less side products, is a perfect option. After that, synthesis derives from two main ways toward targets. The prenyl moiety is especially sensitive to acid in the presence of an ortho hydroxy group due to intramolecular cyclisation. The deprotection of a phenol ether or hydrolysis of an ester would both increase the acidity resulting in lower yields due to cyclisation. Prenyl was furnished by a cross metathesis reaction, which is generally considered to not proceed on a structure with free hydroxy groups, which means the protection of hydroxy is necessary for such structures prior to undergo cross metathesis. However, the protection and later deprotection were shown to be unnecessary by our research. The direct cross metathesis reaction gave a higher yield (99%) within one step. The reaction of methylating phenol not only generates regioisomers but also affords mono and di methylation products in one step, so it should be effected after prenylation. So the general synthetic route was taken in the described order.

Compounds 3.1-3.7, esters 3.45, 3.47, 3.57, 3.58 and 3.59 are acid sensitive (decomposed in CHCl₃) mainly due to their common prenyl structures, their self-cyclised products under acidic condition were also observed during the investigation. However, acid sensitivities of grifolin, neogrifolin, grifolic acid and other analogues possessing two
or three prenyl units’ side chains haven’t been mentioned by literatures so far, the
greater steric hindrance of longer side chain may be the reason.

3.4 Experimental

3.4.1 Equipment and Chemical Reagents

For General Experimental conditions see chapter two experimental section.

3.4.2 Experimental Data

1,3-dimethoxy-5-methylbenzene (3.16). A solution of orcinol (1.00 g, 8.12 mmol) in acetone (20 mL) was added to a round bottom flask. Then potassium carbonate (4.3 eq., 4.86 g, 35.24 mmol) was added to the flask. After that methyl iodide (4.3 eq., 2.20 mL, 35.24 mmol) was added to the flask, and the reaction mixture was stirred and refluxed. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo and the residue was suspended in water and acidified by 1 M HCl, followed by extraction with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na₂SO₄ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (95%, 1.17 g, 7.70 mmol).²⁴ ¹H NMR (400 MHz, CDCl₃): δ 2.32 (3H, s, 5-Me), 3.78 (6H, s, 1,3-OMe), 6.30 (1H, brs, 2-H), 6.35 (2H, brs, 4,6-H). ¹³C NMR (100 MHz, CDCl₃): δ 21.9 (5-Me), 55.3 (1,3-OMe), 97.6 (2-C), 107.2 (4,6-C), 140.3 (5-C), 160.8 (1,3-C). EIMS: m/z 152 [M]+ (100), 123 (33).

2-bromo-1,5-dimethoxy-3-methylbenzene (3.17). A solution of compound 3.16 (1.07 g, 7.03 mmol) in dichloromethane (15 mL) was added to a round bottom flask. Then N-Bromosuccinimide (NBS) (1.4 eq., 1.75 g, 9.83 mmol) was added to the flask, and the reaction mixture was stirred and refluxed for 4 h. Then the reaction mixture was filtered, the filtrate was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 5% EtOAc in petroleum ether as eluent to return the title compound as a low-melting solid (51%, 0.83 g, 3.58 mmol).²⁴ ¹H NMR (400 MHz, CDCl₃): δ 2.39 (3H, s, 3-Me), 3.78 (3H, s, 5-OMe), 3.86 (3H, s, 1-OMe), 6.34 (1H, brs, 6-H), 6.43 (1H, brs, 4-H). ¹³C NMR (100 MHz, CDCl₃): δ 23.7 (3-Me), 55.6 (5-OMe), 56.4 (1-OMe), 97.3 (6-C), 105.2 (2-
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(3'-methylbut-2'-en-1'-yl)benzene (3.19). A solution of phenylboronic acid (100 mg, 0.57 mmol) in anhydrous toluene (5 mL) was added to a oven-dried 2-neck round bottom flask. Then prenyl bromide (1.0 eq., 66 μl, 0.57 mmol) and potassium carbonate (1.0 eq., 79 mg, 0.57 mmol) were added to the flask, and the reaction mixture was stirred and refluxed for 6 h under an argon atmosphere. The reaction mixture was diluted with diethyl ether (5 mL) and washed with deionised water twice. The organic layer was dried over Na₂SO₄ and concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 10% EtOAc in petroleum ether as eluent to return the title compound as a colourless oil (6%, 5 mg, 0.03 mmol).

1'-(2,4-dimethoxy-6-methylphenyl)-3'-methylbut-2'-en-1'-one (3.21). A solution of 3-methylcrotonic acid (1.00 g, 10.00 mmol) in anhydrous DCM (50 mL) was added to a oven-dried 2-neck round bottom flask. Then trifluoroacetic anhydride (TFAA) (2.0 eq., 2.80 mL, 20.00 mmol) was added dropwise to the flask at 0 °C, and the reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was monitored by GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo without further purification to return an anhydride 3.20 as a yellow oil (68%, 615 mg, 3.38 mmol). This product was used immediately for the next reaction. A solution of anhydride 3.20 (610 mg, 3.35 mmol) in anhydrous toluene (5 mL) was added to a oven-dried 2-neck round bottom flask. Then compound 3.16 (0.2 eq., 125 mg, 0.82 mmol) and anhydrous p-toluenesulfonic acid were added to the flask, and the reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until compound 3.16 was consumed. The reaction mixture was washed with potassium carbonate aqueous solution, the organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 10% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil.

\[
\begin{align*}
\text{C, 107.3 (4-C), 139.9 (3-C), 156.7 (5-C), 159.4 (1-C). EI-MS: } & m/z 232 [M+2]^{+} (97), 230 [M]^{+} (100), 189 (19), 187 (23), 149 (41). \\
\end{align*}
\]
(50%, 95 mg, 0.41 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.91 (3H, s, 4'-H), 2.14 (3H, s, 5'-H), 2.22 (3H, s, 6-Me), 3.76 (3H, s, 2-OMe), 3.80 (3H, s, 4-OMe), 6.26 (1H, s, 2'-H), 6.31 (2H, brs, 3,5-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 19.8 (6-Me), 20.8 (5'-C), 28.1 (4'-C), 55.5 (2-OMe), 55.9 (4-OMe), 96.3 (3-C), 107.1 (5-C), 125.7 (1-C), 127.1 (2'-C), 137.6 (6-C), 154.4 (3'-C), 157.9 (2-C), 160.9 (4-C), 196.4 (1'-C). EIMS: m/z 234 [M]$^+$ (36), 219 (100), 203 (24), 191 (26), 179 (47), 83 (19). HREIMS: m/z 234.1257 (calculated for C$_{14}$H$_{18}$O$_3$ 234.1256).

1'-(2,6-dimethoxy-4-methylphenyl)-3'-methylbut-2'-en-1'-one (3.22). This compound was isolated from the same reaction mixture generating 3.21 as a viscous yellow oil (16%, 31 mg, 0.13 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.90 (3H, s, 4'-H), 2.17 (3H, s, 5' -H), 2.34 (3H, s, 4-Me), 3.76 (6H, s, 2,6-OMe), 6.24 (1H, s, 2'-H), 6.36 (2H, s, 3,5-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 20.9 (5'-C), 22.4 (4-Me), 28.0 (4'-C), 56.0 (2,6-OMe), 105.1 (3,5-C), 119.3 (1-C), 126.9 (2'-C), 140.9 (4-C), 154.6 (3'-C), 157.1 (2,6-C), 194.0 (1'-C). EIMS: m/z 234 [M]$^+$ (40), 219 (23), 203 (100), 179 (70), 83 (34). HREIMS: m/z 234.1256 (calculated for C$_{14}$H$_{18}$O$_3$ 234.1256).

1',1''-(4,6-dimethoxy-2-methyl-1,3-phenylene)bis(3'-methylbut-2'-en-1'-one) (3.23). This compound was isolated from the same reaction mixture generating 3.21 as a viscous yellow oil (39%, 101 mg, 0.32 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.90 (6H, s, 4',4''-H), 2.05 (3H, s, 2-Me), 2.14 (6H, s, 5',5''-H), 3.79 (6H, s, 4,6-OMe), 6.20 (2H, s, 2',2''-H), 6.31 (1H, s, 5-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 16.1 (2-Me), 20.9 (5',5''-C), 28.0 (4',4''-C), 55.9 (4,6-OMe), 93.1 (5-C), 126.1 (1,3-C), 126.8 (2',2''-C), 133.6 (2-C), 155.4 (3',3''-C), 157.5 (4,6-C), 196.2 (1',1''-C). EIMS: m/z 316 [M]$^+$ (36), 301 (100), 273 (23), 261 (33), 245 (63), 219 (20), 83 (48). HREIMS: m/z 316.1678 (calculated for C$_{19}$H$_{24}$O$_4$ 316.1675).

1'-(2,4-dimethoxy-6-methylphenyl)-3'-methylbutan-1'-one (3.24). A solution of compound 3.21 (131 mg, 0.56 mmol) in anhydrous ethanol (8 mL) was added to a oven-dried 2-neck round bottom flask. Then triethylsilane (2.0 eq., 182 μl, 1.12 mmol) and palladium (II) chloride (14%, 14 mg, 0.08 mmol) were added to the flask, and the reaction mixture was stirred overnight under an argon atmosphere. The reaction
was quenched by adding water, the reaction mixture was extracted with DCM twice. The combined organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a colourless oil (45%, 59 mg, 0.25 mmol). ¹H NMR (400 MHz, CDCl₃): δ 0.95 (3H, s, 4’ or 5’-H), 0.96 (3H, s, 4’ or 5’-H), 2.19-2.29 (1H, m, 3’-H), 2.23 (3H, s, 6-Me), 2.65 (2H, d, J = 6.8 Hz, 2’-H), 3.78 (3H, s, 2’-OMe), 3.80 (3H, s, 4’-OMe), 6.30 (1H, d, J = 2.2 Hz, 3-H), 6.31 (1H, d, J = 2.2 Hz, 5-H). ¹³C NMR (100 MHz, CDCl₃): δ 19.9 (6-Me), 22.9 (4’,5’-C), 47.3 (3’-C), 54.0 (2’-C), 55.5 (4’-OMe), 55.6 (4’-OMe), 96.2 (3-C), 107.3 (5-C), 124.7 (1-C), 137.7 (6-C), 158.3 (2-C), 161.1 (4-C), 207.2 (1’-C). EIMS: m/z 236 [M⁺] (11), 221 (4), 193 (14), 179 (100), 136 (7). HREIMS: m/z 236.1411 (calculated for C₁₄H₂₀O₃ 236.1412).

2-isopentyl-1,5-dimethoxy-3-methylbenzene (3.25). This compound was isolated from the same reaction mixture generating 3.24 as a viscous yellow oil (46%, 57 mg, 0.25 mmol).

¹H NMR (400 MHz, CDCl₃): δ 0.94 (3H, s, 4’ or 5’-H), 0.96 (3H, s, 4’ or 5’-H), 1.23-1.27 (2H, m, 2’-H), 1.57-1.65 (1H, m, 3’-H), 2.28 (3H, s, 3-Me), 2.52-2.56 (2H, m, 1’-H), 3.78 (6H, s, 1,5-OMe), 6.32 (2H, brs, 4,6-H). ¹³C NMR (100 MHz, CDCl₃): δ 19.9 (3-Me), 22.7 (4’,5’-C), 23.8 (1’-C), 28.7 (3’-C), 38.9 (2’-C), 55.4 (1 or 5-OMe), 55.6 (1 or 5-OMe), 96.2 (6-C), 106.5 (4-C), 122.7 (2-C), 137.7 (3-C), 158.1 (1 or 5-C), 158.5 (1 or 5-C). EIMS: m/z 222 [M⁺] (34), 199 (9), 179 (8), 165 (100), 149 (26), 135 (30). HREIMS: m/z 222.1620 (calculated for C₁₄H₂₂O₂ 222.1620).

3-methoxy-5-methylphenol (3.26). A solution of orcinol (5.00 g, 40.30 mmol) in acetone (150 mL) was added to a round bottom flask. Then potassium carbonate (1.1 eq., 6.11 g, 44.30 mmol) was added to the flask. After that methyl iodide (1.1 eq., 2.80 mL, 44.30 mmol) was added to the flask, and the reaction mixture was stirred and refluxed for 2 h. The reaction mixture was concentrated in vacuo and the residue was suspended in water and acidified by 1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na₂SO₄ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (31%, 1.73 g, 12.58 mmol). ¹H NMR (400 MHz, CDCl₃): δ 2.27 (3H, s, 5-Me), 3.76 (3H, s, 3-OMe), 5.12 (1H,
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brs, 1-OH), 6.24 (1H, dd, J= 2.3, 1.8 Hz, 2-H), 6.27 (1H, brs, 6-H), 6.33 (1H, brs, 4-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 21.7 (5-Me), 55.4 (3-OMe), 98.8 (2-C), 107.5 (4-C), 108.8 (6-C), 140.8 (5-C), 156.6 (1-C), 160.9 (3-C). EIMS: m/z 138 [M]$^+$ (100), 121 (8), 109 (33).

1-(allyloxy)-3-methoxy-5-methylbenzene (3.27). A solution of compound 3.26 (1.73 g, 12.58 mmol) in acetone (50 mL) was added to a round bottom flask. Then potassium carbonate (1.5 eq., 2.60 g, 18.87 mmol) and that allyl bromide (1.5 eq., 1.60 mL, 18.87 mmol) were added to the flask, and the reaction mixture was stirred and refluxed for 6 h. The reaction mixture was concentrated in vacuo, the residue was suspended in water and extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (92%, 2.06 g, 11.59 mmol).$^{31}$ $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 2.30 (3H, s, 5-Me), 3.77 (3H, s, 3-OMe), 4.50 (2H, d, J= 5.6 Hz, 1'-H), 5.28 (1H, d, J= 10.5 Hz, 3'-Ha), 5.40 (1H, d, J= 17.3 Hz, 3'-Hb), 6.00-6.10 (1H, m, 2'-H), 6.31 (1H, dd, J= 2.3, 2.1 Hz, 2-H), 6.35 (2H, brs, 4,6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 22.0 (5-Me), 55.4 (3-OMe), 68.9 (1'-C), 98.4 (2-C), 107.5 (4 or 6-C), 108.0 (4 or 6-C), 117.7 (3'-C), 133.5 (2'-C), 140.3 (5-C), 159.8 (1-C), 160.8 (3-C). EIMS: m/z 178 [M]$^+$ (100), 163 (36), 135 (25), 123 (11), 109 (36).

2-allyl-5-methoxy-3-methylphenol (3.28). The neat compound 3.27 (2.06 g, 11.59 mmol) was rapidly heated up to 220 °C in a round bottom flask under an argon atmosphere. The reaction was monitored by TLC, and was continued until the starting material was consumed. The reaction mixture was recovered after it cooled down to room temperature, then was subjected to flash column chromatography on silica using 10% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (19%, 401 mg, 2.25 mmol).$^{31}$ $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 2.26 (3H, s, 3-Me), 3.36 (2H, dt, J= 5.8, 1.8 Hz, 1'-H), 3.75 (3H, s, 5-OMe), 4.93 (1H, brs, 1-OH), 5.00-5.09 (2H, m, 3'-H), 5.90-6.00 (1H, m, 2'-H), 6.28 (1H, d, J= 2.5 Hz, 4 or 6-H), 6.37 (1H, d, J= 2.5 Hz, 4 or 6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 20.1 (3-Me), 30.2 (1'-C), 55.5 (5-OMe), 99.6 (6-C), 108.7 (4-C), 115.4 (3'-C), 116.1 (2-C), 136.1 (2'-C), 139.0 (3-C), 155.1 (1-C), 158.7 (5-C). EIMS: m/z 178 [M]$^+$ (100), 163 (56), 151 (58), 135 (28), 117 (13), 91 (28).
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6-methoxy-2,4-dimethyl-2,3-dihydrobenzofuran (3.29). This compound was isolated from the same reaction mixture generating 3.28 as a viscous yellow oil (16%, 325 mg, 1.82 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.46 (3H, d, $J$ = 6.2 Hz, 2-Me), 2.19 (3H, s, 4-Me), 2.63-2.68 (1H, m, 3-Ha), 3.15-3.21 (1H, m, 3-Hb), 3.75 (3H, s, 6-OMe), 4.90-4.98 (1H, m, 2-H), 6.22 (1H, d, $J$ = 2.3 Hz, 5 or 7-H), 6.23 (1H, d, $J$ = 2.3 Hz, 5 or 7-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 19.4 (4-Me), 22.1 (2-Me), 35.6 (3-C), 55.6 (6-OMe), 80.3 (2-C), 93.6 (7-C), 106.8 (5-C), 118.1 (4a-C), 135.1 (4-C), 160.4 (6,7a-C). EIMS: $m/z$ 178 [M]$^+$ (100), 163 (45), 151 (16), 135 (19), 119 (7), 91 (19). HREIMS: $m/z$ 178.0994 (calculated for C$_{11}$H$_{14}$O$_2$ 178.0994).

2-allyl-3-methoxy-5-methylphenol (3.30). This compound was isolated from the same reaction mixture generating 3.28 as a viscous yellow oil (34%, 701 mg, 3.94 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 2.29 (3H, s, 5-Me), 3.43 (2H, d, $J$ = 6.1 Hz, 1'-H), 3.80 (3H, s, 3-OMe), 4.97 (1H, brs, 1-OH), 5.05-5.13 (2H, m, 3'-H), 5.92-6.02 (1H, m, 2'-H), 6.33 (2H, brs, 4,6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 21.7 (5-Me), 27.3 (1'-C), 55.9 (3-OMe), 104.5 (4 or 6-C), 109.6 (4 or 6-C), 110.6 (2-C), 115.4 (3'-C), 136.7 (2'-C), 137.9 (5-C), 155.1 (1-C), 158.1 (3-C). EIMS: $m/z$ 178 [M]$^+$ (100), 149 (42), 135 (36), 121 (44), 91 (33).

2'-(2-hydroxy-4-methoxy-6-methylphenyl)acetaldehyde (3.31). A solution of compound 3.28 (82 mg, 0.46 mmol) in DCM/MeOH (1:1) (15 mL) was added to a round bottom flask. Then O$_2$/O$_3$ (30:70) stream was bubbled into the solution for 10 min with the flow rate 1 L/min at -78 °C, then N$_2$ stream was bubbled for 10 min to remove O$_3$ residue. After that, dimethyl sulfide (1.3 eq., 46 μl, 0.59 mmol) was added dropwise into the solution and the reaction mixture was stirred for 0.5 h at room temperature. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (21%, 17.2 mg, 0.10 mmol). This compound is represented as a mixture of aldehyde and semi-acetal. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 2.21 (3H, s, 6-Me), 2.89 (1H, dd, $J$ = 16.2, 2.0 Hz, 2''-Ha), 3.10 (2H, d, $J$ = 7.0 Hz, 2'-H), 3.23 (1H, dd, $J$ = 16.2, 6.5 Hz, 2''-Hb), 3.36 (1H, d, $J$ = 5.4 Hz, 1''-OH), 3.75 (3H, s, 4-OMe), 6.07 (1H, m, 1''-H), 6.29 (2H, brs, 3,5-H), 9.77 (1H, s, 1'-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 19.4 (4-Me), 22.1 (2-Me), 35.6 (3-C), 55.6 (6-OMe), 80.3 (2-C), 93.6 (7-C), 106.8 (5-C), 118.1 (4a-C), 135.1 (4-C), 160.4 (6,7a-C). EIMS: $m/z$ 178 [M]$^+$ (100), 163 (45), 151 (16), 135 (19), 119 (7), 91 (19). HREIMS: $m/z$ 178.0994 (calculated for C$_{11}$H$_{14}$O$_2$ 178.0994).
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2-allyl-1,5-dimethoxy-3-methylbenzene (3.32). A solution of compound 3.28 (500 mg, 2.81 mmol) in acetone (15 mL) was added to a round bottom flask. Then potassium carbonate (1.5 eq., 582 mg, 4.22 mmol) and methyl iodide (3.0 eq., 525 μl, 8.43 mmol) were added to the flask, and the reaction mixture was stirred and refluxed overnight. The reaction mixture was concentrated in vacuo, then the residue was suspended in water and extracted with EtOAc three times. The combined organic layer was dried over Na₂SO₄ and concentrated in vacuo, and the residue was subjected to flash column chromatography on silica using 15% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (95%, 513 mg, 2.67 mmol).¹ H NMR (400 MHz, CDCl₃): δ 2.28 (3H, s, 3-Me), 3.35 (2H, m, 1'-H), 3.80 (6H, s, 1,5-OMe), 4.88-4.96 (2H, m, 3'-H), 5.84-5.95 (1H, m, 2'-H), 6.35 (2H, brs, 4,6-H). ¹³C NMR (100 MHz, CDCl₃): δ 19.9 (3-Me), 30.0 (1'-C), 55.4 (1 or 5-OMe), 55.8 (1 or 5-OMe), 96.3 (6-C), 106.7 (4-C), 114.1 (3'-C), 119.0 (2-C), 136.8 (2'-C), 138.6 (3-C), 158.5 (1 or 5-C), 158.7 (1 or 5-C). EIMS: m/z 192 [M]+ (100), 177 (65), 165 (47), 135 (41), 115 (13), 91 (28).

2'-(2,4-dimethoxy-6-methylphenyl)-1'-methoxyethan-1'-ol (3.33). A solution of compound 3.32 (135 mg, 0.70 mmol) in DCM/MeOH (1:1) (15 mL) was added to a round bottom flask. Then O₃/O₃ (30:70) stream was bubbled into the solution for 10 min with the flow rate 1 L/min at -78 °C, then N₂ stream was bubbled for 10 min to remove O₃ residue. After that, dimethyl sulfide (1.3 eq., 72 μl, 0.91 mmol) was added dropwise into the solution and the reaction mixture was stirred for 0.5 h at room temperature. The reaction mixture was concentrated in vacuo, and the residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (28%, 45 mg, 0.20 mmol).¹ H NMR (400 MHz, CDCl₃): δ 2.31 (3H, s, 6-Me), 2.86 (1H, dd, J= 14.5, 4.0 Hz, 2'-Ha), 3.18 (1H, dd, J= 14.5, 7.8 Hz, 2'-Hb), 3.49 (3H, s, 1'-'OMe), 3.79 (3H, s, 4-OMe), 3.85 (3H, s, 2-OMe), 4.75-4.79 (1H, m, 1'-'OH), 6.37 (1H, d, J= 2.6 Hz, 3 or 5-H), 6.38 (1H, d, J= 2.6 Hz, 3 or 5-H), 8.90 (1H, s, 1'-'OH). ¹³C NMR (100 MHz, CDCl₃): δ 20.4 (6-Me), 28.5 (2'-C), 55.4 (4-OMe), 56.0 (1'-'
OMe), 56.1 (2-OMe), 96.6 (3-C), 107.5 (5-C), 107.7 (1’-C), 115.5 (1-C), 139.5 (6-C), 158.7 (4-C), 159.2 (2-C). ESIMS: m/z 265 [M+K]+ (19), 247 (19), 233 (22), 209 (14), 193 (21), 165 (100).

3-(allyloxy)-5-methylphenol (3.34). A solution of orcinol (1.00 g, 8.06 mmol) in acetone (30 mL) was added to a round bottom flask. Then potassium carbonate (1.3 eq., 1.45 g, 10.48 mmol) was added to the solution. After that allyl bromide (1.3 eq., 888 μl, 10.48 mmol) was added to a dropping funnel containing 10 mL acetone, and added dropwise to the flask, and the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo, then the residue was suspended in water and acidified by 0.1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na2SO4 and concentrated in vacuo, the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a sticky yellow oil (67%, 595 mg, 3.62 mmol). 1H NMR (400 MHz, CDCl3): δ 2.26 (3H, s, 5-Me), 4.48 (2H, dt, J = 5.3, 1.5 Hz, 1’-H), 5.29 (1H, dt, J = 10.5, 1.5 Hz, 3’-Ha), 5.41 (1H, dt, J = 17.3, 1.5 Hz, 3’-Hb), 5.99-6.09 (1H, m, 2’-H), 6.30 (2H, m, 2, 6-H), 6.37 (1H, brs, 4-H). 13C NMR (100 MHz, CDCl3): δ 21.6 (5-Me), 69.0 (1’-C), 99.6 (2-C), 108.4 (4-C), 109.2 (6-C), 117.9 (3’-C), 133.2 (2’-C), 140.7 (5-C), 156.4 (1-C), 159.7 (3-C). EIMS: m/z 164 [M]+ (100), 149 (47), 121 (39), 109 (29), 95 (31), 77 (24). HREIMS: m/z 164.0837 (calculated for C10H12O2 164.0837).

4-allyl-5-methylbenzene-1,3-diol (3.35). A solution of compound 3.34 (595 mg, 3.62 mmol) in N,N-diethylaniline (1 mL) was added to a reaction vessel. Then the solution was heated up to 250 °C by microwave followed by the procedure (250 W, ramp 5 min) and kept 250 °C for 20 min. The reaction mixture was diluted by EtOAc (50 mL) after cooling down to the room temperature, then washed with 1 M HCl twice. The organic layer was washed with brine and dried over Na2SO4 before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a viscous colourless oil (53%, 316 mg, 1.93 mmol). 1H NMR (400 MHz, CDCl3): δ 2.21 (3H, s, 5-Me), 3.33 (2H, d, J = 5.8 Hz, 1’-H), 4.98-5.06 (2H, m, 3’-
H), 5.88-5.98 (1H, m, 2'-H), 6.22 (1H, d, J= 2.3 Hz, 2-H), 6.28 (1H, d, J= 2.3 Hz, 6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 19.8 (5-Me), 30.1 (1'-C), 101.1 (2-C), 110.0 (6-C), 115.3 (3'-C), 116.4 (4-C), 136.1 (2'-C), 139.3 (5-C), 154.4 (1-C), 155.0 (3-C). EIMS: m/z 164 [M]$^+$ (100), 153 (19), 149 (49), 137 (50), 123 (34), 91 (13), 77 (16). HREIMS: m/z 164.0837 (calculated for C$_{10}$H$_{12}$O$_2$ 164.0837).

2,4-dimethyl-2,3-dihydrobenzofuran-6-ol (3.36). This compound was isolated from the same reaction mixture generating 3.35 as a pale yellow solid (5%, 30 mg, 0.18 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.45 (3H, d, J= 6.3 Hz, 2-Me), 2.16 (3H, s, 4 -Me), 2.61-2.64 (1H, m, 3-Ha), 3.12-3.18 (1H, m, 3-Hb), 4.89-4.97 (1H, m, 7-H), 6.13 (1H, d, J= 1.9 Hz, 2-H), 6.14 (1H, d, J= 1.9 Hz, 5-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 19.2 (2-Me), 22.1 (4-Me), 35.6 (3-C), 80.4 (2-C), 95.0 (7-C), 108.0 (5-C), 118.3 (4a-C), 135.4 (4-C), 156.0 (6-C), 160.5 (7a-C). EIMS: m/z 164 [M]$^+$ (100), 149 (56), 137 (19), 121 (28), 91 (19). HREIMS: m/z 164.0836 (calculated for C$_{10}$H$_{12}$O$_2$ 164.0837).

2-allyl-5-methylbenzene-1,3-diol (3.37). This compound was isolated from the same reaction mixture generating 3.33 as a viscous yellow oil (31%, 185 mg, 1.13 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 2.22 (3H, s, 5-Me), 3.44 (2H, dt, J= 6.0, 1.8 Hz, 1'-H), 5.11-5.19 (2H, m, 3'-H), 5.95-5.05 (1H, m, 2'-H), 6.26 (2H, s, 4,6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 21.3 (5-Me), 27.5 (1'-C), 108.9 (2-C), 109.3 (4,6-C), 116.1 (3'-C), 116.4 (6-C), 136.3 (2'-C), 138.2 (5-C), 154.9 (1,3-C). EIMS: m/z 164 [M]$^+$ (100), 149 (39), 137 (36), 121 (30), 91 (20). HREIMS: m/z 164.0836 (calculated for C$_{10}$H$_{12}$O$_2$ 164.0837).

5-methyl-2-(3'-methylbut-2'-en-1'-yl)benzene-1,3-diol (3.1). A solution of compound 3.37 (51 mg, 0.31 mmol) in anhydrous trimethylethylene solution (2M in THF, 100 eq., 15.5 mL, 31 mmol) was added to a oven-dried and air-free 2-neck round bottom flask, the solution at 0 °C was then exposed to a stream of argon. After that, Grubb’s 2nd generation catalyst (5%, 13 mg, 0.02 mmol) was added, and the reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 50% EtOAc in petroleum ether as eluent to return the
title compound as a viscous green oil (99%, 60 mg, 0.31 mmol).\textsuperscript{44} \( ^1H \) NMR (400 MHz, CD\(_3\)OD): \( \delta \) 1.64 (3H, s, 4'-H), 1.74 (3H, s, 5'-H), 2.13 (3H, s, 5-Me), 3.23 (2H, d, J= 7.1 Hz, 1'-H), 5.21 (1H, t, J= 7.1 Hz, 2'-H), 6.12 (2H, s, 4,6-H). \( ^{13} \)C NMR (100 MHz, CD\(_3\)OD): \( \delta \) 17.9 (5'-C), 21.3 (5-Me), 23.0 (1'-C), 26.0 (4'-C), 108.5 (4,6-C), 113.3 (2-C), 125.0 (2'-C), 130.8 (3'-C), 137.1 (5-C), 156.9 (1,3-C). EIMS: \( m/z \) 192 [M] \(^+ \) (61), 177 (31), 137 (100), 136 (31), 121 (16), 108 (18).

**3-methoxy-5-methyl-2-(3'-methylbut-2'-en-1'-yl)phenol (3.2).** A solution of compound 3.1 (54 mg, 0.28 mmol) in anhydrous acetone (8 mL) was added to a oven-dried 2-neck round bottom flask. Then potassium carbonate (1.3 eq., 52 mg, 0.37 mmol) was added to the flask. After that dimethylsulfate (1.3 eq., 35 \( \mu \)l, 0.37 mmol) was added to a dropping funnel containing 5 mL anhydrous acetone, and added dropwise to the flask, and the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated \textit{in vacuo}, the residue was suspended in water and acidified by 0.1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water, dried over Na\(_2\)SO\(_4\) and concentrated \textit{in vacuo}, the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (26%, 12 mg, 0.06 mmol). \( ^1H \) NMR (400 MHz, CD\(_3\)OD): \( \delta \) 1.63 (3H, s, 4'-H), 1.73 (3H, s, 5'-H), 2.21 (3H, s, 5-Me), 3.23 (2H, d, J= 7.1 Hz, 1'-H), 3.74 (3H, s, 3-OMe), 5.16 (1H, t, J= 7.1 Hz, 2'-H), 6.24 (2H, brs, 4,6-H). \( ^{13} \)C NMR (100 MHz, CD\(_3\)OD): \( \delta \) 17.8 (5'-C), 21.7 (5-Me), 22.9 (1'-C), 26.0 (4'-C), 56.0 (3-OMe), 104.3 (4-C), 109.8 (6-C), 114.7 (2-C), 124.9 (2'-C), 130.8 (3'-C), 137.4 (5-C), 156.5 (1-C), 159.7 (3-C). EIMS: \( m/z \) 206 [M] \(^+ \) (73), 191 (41), 151 (100), 150 (25), 138 (17), 121 (27). HREIMS: \( m/z \) 206.1307 (calculated for C\(_{13}\)H\(_{18}\)O\(_2\) 206.1307).

**1,3-dimethoxy-5-methyl-2-(3'-methylbut-2'-en-1'-yl)benzene (3.3).** This compound was isolated from the same reaction mixture generating 3.2 as a viscous yellow oil (51%, 26 mg, 0.12 mmol).\textsuperscript{18} \( ^1H \) NMR (400 MHz, CD\(_3\)OD): \( \delta \) 1.62 (3H, s, 4'-H), 1.72 (3H, s, 5'-H), 2.29 (3H, s, 5-Me), 3.23 (2H, d, J= 7.1 Hz, 1'-H), 3.76 (6H, s, 1,3-OMe), 5.10 (1H, t, J= 7.1 Hz, 2'-H), 6.39 (2H, s, 4,6-H). \( ^{13} \)C NMR (100 MHz, CD\(_3\)OD): \( \delta \) 17.8 (5'-C), 22.0 (5-Me), 22.8 (1'-C), 25.9 (4'-C), 56.0 (3-OMe), 104.3 (4-C), 109.8 (6-C), 114.7 (2-C), 124.9 (2'-C), 130.8 (3'-C), 137.4 (5-C), 156.5 (1-C), 159.7 (3-C). EIMS: \( m/z \) 206 [M] \(^+ \) (73), 191 (41), 151 (100), 150 (25), 138 (17), 121 (27). HREIMS: \( m/z \) 206.1307 (calculated for C\(_{13}\)H\(_{18}\)O\(_2\) 206.1307).
56.1 (1,3-OMe), 105.7 (4,6-C), 116.2 (2-C), 124.8 (2'-C), 130.9 (3'-C), 137.9 (5-C), 159.2 (1,3-C). EIMS: \( m/z \) 220 [M]+ (81), 205 (100), 189 (22), 165 (29), 152 (36), 135 (16). ESIMS: \( m/z \) 243 [M+Na]+ (66), 221 (35), 167 (41), 153 (100), 149 (60), 113 (45). HREIMS: \( m/z \) 220.1465 (calculated for C_{14}H_{20}O_{2} 220.1463). HRESIMS: \( m/z \) 243.1360 (calculated for C_{14}H_{20}O_{2}Na 243.1361).

### 5-methyl-4-(3'-methylbut-2'-en-1'-yl)benzene-1,3-diol (3.4)

A solution of compound 3.35 (118 mg, 0.72 mmol) in anhydrous trimethylethylene solution (2M in THF, 100 eq., 36 mL, 72 mmol) was added to an oven-dried and air-free 2-neck round bottom flask, the solution at 0 °C was then exposed to a stream of argon. After that, Grubb’s 2nd generation catalyst (10%, 61 mg, 0.07 mmol) was added, the reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 40% EtOAc in petroleum ether as eluent to return the title compound as a viscous green oil (94%, 130 mg, 0.67 mmol).\(^{44}\) 

1H NMR (400 MHz, CD_{3}OD): \( \delta \) 1.66 (3H, s, 4'-H), 1.74 (3H, s, 5'-H), 2.14 (3H, s, 5-Me), 3.22 (2H, d, \( J = 6.5 \) Hz, 1'-H), 5.04 (1H, t, \( J = 6.5 \) Hz, 2'-H), 6.11 (1H, d, \( J = 2.1 \) Hz, 6-H), 6.13 (1H, d, \( J = 2.1 \) Hz, 2-H). \( ^{13} \)C NMR (100 MHz, CD_{3}OD): \( \delta \) 17.9 (5'-C), 20.0 (5-Me), 25.6 (1'-C), 25.9 (4'-C), 101.2 (2-C), 109.4 (6-C), 119.3 (4-C), 125.1 (2'-C), 130.8 (3'-C), 139.2 (5-C), 156.4 (1-C), 156.8 (3-C). EIMS: \( m/z \) 192 [M]+ (40), 177 (29), 138 (28), 137 (100), 124 (28). HREIMS: \( m/z \) 192.1152 (calculated for C_{12}H_{16}O_{2} 192.1150).

### 5-methoxy-3-methyl-2-(3'-methylbut-2'-en-1'-yl)phenol (3.5)

A solution of compound 3.4 (146 mg, 0.76 mmol) in anhydrous acetone (25 mL) was added to a oven-dried 2-neck round bottom flask. Then potassium carbonate (1.3 eq., 137 mg, 0.99 mmol) was added to the flask. After that dimethylsulfate (1.3 eq., 94 \( \mu \)l, 0.99 mmol) was added to a dropping funnel containing 10 mL anhydrous acetone, and added dropwise to the flask, and the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo, the residue was suspended in water and acidified by 0.1 M HCl, then extracted with EtOAc three times. The
combined organic layer was washed with deionised water, dried over Na₂SO₄ and concentrated in vacuo, the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (7%, 11 mg, 0.06 mmol).¹⁴⁻¹ ¹H NMR (400 MHz, CD₃OD): δ 1.66 (3H, s, 4'-H), 2.18 (3H, s, 3-Me), 3.24 (2H, d, J = 6.7 Hz, 1'-H), 3.69 (3H, s, 5-OMe), 5.04 (1H, t, J = 6.7 Hz, 2'-H), 6.22 (2H, brs, 4,6-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.9 (5'-C), 25.6 (1'-C), 25.9 (4'-C), 55.4 (5-OMe), 99.9 (6-C), 107.8 (4-C), 120.4 (2-C), 124.8 (2'-C), 131.0 (3'-C), 139.3 (3-C), 156.8 (1-C), 159.4 (5-C). ESIMS: m/z 245 [M+K⁺] (8), 229 [M+Na⁺] (14), 221 (23), 207 (100), 205 (12), 183 (47), 165 (13). HRESIMS: m/z 245.0945 (calculated for C₁₃H₁₈O₂K 245.0944), 229.1205 (calculated for C₁₃H₁₈O₂Na 229.1204).

3-methoxy-5-methyl-4-(3'-methylbut-2'-en-1'-yl)phenol (3.6). This compound was isolated from the same reaction mixture generating 3.5 as a viscous yellow oil (22%, 34 mg, 0.17 mmol). ¹H NMR (400 MHz, CD₃OD): δ 1.63 (3H, s, 4'-H), 1.74 (3H, s, 5'-H), 2.16 (3H, s, 5-Me), 2.22 (2H, d, J = 6.9 Hz, 1'-H), 3.73 (3H, s, 3-OMe), 4.98 (1H, t, J = 6.9 Hz, 2'-H), 6.20 (1H, d, J = 2.0 Hz, 6-H), 6.25 (1H, d, J = 2.0 Hz, 2-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.9 (5'-C), 20.1 (3-Me), 25.6 (1'-C), 25.9 (4'-C), 55.6 (5-OMe), 97.6 (2-C), 109.9 (6-C), 120.6 (4-C), 124.9 (2'-C), 130.9 (3'-C), 138.9 (5-C), 156.9 (1-C), 159.6 (3-C). EI-MS: m/z 206 [M⁺] (84), 192 (53), 191 (100), 177 (19), 151 (50), 138 (52), 121 (31). HREIMS: m/z 206.1309 (calculated for C₁₃H₁₈O₂ 206.1307).

1,5-dimethoxy-3-methyl-2-(3'-methylbut-2'-en-1'-yl)benzene (3.7). This compound was isolated from the same reaction mixture generating 3.5 as a viscous yellow oil (34%, 57 mg, 0.26 mmol). ¹H NMR (400 MHz, CD₃OD): δ 1.64 (3H, s, 4'-H), 1.74 (3H, s, 5'-H), 2.21 (3H, s, 3-Me), 3.24 (2H, d, J = 6.7 Hz, 1'-H), 3.73 (3H, s, 5-OMe), 3.75 (3H, s, 1-OMe), 4.98 (1H, t, J = 6.7 Hz, 2'-H), 6.31 (1H, d, J = 2.2 Hz, 4-H), 6.33 (1H, d, J = 2.2 Hz, 6-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.9 (5'-C), 20.1 (3-Me), 25.6 (1'-C), 25.9 (4'-C), 55.6 (5-OMe), 55.9 (1-OMe), 97.0 (6-C), 107.9 (4-C), 121.9 (2-C), 124.6 (2'-C), 131.1 (3'-C), 138.9 (3-C), 159.5 (1-C), 159.8 (5-C). ESIMS: m/z 259 [M+K⁺] (5), 243 [M+Na⁺] (13), 221 (11), 188 (44), 153 (100), 108 (13). HRESIMS: m/z 259.1103 (calculated for C₁₄H₂₀O₂K 259.1100), 243.1361 (calculated for C₁₄H₂₀O₂Na 243.1361).
**methyl 2,4-dihydroxy-6-methylbenzoate (3.38).** A solution of orsellinic acid (1.50 g, 8.92 mmol) in acetone (50 mL) was added to a round bottom flask. Then potassium carbonate (0.5 eq., 616 mg, 4.46 mmol) and methyl iodide (10.0 eq., 5.55 mL, 89.2 mmol) were added to the solution, the reaction mixture was stirred and refluxed overnight. The reaction mixture was concentrated *in vacuo*, the residue was suspended in a saturated NaHCO₃ aqueous solution and extracted with EtOAc three times. The combined organic layer was washed with deionised water, dried over Na₂SO₄ and concentrated *in vacuo* without further purification to return the title compound as white crystals (99%, 1.62 g, 8.92 mmol).

1H NMR (400 MHz, CDCl₃): δ 2.49 (3H, s, 6-Me), 3.93 (3H, s, 1-COOCH₃), 5.27 (1H, s, 4-OH), 6.23 (1H, s, 3-H), 6.27 (1H, s, 5-H), 11.73 (1H, s, 2-OH). 13C NMR (100 MHz, CDCl₃): δ 24.4 (6-Me), 52.0 (1-COOCH₃), 101.4 (3-C), 105.9 (1-C), 111.5 (5-C), 144.2 (6-C), 160.3 (2-C), 165.5 (4-C), 172.3 (1-COOCH₃). EIMS: *m/z* 182 [M]⁺ (51), 151 (36), 150 (100), 122 (59), 94 (17). HREIMS: *m/z* 182.0586 (calculated for C₉H₁₀O₄ 182.0579).

**methyl 4-(allyloxy)-2-hydroxy-6-methylbenzoate (3.39).** A solution of compound 3.38 (392 mg, 2.15 mmol) in acetone (30 mL) was added to a round bottom flask. Then potassium carbonate (1.0 eq., 297 mg, 2.15 mmol) and allyl bromide (1.0 eq., 182 μl, 2.15 mmol) were added to the solution, and the reaction mixture was stirred and refluxed overnight. The reaction mixture was diluted in 200 mL DCM, then filtered. The filtrate was concentrated *in vacuo*, and the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a white solid (99%, 472 mg, 2.13 mmol). 1H NMR (400 MHz, CDCl₃): δ 2.49 (3H, s, 6-Me), 3.92 (3H, s, 1-COOCH₃), 4.53 (2H, d, *J* = 5.3 Hz, 1'-H), 5.29-5.43 (2H, m, 3'-H), 5.97-6.07 (1H, m, 2'-H), 6.31 (1H, s, 5-H), 6.33 (1H, s, 3-H), 11.75 (1H, s, 2-OH). 13C NMR (100 MHz, CDCl₃): δ 24.5 (6-Me), 52.0 (1-COOCH₃), 68.8 (1'-C), 99.6 (3-C), 105.5 (1-C), 111.8 (5-C), 118.2 (3'-C), 132.6 (2'-C), 143.3 (6-C), 163.0 (4-C), 165.6 (2-C), 172.3 (1-COOCH₃). EIMS: *m/z* 222 [M]⁺ (37), 190 (100), 175 (28), 162 (75), 121 (26), 91 (24). HREIMS: *m/z* 222.0893 (calculated for C₁₂H₁₄O₄ 222.0892).

**methyl 3-allyl-4,6-dihydroxy-2-methylbenzoate (3.40).** A solution of compound 3.39 (105 mg, 0.47 mmol) in *N*,*N*-diethylaniline (1 mL) was added to a reaction vessel. Then the solution was heated
up to 250 °C by microwave followed by the procedure (250 W, ramp 5 min) and kept 250 °C for 20 min. The reaction mixture was diluted by EtOAc (50 mL) after cooling down to the room temperature, then washed with 1 M HCl twice. The organic layer was washed with brine and dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (86%, 90 mg, 0.41 mmol). \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) 2.44 (3H, s, 2-Me), 3.40 (2H, dt, \(J = 5.5, 2.0\) Hz, 1'-H), 3.93 (3H, s, 1-COOCH₃), 4.91-5.05 (2H, m, 3'-H), 5.60 (1H, s, 4-OH), 5.88-5.97 (1H, m, 2'-H), 6.31 (1H, s, 5-H), 11.31 (1H, s, 6-OH). \(^1\)C NMR (100 MHz, CDCl₃): \(\delta\) 18.7 (2-Me), 30.1 (1'-C), 52.1 (1-COOCH₃), 101.5 (5-C), 107.0 (1-C), 115.3 (3'-C), 117.8 (3-C), 135.8 (2'-C), 141.9 (2-C), 159.1 (4-C), 162.5 (6-C), 172.3 (1-COOCH₃). EIMS: \(m/z\) 222 [M]⁺ (56), 190 (100), 175 (15), 162 (25), 134 (19), 91 (21). HREIMS: \(m/z\) 222.0892 (calculated for C₁₂H₁₄O₄ 222.0892).

**methyl 4-(allyloxy)-2-((tert-butyldimethylsilyl)oxy)-6-methylbenzoate (3.41).** A solution of compound 3.39 (1.06 g, 4.77 mmol) in anhydrous dimethylformamide (DMF) (20 mL) was added to a oven-dried 2-neck round bottom flask. Then tert-Butyldimethylsilyl chloride (2.0 eq., 1.44 g, 9.53 mmol) was added to the solution, after that \(N,N\)-diisopropylethylamine (2.0 eq., 1.66 mL, 9.53 mmol) was added dropwise to the flask at 0 °C, the reaction mixture was stirred for 4 h under an argon atmosphere. The reaction was quenched by adding brine, the reaction mixture was extracted with DCM three times. The combined organic layer was dried over Na₂SO₄ and concentrated in vacuo, and the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a pale yellow oil (99%, 1.61 g, 4.77 mmol). \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) 0.21 (6H, s, 2-OSi(CH₃)₂C(CH₃)₃), 0.96 (9H, s, 2-OSi(CH₃)₂C(CH₃)₃), 2.26 (3H, s, 6-Me), 3.84 (3H, s, 1-COOCH₃), 4.49 (2H, dt, \(J = 5.3, 1.6\) Hz, 1'-H), 5.27-5.41 (2H, m, 3'-H), 5.97-6.07 (1H, m, 2'-H), 6.23 (1H, s, 3-H), 6.36 (1H, s, 5-H). \(^1\)C NMR (100 MHz, CDCl₃): \(\delta\) -4.2 (2-OSi(CH₃)₂C(CH₃)₃), 18.2 (2-OSi(CH₃)₂C(CH₃)₃), 20.2 (6-Me), 25.7 (2-OSi(CH₃)₂C(CH₃)₃), 52.0 (1-COOCH₃), 69.0 (1'-C), 103.8 (3-C), 109.3 (5-C), 118.0 (3'-C), 119.5 (1-C), 133.1 (2'-C), 138.4 (6-C), 154.2 (2-C), 160.0 (4-C), 168.9 (1-COOCH₃). EIMS: \(m/z\) 321 (7), 305 (14), 279 (100), 210 (23). ESIMS: \(m/z\) 359 [M+Na]\(^+\) (99), 337 (38), 305 (100). HREIMS: \(m/z\) 321.1523 (calculated for C₁₇H₂₅O₄Si 321.1522), 305.1575 (calculated...
for C_{17}H_{25}O_{3}Si 305.1573). HRESIMS: m/z 359.1661 (calculated for C_{18}H_{28}O_{4}SiNa 359.1655), 337.1838 (calculated for C_{18}H_{29}O_{4}Si 337.1835).

**methyl 3-allyl-6-((tert-butyldimethylsilyl)oxy)-4-hydroxy-2-methylbenzoate (3.42).** A solution of compound 3.41 (860 mg, 2.56 mmol) in N,N-diethylaniline (3 mL) was added to a reaction vessel. Then the solution was heated up to 250 °C by microwave followed by the procedure (250 W, ramp 5 min) and kept 250 °C for 20 min. The reaction mixture was diluted by EtOAc (50 mL) after cooling down to the room temperature, then washed with 1 M HCl twice. The organic layer was washed with brine and dried over Na_{2}SO_{4} before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a viscous pale yellow oil (63%, 542 mg, 1.61 mmol). \(^1\)H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 0.20 (6H, s, 6-OSi(C\textsubscript{3}H\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 0.95 (9H, s, 6-OSi(CH\textsubscript{3})\textsubscript{2}C(C\textsubscript{3}H\textsubscript{3})\textsubscript{3}), 2.16 (3H, s, 2-Me), 3.34 (2H, dt, \(J= 5.6, 1.8\) Hz, 1'-H), 3.84 (3H, s, 1-COOC\textsubscript{3}H\textsubscript{3}), 4.96-5.06 (2H, m, 3'-H), 5.26 (1H, s, 4-OH), 5.85-5.95 (1H, m, 2'-H), 6.23 (1H, s, 5-H). \(^{13}\)C NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) -4.3 (6-OSi(C\textsubscript{3}H\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 16.7 (2-Me), 18.1 (6-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 25.6 (6-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 30.1 (1'-C), 52.1 (1-COOCH\textsubscript{3}), 104.5 (5-C), 115.7 (3'-C), 117.1 (3-C), 120.5 (1-C), 135.5 (2'-C), 136.1 (2-C), 151.8 (6-C), 155.5 (4-C), 169.8 (1-COOCH\textsubscript{3}). EIMS: m/z 359 [M+Na]^+ (18), 337 (5), 305 (100). HRESIMS: m/z 359.1659 (calculated for C_{18}H_{28}O_{4}SiNa 359.1655), 337.1835 (calculated for C_{18}H_{29}O_{4}Si 337.1835).

**methyl 3-allyl-2-((tert-butyldimethylsilyl)oxy)-4-hydroxy-6-methylbenzoate (3.43).** This compound was isolated from the same reaction mixture generating 3.42 as white crystals (29%, 253 mg, 0.69 mmol). \(^1\)H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 0.09 (6H, s, 2-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 1.01 (9H, s, 2-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 2.24 (3H, s, 6-Me), 3.41 (2H, d, \(J= 6.0\) Hz, 1'-H), 3.83 (3H, s, 1-COOCH\textsubscript{3}), 5.16-5.23 (2H, m, 3'-H), 5.50 (1H, s, 4-OH), 5.88-5.98 (1H, m, 2'-H), 6.38 (1H, s, 5-H). \(^{13}\)C NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) -3.8 (2-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 18.5 (2-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 20.5 (6-Me), 26.0 (2-OSi(CH\textsubscript{3})\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3}), 28.9 (1'-C), 51.8 (1-COOCH\textsubscript{3}), 112.1 (5-C), 114.4 (3-C), 117.1 (3'-C), 119.1 (1-C), 136.2 (2'-C), 137.1 (6-C), 151.8 (2-C), 157.2 (4-C), 168.9 (1-COOCH\textsubscript{3}). EIMS: m/z 335 (1), 321 (10), 305 (13), 279 (100), 249 (9), 239 (10), 205 (8). HREIMS: m/z 335.1668 (calculated for C_{18}H_{27}O_{4}Si...
methyl 2-((tert-butyldimethylsilyl)oxy)-4-hydroxy-6-methyl-3-(3’-methylbut-2’-en-1’-yl)benzoate (3.44). A solution of compound 3.43 (148 mg, 0.44 mmol) in anhydrous trimethylethylene solution (2M in THF, 100 eq., 22.0 mL, 44.00 mmol) was added to a oven-dried and air-free 2-neck round bottom flask, the solution at 0 °C was then exposed to a stream of argon. After that, Grubb’s 2nd generation catalyst (5%, 19 mg, 0.02 mmol) was added, and the reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a white solid (99%, 214 mg, 0.59 mmol). 1H NMR (400 MHz, CD3OD): δ 0.08 (6H, s, 2-OSi(CH3)2C(CH3)3), 1.00 (9H, s, 2-OSi(CH3)2C(CH3)3), 1.64 (3H, s, 4’-H), 1.70 (3H, s, 5’-H), 2.19 (3H, s, 6-Me), 3.25 (2H, d, J= 6.3 Hz, 1’-H), 3.79 (3H, s, 1-COOCH3), 5.13 (1H, t, J= 6.3 Hz, 2’-H), 6.33 (1H, s, 5-H). 13C NMR (100 MHz, CD3OD): δ -3.6 (2-OSi(CH3)2C(CH3)3), 18.2 (5’-C), 19.3 (2-OSi(CH3)2C(CH3)3), 20.5 (6-Me), 24.2 (1’-C), 25.8 (4’-C), 26.5 (2-OSi(CH3)2C(CH3)3), 52.1 (1-COOCH3), 111.9 (5-C), 118.8 (1-C), 119.1 (3-C), 124.6 (2’-C), 131.7 (3’-C), 136.7 (6-C), 153.2 (2-C), 159.1 (4-C), 171.0 (1-COOCH3). EIMS: m/z 349 (5), 333 (6), 307 (100), 293 (11), 251 (36). ESIMS: m/z 387 [M+Na]+ (44), 365 (36), 333 (100), 289 (14). HREIMS: m/z 349.1835 (calculated for C19H29O4Si 349.1835), 307.1364 (calculated for C16H23O4Si 307.1364). HRESIMS: m/z 387.1970 (calculated for C20H32O4SiNa 387.1968), 365.2148 (calculated for C20H33O4Si 365.2148).

methyl 2,4-dihydroxy-6-methyl-3-(3’-methylbut-2’-en-1’-yl)benzoate (3.45). A solution of compound 3.44 (151 mg, 0.42 mmol) in anhydrous THF (9 mL) was added to a oven-dried 2-neck round bottom flask. Then tetra-n-butylammonium fluoride solution (1M in THF, 1.5 eq., 0.68 mL, 0.68 mmol) was added dropwise to the flask, and the reaction mixture was stirred for 0.5 h under an argon atmosphere. The reaction was quenched by adding water, the reaction mixture was extracted with EtOAc three times. The combined organic layer was washed with saturated NH4Cl aqueous solution, dried
over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a white solid (79%, 83 mg, 0.33 mmol).¹⁹ ¹H NMR (400 MHz, CD₃OD): δ 1.64 (3H, s, 4'-H), 1.75 (3H, s, 5'-H), 2.41 (3H, s, 6-Me), 3.26 (2H, d, J = 7.1 Hz, 1'-H), 3.87 (3H, s, 1-COOCH₃), 5.19 (1H, t, J = 7.1 Hz, 2'-H), 6.20 (1H, s, 5-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.9 (5'-C), 22.8 (1'-C), 24.4 (6-Me), 25.9 (4'-C), 52.0 (1-COOCH₃), 105.1 (1-C), 111.7 (5-C), 114.1 (3-C), 124.0 (2'-C), 131.5 (3'-C), 141.2 (6-C), 161.2 (4-C), 164.0 (2-C), 174.0 (1-COOCH₃). EIMS: m/z 250 [M⁺]⁺ (76), 218 (76), 203 (52), 190 (90), 175 (100), 163 (94), 147 (16). HREIMS: m/z 250.1205 (calculated for C₁₄H₁₈O₄ 250.1205).

**methyl 6-(tert-butyldimethylsilyloxy)-4-hydroxy-2-methyl-3-(3'-methylbut-2'-en-1'-yl)benzoate (3.46).** A solution of compound 3.42 (140 mg, 0.42 mmol) in anhydrous trimethylethylene solution (2M in THF, 100 eq., 21.0 mL, 42.00 mmol) was added to a oven-dried and air-free 2-neck round bottom flask, the solution at 0 °C was then exposed to a stream of argon. After that, Grubb’s 2nd generation catalyst (5%, 18 mg, 0.02 mmol) was added, and the reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a viscous yellow oil (99%, 153 mg, 0.42 mmol). ¹H NMR (400 MHz, CD₃OD): δ 0.21 (6H, s, 6-OSi(CH₃)₂C(CH₃)₃), 0.96 (9H, s, 6-OSi(CH₃)₂C(CH₃)₃), 1.67 (3H, s, 4'-H), 1.75 (3H, s, 5'-H), 2.10 (3H, s, 2-Me), 3.27 (2H, d, J = 6.7 Hz, 1'-H), 3.80 (3H, s, 1-COOCH₃), 5.02 (1H, t, J = 6.7 Hz, 2'-H), 6.28 (1H, s, 5-H). ¹³C NMR (100 MHz, CD₃OD): δ -4.2 (6-OSi(CH₃)₂C(CH₃)₃), 16.7 (2-Me), 17.9 (5'-C), 18.9 (6-OSi(CH₃)₂C(CH₃)₃), 25.6 (1'-C), 25.9 (4'-C), 26.1 (6-OSi(CH₃)₂C(CH₃)₃), 52.4 (1-COOCH₃), 104.6 (5-C), 120.0 (1-C), 121.5 (3-C), 124.2 (2'-C), 131.7 (3'-C), 136.5 (2-C), 152.2 (6-C), 157.7 (4-C), 171.9 (1-COOCH₃). EIMS: m/z 387 [M+Na⁺]⁺ (66), 365 (23), 333 (100), 319 (13), 289 (5). HRESIMS: m/z 387.1969 (calculated for C₂₀H₃₂O₄SiNa 387.1968), 365.2145 (calculated for C₂₀H₃₃O₄Si 365.2148).
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**methyl 4,6-dihydroxy-2-methyl-3-(3’-methylbut-2’-en-1’-yl)benzoate (3.47).** A solution of compound 3.46 (148 mg, 0.41 mmol) in anhydrous THF (8 mL) was added to a oven-dried 2-neck round bottom flask. Then tetra-n-butylammonium fluoride solution (1M in THF, 2.0 eq., 0.82 mL, 0.82 mmol) was added dropwise to the flask, and the reaction mixture was stirred for 0.5 h under an argon atmosphere. The reaction was quenched by adding water, the reaction mixture was extracted with EtOAc three times. The combined organic layer was washed with saturated NH₄Cl aqueous solution, dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a white solid (99%, 105 mg, 0.42 mmol). ¹H NMR (400 MHz, CD₃OD): δ 1.67 (3H, s, 4’-H), 1.75 (3H, s, 5’-H), 2.34 (3H, s, 2-Me), 3.29 (2H, d, J= 6.9 Hz, 1’-H), 3.88 (3H, s, 1-COOC₃H₃), 5.00 (1H, t, J= 6.9 Hz, 2’-H), 6.22 (1H, s, 5-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.9 (5’-C), 18.4 (2-Me), 25.7 (1’-C), 25.9 (4’-C), 52.1 (1-COOCH₃), 101.2 (5-C), 108.3 (1-C), 121.6 (3-C), 124.4 (2’-C), 131.6 (3’-C), 140.8 (2-C), 161.1 (4-C), 161.5 (6-C), 173.3 (1-COOCH₃). EIMS: m/z 250 [M]+ (77), 218 (83), 203 (43), 175 (20), 163 (100), 150 (14). ESIMS: m/z 251 [M+H]+ (42), 219 (100), 207 (20), 195 (15), 163 (11). HREIMS: m/z 250.1212 (calculated for C₁₄H₁₈O₄ 250.1205). HRESIMS: m/z 251.1284 (calculated for C₁₄H₁₉O₄ 251.1283).

**methyl 7-hydroxy-2,2,5-trimethylchromane-6-carboxylate (3.48).** A solution of compound 3.47 (22 mg, 0.09 mmol) in anhydrous THF (2 mL) was added to a round bottom flask. Then 1M HCl aqueous solution (2 mL) was added to the flask, and the reaction mixture was stirred and refluxed overnight. The reaction mixture was diluted by water and extracted with EtOAc. The combined organic layer was washed with deionised water, dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 15% EtOAc in petroleum ether as eluent to return the title compound as a white solid (92%, 20 mg, 0.08 mmol). ¹H NMR (400 MHz, CD₃OD): δ 1.29 (6H, s, 2-Me), 1.82 (2H, t, J= 6.9 Hz, 3-H), 2.31 (3H, s, 5-Me), 2.61 (2H, t, J= 6.9 Hz, 4-H), 3.88 (3H, s, 1-COOCH₃), 6.12 (1H, s, 8-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.6 (5-Me), 21.4 (4-C), 26.8 (2-Me), 34.0 (3-C), 52.3 (1-COOCH₃), 75.4 (2-C), 103.2 (8-C), 110.4 (6-C), 113.8 (5a-C), 140.3 (5-C), 159.3 (8a-C), 160.2 (7-C), 172.8 (1-COOCH₃). EIMS: m/z 250 [M]+ (53), 232
methyl 3-allyl-2,4-dihydroxy-6-methylbenzoate (3.49). A solution of compound 3.43 (220 mg, 0.65 mmol) in anhydrous THF (13 mL) was added to a oven-dried 2-neck round bottom flask. Then tetra-n-butylammonium fluoride solution (1M in THF, 1.5 eq., 0.98 mL, 0.98 mmol) was added dropwise to the flask, and the reaction mixture was stirred for 0.5 h under an argon atmosphere. The reaction was quenched by adding water, the reaction mixture was extracted with EtOAc three times. The combined organic layer was washed with saturated NH₄Cl aqueous solution, dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 40% EtOAc in petroleum ether as eluent to return the title compound as a white solid (98%, 141 mg, 0.64 mmol). ¹H NMR (400 MHz, CDCl₃): δ 2.47 (3H, s, 6-Me), 3.46 (2H, d, J= 6.1 Hz, 1'-H), 3.92 (3H, s, 1-COOCH₃), 5.09-5.17 (2H, m, 3'-H), 5.48 (1H, s, 4-OH), 5.94-6.04 (1H, m, 2'-H), 6.24 (1H, s, 5-H), 12.07 (1H, s, 2-OH). ¹³C NMR (100 MHz, CDCl₃): δ 24.3 (6-Me), 27.3 (1'-C), 52.0 (1-COOCH₃), 105.6 (1-C), 110.1 (3-C), 111.3 (5-C), 115.9 (3'-C), 136.0 (2'-C), 141.4 (6-C), 159.0 (4-C), 162.9 (2-C), 172.7 (1-COOCH₃). EIMS: m/z 222 [M⁺] (75), 190 (65), 175 (67), 162 (100), 147 (17), 91 (45). HREIMS: m/z 222.0893 (calculated for C₁₂H₁₄O₄ 222.0892).

4-hydroxy-2,6-dimethyl-2,3-dihydrobenzofuran-7-carboxylic acid (3.50). Compound 3.49 (50 mg, 0.23 mmol) was dissolved in concentrated H₂SO₄ (5 mL) at 0 °C, this solution was stirred at the same temperature overnight. The reaction mixture was poured into ice-water (20 mL) and rapidly extracted with DCM three times. The combined organic layer was washed with saturated Na₂CO₃ aqueous solution. The aqueous layer was acidified by 2 M HCl to pH=1, then extracted with DCM three times. The combined organic layer was washed with deionised water, dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 55% EtOAc in petroleum ether containing 0.2% acetic acid as eluent to return the title compound as a pale yellow solid (trace amount, less than 2%). ¹H NMR (800 MHz, CDCl₃): δ 1.57 (3H, d, J= 6.3 Hz, 2-Me), 2.60 (3H, s, 6-Me), 2.80-2.82 (1H, m, 3-Ha), 3.32-3.35 (1H, m, 3-Hb), 5.21-5.24 (1H, m, 2-H), 6.30 (1H, s, 5-H). ¹³C NMR (200 MHz, CDCl₃): δ 22.0 (2-Me), 22.8
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(6-Me), 33.5 (3-C), 83.7 (2-C), 105.1 (7-C), 110.2 (4a-C), 113.1 (5-C), 145.5 (6-C), 155.5 (4-C), 161.4 (7a-C), 165.0 (1-COOH). ESIMS: \( m/z \) 231 [M+Na\(^+\)] (100), [M+H\(^+\)] 209 (17), 191 (17). HREIMS: \( m/z \) 231.0633 (calculated for C\(_{11}H_{12}O_4\)Na 231.0633).

6-hydroxy-2,4-dimethyl-2,3-dihydrobenzofuran-5-carboxylic acid (3.51).

Compound 3.40 (50 mg, 0.23 mmol) was dissolved in concentrated H\(_2\)SO\(_4\) (5 mL) at 0 °C, this solution was stirred at the same temperature overnight. The reaction mixture was poured into ice-water (20 mL) and rapidly extracted with DCM three times. The combined organic layer was washed with saturated NaCO\(_3\) aqueous solution. The aqueous layer was acidified by 2 M HCl to pH=1, then extracted with DCM three times. The combined organic layer was washed with deionised water, dried over Na\(_2\)SO\(_4\) before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 55% EtOAc in petroleum ether containing 0.2% acetic acid as eluent to return the title compound as a pale yellow solid (trace amount, less than 2%). \(^1\)H NMR (800 MHz, CDCl\(_3\)): \( \delta \) 1.48 (3H, d, \( J = 6.3 \) Hz, 2-Me), 2.47 (3H, s, 4-Me), 2.67-2.73 (1H, m, 3-Ha), 3.21-3.26 (1H, m, 3-Hb), 4.96-5.01 (1H, m, 2-H), 6.26 (1H, s, 7-H), 11.56 (1H, s, 6-OH). \(^{13}\)C NMR (200 MHz, CDCl\(_3\)): \( \delta \) 20.5 (4-Me), 22.1 (2-Me), 35.9 (3-C), 81.0 (2-C), 96.3 (7-C), 103.4 (5-C), 120.5 (4a-C), 139.1 (4-C), 165.2 (7a-C), 167.0 (6-C), 176.2 (1-COOH). ESIMS: \( m/z \) 231 [M+Na\(^+\)] 79, [M+H\(^+\)] 209 (55), 191 (100). HREIMS: \( m/z \) 231.0633 (calculated for C\(_{11}H_{12}O_4\)Na 231.0633).

4-hydroxy-2,6-dimethyl-2,3-dihydrobenzofuran-5-carboxylic acid (3.52).

This compound was isolated from the same reaction mixture generating 3.51 as a pale yellow solid (tiny amount, less than 2%). \(^1\)H NMR (800 MHz, CDCl\(_3\)): \( \delta \) 1.48 (3H, d, \( J = 6.3 \) Hz, 2-Me), 2.57 (3H, s, 6-Me), 2.73-2.78 (1H, m, 3-Ha), 3.27-3.33 (1H, m, 3-Hb), 5.02-5.08 (1H, m, 2-H), 6.26 (1H, s, 7-H), 11.52 (1H, s, 4-OH). \(^{13}\)C NMR (200 MHz, CDCl\(_3\)): \( \delta \) 22.1 (2-Me), 24.9 (6-Me), 33.8 (3-C), 82.1 (2-C), 104.1 (5-C), 106.3 (7-C), 111.1 (4a-C), 145.8 (6-C), 161.4 (4-C), 165.8 (7a-C), 175.6 (1-COOH). ESIMS: \( m/z \) 231 [M+Na\(^+\)] 78, [M+H\(^+\)] 209 (60), 191 (100). HREIMS: \( m/z \) 209.0814 (calculated for C\(_{11}H_{13}O_4\) 209.0814).

methyl 3-allyl-2-hydroxy-4-methoxy-6-methylbenzoate (3.53).

A solution of compound 3.49 (107 mg, 0.48 mmol) in anhydrous acetone (25 mL) was added to a oven-dried 2-neck round bottom
flask. Then potassium carbonate (1.3 eq., 97 mg, 0.70 mmol) was added to the flask. After that dimethylsulfate (1.3 eq., 67 μl, 0.70 mmol) was added to a dropping funnel containing 10 mL anhydrous acetone, and added dropwise to the flask, and the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo, the residue was suspended in water and acidified by 1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as white crystals (76%, 86 mg, 0.36 mmol). ¹H NMR (400 MHz, CDCl₃): δ 2.53 (3H, s, 6-Me), 3.39 (2H, dt, J= 6.1, 1.6 Hz, 1’-H), 3.85 (3H, s, 4-OMe), 3.92 (3H, s, 1-COOCH₃), 4.93-5.01 (2H, m, 3’-H), 5.91-6.01 (1H, m, 2’-H), 6.29 (1H, s, 5-H), 11.81 (1H, s, 2-OH). ¹³C NMR (100 MHz, CDCl₃): δ 24.8 (6-Me), 27.1 (1’-C), 52.0 (1-COOCH₃), 55.7 (4-OMe), 105.9 (1-C), 106.3 (5-C), 113.0 (3-C), 114.3 (3’-C), 136.5 (2’-C), 141.2 (6-C), 161.4 (4-C), 162.1 (2-C), 172.6 (1-COOCH₃). EIMS: m/z 236 [M]+ (91), 221 (5), 204 (100), 189 (84), 176 (98), 161 (75). HREIMS: m/z 236.1048 (calculated for C₁₃H₁₆O₄ 236.1049).

3-allyl-2-hydroxy-4-methoxy-6-methylbenzoic acid (3.54). A solution of compound 3.53 (29 mg, 0.12 mmol) in H₂O/MeOH/THF (1:1:4) (6 mL) was added to a round bottom flask. Then potassium hydroxide (20 eq., 134 mg, 2.4 mmol) was added to the flask, and the reaction mixture was stirred and refluxed for 4 h. The reaction mixture was diluted by water and acidified by 1 M HCl, then extracted with DCM three times. The combined organic layer was washed with saturated NaCO₃ aqueous solution three times. The combined aqueous layer was acidified by 2 M HCl to pH=1 then extracted with DCM three times. The combined organic layer was dried over Na₂SO₄ and concentrated in vacuo, the residue was subjected to flash column chromatography on silica using 60% EtOAc in petroleum ether containing 0.2% acetic acid as eluent to return the title compound as a white solid (99%, 28 mg, 0.12 mmol). ¹H NMR (400 MHz, CDCl₃): δ 2.60 (3H, s, 6-Me), 3.40 (2H, d, J= 6.1 Hz, 1’-H), 3.87 (3H, s, 4-OMe), 4.94-5.02 (2H, m, 3’-H), 5.90-6.00 (1H, m, 2’-H), 6.34 (1H, s, 5-H), 11.61 (1H,s, 2-OH). ¹³C NMR (100 MHz, CDCl₃): δ 24.9 (6-Me), 27.0 (1’-C), 55.8 (4-OMe), 104.4 (1-C), 106.7 (5-C), 113.2 (3-C), 114.4 (3’-C), 136.3 (2’-C), 142.9 (6-C),
162.4 (4-C), 163.1 (2-C), 175.6 (1-COOH). ESIMS: \textit{m}/z 245 [M+Na$^+$] (47), 227 (25), 205 (100), 130 (31). HREIMS: \textit{m}/z 245.0790 (calculated for C$_{12}$H$_{14}$O$_{4}$Na 245.0790).

\textit{methyl 3-allyl-6-hydroxy-4-methoxy-2-methylbenzoate (3.55)}. A solution of compound 3.40 (10 mg, 0.05 mmol) in anhydrous acetone (10 mL) was added to a oven-dried 2-neck round bottom flask. Then potassium carbonate (1.3 eq., 9 mg, 0.07 mmol) was added to the flask. After that dimethylsulfate (1.3 eq., 7 μl, 0.07 mmol) was added to a dropping funnel containing 5 mL anhydrous acetone, and added dropwise to the flask, and the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated \textit{in vacuo}, the residue was suspended in water and acidified by 1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na$_2$SO$_4$ before concentration \textit{in vacuo}, the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a pale yellow oil (99%, 11 mg, 0.05 mmol). $^1$H NMR (400 MHz, CDCl$_3$): δ 2.43 (3H, s, 2-Me), 3.38 (2H, dt, $J$ = 5.7, 1.6 Hz, 1'-H), 3.81 (3H, s, 4-OMe), 3.92 (3H, s, 1-COOC$_3$H$_3$), 4.83-4.96 (2H, m, 3'-H), 5.81-5.91 (1H, m, 2'-H), 6.36 (1H, s, 5-H), 11.43 (1H, s, 6-OH). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 18.5 (2-Me), 29.8 (1'-C), 52.0 (1-COOC$_3$H$_3$), 55.7 (4-OMe), 97.2 (5-C), 106.0 (1-C), 114.4 (3'-C), 119.6 (3-C), 136.3 (2'-C), 140.6 (2-C), 162.4 (4-C), 163.2 (6-C), 172.5 (1-COOC$_3$H$_3$). EIMS: \textit{m}/z 236 [M$^+$] (56), 204 (100), 189 (24), 177 (18), 161 (15). HREIMS: \textit{m}/z 236.1050 (calculated for C$_{13}$H$_{16}$O$_{4}$ 236.1049).

\textit{3-allyl-6-hydroxy-4-methoxy-2-methylbenzoic acid (3.56)}. A solution of compound 3.55 (32 mg, 0.14 mmol) in H$_2$O/MeOH/THF (1:1:4) (6 mL) was added to a round bottom flask. Then potassium hydroxide (20 eq., 157 mg, 2.8 mmol) was added to the flask, and the reaction mixture was stirred and refluxed for 4 h. The reaction mixture was diluted by water and acidified by 1 M HCl, then extracted with DCM three times. The combined organic layer was washed with saturated Na$_2$CO$_3$ aqueous soluton three times. The combined aqueous layer was acidified by 2 M HCl to pH=1 then extracted with DCM three times. The combined organic layer was dried over Na$_2$SO$_4$ and concentrated \textit{in vacuo}, the residue was subjected to flash column chromatography on silica using 60%
EtOAc in petroleum ether containing 0.2% acetic acid as eluent gives the title compound as a white solid (99%, 31 mg, 0.14 mmol). $^1$H NMR (400 MHz, CDCl$_3$): δ 2.53 (3H, s, 2-Me), 3.40 (2H, d, $J = 5.5$ Hz, 1'-H), 3.84 (3H, s, 4-OMe), 4.85-4.98 (2H, m, 3'-H), 5.83-5.92 (1H, m, 2'-H), 6.39 (1H, s, 5-H), 11.35 (1H, s, 6-OH). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 18.6 (2-Me), 29.8 (1'-C), 55.9 (4-OMe), 97.4 (5-C), 104.5 (1-C), 114.6 (3'-C), 120.3 (3-C), 136.2 (2'-C), 142.3 (2-C), 163.5 (4-C), 164.6 (6-C), 176.4 (1-COOH). EIMS: $m/z$ 245 [M+Na]$^+$ (53), 227 (66), 205 (100). HRESIMS: $m/z$ 245.0793 (calculated for C$_{12}$H$_{14}$O$_4$Na 245.0790).

**methyl 2-hydroxy-4-methoxy-6-methyl-3-(3'-methylbut-2'-en-1'-yl)benzoate (3.57)**. A solution of compound 3.45 (83 mg, 0.33 mmol) in anhydrous acetone (10 mL) was added to a oven-dried 2-neck round bottom flask. Then potassium carbonate (1.0 eq., 46 mg, 0.33 mmol) was added to the flask. After that dimethylsulfate (1.0 eq., 31 μl, 0.33 mmol) was added to a dropping funnel containing 5 mL anhydrous acetone, and added dropwise to the flask, the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated _in vacuo_, the residue was suspended in water and acidified by 1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na$_2$SO$_4$ before concentration _in vacuo_, the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent to return the title compound as a white solid (70%, 61 mg, 0.23 mmol). $^1$H NMR (400 MHz, CD$_3$OD): δ 1.63 (3H, s, 4'-H), 1.74 (3H, s, 5'-H), 2.50 (3H, s, 6-Me), 3.26 (2H, d, $J = 7.2$ Hz, 1'-H), 3.83 (3H, s, 4-OMe), 3.90 (3H, s, 1-COOCH$_3$), 5.14 (1H, t, $J = 7.2$ Hz, 2'-H), 6.38 (1H, s, 5-H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 17.8 (5'-C), 22.7 (1'-C), 24.7 (6-Me), 25.9 (4'-C), 52.2 (1-COOCH$_3$), 56.0 (4-OMe), 106.7 (1-C), 107.2 (5-C), 115.6 (3-C), 123.8 (2'-C), 131.7 (3'-C), 141.9 (6-C), 162.6 (4-C), 162.7 (2-C), 173.8 (1-COOCH$_3$). EIMS: $m/z$ 264 [M]$^+$ (59), 250 (19), 232 (57), 217 (57), 204 (79), 189 (100), 177 (74), 91 (16). HREIMS: $m/z$ 264.1362 (calculated for C$_{15}$H$_{20}$O$_4$ 264.1362).

**methyl 6-hydroxy-4-methoxy-2-methyl-3-(3'-methylbut-2'-en-1'-yl)benzoate (3.58)**. A solution of compound 3.47 (102 mg, 0.41 mmol) in anhydrous acetone (10 mL) was added to a oven-dried 2-neck round bottom flask. Then potassium carbonate (1.0 eq.,
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57 mg, 0.41 mmol) was added to the flask. After that dimethylsulfate (1.0 eq., 39 μl, 0.41 mmol) was added to a dropping funnel containing 5 mL anhydrous acetone, and added dropwise to the flask, the reaction mixture was stirred and refluxed under an argon atmosphere. The reaction was monitored by TLC and GC-MS, and was continued until the starting material was consumed. The reaction mixture was concentrated in vacuo, the residue was suspended in water and acidified by 1 M HCl, then extracted with EtOAc three times. The combined organic layer was washed with deionised water and dried over Na₂SO₄ before concentration in vacuo, the residue was subjected to flash column chromatography on silica using 20% EtOAc in petroleum ether as eluent gives the title compound as a transparent oil (57%, 62 mg, 0.23 mmol). ¹H NMR (400 MHz, CD₃OD): δ 1.64 (3H, s, 4’-H), 1.73 (3H, s, 5’-H), 2.31 (3H, s, 2-Me), 3.26 (2H, d, J= 6.5 Hz, 1’-H), 3.78 (3H, s, 4-OMe), 3.87 (3H, s, 1-COOCH₃), 4.93 (1H, t, J= 6.5 Hz, 2’-H), 6.33 (1H, s, 5-H). ¹³C NMR (100 MHz, CD₃OD): δ 17.9 (5’-C), 18.3 (2-Me), 25.6 (1’-C), 25.9 (4’-C), 52.3 (1-COOCH₃), 56.0 (4-OMe), 97.8 (5-C), 109.2 (1-C), 122.5 (3-C), 124.1 (2’-C), 131.8 (3’-C), 139.8 (2-C), 161.6 (6-C), 162.6 (4-C), 173.1 (1-COOCH₃). EIMS: m/z 264 [M]+ (83), 249 (17), 232 (100), 217 (99), 196 (13), 189 (34), 177 (22), 164 (37), 149 (37). HREIMS: m/z 264.1363 (calculated for C₁₅H₂₀O₄).

methyl 4,6-dimethoxy-2-methyl-3-(3’-methylbut-2’-en-1’-yl)benzoate (3.59). This compound was isolated from the same reaction mixture generating 3.58 as a transparent oil (4%, 5 mg, 0.02 mmol). ¹H NMR (400 MHz, CD₃OD): δ 1.66 (3H, s, 4’-H), 1.75 (3H, s, 5’-H), 2.12 (3H, s, 2-Me), 3.28 (2H, d, J= 6.7 Hz, 1’-H), 3.80 (3H, s, 6-OMe), 3.83 (3H, s, 1-COOCH₃), 3.84 (3H, s, 4-OMe), 4.96 (1H, t, J= 6.5 Hz, 2’-H), 6.50 (1H, s, 5-H). ¹³C NMR (100 MHz, CD₃OD): δ 16.6 (2-Me), 17.9 (5’-C), 25.5 (1’-C), 25.9 (4’-C), 52.5 (1-COOCH₃), 56.2 (4-OMe), 56.4 (6-OMe), 94.4 (5-C), 118.1 (1-C), 122.2 (3-C), 123.9 (2’-C), 131.9 (3’-C), 136.1 (2-C), 157.1 (6-C), 160.3 (4-C), 171.6 (1-COOCH₃). EIMS: m/z 278 [M]+ (100), 263 (91), 247 (50), 231 (33), 210 (33), 189 (26), 179 (26), 149 (6). HREIMS: m/z 278.1519 (calculated for C₁₆H₂₂O₄).

3-methoxy-2,5-dimethylphenol (3.60). This compound was isolated from the same reaction mixture generating 3.16 as a transparent oil (trace amount, less than 2%). ¹H NMR (400 MHz, CDCl₃): δ 2.07 (3H, s, 2-Me), 2.27 (3H, s, 5-Me), 3.80 (3H, s, 3-OMe), 6.29 (2H, s, 4,6-H). ¹³C NMR (100 MHz,
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CDCl₃): δ 7.9 (2-Me), 21.6 (5-Me), 55.8 (3-OMe), 104.2 (4-C), 108.8 (6-C), 109.0 (2-C), 136.7 (5-C), 154.4 (1-C), 158.6 (3-C). EIMS: m/z 152 [M]+ (100), 137 (41), 121 (31).

2,6-dimethyl-2,3-dihydrobenzofuran-4-ol (3.61). This compound was isolated from the same reaction mixture generating 3.36 as a pale yellow solid (trace amount, less than 1%).[^1] ¹H NMR (800 MHz, CDCl₃): δ 1.46 (3H, d, J = 6.3 Hz, 2-Me), 2.24 (3H, s, 6-Me), 2.69-2.71 (1H, m, 3-Ha), 3.21-3.24 (1H, m, 3-Hb), 4.93-4.96 (1H, m, 2-H), 6.13 (1H, s, 6-H), 6.22 (1H, s, 7-H). ¹³C NMR (200 MHz, CDCl₃): δ 21.7 (6-Me), 22.0 (2-Me), 34.0 (3-C), 80.3 (2-C), 103.4 (7-C), 108.3 (5-C), 109.6 (4a-C), 139.8 (6-C), 152.2 (4-C), 161.5 (7a-C). EIMS: m/z 164 [M]+ (36), 147 (100), 103 (86). HREIMS: m/z 164.0839 (calculated for C₁₀H₁₂O₂ 164.0837).

methyl 2-hydroxy-4-methoxy-6-methylbenzoate (3.62). This compound was isolated from the same reaction mixture generating 3.38 as a white solid (trace amount, less than 1%).[^2] ¹H NMR (400 MHz, CDCl₃): δ 2.49 (3H, s, 6-Me), 3.79 (3H, s, 4-OMe), 3.92 (3H, s, 1-COOCH₃), 6.28 (1H, s, 3-H), 6.32 (1H, s, 5-H). ¹³C NMR (100 MHz, CDCl₃): δ 24.5 (6-Me), 51.9 (1-COOCH₃), 55.4 (4-OMe), 98.8 (3-C), 105.4 (1-C), 111.5 (5-C), 143.2 (6-C), 164.1 (2-C), 165.7 (4-C), 172.3 (1-COOCH₃). EIMS: m/z 196 [M]+ (50), 164 (100), 136 (46), 121 (24), 93 (25). HREIMS: m/z 196.0737 (calculated for C₁₀H₁₂O₄ 196.0736).

methyl 2,4-dimethoxy-6-methylbenzoate (3.63). This compound was isolated from the same reaction mixture generating 3.38 as a viscous pale yellow oil (trace amount, less than 1%).[^3] ¹H NMR (400 MHz, CDCl₃): δ 2.28 (3H, s, 6-Me), 3.79 (6H, s, 2,4-OMe), 3.88 (3H, s, 1-COOCH₃), 6.31 (2H, s, 3,5-H). ¹³C NMR (100 MHz, CDCl₃): δ 20.1 (6-Me), 52.1 (1-COOCH₃), 55.5 (2 or 4-OMe), 56.0 (2 or 4-OMe), 96.3 (3-C), 106.8 (1-C), 116.5 (5-C), 138.4 (6-C), 158.3 (2 or 4-C), 161.5 (2 or 4-C), 168.8 (1-COOCH₃). EIMS: m/z 210 [M]+ (43), 179 (100). HREIMS: m/z 210.0892 (calculated for C₁₁H₁₄O₄ 210.0892).

3.5 References

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(22) Beekman, A. M.; Wossa, S. W.; Kevo, O.; Ma, P.; Barrow, R. A. J. Nat. Prod. 2015, 78, 2133.


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3.6 Appendix NMR Spectra of Novel Compounds

Figure 3-4 $^1$H NMR Spectrum of Compound 3.21 (400 MHz, CDCl$_3$)

Figure 3-5 $^{13}$C NMR Spectrum of Compound 3.21 (100 MHz, CDCl$_3$)

Figure 3-6 $^1$H NMR Spectrum of Compound 3.22 (400 MHz, CDCl$_3$)

Figure 3-7 $^{13}$C NMR Spectrum of Compound 3.22 (100 MHz, CDCl$_3$)

Figure 3-8 $^1$H NMR Spectrum of Compound 3.23 (400 MHz, CDCl$_3$)

Figure 3-9 $^{13}$C NMR Spectrum of Compound 3.23 (100 MHz, CDCl$_3$)

Figure 3-10 $^1$H NMR Spectrum of Compound 3.24 (400 MHz, CDCl$_3$)

Figure 3-11 $^{13}$C NMR Spectrum of Compound 3.24 (100 MHz, CDCl$_3$)

Figure 3-12 $^1$H NMR Spectrum of Compound 3.25 (400 MHz, CDCl$_3$)

Figure 3-13 $^{13}$C NMR Spectrum of Compound 3.25 (100 MHz, CDCl$_3$)

Figure 3-14 $^1$H NMR Spectrum of Compound 3.29 (400 MHz, CDCl$_3$)

Figure 3-15 $^{13}$C NMR Spectrum of Compound 3.29 (100 MHz, CDCl$_3$)

Figure 3-16 $^1$H NMR Spectrum of Compound 3.31 (400 MHz, CDCl$_3$)

Figure 3-17 $^{13}$C NMR Spectrum of Compound 3.31 (100 MHz, CDCl$_3$)

Figure 3-18 $^1$H NMR Spectrum of Compound 3.33 (400 MHz, CDCl$_3$)

Figure 3-19 $^{13}$C NMR Spectrum of Compound 3.33 (100 MHz, CDCl$_3$)

Figure 3-20 $^1$H NMR Spectrum of Compound 3.35 (400 MHz, CDCl$_3$)

Figure 3-21 $^{13}$C NMR Spectrum of Compound 3.35 (100 MHz, CDCl$_3$)

Figure 3-22 $^1$H NMR Spectrum of Compound 3.36 (400 MHz, CDCl$_3$)

Figure 3-23 $^{13}$C NMR Spectrum of Compound 3.36 (100 MHz, CDCl$_3$)

Figure 3-24 $^1$H NMR Spectrum of Compound 3.2 (400 MHz, CD$_3$OD)

Figure 3-25 $^{13}$C NMR Spectrum of Compound 3.2 (100 MHz, CD$_3$OD)

Figure 3-26 $^1$H NMR Spectrum of Compound 3.6 (400 MHz, CD$_3$OD)
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Figure 3-27 13C NMR Spectrum of Compound 3.6 (100 MHz, CD3OD)
Figure 3-28 1H NMR Spectrum of Compound 3.7 (400 MHz, CD3OD)
Figure 3-29 13C NMR Spectrum of Compound 3.7 (100 MHz, CD3OD)
Figure 3-30 1H NMR Spectrum of Compound 3.40 (400 MHz, CDCl3)
Figure 3-31 13C NMR Spectrum of Compound 3.40 (100 MHz, CDCl3)
Figure 3-32 1H NMR Spectrum of Compound 3.41 (400 MHz, CDCl3)
Figure 3-33 13C NMR Spectrum of Compound 3.41 (100 MHz, CDCl3)
Figure 3-34 1H NMR Spectrum of Compound 3.42 (400 MHz, CDCl3)
Figure 3-35 13C NMR Spectrum of Compound 3.42 (100 MHz, CDCl3)
Figure 3-36 1H NMR Spectrum of Compound 3.43 (400 MHz, CDCl3)
Figure 3-37 13C NMR Spectrum of Compound 3.43 (100 MHz, CDCl3)
Figure 3-38 1H NMR Spectrum of Compound 3.44 (400 MHz, CD3OD)
Figure 3-39 13C NMR Spectrum of Compound 3.44 (100 MHz, CD3OD)
Figure 3-40 1H NMR Spectrum of Compound 3.46 (400 MHz, CD3OD)
Figure 3-41 13C NMR Spectrum of Compound 3.46 (100 MHz, CD3OD)
Figure 3-42 1H NMR Spectrum of Compound 3.47 (400 MHz, CD3OD)
Figure 3-43 13C NMR Spectrum of Compound 3.47 (100 MHz, CD3OD)
Figure 3-44 1H NMR Spectrum of Compound 3.48 (400 MHz, CD3OD)
Figure 3-45 13C NMR Spectrum of Compound 3.48 (100 MHz, CD3OD)
Figure 3-46 1H NMR Spectrum of Compound 3.49 (400 MHz, CDCl3)
Figure 3-47 13C NMR Spectrum of Compound 3.49 (100 MHz, CDCl3)
Figure 3-48 1H NMR Spectrum of Compound 3.50 (800 MHz, CDCl3)
Figure 3-49 13C NMR Spectrum of Compound 3.50 (200 MHz, CDCl3)
Figure 3-50 1H NMR Spectrum of Compound 3.51 (800 MHz, CDCl3)
Figure 3-51 $^{13}$C NMR Spectrum of Compound 3.51 (200 MHz, CDCl$_3$)

Figure 3-52 $^1$H NMR Spectrum of Compound 3.52 (800 MHz, CDCl$_3$)

Figure 3-53 $^{13}$C NMR Spectrum of Compound 3.52 (200 MHz, CDCl$_3$)

Figure 3-54 $^1$H NMR Spectrum of Compound 3.53 (400 MHz, CDCl$_3$)

Figure 3-55 $^{13}$C NMR Spectrum of Compound 3.53 (100 MHz, CDCl$_3$)

Figure 3-56 $^1$H NMR Spectrum of Compound 3.54 (400 MHz, CDCl$_3$)

Figure 3-57 $^{13}$C NMR Spectrum of Compound 3.54 (100 MHz, CDCl$_3$)

Figure 3-58 $^1$H NMR Spectrum of Compound 3.55 (400 MHz, CDCl$_3$)

Figure 3-59 $^{13}$C NMR Spectrum of Compound 3.55 (100 MHz, CDCl$_3$)

Figure 3-60 $^1$H NMR Spectrum of Compound 3.56 (400 MHz, CDCl$_3$)

Figure 3-61 $^{13}$C NMR Spectrum of Compound 3.56 (100 MHz, CDCl$_3$)

Figure 3-62 $^1$H NMR Spectrum of Compound 3.58 (400 MHz, CD$_3$OD)

Figure 3-63 $^{13}$C NMR Spectrum of Compound 3.58 (100 MHz, CD$_3$OD)

Figure 3-64 $^1$H NMR Spectrum of Compound 3.59 (400 MHz, CD$_3$OD)

Figure 3-65 $^{13}$C NMR Spectrum of Compound 3.59 (100 MHz, CD$_3$OD)
Figure 3-4 $^1$H NMR Spectrum of Compound 3.21 (400 MHz, CDCl$_3$)
Figure 3-5: $^{13}$C NMR Spectrum of Compound 3.21 (100 MHz, CDCl$_3$)
Figure 3-6 $^1$H NMR Spectrum of Compound 3.22 (400 MHz, CDCl$_3$)
Figure 3-7 $^{13}$C NMR Spectrum of Compound 3.22 (100 MHz, CDCl$_3$)
Figure 3-8 $^1$H NMR Spectrum of Compound 3.23 (400 MHz, CDCl$_3$)
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Figure 3-9 $^{13}$C NMR Spectrum of Compound 3.23 (100 MHz, CDCl$_3$)
Figure 3-10 $^1$H NMR Spectrum of Compound 3.24 (400 MHz, CDCl$_3$)
Figure 3-11 $^{13}$C NMR Spectrum of Compound 3.24 (100 MHz, CDCl$_3$)
Figure 3-12 $^1$H NMR Spectrum of Compound 3.25 (400 MHz, CDCl$_3$)
Figure 3-13 $^{13}$C NMR Spectrum of Compound 3.25 (100 MHz, CDCl$_3$)
Figure 3-14 $^1$H NMR Spectrum of Compound 3.29 (400 MHz, CDCl$_3$)
Figure 3-15 $^{13}$C NMR Spectrum of Compound 3.29 (100 MHz, CDCl$_3$)
Figure 3-16 $^1$H NMR Spectrum of Compound 3.31 (400 MHz, CDCl$_3$)
Figure 3-17 \(^{13}\)C NMR Spectrum of Compound 3.31 (100 MHz, CDCl\(_3\))
Figure 3-18 $^1$H NMR Spectrum of Compound 3.33 (400 MHz, CDCl$_3$)
Figure 3-19 $^{13}$C NMR Spectrum of Compound 3.33 (100 MHz, CDCl$_3$)
Figure 3-20 $^1$H NMR Spectrum of Compound 3.35 (400 MHz, CDCl$_3$)
Figure 3-21 $^{13}$C NMR Spectrum of Compound 3.35 (100 MHz, CDCl$_3$)
Figure 3-22 $^1$H NMR Spectrum of Compound 3.36 (400 MHz, CDCl$_3$)
Figure 3-23 $^{13}$C NMR Spectrum of Compound 3.36 (100 MHz, CDCl$_3$)
**Figure 3-24** $^1$H NMR Spectrum of Compound 3.2 (400 MHz, CD$_3$OD)
Figure 3-25 $^{13}$C NMR Spectrum of Compound 3.2 (100 MHz, CD$_3$OD)
Figure 3-26 $^1$H NMR Spectrum of Compound 3.6 (400 MHz, CD$_3$OD)
Figure 3-27 $^{13}$C NMR Spectrum of Compound 3.6 (100 MHz, CD$_3$OD)
**Figure 3-28** $^1$H NMR Spectrum of Compound 3.7 (400 MHz, CD$_3$OD)
Figure 3.29 $^{13}$C NMR Spectrum of Compound 3.7 (100 MHz, CD$_3$OD)
Figure 3-30 $^1$H NMR Spectrum of Compound 3.40 (400 MHz, CDCl$_3$)
Figure 3-31 $^{13}$C NMR Spectrum of Compound 3.40 (100 MHz, CDCl$_3$)
Figure 3-32 $^1$H NMR Spectrum of Compound 3.41 (400 MHz, CDCl$_3$)
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Figure 3-33 $^{13}$C NMR Spectrum of Compound 3.41 (100 MHz, CDCl$_3$)
Figure 3-34 $^1$H NMR Spectrum of Compound 3.42 (400 MHz, CDCl$_3$)
Figure 3-35 $^{13}$C NMR Spectrum of Compound 3.42 (100 MHz, CDCl$_3$)
Figure 3-36 $^1$H NMR Spectrum of Compound 3.43 (400 MHz, CDCl$_3$)
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Figure 3-37 $^{13}$C NMR Spectrum of Compound 3.43 (100 MHz, CDCl$_3$)
Figure 3-38 \( ^1\text{H} \) NMR Spectrum of Compound 3.44 (400 MHz, CD\(_2\)OD)
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Figure 3-39 $^{13}$C NMR Spectrum of Compound 3.44 (100 MHz, CD$_3$OD)
Figure 3-40 $^1$H NMR Spectrum of Compound 3.46 (400 MHz, CD$_3$OD)
Figure 3-41 $^{13}$C NMR Spectrum of Compound 3.46 (100 MHz, CD$_3$OD)
Figure 3-42 $^1$H NMR Spectrum of Compound 3.47 (400 MHz, CD$_3$OD)
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Figure 3-43 $^{13}$C NMR Spectrum of Compound 3.47 (100 MHz, CD$_3$OD)
Figure 3-44 $^1$H NMR Spectrum of Compound 3.48 (400 MHz, CD$_3$OD)
Figure 3-45 $^{13}$C NMR Spectrum of Compound 3.48 (100 MHz, CD$_3$OD)
Figure 3-46 $^1\text{H}$ NMR Spectrum of Compound 3.49 (400 MHz, CDCl$_3$)
Figure 3-47 $^{13}$C NMR Spectrum of Compound 3.49 (100 MHz, CDCl$_3$)
Figure 3-48 $^1$H NMR Spectrum of Compound 3.50 (800 MHz, CDCl$_3$)
Figure 3-49 $^{13}$C NMR Spectrum of Compound 3.50 (200 MHz, CDCl$_3$)
Figure 3-50 $^1$H NMR Spectrum of Compound 3.51 (800 MHz, CDCl$_3$)
Figure 3-51 $^{13}$C NMR Spectrum of Compound 3.51 (200 MHz, CDCl$_3$)
Figure 3-52 $^1$H NMR Spectrum of Compound 3.52 (800 MHz, CDCl$_3$)
Figure 3-53 $^{13}$C NMR Spectrum of Compound 3.52 (200 MHz, $CDCl_3$)
Figure 3-54 $^1$H NMR Spectrum of Compound 3.53 (400 MHz, CDCl$_3$)
Figure 3-55 $^{13}$C NMR Spectrum of Compound 3.53 (100 MHz, CDCl₃)
Figure 3-56 $^1$H NMR Spectrum of Compound 3.54 (400 MHz, CDCl$_3$)
**Figure 3-57** $^{13}$C NMR Spectrum of Compound 3.54 (100 MHz, CDCl$_3$)
Figure 3-58 $^1$H NMR Spectrum of Compound 3.55 (400 MHz, CDCl$_3$)
Figure 3-59 $^{13}$C NMR Spectrum of Compound 3.55 (100 MHz, CDCl$_3$)
Figure 3-60 $^1$H NMR Spectrum of Compound 3.56 (400 MHz, CDCl$_3$)
Figure 3-61 $^{13}$C NMR Spectrum of Compound 3.56 (100 MHz, CDCl$_3$)
Figure 3-62 $^1$H NMR Spectrum of Compound 3.58 (400 MHz, CD$_3$OD)
Figure 3-63 $^{13}$C NMR Spectrum of Compound 3.58 (100 MHz, CD$_3$OD)
Figure 3-64 $^1$H NMR Spectrum of Compound 3.59 (400 MHz, CD$_3$OD)
Figure 3-65 $^{13}$C NMR Spectrum of Compound 3.59 (100 MHz, CD$_3$OD)
Chapter 4: Antioxidant Assay of Grifolin Analogues

4.1 Introduction

4.1.1 Antioxidant Assay Mechanisms and Classifications

With the increasing interest in antioxidant research, numerous methods have been established and developed to assess antioxidant activity, so far a large number of reviews and monographs regarding antioxidant assays are available.1-32 The brief description of various antioxidant assays as well as their advantages and disadvantages is given in this section, for more details the reader is referred to aforementioned references, in particular those cited here.9,10,12,17,19,24,26,27 Reagents, instruments and procedures vary a lot in antioxidant assays, however, all of them are based on two major reaction mechanisms, namely hydrogen atom transfer (HAT) and electron transfer (ET). As discussed in chapter one, an antioxidant has the property to inhibit formation of free radicals or interrupt propagation of the free radical.33 So the radical can be neutralised either by directly receiving a hydrogen from the antioxidant, which is the HAT mechanism, or by receiving an electron from the antioxidant, which is the ET mechanism.

In general, X· is symbolised as a radical, AH is symbolised as a hydrogen donor, M is symbolised as a transition metal. The reaction in a HAT based antioxidant assay can be described as below (Scheme 4-1), in which the working radical X· is directly quenched to XH by the antioxidant.10,12,24,27

\[
X^- + AH \rightarrow XH + A^+\]

Scheme 4-1 HAT Based Antioxidant Reaction

The reaction in a ET based antioxidant assay is shown in Scheme 4-2, where the working radical X is eventually quenched to XH, however, the progress is different from that in HAT mechanism and a molecule of water is involved in this reaction. In some ET based antioxidant assays, a transition metal ion rather than a radical is reduced by the antioxidant.10,12,24,27

\[
X^- + AH \rightarrow X^- + [AH]^+ \stackrel{H_2O}{\rightarrow} A^+ \downarrow H_3O^+ \rightarrow XH + H_2O
\]

Or

\[
AH + M^{3+} \rightarrow AH^+ + M^{2+}
\]

Scheme 4-2 ET Based Antioxidant Reactions
Therefore, antioxidant assays are divided into two major classifications based on their reaction mechanisms. The HAT based methods including oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), inhibition of induced low density lipoprotein (LDL) oxidation, total oxyradical scavenging capacity assay (TOSCA), crocin-bleaching assays, chemiluminescent assay, lipid peroxidation inhibition capacity (LPIC) assay and inhibited oxygen uptake (IOC); the ET based method including total phenolics assay by Folin-Ciocalteu reagent assay, trolox equivalence antioxidant capacity (TEAC) assay, ferric ion reducing antioxidant power (FRAP) assay, total antioxidant potential assay (using a Cu^{2+}-complex as an oxidant), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2-azinobis 3-ethylbenzthiazoline-6-sulphonic acid radical (ABTS) scavenging assay, N,N-dimethyl-p-phenylenediamine radical (DMPD) scavenging assay, cupric (Cu^{2+}) reducing antioxidant capacity (CUPRAC) assay. It is worth noting that TEAC, DPPH and ABTS assays are argued as mixed HAT and ET based assays. In addition, in terms of samples’ sources, antioxidant assays can also be classified as in vitro and in vivo. Essentially many methods are applicable to both physiological and nonphysiological testing samples, so this classification isn’t well-defined, however, the results of in vivo antioxidant methods are generally regarded as more accurate than their in vitro counterparts because in vivo assays are conducted under conditions with more biological relevance, which approximate interactions between antioxidants in the body. Since the first assay evaluating a compound antioxidant potential, by mean of the stable radical DPPH in 1958, a great number of approaches have been established to measure antioxidant capacity. In the last few decades, the application of combining various chromatography technique including thin-layer chromatography (TLC), gas-chromatography (GC), liquid-chromatography (LC) with traditional determination methods has enabled researchers to eliminate interference and achieve higher accuracy. In this chapter, the classification of antioxidant assay follows the standard demonstrated in the International Union of Pure and Applied Chemistry (IUPAC) technical report. Several common methods with their advantages and drawbacks are briefly introduced in the following section.
4.1.2 Antioxidant Assays

4.1.2.1 ORAC Assay

The oxygen radical absorbance capacity (ORAC) assay is to measure the antioxidant capacity of inhibiting peroxyl radical produced by thermal decomposition of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) (Scheme 4-3) in aqueous buffer in the presence of sufficient oxygen. The generated peroxyl radical reacts with a fluorescent probe such as the protein \(\beta\)-phycoerythrin or fluorescein which quenches the fluorescence. The antioxidant can scavenge the peroxyl radical and delay the decay of fluorescence. The mechanism is outlined below (Scheme 4-3).10,24,26,27

\[
\begin{align*}
\text{R} & \equiv N \equiv N \equiv \text{R} \quad \overset{O_2}{\longrightarrow} \quad \text{N}_2 \quad + \quad 2\text{ROO}^\cdot \\
\text{ROO}^\cdot & + \quad \text{Probe(fluorescent)} \quad \longrightarrow \quad \text{ROOH} \quad + \quad \text{Probe(non-fluorescent)} \\
\text{ROO}^\cdot & + \quad \text{AH} \quad \longrightarrow \quad \text{ROOH} \quad + \quad \text{A}^\cdot \\
\text{ROO}^\cdot & + \quad \text{A}^\cdot \quad \longrightarrow \quad \text{ROO-A}
\end{align*}
\]

Scheme 4-3 AAPH Structure and the ORAC Assay Mechanism

The net integrated areas under fluorescence decay curves indicate test samples antioxidant capacity which can be used to make the comparison and assessment. Values of net area under curve (AUC) from different samples are normally converted to Trolox (an aqueous congener of tocopherol) equivalents (TE) by comparing a standard curve of Trolox following linear or quadratic relationships. This standard curve is formed by using a series of Trolox concentrations with their corresponding net AUC.10,24,26,27

ORAC assay supplies a controllable source of various peroxyl radicals, which are biological relevant. This assay can be adapted to measure both hydrophilic and hydrophobic antioxidants by varying the radical source and solvent, and it can be easily developed to an automated and high-throughput assay coupling with other instruments.10,19,24 This assay is recommended by IUPAC as a standardised method for antioxidant evaluation.27 However, like other assays, the ORAC assay is sensitive to
temperature, and the equipment requirement of this assay is relatively high, since a fluorometer may not be routinely accessed in analytical laboratories.\textsuperscript{10,19,24,27}

### 4.1.2.2 TRAP Assay

Total radical-trapping antioxidant parameter (TRAP) assay also measures an antioxidant ability against peroxyl radicals. Essentially, TRAP assays are variants of ORAC assays in principle with a broader range of initiator, probes and endpoint measurements.\textsuperscript{27} Likewise, the peroxyl radical is initiated by different initiators such as AAPH, enzymes like horseradish peroxidase, H\textsubscript{2}O\textsubscript{2}-hemin, nitric oxide radical NO or singlet oxygen \textsuperscript{1}O\textsubscript{2}. Then the generated peroxyl radical reacts with a target probe. The method has different variations to verify the target probe, so oxygen uptake, fluorescence of \textit{R}-phycoerythrin or absorbance of 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) can be measured to evaluate a test sample’s antioxidant capacities.\textsuperscript{10,24,27} TRAP assay is mostly applied to measure \textit{in vivo} antioxidant capacity of serum or plasma samples. Among them, measuring the oxygen uptake during a controlled lipid peroxidation reaction is the most widely used method.\textsuperscript{24} Generally, rather than net AUC in the ORAC assay, the lag-phase duration of oxidant inhibition is measured in the TRAP assay to quantify antioxidant capacity. This is based on the assumption that all antioxidant reactions show a lag phase and the length of the lag phase is proportional to samples’ antioxidant capacities. As in the ORAC assay, this value is also usually converted to a TE.\textsuperscript{10,24}

The TRAP assay utilises the biologically relevant testing radical and is conducted in systems similar to physiological conditions, the antioxidant capacity measured by this method may more closely reflect \textit{in vivo} action.\textsuperscript{10} This assay is usually recommended as a standardised measurement of antioxidant evaluation.\textsuperscript{10,27} Nevertheless, this assay requires sophisticated instruments and a time consuming preparation is needed to achieve the measurement. Additionally, because too many different endpoints have been used, comparisons between laboratories are challenging. The most significant disadvantage is that the measured value is often underestimated since not every antioxidant has an obvious lag phase, and the antioxidant profile before or after the lag phase is totally ignored in the TRAP assay.\textsuperscript{10,19,27}

### 4.1.2.3 TEAC Assay and ABTS Assay

Trolox equivalent antioxidant capacity (TEAC) assay uses the long-life 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical anion ABTS\textsuperscript{+} initiated by the
oxidation of ABTS (Scheme 4-4) with peroxyl radicals or other oxidants. The generation of anion ABTS+ includes chemical reactions with oxidised reagents such as hydrogen peroxide, manganese dioxide, AAPH and potassium persulfate (K₂S₂O₈), enzyme reactions with enzymes like metmyoglobin, hemoglobin and horseradish peroxidase.¹⁰,²⁷ The produced ABTS+ possessing intense colour can be subsequently quenched to ABTS by the post added testing antioxidant with loss of colour intensity. The mechanism of TEAC assay initiated by K₂S₂O₈ is shown below (Scheme 4-4).

**Scheme 4-4 ABTS Structure and the TEAC Assay Mechanism**

The ABTS+ radical possesses maximum absorptions at wavelengths of 415, 645, 734 and 815 nm while ABTS has nearly no absorption at these wavelengths. Therefore the absorptions of a test sample after a fixed time at these wavelengths (in particular 734 nm) because both interference from sample can be minimised and the extinction coefficient of ABTS+ is higher at this wavelength, is proportional to the ABTS+ radical concentration, which is used to quantify samples’ antioxidant capacities after a fixed reaction time. The final value of the TEAC assay is expressed as a TE, representing the concentration of Trolox that can eliminate the same portion of ABTS+ at the same temperature after the same fixed reaction time.¹⁰,²⁴,²⁶,²⁷ The ABTS assay and TEAC assay are similar, the only difference is the value of ABTS assay isn’t necessary to process TE conversion.

TEAC and ABTS assays are simple, rapid assays and run over a wide range of pH, are compatible for both hydrophilic and hydrophobic antioxidants without being affected by ionic strength. They can be easily developed to high–throughput assays. The results of TEAC and ABTS assays are comparable between different laboratories.⁴,¹⁰,²⁴,²⁶,²⁷ They are recommended for antioxidant capacity measurement by IUPAC.²⁷ However, the testing radical ABTS+ is a nitrogen radical without biological relevance. The results of
these assays are dependent on time of analysis, so the values may be underestimated for the slow processed antioxidant reactions.\textsuperscript{24} Moreover, the high extinction coefficient of ABTS\textsuperscript{+} at working wavelength limits the evaluated antioxidant concentration range. In order to get accurate results based on spectrophotometer reading, the concentration of generated ABTS\textsuperscript{+} should be within 1.5 to 70 µM, which means the concentration of test sample should also be within the same range.\textsuperscript{27}

4.1.2.4 FRAP Assay

Ferric reducing antioxidant power (FRAP) assay is to measure the ability of an antioxidant to reduce the ferric 2,4,6-tripyridyl-s-triazine complex [Fe(TPTZ)\textsubscript{2}]\textsuperscript{3+} (Scheme 4-5) to the corresponding ferrous complex in acidic medium, the mechanism of which is described in Scheme 4-5. Because the redox potential of the reaction [Fe(TPTZ)\textsubscript{2}]\textsuperscript{3+} is 0.7 V, the FRAP assay can react with compounds with smaller redox potentials, these compound are regarded as antioxidants which may also maintain their reducing potentials and function as real antioxidants in cells or tissues.\textsuperscript{10}

\[ [\text{Fe(TPTZ)}_2]^{3+} + \text{ArOH} \rightarrow [\text{Fe(TPTZ)}_2]^{2+} + \text{ArO}^- + \text{H}^+ \]

\textit{Scheme 4-5 [Fe(TPTZ)\textsubscript{2}]\textsuperscript{3+} Structure and the TRAP Assay Mechanism}

The generated ferrous complex is an intense blue colour with a maximum absorption at 595 nm wavelength. The increased absorbance at 595 nm is proportional to the concentration of generated ferrous ion after calibration with that of a standard ferrous ion solution so it can be used to quantify samples’ antioxidant abilities after a fixed reaction time.\textsuperscript{10,24,27} The values of samples’ antioxidant activities are generally processed to IC\textsubscript{50} (concentration of an antioxidant where the testing radical or ion is reduced by half) for evaluation. The FRAP assay was originally designed to measure reducing power in plasma, but it was later adapted and used for botanical samples’ testing.\textsuperscript{10,24} The acidic pH is essential for this method to maintain iron solubility.
The FRAP assay is simply, speedy, economic, robust and does not require specialised instruments, it can be easily developed to automated and high-throughput operation. However, one drawback of the assay is that the required acidic operational condition limits its application. Also, the TRAP assay only detects the compounds with reducing capacity while the compounds that act as antioxidants, like thiols and proteins, by quenching radicals cannot be measured. Therefore the results of antioxidant capacity determined by this method are always underestimated. In addition, this assay relies on the hypothesis that all antioxidant reactions finish within a fixed testing time, however, that is not true for many cases because many compounds, especially polyphenols such as caffeic acid, tannic acid, ferulic acid, ascorbic acid and quercetin react very slowly with ferric ion. The reduction reactions between those polyphenols and ferric ion usually take several hours to complete while the practical operation time is normally within half an hour, which results in the underestimation as well.10,19,24,26

4.1.2.5 DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay measure the abilities of antioxidants to scavenge a stable nitrogen radical DPPH· (Scheme 4-6). The DPPH· radical is commercially available compound with a deep purple colour, its neutral form becomes pale yellow after reacting with an antioxidant (Scheme 4-6).10,19,24,26

\[
\text{DPPH}^+ + \text{ArOH} \rightarrow \text{DPPH} + \text{ArO}^- + \text{H}^+ \\
\text{DPPH}^+ \text{ (purple)} \quad \text{DPPH} \quad \text{ (yellow)}
\]

Scheme 4-6 DPPH Radical Structure and the DPPH Assay Mechanism

The DPPH· radical has a maximum absorption at 515 nm wavelength while its neutral form possesses almost no absorption at this wavelength (details in chapter two section 2.2.2). So the absorbance at this wavelength is proportional to DPPH· radical concentration, which can be used to quantify the antioxidant effect of a test sample after a fixed reaction time.10,19,24,26 As in the FRAP assay, the results of the DPPH assay are always processed to return an IC50 value for evaluation and comparison.
The DPPH assay is the oldest method to directly measure antioxidant activity, it was first reported in 1958.\textsuperscript{1} Since DPPH radical is commercially available, it can be directly applied for assessment, it is the simplest, most rapid and inexpensive antioxidant assay, that is the reason why it is the most widely used assay in antioxidant screening. The DPPH assay is recommended as a standard method by IUPAC, it can be easily developed to various automated and high-throughput assays. The DPPH assay is compatible for hydrophilic and hydrophobic antioxidants.\textsuperscript{3,4,6-8,10,19,24,26,27} However, the DPPH radical is not biologically relevant, as it cannot be found in any cell or tissue. Compared with highly active radicals in organism, this stable radical reacts much slower with antioxidants, so the results achieved within the fixed time (normally no more than 0.5 h) may be underestimated. Also, the steric accessibility is another cause to generate underestimation of results in this assay, only antioxidants with small molecular structures can easily get close to reaction site and rapidly complete the reaction while the big molecules undergo very slow reaction.\textsuperscript{10,19,24,26,27}

4.1.2.6 CUPRAC Assay
Cupric ion reducing antioxidant capacity (CUPRAC) assay is to measure an antioxidant’s ability of reducing Cu\textsuperscript{2+} to Cu\textsuperscript{+} in aqueous-ethanolic medium. The light blue CUPRAC reagent (Scheme 4-7), bis(neocuproine)copper (II) cation \([Cu(Nc)_2]^{2+}\) is able to be reduced to the yellow-orange CUPRAC chromophore bis(neocuproine)copper (I) cation \([Cu(Nc)_2]^{+}\) by receiving an electron from the antioxidants. For example phenols are commonly oxidised to the corresponding quinones (Scheme 4-7).\textsuperscript{10,19,24,26,27} The efficiency of the CUPRAC reduction depends on the number and position of hydroxyl groups on the aromatic ring. It is also affected by the overall conjugation level of the polyphenolic molecule.\textsuperscript{27}
The reduction product bis(neocuproine)copper (I) \([\text{Cu(Nc)}_2]^{2+}\) chelate possesses the maximum absorption at 450 nm. So this can be used to measure the antioxidant capacity after a fixed reaction time (normally within 30 min). The absorbance at 450 nm wavelength is proportional to the concentration of this generated CUPRAC chromophore cation. Sometimes a curve generated by uric acid or Trolox standards is used to convert sample antioxidant value to uric acid equivalents.\(^{10,26}\)

The CUPRAC assay is simultaneously cost-effective, rapid, stable, selective and compatible for both hydrophilic and hydrophobic antioxidants. It is recommended by IUPAC as a standard method for evaluation of antioxidant capacity.\(^{27}\) Compared with the FRAP assay, copper ions have a lower redox potential than iron ions, so the CUPRAC assay can detect some antioxidants including thiols which cannot be measured in the FRAP assay. Also, the copper reaction kinetics are faster than iron. Moreover, many interfering compounds like simple sugars and citric acid in the FRAP assay can be oxidised by the CUPRAC reagent. The operational pH in CUPRAC assay is 7.0 which is close to the physiological pH. However, the preparation of CUPRAC reagent is time intensive, the operational procedure is not simple and the instrument requirement is high. As with any method using a fixed time as the endpoint, the low reaction rates with less active antioxidants may induce the underestimation of antioxidants values in the CUPRAC assay.\(^{10,19,24,26,27}\)

**4.1.2.7 Overview**

Numerous variations of antioxidant evaluation methods and abundant new measurements applying completely new mechanisms, and technology, have arisen in the last few decades. Therefore it was pointless to list all antioxidant assays and only the most common and representative methods were discussed above. It is worth noting that
there is no single method that can offer comprehensive results for the variety of antioxidants that exist. It is recommended to combine the results of at least two different methods to give reliable and accurate values for antioxidant assessment. However, from these a best method can be selected based on testing sample structure, properties and reaction mechanisms.\textsuperscript{4,10,17}

In order to evaluate the number of free hydroxy groups on the effect of antioxidant capacity in grifolin (2.3) and its analogues, TEAC, ATBS and DPPH assays are the best choices since they are mixed HAT and ET based assays, with the DPPH being the first option among these three candidates. As the oldest, simplest and the most economical method, the DPPH assay is the most frequently and widely used \textit{in vitro} antioxidant assessment.\textsuperscript{1,23,26} Various developments to the DPPH assays have overcome its limitations and give reliable and high throughput results. The adjustments in the previous DPPH assay have resulted in a method suitable for grifolin and its analogues. All the test samples are small molecules not anticipated to present steric obstruction to the reactive site on the DPPH radical and consequently samples are expected to react rapidly with the DPPH radical. Therefore, a modified DPPH assay was chosen to evaluate grifolin and its analogues’ antioxidant capacities.

\section*{4.2 Development of the DPPH Assay}

The DPPH assay described here has been developed from published methods.\textsuperscript{34-36} The DPPH assay and the accuracy of the results have been reported to be sensitive to some Lewis bases, light, oxygen, p\textit{H} and solvent types.\textsuperscript{1,4,23,37-41} In addition the selection of a positive standard reference compound, which is used to calculate the test samples’ antioxidant activities, also plays an important role in the assay. In order to gain a rapid, high-throughput and accurate antioxidant assay to evaluate grifolin and its analogues, a plate reader assisted DPPH assay was developed after investigation of the following assay factors.

\subsection*{4.2.1 Solvent}

DPPH is only soluble in organic solvent.\textsuperscript{1,37} Generally alcoholic media like methanol and ethanol are first considered, with many reported antioxidant assays using these alcoholic solvents.\textsuperscript{34-36} The solvent \textit{tert}-butyl alcohol is reported to increase the reactivity of the DPPH radical with antioxidants.\textsuperscript{38,40} However, the application of an
alcoholic solvent has two significant limitations. First is the solubility, where some of the
test samples, grifolin and its analogues, possessing low polarities have difficulty
dissolving in the alcoholic solvents while they are quite soluble in solvents such as CH₂Cl₂
or EtOAc. Unsurprisingly precipitation in the wells of the 96-well plate deleteriously
interferes with absorption readings. Previously antioxidant screening of mushroom
crude extracts within the group encountered solubility issues when methanol was used
as solvent. Loss of volume in wells is another critical problem due to the volatility of the
alcoholic solvents, but the loss of volume is inevitable during the operation, which
involves 30 minutes incubation at 37 °C. Even a small loss of volume will significantly
impact the results when only 200 μL of solution is added to each well. Because of the
solubility to most structural classes and the very low volatility, DMSO represents a
perfect alternative solvent. Figure 4-1 shows the volumes of DMSO versus methanol
solutions in wells kept at room temperature overnight. The volume change in the DMSO
wells was not noticeable while at the same time methanol solutions had become dry. As
such all the testing of compounds in our DPPH assay were conducted in DMSO.

![Figure 4-1 Volume Changes of DMSO and Methanol Solvent in a 96-Well Plate](image)

### 4.2.2 DPPH Solution Concentration

DPPH solution is intensely coloured and has an absorption maximum at 515 nm
wavelength. As such the concentration of DPPH solution should be set in a range in order
to obtain a reliable and accurate value, within which the Beer-Lambert law is obeyed.⁴²
A 500 μM alcoholic solution of DPPH is regarded as the maximum concentration for
quantitative analysis, while a range of 50-100 μM is a normal working concentration.
The absorbance value of DPPH solution is recommended to be no more than 1.0.⁴¹,²³,³⁴-³⁷,⁴³ As such DPPH DMSO solutions with two different concentrations, 100 μM and 137
μM respectively were used, the total volume of each well was set as 200 μL following
reference suggestions.³⁴-³⁶ The absorbance values of wells with various volumes DPPH
DMSO solution (using DMSO to make up to 200 μL in each well) were measured, the test wells were duplicated in each measurement. The results are shown in Table 4-1.

**Table 4-1 Absorbance Values of Wells with Various Volumes DPPH Solutions**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>190 μL DPPH</td>
<td></td>
</tr>
<tr>
<td>180 μL DPPH</td>
<td></td>
</tr>
<tr>
<td>160 μL DPPH</td>
<td></td>
</tr>
<tr>
<td>120 μL DPPH</td>
<td></td>
</tr>
<tr>
<td>40 μL DPPH</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>100 μM</th>
<th>0.553 ± 0.004</th>
</tr>
</thead>
<tbody>
<tr>
<td>137 μM</td>
<td>0.654 ± 0.001</td>
</tr>
</tbody>
</table>

By using DPPH DMSO solution with these two concentrations, the absorbance values of all the wells are within the spectrometer’s working range, reliable and reproducible. Further linear analysis of the relationship between absorbance value and concentration offers the values of $R^2$ with 1 and 0.9998 at 100 μM and 137 μM respectively. The repeated measurements using different sources of DPPH at the two specified concentrations also confirmed the linear relationship between absorbance and concentration.

### 4.2.3 Volume Composition

According to the manufacture’s description, the working volume of 96-well plates is 50-200 μL and the recommendation of working volume is at least 100 μL. 44,45 200 μL is set as the working volume for optimum performance, this working volume was also applied in the reported assays. 34-36 As shown in Figure 2-6 and as discussed in the introduction of this chapter, the DPPH radical has a maximum absorption at 515 nm while its neutral form possesses very weak (nearly zero absorption) at this wavelength. The positive control group using excess reference compound, ascorbic acid, should achieve nearly 100% scavenging activity but the actual result is in conflict with this assumption. The highest scavenging activity achieved in experiments was below 90% while absorbance values of various ascorbic acid DMSO solutions with different concentrations have been confirmed to be almost zero at 515 nm. Figure 4-2 shows that the measured absorbance value of the positive control, which is comprised of 190 μL of a 100 μM DPPH DMSO solution and 10 μL of a 10 mM ascorbic acid DMSO solution, is always higher than the blank, which is comprised of 200 μL pure DMSO solvent and used for absorption calibration. According to theoretical calculation and experimental data, the stoichiometry between DPPH and ascorbic acid is 2:1, therein ascorbic acid is in excess. 23 As such the absorption difference between the blank and positive control cannot be
attributed to unreacted DPPH radical. Therefore interactions between the DPPH radical and ascorbic acid solutions must cause interference which induces a background absorption.

![Figure 4-2 Spectra of DPPH + Ascorbic Acid and Blank Solutions](image)

In order to understand how these interactions impact results and minimise background absorption, different volumes of DPPH solution were added to wells, with volumes topped up to the working volume, 200 μL in each well, with ascorbic acid solution. DPPH solutions were at concentrations of 100 μM and 137 μM, the concentration of ascorbic acid solution was set as 10 mM following reference suggestions, the testing wells were duplicated and their mean absorbance values were used to process scavenging activities as shown in Table 4-2.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>190 μL</th>
<th>180 μL</th>
<th>160 μL</th>
<th>120 μL</th>
<th>40 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μM</td>
<td>85.1</td>
<td>84.8</td>
<td>84.1</td>
<td>81.7</td>
<td>74.1</td>
</tr>
<tr>
<td>137 μM</td>
<td>87.9</td>
<td>87.6</td>
<td>86.9</td>
<td>85.4</td>
<td>79.6</td>
</tr>
</tbody>
</table>

These results show the scavenging activity reduces with the decrease of added DPPH. This suggests the background absorption from interactions between DPPH and ascorbic acid solutions is not from DPPH-ascorbic acid reaction products and therefore isn’t able to be eliminated by normalization. This interference increases its significance in a solution with a small absorbance value. The larger the volume of added DPPH solution
is, the higher the absorbance value of the solution, and consequently the less the system error is. So the maximum volume of DPPH solution should be used in an assay in order to acquire an accurate result and this is possibly the reason why a volume of 195 μL of DPPH solution was applied in the reported method. However, our results show the difference of scavenging activity between experiments using 190 μL and 160 μL of added DPPH solution was only 1% (Table 4-2). In practice, the manipulation of adding the 40 μL of solution has been confirmed to be easier, faster, more accurate and stable than that of adding 10 or 20 μL, especially when the absorption of DPPH decreases markedly with time, which will be discussed later. In preliminary studies the scavenging activity of adding 195 μL DPPH solution was very close to that of adding 160 μL while air bubbles were always trapped during 5 μL liquid transfer, which makes the manipulation of adding 195 μL DPPH solution impractical. Therefore, the working volume in each well is set as 200 μL, which is comprised of 160 μL DPPH solution, for acquiring an accurate, stable and reproducible result.

### 4.2.4 Ascorbic Acid Concentration

As mentioned in section 4.2.3, interactions between DPPH and ascorbic acid solutions cause background absorption and interfere with the accuracy of measurements. Undoubtedly, not only volume composition but also the concentration of ascorbic acid solution will affect such interactions. 10 mM ascorbic acid solution was applied in reported methods, it is used to assess the concentration effect as well as its tenfold dilution. While recognising the optimised working volume of 200 μL, different volumes of ascorbic acid solutions with concentrations of 10 mM or 1 mM and compensating volumes of 100 μM DPPH solutions, were added into wells. The ascorbic acid in each well was in excess based on the aforementioned stoichiometry. The testing wells were duplicated and their mean absorbance values were used to process scavenging activities, the results are shown in Table 4-3.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Scavenging Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 μL</td>
</tr>
<tr>
<td>10 mM</td>
<td>85.1</td>
</tr>
<tr>
<td>1 mM</td>
<td>87.8</td>
</tr>
</tbody>
</table>
The result shows the diluted ascorbic acid solution (1 mM) always gives a higher scavenging activity than the concentrated solution (10 mM), which suggests the high concentration ascorbic acid solution increases the interactions with DPPH solution and consequently causes a high background absorption. In order to acquire an accurate result, the concentration of ascorbic acid solution is set as 1 mM. It is worth noting that the differences of scavenging activity between 10 μL and 40 μL ascorbic acid solutions, at the two concentrations of 10 mM and 1 mM, are very small (less than 1%), affirming previously optimised volume composition employed by us. Subsequently, 80 μL of 1 mM ascorbic acid solution was added into a well, followed by 7-level series dilution, then 160 μL of 100 μM DPPH solution was added into each well. The negative control comprised of 160 μL of 100 μM DPPH DMSO solution and 40 μL DMSO solvent was used as a reference for spectra comparison. The testing wells were duplicated and their mean absorbance values were used to process scavenging activities, the results are shown in Table 4-4, their spectra are displayed in Figure 4-3.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Scavenging Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000.0</td>
<td>87.5</td>
</tr>
<tr>
<td>500.0</td>
<td>87.0</td>
</tr>
<tr>
<td>250.0</td>
<td>87.1</td>
</tr>
<tr>
<td>125.0</td>
<td>58.6</td>
</tr>
<tr>
<td>62.5</td>
<td>28.7</td>
</tr>
<tr>
<td>31.3</td>
<td>14.9</td>
</tr>
<tr>
<td>15.6</td>
<td>7.9</td>
</tr>
<tr>
<td>7.8</td>
<td>4.9</td>
</tr>
</tbody>
</table>
According to the stoichiometry between DPPH and ascorbic acid, ascorbic acid is still in excess after 2\textsuperscript{nd} level dilution. Based on Table 4-4 and Figure 4-3, after 2\textsuperscript{nd} level dilution, the scavenging activities of ascorbic acid solutions are almost the same, and their spectra are almost completely overlapped (the lowest absorbance at 515 nm). Herein the 100\% radical scavenging can be triple checked and the concentration of ascorbic acid solution has a negligible effect on background absorption. The DPPH radical is partially scavenged from 3\textsuperscript{rd} level dilution onwards, the relationship between concentration of ascorbic acid and its scavenging activity afterward is perfectly linear, the \( R^2 \) value is 0.9994. The spectra (Figure 4-3) clearly show solution absorption at 515 nm continuously increases with the ongoing dilution of ascorbic acid and finalises the value lower than absorption of reference DPPH solution. The scavenging activities and spectra (Table 4-4 and Figure 4-3) confirm the usage of 1mM ascorbic acid solution used as the positive control is able to offer an accurate, constant and reliable result.

4.2.5 DPPH Stability

DPPH radical is a stable radical which is commercially available and ready for use. For convenience, the working DPPH solution is generally prepared at high concentration and stored in an amber container covered with foil and placed in a refrigerator. It is further diluted to the required concentration before usage. However, the solvated DPPH is less stable than non-solvated material, in addition to its sensitivity to light, oxygen and temperature,\textsuperscript{23,38,40,41,43} resulting in irreversible degradation during the storage of the
stock solution which reduces the absorbance value and consequently affects the final result. Moreover, the melting point of DMSO is only 19 °C, so the stock DPPH solution is frozen in the refrigerator, and the thawing procedure is likely to increase the possibility of DPPH degradation.\textsuperscript{23,37,41,43} Hence, it is necessary to check the stability of DPPH solution in order to guarantee the accuracy of result. First, two 100 μM DPPH DMSO solutions were compared. One of them was diluted from 50 mM stock solution stored in a refrigerator for four days, another one was freshly prepared. 40 μL of a 1 mM ascorbic acid was 7-level series diluted in a 96-well plate and after making up to 200 μL their absorbance values were measured. An image and absorbance values are displayed in Figure 4-4 and Table 4-5.

![Image of DPPH solutions](image.png)

**Figure 4-4 Four-day Stocked and Fresh DPPH Solutions Antioxidant Assay**

**Table 4-5 Four-day Stocked and Fresh DPPH Solutions Antioxidant Assay**

<table>
<thead>
<tr>
<th>Dilution Level</th>
<th>Scavenging Activity (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluted Stocked DPPH</td>
<td>Fresh DPPH</td>
</tr>
<tr>
<td>0</td>
<td>78.2</td>
<td>87.7</td>
</tr>
<tr>
<td>1</td>
<td>77.9</td>
<td>87.2</td>
</tr>
</tbody>
</table>
The 3rd level diluted ascorbic acid didn’t show any purple colour after mixing with stock DPPH solution while the same level of ascorbic acid was light purple after mixing with fresh DPPH solution (Figure 4-4). The scavenging activities of ascorbic acid (dilution level 0-2) against fresh DPPH solution is higher than against the stock DPPH solution with the same concentration where ascorbic acid is in excess. However, we observed that the scavenging activities of ascorbic acid at lower dilutions, where it is stoichiometrically inadequate (dilution level 3-6), against fresh DPPH solution is lower than the stock DPPH solution (Table 4-5). These observations confirm that DPPH solution degrades over time in the refrigerator. In addition, Figure 4-5 shows spectra of two 100 μM DPPH in DMSO solutions, diluted from 50 mM two-day stock and freshly prepared respectively. Apparently, DPPH solution shows significant degradation after two days. Therefore, we advise that DPPH solution to be used for testing should be prepared immediately before use.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>78.7</td>
<td>86.8</td>
</tr>
<tr>
<td>3</td>
<td>70.1</td>
<td>61.7</td>
</tr>
<tr>
<td>4</td>
<td>34.7</td>
<td>31.2</td>
</tr>
<tr>
<td>5</td>
<td>17.3</td>
<td>16.1</td>
</tr>
<tr>
<td>6</td>
<td>8.3</td>
<td>8.1</td>
</tr>
<tr>
<td>7</td>
<td>4.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Figure 4-5 Spectra of Two-day Stock and Fresh DPPH Solutions*

The DPPH assay is generally incubated at 37 °C for around 30 min before measuring absorbance value. This manipulation is to imitate physiologic condition and allow sufficient reaction time. As mentioned in the introduction section of this chapter, the short reaction time may result in an underestimation, while the longer incubation time...
will increase DPPH degradation. In order to optimise the reaction time, 200 μL freshly prepared 100 μM DPPH in DMSO solution was added to three rows of a 96-well plate, it was then covered and placed under dark conditions at 25 °C. Absorbance values of each well were recorded every 15 min for 14 h. The data shows the absorbance mean reduced by 7%, 12% and 35% after 30 min, 1 h and 14 h respectively. Therefore, incubation time significantly affects result accuracy. Since all testing structures are small molecules, repeated experiments have confirmed their reactions with DPPH were complete within 30 min, thus 30 min is determined as the incubation time. The absorbance value tracking also suggests the operation of whole test should finish as soon as possible to avoid interference from DPPH self-degradation.

4.2.6 Ascorbic Acid Stability

Ascorbic acid is unstable with the most common method of degradation being oxidation to dehydroascorbic acid. Many references have investigated the stability of ascorbic acid confirming that storage temperature and its exposure to air significantly affect its stability. However, the literature gives different suggestions on storage duration ranging from 24 h or 4 days.46-50 For convenience, ascorbic acid is generally prepared and stored under sealed and dark conditions before usage. In order to explore the activity of degradation, two 1 mM ascorbic acid in DMSO solutions, prepared either four days earlier or freshly prepared, were compared for their antioxidant abilities. Following the aforementioned method, 40 μL of 1 mM ascorbic acid solution was subjected to a 7-level series dilution in a 96-well plate and their absorbance values were measured to process the corresponding scavenging activities. All wells were duplicated and the results are displayed in Table 4-6.

<table>
<thead>
<tr>
<th>Dilution Level</th>
<th>Stocked Ascorbic Acid</th>
<th>Fresh Ascorbic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>87.5</td>
<td>87.4</td>
</tr>
<tr>
<td>1</td>
<td>86.9</td>
<td>86.6</td>
</tr>
<tr>
<td>2</td>
<td>86.7</td>
<td>86.4</td>
</tr>
<tr>
<td>3</td>
<td>52.0</td>
<td>63.0</td>
</tr>
<tr>
<td>4</td>
<td>25.6</td>
<td>31.3</td>
</tr>
<tr>
<td>5</td>
<td>12.6</td>
<td>15.2</td>
</tr>
<tr>
<td>6</td>
<td>6.9</td>
<td>8.3</td>
</tr>
<tr>
<td>7</td>
<td>4.6</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Chapter 4: Antioxidant Assay of Grifolin Analogues

From Table 4-6, the scavenging activities of four-day stock ascorbic acid solution (dilution level 0-2) differ marginally from the fresh ascorbic acid solution. As such it is acceptable to prepare the ascorbic acid solution up to four days in advance.

4.2.7 Interactions between Samples and DPPH

As already mentioned, because of the interaction between ascorbic acid and DPPH, the resulting background absorption reduces the maximum achievable scavenging activity of ascorbic acid to around 88%. A similar phenomenon can also be observed in the DPPH assay of some test samples (such as orcinol, compounds 3.5, 3.23, 3.47 and 3.59). We observed that in such cases (Figure 4-6), a high concentration sample solution returned a low or even negative scavenging activity while low concentrations of the same sample solution could show a higher activity (DPPH solution used as a negative control). Figure 4-6 shows that after compound 3.47 with the highest concentration (dilution level 0) reacted with DPPH, the absorption of the reaction mixture at 515 nm was even higher than DPPH solution, the negative control, returning a negative scavenging activity. After the 1st and 2nd level dilution of compound 3.47, the absorptions of the reaction mixture at 515 nm significantly reduced, returning stronger scavenging activities. However, from the 3rd level dilution onwards, the absorptions of the reaction mixture increased at 515 nm, returning weaker scavenging activities, and their spectrum was increasingly close to DPPH’s. The absorptions of test samples such as compound 3.47 have been confirmed to be very weak at 515 nm in the preliminary study. As such from the 3rd level dilution onwards, the increased absorptions observed (Figure 4-6) of the reaction mixture must be from the increasing residual DPPH. In dilution level 0-2, the decreased absorptions of the mixture were from the decreased sample-DPPH complex as a consequence of less test compound being present. Herein DPPH could interact with a test sample or ascorbic acid to generate a complex with strong absorption, inducing the interference, this explains why the maximum achieved scavenging rate of ascorbic acid is less than 90% without illumination. The preliminary study found that continuous illumination from an incandescent lamp (30 W) was able to reduce absorption of residual DPPH while absorption of the generated complex wasn’t changed. Absorption of residual DPPH was found to be completely eliminated after 168 h continuous illumination (Figure 4-7).
As a result, the absorption difference induced by illumination only comes from the residual DPPH, which can be used to calculate the sample’s genuine antioxidant activity. Therefore, the absorption difference before and after illumination is directly proportional to residual DPPH, and it should be used to calculate actual scavenging activity. As a developed method, the absorbance value of each sample’s well is measured after incubation at 37 °C under dark conditions for 30 min, then the 96-well plate was illuminated by the lamp for 168 h, the absorbance value of each sample’s well was measured again. The absorbance difference between the two readings ($\Delta A_{\text{Sample}}$) was from residual DPPH in each well, the mean absorbance of wells ($A_{\text{DPPH}}$) in the first
reading is regarded as pure DPPH which is the equal to the absorption of initially adding DPPH, so the scavenging activity is processed as below (Equation 4-1).

**Equation 4-1 Scavenging Activity Equation**

$$\% \text{ scavenging} = 100 \times \left(1 - \frac{\Delta A_{\text{Sample}}}{A_{\text{DPPH}}} \right)$$

Following the developed method, interference from background absorption of a generated complex can be so eliminated, the scavenging activity of ascorbic acid is able to achieve 100%, which is higher than the counterparts acquired from the same testing material based on published methods (Table 4-7). This confirms the validity and accuracy of the method developed in the work presented here, and confirms ascorbic acid in the first three dilution levels is in excess. In the developed method (Table 4-7), some errors can be seen, these are attributable to variation of solvent volume during the illumination, and the error will become more significant when higher levels of residual DPPH exists. It also confirms the importance of choosing a less volatile solvent like DMSO.

<table>
<thead>
<tr>
<th>Dilution Level</th>
<th>Scavenging Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Published Method\textsuperscript{34,35}</td>
</tr>
<tr>
<td>0</td>
<td>86.2</td>
</tr>
<tr>
<td>1</td>
<td>86.2</td>
</tr>
<tr>
<td>2</td>
<td>85.8</td>
</tr>
<tr>
<td>3</td>
<td>61.1</td>
</tr>
<tr>
<td>4</td>
<td>30.2</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>6.9</td>
</tr>
<tr>
<td>7</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*The working Volume is comprised of a 160 μL DPPH solution and a 40 μL ascorbic acid solution

### 4.3 Antioxidant Result of Synthetic Grifolin’s Analogues

A selection of commercially available and synthetic analogues of grifolin prepared in chapter three (Figure 4-8 to Figure 4-11) were subjected to antioxidant assessment following the DPPH assay developed in this study.
Figure 4-8 EC50 Values (mM) of Neogrifolin and Its Analogues
Chapter 4: Antioxidant Assay of Grifolin Analogues

Figure 4-9 EC₅₀ Values (mM) of Grifolin and Its Analogues
Chapter 4: Antioxidant Assay of Grifolin Analogues

orsellinic acid

\[
\text{EC}_{50} = (24.13 \pm 7.37) \text{ mM}
\]

3.38

(> 10.44)

3.62

(> 22.96)

3.63

(> 270.00)

Figure 4-10 EC_{50} Values (mM) of Neogriofolic Acid's Analogues

orsellinic acid

\[
\text{EC}_{50} = (24.13 \pm 7.37) \text{ mM}
\]

3.40

(5.30 \pm 0.24)

3.47

(1.97 \pm 0.14)

3.48

(2.49 \pm 0.07)

Figure 4-11 EC_{50} Values (mM) of Grijofic Acid's Analogues
Based on a test sample's concentration and its corresponding radical scavenging activity, OriginPro (2015) software was applied for calculating the EC$_{50}$ value of DPPH radical scavenging. The results are listed in Figure 4-8 to Figure 4-11.

In this work an EC$_{50}$ value of 10 mM, which is two orders of magnitude greater than the EC$_{50}$ value of grifolin, is regarded as the threshold value for antioxidant activity. Thus among analogues of neogrifolin and grifolin (Figure 4-8 and Figure 4-9), orcinol, compounds 3.26, 3.16 are inactive, no matter how many free phenols are present. However, the antioxidant activity is extraordinarily enhanced after a carbon chain is appended to the structures (for example compounds 3.35 and 3.4 in Figure 4-8, and compounds 3.37 and 3.1 in Figure 4-9). After being furnished with a carbon chain (propenyl or prenyl), the position of the carbon chain (for example compounds 3.4 and 3.1), and the number of free phenols (for example compounds 3.4, 3.5, 3.6 and 3.7) does not significantly affect the antioxidant activity. It is worth noting that among the carbon chains, those possessing a prenyl group generally possess more activity than their propenyl counterparts (3.5 and 3.28, or 3.2 and 3.30), but clearly oxidation of the aromatic ring plays a role as the structure containing only an aromatic ring and a prenyl substituent (3.19), does not indicate an antioxidant activity. Manipulation of the prenyl sidechain of 3.7 through reduction of the double bond to return 3.25, or oxidation to deliver 3.21, or both to give 3.24, resulted in loss of activity. The increased length of carbon chain (for example 3.4 and neogrifolin, or 3.1 and grifolin) significantly enhances the activity. The increased activity can be attributable to the increased stability of a structure with the longer carbon chain, discussed in section 3.2.2.1 and section 3.3.

The comparison of antioxidant activity of analogues of neogrigolinic acid and grifolinic acid (Figure 4-10 and Figure 4-11), also indicates that the presence of free phenols (compounds 3.38, 3.62 and 3.63) is not correlated to antioxidant activity, contrary to popularly held beliefs. However, as observed previously the addition of a carbon chain (propenyl or prenyl) is sufficient to introduce appreciable levels of antioxidant activity to the previously inactive structures (for example compounds 3.40 and 3.47 compared to 3.38). The position of the carbon chain (for example compounds 3.47 and 3.45), and the number of free phenols (for example compounds 3.47, 3.58 and 3.59) have an irregular effect on activity.
Chapter 4: Antioxidant Assay of Grifolin Analogues

Among analogues prepared and tested here compound 3.6 (EC\textsubscript{50} 0.81 ± 0.03 mM) is the most active compound, however, it is around 20 times less active than neogrigolin (EC\textsubscript{50} 0.034 ± 0.005 mM) containing three prenyl units. Curiously among the side products isolated and tested in this study (3.36, 3.29, 3.31, 3.33, 3.48, 3.60 and 3.61), the hemiacetal derivative (3.33) possessed the highest activity of any compound being slightly more active (EC\textsubscript{50} 0.68 ± 0.05 mM) than 3.6.

4.4 Conclusions

4.4.1 A Developed DPPH Assay

The DPPH assay developed here is a simple, fast, economic and efficient method to offer a reproducible antioxidant evaluation. Several factors have been considered and carried out in the investigation to optimise the DPPH antioxidant assay. As a result, DMSO was chosen as the solvent, the concentration of testing DPPH solution was set as 100 μM, the concentration of the positive control, ascorbic acid solution, was set as 1 mM, the total volume of testing solution in each well was set as 200 μL, comprised of 160 μL DPPH solution and 40 μL test sample solution (at various dilutions). The DPPH solution should be prepared fresh while ascorbic acid solution can be prepared in advance and stored for up to four days. After incubation and shaking thoroughly at 37 °C in the dark for 30 min, the absorbance of each well of the 96-well plate is determined. The formula (Equation 2-1) can be used to calculate a test sample’s DPPH scavenging activity.

\[
\text{% scavenging} = 100 \times \left[ \frac{\text{A}_{\text{negative control}} - \text{A}_{\text{testing sample}}}{\text{A}_{\text{negative control}} - \text{A}_{\text{positive control}}} \right]
\]

However, it was found that sometimes a test sample interacted with DPPH to generate a complex inducing a strong background absorption at 515 nm. In such cases the continuous illumination provided by an incandescent lamp (30 W) represented a solution. Subtraction of the absorbance value after 168 h illumination from the first measured absorbance value returned the absorbance value difference (\(\Delta A_{\text{Sample}}\)), which is the absorption of residual DPPH in each well. Then the exact DPPH scavenging activity of a test sample can be calculated using Equation 4-1 where the absorption of DPPH (\(A_{\text{DPPH}}\)) in the reading obtained prior to illumination.

\[
\text{% scavenging} = 100 \times \left[ 1 - \frac{\Delta A_{\text{Sample}}}{A_{\text{DPPH}}} \right]
\]  
(Equation 4-1)
4.4.1.2 Result of SAR Research

Based on testing results, the SAR of grifolin and its analogues can be elucidated as below. The essential components for a molecule to show an antioxidant activity requires the aromatic ring to be allylated with a carbon chain in addition to the presence of phenols, no matter if the phenols are alkylated or not. The position of the carbon chain, grifolin versus neogrifolin type substitution does not significantly impact on the antioxidant activity. The enhanced activity of a carbon chain substituted with a propenyl or a prenyl, group can be attributed, in part, to the double bond present (Figure 4-12), which can delocalise a radical produced by a HAT mechanism in the DPPH radical scavenging reaction. Clearly the oxygenation of the ring plays a role as the prenylated aromatic (3.19) is inactive. The increased activity generally observed in going from a propenyl to a prenyl can be attributed to the increased stability of a radical with a prenyl structure (Figure 4-12). The increased activity observed with the increased length of a carbon chain, that is more prenyl units, is attributed to the increased stability of the structure itself.

![Figure 4-12 Stabilities of the Generated Radicals](image)

4.4.1.3 Exploitation of Fungal Biosynthesis

As discussed in chapter three section 3.1, based on the biosynthesis of antioxidant metabolites such as grifolin and neogrifolin, it is practicable to add components, such as an aromatic core like orcinol and/or an aliphatic side chain like farnesyl pyrophosphate (FOPP), to a mushroom culture medium to increase the metabolites yields. In practice, even though FOPP is a commercially available reagent, the application of FOPP isn’t industrially valuable due to its high price. However, the analogue of FOPP with a shorter carbon chain, prenyl pyrophosphate (POPP), is around 10 time cheaper. As discussed in the section 4.4.1.2 a structure containing a prenylated orcinol shows antioxidant activity, thus the addition of POPP instead of FOPP into the mushroom culture medium, could also enhance the mushroom antioxidant activity while decreasing the cost of production.
However, the results determined by our DPPH assay show that the antioxidant activity of the alternative product, compound 3.1 (EC$_{50}$ 1.03 ± 0.09 mM), is about 10 time less than the activity of grifolin (EC$_{50}$ 0.13 ± 0.02 mM). As such, the usage of POPP in an aberrant biosynthesis approach does not appear to represent a solution to commercialise the production of antioxidants in mushrooms.

### 4.5 Experimental

#### 4.5.1 General Procedure of the Developed DPPH Assay

**4.5.1.1 Preparation of the DPPH in DMSO solution**

3.9 mg DPPH (MW= 394.32 g/mol) was dissolved in 100 mL freshly distilled DMSO in a volumetric flask, wrapped with aluminium foil (solution concentration $c = 0.1$ mM).

**4.5.1.2 Preparation of Ascorbic acid (positive control)**

8.81 mg L-ascorbic acid (MW= 176.12 g/mol) was dissolved in 50 mL freshly distilled DMSO in a volumetric flask, wrapped with aluminium foil. This solution (concentration $c = 1$ mM) could be stored for up to four days. The volumetric flask is covered with an atmosphere of nitrogen and sealed after each use.

**4.5.1.3 Preparation of Sample**

Add 200 μL DMSO in a vial to dissolve sample (W mg) and mix on the vortex mixer.

**4.5.1.4 Preparation of Sample Control (Optional)**

This is applicable when a sample is highly coloured, most often in a crude extract sample. Preparation of sample control is the same as the procedure conducted in the preparation of sample (4.5.1.3). In order to execute an accurate assay a sample’s background absorption should be eliminated during the absorbance measurement. This can be done by subtracting the absorption of a sample from the absorption of a sample control.

**4.5.1.5 Absorbance Measurement**

Add 200 μL DMSO solvent to wells labelled as “DMSO Solvent” (Figure 4-13 or Figure 4-14).
Add 40 μL DMSO solvent to wells labelled as “DPPH”.

Add 40 μL DMSO solvent to the wells of rows (B-H) labelled as “Sample”, “Sample Control” (optional) and “Ascorbic Acid”.

Add 80 μL of sample, sample control (optional) and ascorbic acid to the corresponding wells in row A of the plate (Figure 4-13 or Figure 4-14).

Take 40 μL from wells in row A of “Sample”, “Sample Control” (optional) and “Ascorbic Acid”, then add to wells in the next row (B) of the same column, then mix thoroughly.

Repeat the previous step until the last row (H) and discard the 40 μL of solution.
Add 160 μL DPPH in DMSO solution to wells labelled as “Sample”, “Sample Control” (optional), “Ascorbic Acid” and “DPPH” (Figure 4-13 or Figure 4-14).

Cover the plate with aluminium foil and place it in an incubator at 37 °C for 30 min.

Place the plate in the plate reader and start absorbance measurement. The wells labelled as “DMSO Solvent” are used as a blank, their mean absorbance values are applied for a calibration of autozero. The wells labelled as “DPPH” are used as the negative control.

For a sample without an intense colour, its scavenging activity is processed by the formula below (Equation 2-1).

\[
\% \text{ scavenging} = 100 \times \left( \frac{A_{\text{negative control}} - A_{\text{test sample}}}{A_{\text{negative control}} - A_{\text{positive control}}} \right) \quad (\text{Equation 2-1})
\]

For a highly coloured sample, the scavenging activity is processed followed by the formula below (Equation 4-2).

\[
\text{Equation 4-2 Scavenging Activity Equation of Coloured Sample}
\]

\[
\% \text{ scavenging} = 100 \times \left( \frac{A_{\text{negative control}} - (A_{\text{test sample}} - A_{\text{sample control}})}{A_{\text{negative control}} - A_{\text{positive control}}} \right)
\]

4.5.1.6 Epoch Plate Reader Protocol

Open Gen 5, “Create a new protocol” or open an existing protocol.

Select plate type from the pull-down menu from the top right window with proper ticking “Use lid”. Then set up “Read” for single quick reading or “Kinetic” for multiple readings under “Procedure”.

In the middle right window under “Description”, choose a step to add by dragging the step from the list of left window, change the order of steps by dragging up and down. Choose the step and either press “delete” or right click then click “Remove” to delete the chosen step. The chosen step can be edited by either double click or right click then click “Edit”.

Edit “Read” step to set up wavelength and read speed.

Double click “Plate Layout” to lay out blank (BLK), assay control including positive control (PC) and agent control (AC), sample (SPL) and sample control (SPLC) groups.
Double click each group (for instance “Sample”) on the list of left window to set up labels and parameters of each group.

Choose type of dilution or concentration in the coming window. On the right bottom “Auto” menu, click “Increment” with typing a number in its right (which means automatically adds the typed number for the next dilution/concentration) or “Factor” with typing a number in its right (which means automatically multiples the typed number for the next dilution/concentration), then set up the first dilution/concentration on the left window, followed by sequent double click to add the dilution/concentration for the rest.

Choose a group (for instance “SPL1”) and choose a “Replicates” model under “Serial Assignment”, then drag in the corresponding wells in the chosen plate, sequent drags enable to assign the rest sample groups. This function is applicable for all the groups.

Double click “Data Reduction” to run automatic result analysis.

Double click analysis steps to add under “Description” and change the order of them by dragging them up and down.

For instance, “Blank” (only available when blank group is laid out) is to run a background subtraction for all the data by simply choose the data being subtracted.

For instance, “Custom” can run the complicated calculation to achieve the final result.

### 4.5.1.7 EC<sub>50</sub> Calculation

A sample’s concentration at the dilution level 0 (row A) is determined by (Equation 4-3);

**Equation 4-3 a Sample’s Concentration at the Dilution Level 0**

\[
c = \frac{W \text{ (mg)} \times 10^{-3} \times 40 \text{ (µL)} \times 10^{3} \text{ (µL)} \times 10^{-3} \times 40 \text{ (µL)} \times 10^{3} \text{ (µL)}}{MW \text{ (g mol}^{-1}) \times 200 \text{ (µL)} \times 10^{-6} \times 200 \text{ (µL)}} \text{ mM}
\]

\[
c = \frac{1000 \times W}{MW} \text{ mM, W is a sample's mass, MW is the sample molecular weight}
\]

A sample’s concentrations in different dilution levels along with their corresponding scavenging activities are analysed by OriginPro (2015) software, followed by sigmoidal fit using category “growth/sigmoidal”, function “dose/response”. EC<sub>50</sub> value of this
sample will be directly given by the conducted simulation, it is reported as mean ± standard error.

4.5.2 General Procedure of the Developed DPPH Assay with Illumination

This first part of the procedure, prior to illumination, is exactly the same as the developed DPPH assay described above. The first reading is measured after incubation under dark conditions for 30 min at 37 °C. Subsequently the 96-well plate was sealed with Scotch tape to prevent evaporation then placed approximately 15 cm from an incandescent lamp (30 W) and subjected to continuous illumination for 168 h after which the absorbance was measured.

The absorption of the DPPH (A_{DPPH}) is measured prior to illumination, \( \Delta A_{\text{Sample}} \) is sample’s mean absorbance value difference before and after illumination. The scavenging activity is determined by Equation 4-1.

\[
\% \text{ scavenging} = 100 \times \left[ 1 - \frac{\Delta A_{\text{Sample}}}{A_{\text{DPPH}}} \right] \quad \text{(Equation 4-1)}
\]

Subsequently, the calculation of each sample’s EC_{50} value is directly output by OriginPro (2015) software as described in previous section.

4.6 References

Chapter 5: Halogenated Natural Products

5.1 Introduction

The natural halogens are comprised of fluorine (F), chlorine (Cl), bromine (Br), iodine (I) and astatine (At), a halogen is normally symbolised as X. However, due to the instability of astatine with the half-lives of all its isotopes being less than one minute,1 generally halogens are regarded to include only the stable elements fluorine, chlorine, bromine and iodine, and this conception of halogen is applied in this chapter. In 1968 the world renowned organic chemist and Nobel laureate, Sir Robert Robinson stated “... present information suggests that organic compounds containing covalently bound halogens are found only infrequently in living organisms although the species known to elaborate these compounds span the Orders of the animal and plant kingdoms.”2 Scientific community used to deny the existence of halogenated natural products, considering them as artefacts formed during the isolation, fifty years later the discovery of thousands of halogenated natural products with various structures negates the previous conclusion while there still remains a persistent controversy about the existence of naturally occurring organohalogens.3-5 The number of natural organohalogen compounds, such as Tyrian purple,6 structurally solved in 1909 and 3,5-dibromotyrosine which had its two dimensional structure solved in in 1913 (Figure 5-1),7 were two of only a dozen compounds known by 1954,8 and were regarded as chemical freaks and largely ignored by the scientific community.9 However, this number has increased significantly, multiplying hundreds of times in the past decades,8,10-12 with more than 5000 naturally occurring organohalogen compounds existing today.13-16 Gribble, in his frequent commentary on the subject,13,14,16,17 suggests this enormous increase in organohalogen discovery results from;

(1) a worldwide revitalisation of natural products research in the search for new medicinal compounds from all aspects of our environment,

(2) improved isolation, separation and identification techniques,

(3) selective bioassays to identify biologically active extracts, and

(4) an awareness of folk medicine and ethnobotany for guidance to potentially important organisms.
Chapter 5: Halogenated Natural Products

Figure 5-1 Early Discovered Natural Organohalogen Compounds

The continual discovery of natural organohalogen compounds described in the primary literature and through a number of relevant reviews affords abundant evidence to end the debate. 18-38 Contrary to early held scientific opinions, organohalogens are not chemical freaks and without doubt occur often in nature.

Naturally occurring organohalogen compounds have both biotic and abiotic origins. 17 This chapter will discuss biotic organohalogen “natural products”, 39-41 that is those which are produced by a living organism. The abiotic organohalogens, which are generated by biomass combustion, sediments and soil production, volcanic and other geophysical phenomena, mining operations and in interstellar space, are beyond the scope of this chapter, and the reader is referred to the literature. 12,17,42-45

Halogenated natural products often express powerful biological effects and in many cases the halogen atoms play the key role in influencing these biological activities. 12,15,17,18,29,46-49 For instance, the antibiotic vancomycin, which is produced by the soil bacterium, Amycolatopsis orientalis, is the last choice of treatment against multiple-drug resistant Staphylococcus aureus infection. In this molecule two chlorine atoms are essential with the antibacterial effect of the drug diminishing 30% after one chlorine atom (Cl*) was substituted by hydrogen and diminishing 50% after both chlorine atoms were substituted by hydrogens. 50
7-Hydroxylaurene, isolated from the red alga *Laurencia okamurai*, shows stronger lethal toxicity against brine shrimp than its natural bromide analogues, while the synthetic non-halogenated anticancer drug tamoxifen expresses strongly hepatocarcinogenic activity in contrast to its chlorinated analogue toremifene. These examples serve to negate “halogenophobia”, the perception that halogenated compounds tend to be more toxic than their non-halogen substituted counterparts.

The structural diversity of halogenated natural products and their various biological activities are attracting increasing attention from chemists and biologists. So far, a large number of literature examples regarding the structures of halogenated natural products are available, Gribble in particular has contributed a lot to this field, including two comprehensive monographs. To be specific, 2448 naturally occurring organohalogen compounds were listed in his review in 1996, while an additional 2266 compounds were identified based in his update review in 2009. As Gribble wrote, intensive research has been ongoing resulting in halogenated compounds amounting to 15-20%
of newly discovered natural products, representing 100-200 compounds per year.\textsuperscript{17} With so many halogenated natural products reported every year, updating all newly discovered halogenated natural products since Gribble’s monograph (2009) is beyond the scope of this PhD thesis. It is worth noting that natural functions and bioactivities of biotic organohalogen compounds were only discussed quite briefly in Gribble’s two monographs before 2010, and after 2010 the bioactive halogenated natural products described in his other reviews were basically classified by bioactivities rather than structures. As such, instead of a comprehensive description, selected examples discovered from 2010 onwards will be given in this chapter following a structural classification, in addition to their bioactivities, in order to showcase the structural diversity and the bioactive variety of halogenated natural products. The biosynthetic mechanisms of halogenated natural products will be detailed in the end of this chapter.

### 5.2 Structures

Halogenated natural products possess a broad range of structures, distributed across many chemical classes.\textsuperscript{17} As mentioned in the previous section, Gribble has given comprehensive structural information of halogenated natural products in his two monographs,\textsuperscript{12,17} in which he has applied a specific and consistent structural classification system. In order to keep the consistency, the updated structures presented herein are based on the format used in Gribble’s two monographs. It is worth noting that Gribble’s category system may induce arguments since the classified structures are easily overlapped, resulting in confusion, such as in the categories of alkaloids, heterocycles, aromatics and phenols. In addition, this structural classification system produces confusion in some of the naming, therefore some amendments have been applied. The category of ‘polyacetylenes’ in Gribble’s original format is replaced by the term polyynes in the following format to avoid confusion with the classification of polymers of acetylene possessing the general molecular formula \((\text{C}_2\text{H}_2)_n\). The category of ‘simple alkanes’ used by Gribble’s is replaced by the term simple hydrocarbons to properly refer to the halogenated alkanes, alkenes and alkynes. The categories of ‘simple phenols’ and ‘complex phenols’ in Gribble’s monograph are merged into the term phenols described below to avoid unnecessary and undemanding subdivision.

As such, categories of the updated structures include simple hydrocarbons, simple functionalised acyclic organohalogens and simple functionalised cyclic organohalogens,
terpenes, steroids, marine nonterpenes: acetogenins, iridoids, lipids and fatty acids, furanones, amino acids and peptides, alkaloids, heterocycles, polyynes, enediynes, macrolides and polyethers, naphthoquinones, higher quinones and related compounds, aromatics, phenols, glycopeptides, dioxins and dibenzofurans.

5.2.1 Simple Hydrocarbons

It is estimated that around 23,000 tonnes of chloroform (CHCl₃) is produced every year from microalgae, while industry contributes approximately 66,000 tonnes.⁵⁶,⁵⁷ Indeed, marine algae and salt marsh organisms, and some terrestrial organisms are capable of generating enormous amounts of simple haloalkanes. For instance, the essential oil of marine red alga Asparagopsis taxiformis, which is the favourite Hawaiian edible seaweed ‘limu kohu’, is comprised of 80% CHBr₃ by weight.¹¹,⁵⁸ Numerous other simple halohydrocarbons have been confirmed to be of biotic origins, some representative examples are shown in Figure 5-2.¹⁷,²⁶

\[
\text{CH}_3\text{F} \quad \text{CH}_3\text{Cl} \quad \text{CH}_2\text{Cl}_2 \quad \text{CHCl}_3 \quad \text{CCl}_4 \quad \text{CH}_3\text{Br} \quad \text{CH}_2\text{Br}_2 \quad \text{CHBr}_3 \quad \text{CBr}_4 \quad \text{CH}_3\text{I} \quad \text{CH}_2\text{I}_2 \\
\text{CH}_2\text{BrCl} \quad \text{CHBrCl}_2 \quad \text{CHBr}_2\text{Cl} \quad \text{CBrCl}_3 \quad \text{CBr}_2\text{Cl}_2 \quad \text{CBr}_3\text{Cl} \quad \text{CHI}_2\text{Br} \quad \text{CH}_{12}\text{I}_2 \quad \text{CHBrCl} \\
\text{CH}_3\text{CH}_2\text{Br} \quad \text{BrCH}_2\text{CH}_2\text{Br} \quad \text{BrCH}_2\text{CH}_2\text{I} \quad \text{ClCHCHCl} \quad \text{ClCH}_2\text{Cl}_2 \quad \text{CCl}_3\text{Cl}_2 \quad \text{CBr}_2\text{Cl}_2 \quad \text{CF}_3\text{CF}_2\text{CF}_2\text{H} \\
n\text{C}_{12}\text{H}_{25}\text{Cl} \quad n\text{C}_{18}\text{H}_{37}\text{Cl} \quad n\text{C}_{24}\text{H}_{49}\text{Cl} \quad n\text{C}_{29}\text{H}_{59}\text{Cl} \quad \text{ClCHCHBr} \quad \text{BrCHC}_2\text{Cl} \quad \text{BrCHCHBr} \quad \text{BrCHCB}_2
\]

Figure 5-2 Representative Examples of Biotic Halohydrocarbons

The simple hydrocarbons discovered from marine organisms are mainly substituted by chlorine, rather than fluorine, bromine or iodine with the exception that the predominant volatile metabolites of brown and green algae are bromoform.⁵⁹

5.2.2 Simple Functionalised Acyclic Organohalogens

The cyanobacterium Leptolyngbya crosbyana⁴ which is found on Hawaiian coral was reported to produce three new simple functionalised acyclic organohalogens, the

---

¹ Leptolyngbya crossbyana is used in this reference, it is the misspelling of Leptolyngbya crosbyana.
Two novel biologically active lipids, pitinoic acid B and C (5.4-5.5), were isolated from an unidentified Guamanian cyanobacterium. Six novel iodinated acetylenic acid metabolites (5.6-5.9) were isolated from the South Korean marine sponges, the former two metabolites (5.6-5.7) were produced by *Suberites mammilaris* and the latter two metabolites (5.8-5.9) were produced by *Suberites japonicus*.

Despite honaucin A (5.1) incorporating a lactone, it is classified as acyclic based on Gribble’s classification due to its skeleton.
5.2.3 Simple Functionalised Cyclic Organohalogens

The aforementioned red alga ‘limu kohu’ *(Asparagopsis taxiformis)* was reported to generate the first examples of natural 2,3-dibromocyclopentenone derivatives, two new highly brominated cyclopentenones, mahorone and 5-bromomahorone *(5.10-5.11)*.63

![Mahorone (5.10) and 5-Bromomahorone (5.11)](image)

Polyporapyranone D *(5.12)* has been isolated from a seagrass *(Thalassia hemprichii)*-derived fungi of the order Polyporales PSU-ES44,64 a new azaphilone, chaetomugilin S *(5.13)* has been isolated from a marine fish *(Mugil cephalus)* derived fungi Chaetomium globosum.65

![Polyporapyranone D (5.12) and Chaetomugilin S (5.13)](image)

A chloroazaphilone, (+)-isochromophilone X *(5.14)*, was isolated together with (+)-isochromophilone XI and (+)-isochromophilone XII from fungus *Diaporthe* sp. in 2012, which was itself isolated from the Chinese mangrove *Rhizophora stylosa*.66 In 2013 the fungus *Bartalinia robillardoides* strain LF550, which was isolated from the Mediterranean sponge *Tethya aurantium*, was reported to produce two novel chloroazaphilones which the authors also named isochromophilone X and isochromophilone XI *(5.15)*.67 Given these numbers were already assigned, a renaming of the second occurrence of isochromophilone X and isochromophilone XI to isochromophilone XIII and isochromophilone XIV *(5.15)* is required.
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5.2.4 Terpenes

Two new polyhalogenated monoterpenes, (-)-plocamenone and a reportedly unstable isoplocamenone (5.16-5.17) were isolated from marine alga *Plocamium angustum*. Curiously the enantiomer of the reported structure of (-)-plocamenone was synthesised in 2015 and also reported to possess a negative specific rotation, raising doubt as to the stereochemistry reported. The synthesis paper also noted a light mediated interconversion between (-)-plocamenone and (-)-isoplocamenone. A polychlorinated monoterpane (5.18) was isolated from a red alga *Plocamium cartilagineum* collected from eastern coast of South Africa.

Three monobrominated sesquiterpenes (5.19-5.21) have been isolated from the red alga *Laurencia okamurai* Yamada collected from the coast of Nanji Island, China. A tribrominated sesquiterpene tristichol A (5.22) was isolated from a red alga *Laurencia tristicha* collected from the coast of Hsiao Liuchiu Island on Taiwan southwestern coast.
A new briarane type diterpenoid fragilisinin J (5.23) was isolated from the South China Sea gorgonian Junceella fragilis.\textsuperscript{73} A new briarane type diterpenoid gemmacolide O (5.24) was isolated from the South China Sea gorgonian Dichotella gemmacea,\textsuperscript{74} while in the later studies further new briarane type diterpenoid dichotellide H (5.25),\textsuperscript{75} as well as gemmacolide T-Y (5.26-5.31) were revealed.\textsuperscript{76} It is worth noting that the name of gemmacolide U refers to the structure of 5.27,\textsuperscript{76,77} while it also refers to a different diterpenoid, 12-\textit{epi}-fragilide G.\textsuperscript{78,79} Here gemmacolide U only refers to the structure of 5.27.
gemmacolide O \((5.24)\)

dichotellide H \((5.25)\)

gemmacolide T \((5.26)\)

gemmacolide U \((5.27)\)

12-epi-fragilide G

gemmacolide V \((5.28)\)

gemmacolide W \((5.29)\)

gemmacolide X \((5.30)\)

gemmacolide Y \((5.31)\)
Eight new diterpenoids, kalihinol M-T (5.32-5.39) were isolated from the South China Sea sponge *Acanthella cavernosa*.\(^80\)

\[\text{Figure: Kalihinol M (5.32)}\]
\[\text{Figure: Kalihinol N (5.33)}\]
\[\text{Figure: Kalihinol O (5.34)}\]
\[\text{Figure: Kalihinol P (5.35)}\]
\[\text{Figure: Kalihinol Q (5.36)}\]
\[\text{Figure: Kalihinol R (5.37)}\]
\[\text{Figure: Kalihinol S (5.38)}\]
\[\text{Figure: Kalihinol T (5.39)}\]

### 5.2.5 Steroids

A new chlorinated steroid, 4-chloro-\(\beta\)-sitosterone (5.40) was obtained from the tuber of *Bletilla striata* used in Traditional Chinese Medicine.\(^81\)

\[\text{Figure: 4-chloro-\(\beta\)-sitosterone (5.40)}\]
5.2.6 Marine Nonterpenes: Acetogenins

The red alga Laurencia sp., collected in Omaezaki, Japan, was reported to yield three brominated C₁₅-acetogenins, omaezallene and two unnamed analogues (5.41-5.43).⁸²

![Omaezallene](image)

5.2.7 Iridoids

Two chlorinated iridoid glucosides, longifolioside A and B (5.44-5.45) were isolated from the plant Veronica longifolia.⁸³

![Longifolioside](image)

5.2.8 Lipids and Fatty Acids

The marine sponge Melophlus sp., collected along the coast of Cicia Island, Fiji, was reported to produce a new chlorinated tetramic acid glycoside aurantoside K (5.46).⁸⁴
Two novel polyketide lactones coibacin C-D \( (\text{5.47-5.48}) \) were isolated from a Panamanian marine cyanobacterium, \textit{Oscillatoria} sp.,\textsuperscript{85} another new chlorinated polyketide lactone PM050489 \( (\text{5.49}) \) was isolated from the Madagascan sponge \textit{Lithoplocamia lithistoides}.\textsuperscript{86}

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{Cl} \\
\text{MeO} & \quad \text{O} & \quad \text{N} & \quad \text{O} & \quad \text{CONH}_2 \\
\text{O} & \quad \text{O} & \quad \text{H} & \quad \text{N} & \quad \text{O} & \quad \text{Cl} & \quad \text{CONH}_2 \\
\text{PM050489 (5.49)}
\end{align*}
\]

A Papua New Guinea collection of the marine cyanobacterium \textit{Lyngbya sordida} yielded a new compound malyngamide 2 \( (\text{5.50}) \),\textsuperscript{87} the marine cyanobacterium \textit{Lyngbya majuscula} collected from Cocos Lagoon, Guam yielded another new compound malyngamide 3 \( (\text{5.51}) \).\textsuperscript{88}

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{Cl} \\
\text{O} & \quad \text{O} & \quad \text{Me} & \quad \text{Cl} & \quad \text{CONH}_2 \\
\text{O} & \quad \text{O} & \quad \text{HO} & \quad \text{OH} & \quad \text{HO} & \quad \text{OH} & \quad \text{Me} & \quad \text{CONH}_2 \\
\text{malyngamide 2 (5.50)}
\end{align*}
\]

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{Me} & \quad \text{Cl} \\
\text{O} & \quad \text{O} & \quad \text{Me} & \quad \text{Cl} & \quad \text{CONH}_2 \\
\text{O} & \quad \text{O} & \quad \text{HO} & \quad \text{OH} & \quad \text{HO} & \quad \text{OH} & \quad \text{Me} & \quad \text{CONH}_2 \\
\text{malyngamide 3 (5.51)}
\end{align*}
\]

\subsection*{5.2.9 Furanones}

Two new brominated furanones, 3’-bromorubrolide E and 3’,3’’-dibromorubrolide E \( (\text{5.52-5.53}) \) were isolated from a South African tunicate \textit{Synoicum globosum} Parker-Nance sp. nov. (Ascidiae, Aplousobranchia).\textsuperscript{89} Six new brominated furanones, cadiolide C-F, and rubrolide P-Q \( (\text{5.54-5.59}) \), were isolated from the Korean ascidian \textit{Pseudodistoma antinboja},\textsuperscript{90} another three new brominated furanones, cadiolide G-I
(5.60-5.62) were reported to be generated by the ascidian *Synoicum* sp. collected off the Korean coast of Chuja-do.\textsuperscript{91}

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {3'-bromorubrolide E (5.52)}; \hspace{2cm} \node (B) at (3,0) {3',3''-dibromorubrolide E (5.53)};
\draw (A) -- (B);
\end{tikzpicture}
\end{center}

\begin{center}
\begin{tikzpicture}
\node (C) at (0,0) {cadiolide C (5.54) \text{ } R_1=R_2=R_3=H}; \hspace{2cm} \node (D) at (3,0) {rubrolide P (5.58) \text{ } R_1=Br};
\draw (C) -- (D);
\end{tikzpicture}
\end{center}

\begin{center}
\begin{tikzpicture}
\node (E) at (0,0) {cadiolide D (5.55) \text{ } R_1=Br, R_2=R_3=H}; \hspace{2cm} \node (F) at (3,0) {rubrolide Q (5.59) \text{ } R_1=H};
\draw (E) -- (F);
\end{tikzpicture}
\end{center}

\begin{center}
\begin{tikzpicture}
\node (G) at (0,0) {cadiolide G (5.60) \text{ } R_1=R_2=R_3=H, R_2=Br}; \hspace{2cm} \node (H) at (3,0) {cadiolide I (5.62) \text{ } R_1=Br, R_2=R_3=H, R_3=Me};
\draw (G) -- (H);
\end{tikzpicture}
\end{center}

\begin{center}
\begin{tikzpicture}
\node (I) at (0,0) {cadiolide H (5.61) \text{ } R_1=Br, R_2=R_3=Me}; \hspace{2cm} \node (J) at (3,0) {cadiolide C (5.54) \text{ } R_1=R_2=R_3=H}
\draw (I) -- (J);
\end{tikzpicture}
\end{center}

\section*{5.2.10 Amino Acids and Peptides}

A new brominated amino acid herdmamine D (5.63) was isolated from the Korean marine ascidian *Herdmania momus*.\textsuperscript{92}
The cyanobacterium *Anabaena cylindrica* strain Bio33 was isolated from a water sample collected from the Rugen Island in the Baltic Sea, its laboratory cultivation yielded two chlorinated lipopeptides, balticidin A and B (5.64-5.65) with partial stereochemistry being assigned.93,94
5.2.11 Alkaloids

A new bisindole derived alkaloid indimicin B (5.66) was isolated from a deep-sea derived Streptomyces sp. SCSIO 03032.\(^{95}\)

![indimicin B (5.66)]

A new brominated pyrrole alkaloid aspidostomide E (5.67) was isolated from the Patagonian bryozoan Aspidostoma giganteum.\(^{96}\) A new chlorinated alkaloid chloromangiferamide (5.68) was isolated from the Sri Lanka endemic plant Mangifera zeylanica Hook.f. (Anacardiaceae), which is traditionally used in Ayurvedic medicine for the treatment of various diseases.\(^{97}\)

![aspidostomide E (5.67)]

![chloromangiferamide (5.68)]

* unknown configuration

Two new dibrominated alkaloids pulmonarin A and B (5.69-5.70) were isolated from the sub-Arctic ascidian Synoicum pulmonaria collected off the Norwegian coast.\(^{98}\)

![pulmonarin A (5.69)]

![pulmonarin B (5.70)]

Two structurally unique dimeric brominated pyrrole alkaloids, agelamadin A and B (5.71-5.72) were isolated from a marine sponge Agelas sp.,\(^{99}\) another three brominated pyrrole alkaloids, agelamadin C-E (5.73-5.75), were isolated from the same sponge.\(^{100}\)
5.2.12 Heterocycles

The Mediterranean gorgonian *Paramuricea clavata* was reported to produce a new monobrominated indole (5.76),\textsuperscript{101} the marine bryozoan cheilostomatous species *Chartella (Terminoflustra) membranaceatunruncata* (Smitt, 1868) was reported to yield a new tribrominated indole (5.77).\textsuperscript{102}

A Fijian red alga *Callophycus* sp. produced five new brominated diterpene-benzoates bromophycoic acid A-E (5.78-5.82).\textsuperscript{103}
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5.2.13 Polynyes

The Korean marine sponge *Placospongia* sp. generated an iodinated diacetylene placotylene A (5.83).\(^{104}\)

![Placotylene A](image)

The Red Sea sponge, *Haliclona* sp. yielded two new brominated acetylenes, a diacetylenic acid and its methyl ester (5.84-5.85),\(^{105}\) while the Japanese marine sponge *Theonella swinhoei*, collected off the coast of Tanegashima, Kagoshima Prefecture, produced a new brominated C\(_{17}\)-diacetylenic acid, bromotheoynic acid (5.86).\(^{106}\)
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5.2.14 Enediynes

A nitrogenous halogenated long-chain enediyne, mollenyne A (5.87) was isolated from the sponge *Spirastrella mollis* collected off the coast of Bahamas.\(^{107}\)

5.2.15 Macrolides and Polyethers

The marine-derived fungus *Humicola fuscoatra* (UCSC strain no. 108111A) produced a new resorcylic acid lactone radicicol B (5.88),\(^{108}\) a marine sponge of the Petrosiidae family collected off the coast of Pemba (Tanzania) furnished two new polyketide macrolides phormidolide B and C (5.89-5.90), with partial stereochemistry being assigned.\(^{109}\)
Three new brominated polyethers, 15-dehydroxythyrsenol A, prethyrsenol A and 13-hydroxyprethyrsenol A (5.91-5.93) have been isolated from the red alga Laurencia viridis,\textsuperscript{110} another two brominated polyethers, iubol and 22-hydroxy-15(28)-dehydrovenustatriol (5.94-5.95) were isolated from the same red alga,\textsuperscript{111} as well as two new brominated oxasqualenoids, saiyacenol A and B (5.96-5.97).\textsuperscript{112}
5.2.16 Naphthoquinones, Higher Quinones and Related Compounds

Four new dihalogenated napyradiomycins (5.98-5.101) were isolated from the marine-derived actinomycete strain CNQ525, which was collected from California.\textsuperscript{113} Three new napyradiomycins (5.102-5.104) were isolated from a marine-derived actinomycete Streptomyces strain SCSIO 10428.\textsuperscript{114} A new chlorinated metabolite guignardin E (5.105)
was isolated from the endophytic fungus *Guignardia* sp. KcF8 derived from a mangrove *Kandelia candel*.[115]

![Chemical structures](attachment:image.png)

A cultured marine-derived actinomycete *Streptomyces* strain CNQ-329, isolated from marine sediments collected in California, was reported to produce the novel chlorinated compound napyradiomycin A (5.106).[116]
5.2.17 Aromatics

Two new paracyclophanes carbamidocyclophane F and G (5.107-5.108) were isolated from a cultured freshwater cyanobacterium Nostoc sp. (UIC 10274), collected near Des Plaines, Illinois.\textsuperscript{117}

5.2.18 Phenols

Three new brominated polyphenols (5.109-5.111) were isolated from a marine red alga Symphyocladia latiuscula, which was collected from the coast of Qingdao, China.\textsuperscript{118}

The southern Australian sponge Pseudoceratina sp. yielded two new bromotyrosine-derived metabolites, (-)-purealin B and purealin C (5.112-5.113).\textsuperscript{119}
Fourteen new chlorinated depsidone-based compounds spiromastixone B-O (5.114-5.127) were isolated from the deep-sea fungus *Spiromastix* sp. 120
5.2.19 Glycopeptides

A new bicyclic glycopeptide theonellamide G (5.128) was isolated from the Red Sea sponge Theonella swinhoei, with partial stereochemistry being assigned.\textsuperscript{121}

\[
\text{theonellamide G (5.128)}
\]

5.2.20 Dioxins and Dibenzofurans

No bioactive halogenated dioxins with a biotic source have been discovered since 2010 based on the literature search. A new chlorinated dibenzofuran Pf-1 (5.129) was isolated from fruiting bodies of the cellular slime mould Polysphondylium filamentosum.\textsuperscript{122}

\[
Pf-1 (5.129)
\]

Two polybrominated dibenzofurans, boletopsin 13 and 14 (5.130-5.131) were isolated from a terrestrial macrofungi Boletopsis sp., a traditionally used mushroom as a food source and as a treatment for gastrointestinal disorders by the Kiovi Tribe of the Lufa district in the Eastern Highlands province of Papua New Guinea.\textsuperscript{123}
5.3 Bioactivities

The large variety and complexity of structures of natural halogenated products has resulted in them displaying a variety of bioactivities. Halogenated natural products have demonstrated antibacterial activity, antifouling activity, antifungal activity, antiparasitic activity, antiviral activity, antitumour activity, antioxidant activity, anti-inflammatory activity, enzymatic and molecular regulation activity.13-18,23,26,29,124

5.3.1 Antibacterial Activity

The simple functionalised acyclic organohalogens the honaucin A-C (5.1-5.3) show activity against Gram-negative Escherichia coli (JB525) and the marine derived bioluminescent bacterium Vibrio harveyi (BB120).60 Pitinoic acid B (5.4) and C (5.5) are active against the Gram-negative Pseudomonas aeruginosa.61

The simple functionalised cyclic organohalogens, mahorone (5.10) and 5-bromomahorone (5.11) exhibit broad spectrum activity being active against the Gram-positive Staphylococcus aureus and the Gram-negative V. fisheri, and the Gram-negative Acinetobacter baumannii and E. coli.63 Isochromophilone XIV (5.15) also shows broad spectrum activity being inhibitory toward B. subtilis and S. lentus.67

The monoterpene (-)-plocamenone (5.16) displays activity against the Gram-positive Bacillus subtilis.68 The sesquiterpene tristichol A (5.22) display activity against the Gram-negative Enterobacter aerogenes, Serratia marcescens, and Yersinia enterocolitica.72

The diterpene gemmacolide O (5.24) shows activity against E. coli and is the most antibacterial active compound among the gemmacolides.74 The diterpene gemmacolide T-Y (5.26-5.31) show activity against E. coli.

The furanones 3'-bromorubrolide E and 3',3''-dibromorubrolide E (5.52-5.53) show activity against methicillin-resistant S. aureus (MRSA) and S. epidermidis.89 The furanones cadiolide C-I and rubrolide P-Q (5.54-5.62) show broad spectrum antibacterial activity, with 5.54-5.59 possessing activity against S. aureus, B. subtilis, S. epidermidis and Kocuria rhizophila while 5.60-5.62 have activity against B. subtilis, K. rhizophila and Salmonella enterica.90,91

The alkaloids agelamadin A-E (5.71-5.75) are active against B. subtilis and the Gram-positive to Gram-variable Micrococcus luteus.99,100
The heterocyclic compound 5.76 shows strong antibacterial activity against *Pseudoalteromonas* sp. D41 and TC8, and *Paracoccus* sp. 4M6. The heterocyclic compounds bromophycoic acid A-E (5.78-5.82) all exhibit activity against MRSA, with bromophycoic acid A (5.78) being more active than vancomycin. In addition, bromophycoic acid A (5.78) and E (5.82) are active against vancomycin-resistant *Enterococcus faecium* (VRE).

The naphthoquinones 5.102-5.103 are active against *S. aureus* (ATCC 29213), *B. thuringiensis* (SCS10 BT01) and *B. subtilis* (SCS10 BS01) while 5.104 is only active against the latter two bacteria. Napyradiomycin A (5.106) is strongly active against MRSA.

The phenol purealin C (5.113) shows significant activity against *S. aureus* and *B. subtilis*, while (-)-purealin B (5.112) only shows activity against *B. subtilis*. Spiromastixone B-O (5.114-5.127) display activity against *S. aureus* (ATCC 29213), *B. thuringiensis* (SCS10 BT01) and *B. subtilis* (SCS10 BT01).

The dibenzofurans boletopsin 13 and 14 (5.130-5.131) show broad spectrum activity being active against the Gram-positive *S. epidermidis* and the Gram-negative *E. coli*.

### 5.3.2 Antifouling Activity

The diterpenoids fragilisinin J (5.23) and dichotellide H (5.25) exhibit antifouling activity inhibiting larval settlement of the barnacle *Balanus amphitrite*. Kalihinol O-T (5.34-5.39) also display antifouling activity against the same barnacle larvae.

The acetogenin omaezallene (5.41) shows the strongest antifouling activity against the barnacle *Balanus amphitrite* while the analogues 5.42-5.43 exhibit weak antifouling activity.

### 5.3.3 Antifungal Activity

The simple functionalised cyclic organohalogen isochromophilone XIV (5.15) shows activity against *Trichophyton rubrum*. The monoterpene (-)-plocamenone (5.16) shows activity against *Candida albicans* and *Cladosporium resinae*. The sesquiterpenes laurepoxyene (5.19) and 3β-hydroperoxyaplysin (5.20) show activity against *C. glabrata*, while 3β-hydroperoxyaplysin (5.20) and 3α-hydroperoxy-3-epiaplysin (5.21) display activity...
against *Cryptococcus neoformans*.71 The diterpenes gemmacolide T-Y (5.26-5.31) display activity against *Microbotryum violaceum* and *Septoria tritici*.76

The lipid and fatty acid classified aurantoside K (5.46) shows broad antifungal activity against wild type *C. albicans*, amphotericin-resistant *C. albicans*, *C. neoformans*, *Aspergillus niger*, *Penicillium sp.*, *Rhizopus sporangia* and *Sordaria sp.*84

The peptides balticidin A-B (5.64-5.65) show antifungal activity against *C. maltosa*, *C. albicans*, *C. krusei*, *A. fumigatus*, *Microsporum gypseum*, *M. canis*, and *Mucor sp.*93,94

The alkaloids agelamadin A-E (5.71-5.75) are active against *C. neoformans*.99,100

The heterocyclic compound 5.77 displays activity against *C. albicans* and *Saccharomyces cerevisiae*.102

The phenols 5.109-5.111 exhibit activity against *C. albicans*.118

The glycopeptide theonellamide G (5.128) possesses activity against both wild type and amphotericin B-resistant strains of *C. albicans*.121

### 5.3.4 Antiparasitic Activity

The lipid and fatty acid classified compounds coibacin C (5.47) and D (5.48) are active against the parasite *Leishmania donovani* responsible for the debilitating disease leishmaniasis.85

### 5.3.5 Antiviral Activity

The macrolide radicicol B (5.88) shows activity against human immunodeficiency virus type-1 (HIV-1).108

### 5.3.6 Antitumour Activity

The simple functionalised cyclic organohalogens polyporapyranone D (5.12), chaetomugilin S (5.13) and (+)-isochromophilone X (5.14) demonstrate antitumor activity. Compound 5.12 exhibits cytotoxic activity against African green monkey kidney fibroblasts (Vero) cell line,64 5.13 inhibits P388, HL-60, L1210 and KB cell lines,65 while 5.14 shows activity against MCF-7 (breast), SGC-7901 (gastric), SW1116 (colorectal), A549 (lung) and A375 (melanoma) human cancer cell lines.66

The monoterpenes (-)-plocamenone and isoplocamenone (5.16-5.17) exhibit cytotoxicity against P388 mouse leukemia cells.68 The monoterpene 5.18 exhibits
activity against the human lung cancer (NCI-H460) and the mouse neuro-2a neuroblastoma cell lines. The sesquiterpene 3β-hydroperoxyaplysin (5.20) shows cytotoxicity against the A-549 (lung) cell line. The diterpenoids gemmacolide T-Y (5.26-5.31) show activity against A549 and MG63 human cancer cell lines, with 5.28 and 5.31 possessing stronger cytotoxicity than other analogues. The diterpenoids kalihinol O-R (5.34-5.37) show activity against HCT-116 human colon cancer cell. The steroid 4-chloro-β-sitosterone (5.40) shows weak toxicity to 293A cells of human.

The lipid and fatty acid classified compound PM050489 (5.49) is a tubulin-binder and inhibits HT-29 (colon), A-549 (lung) and MDA-MB-231 (breast) human tumor cells. Malyngamide 2 (5.50) is active against H-460 (lung) cells, malyngamide 3 (5.51) is weakly inhibitory to MCF-7 (breast) and HT-29 (colon) cells.

The alkaloid Indimicin B (5.66) displays cytotoxic activity against human breast cancer MCF-7 cells, aspidostomide E (5.67) is active against the human renal cancer 786-O cells, while chloromangiferamide (5.68) shows cytotoxicity against the human breast cancer MDA-MB-231 cell line.

The polyyynes 5.84-5.85 show activity against MCF-7 human breast cancer cells. Bromotheoynic acid (5.86) inhibits the proliferation of U937, HL60, A549, H1299, HEK293 human cell lines.

The enediyne mollenyne A (5.87) is highly active against HCT-116 human colon cancer cells.

The macrolides phormidolide B and C (5.89-5.90) show significant inhibition to the growth of A-549(lung), HT-29 (colon) and MDA-MB-231 (breast) cell lines. Polyether compounds 5.91-5.97 all exhibit antitumor activity against MM144, HeLa, CAD-ES-1 human cancer cell lines with the exception that compound 5.95 only inhibits HeLa cells.

The naphthoquinone compounds napyradiomycins CNQ525.510B (5.98), CNQ525.538 (5.99), CNQ525.554 (5.100) and CNQ525.600 (5.101) can induce apoptosis in the human colon cancer cell line HCT-116. Guignardin E (5.105) shows cytotoxicity towards K562, A549, Huh-7, H1975, MCF-7, U937, BGC823, HL60, HeLa, MOLT-4 human cancer cells.
The aromatic compounds carbamidocyclophane F (5.107) and G (5.108) show inhibition of the growth of MDA-MB-435 (breast) and HT-29 (colon) human cancer cell lines.\textsuperscript{117}

The dibenzofuran Pf-1 (5.129) exhibits cytotoxicity against human K562 and HeLa cell lines, and the mouse embryo fibroblast 3T3-L1 cell line.\textsuperscript{122}

5.3.7 Antioxidant Activity
The iridoids longifolioside A and B (5.44-5.45) show antioxidant activity against nitric oxide, superoxide and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.\textsuperscript{83}

5.3.8 Anti-inflammatory Activity
The simple functionalised acyclic organohalogens 5.6 and 5.7 display strong inhibition of nitric oxide production from RAW 264.7 cells, while 5.8 and 5.9 show stronger inhibition of nitric oxide production from BV2 cells than the former compounds. All four compounds attenuate PGE\textsubscript{2} (prostaglandin E2) production from RAW 264.7 and BV2 cells.\textsuperscript{62}

The sesquiterpene tristichol A (5.22) inhibits N-formylmethionyl-leucyl-phenylalanine/cytochalasin B (fMLP/CB)-induced superoxide anion generation.\textsuperscript{72}

The amino acid herdmanine D (5.63) is able to suppress the nitric oxide production of RAW 264.7 cells induced by lipopolysaccharide (LPS), resulting from inhibiting the mRNA expression of the inducible nitric oxide synthase (i-NOS).\textsuperscript{92}

5.3.9 Enzymatic and Molecular Regulation Activity
The simple functionalised cyclic organohalogen isochromophilone XIV (5.15) shows phosphodiesterase 4 (PDE4) inhibition activity.\textsuperscript{67}

The alkaloids pulmonarin A and B (5.69-5.70) both show reversible, non-competitive acetylcholinesterase inhibition activity.\textsuperscript{98}

The polyyne placotylene A (5.83) suppresses receptor activator of nuclear factor κB ligand (RANKL) induced TRAP-positive osteoclast differentiation.\textsuperscript{104} Compounds 5.84-5.85 are able to stimulate differentiation of the ST-13 preadipocytes to adipocytes.\textsuperscript{126} Bromotheoynic acid (5.86) inhibits the maturation of starfish (Asterina pectinifera) oocytes.\textsuperscript{106}

The dibenzofuran Pf-1 (5.129) suppresses the activity of β-galactosidase.\textsuperscript{122}
5.4 Biosynthetic Mechanisms

Since the reality of halogenated natural products is undoubted, their remarkable chemistry and bioactivities in addition to their biosynthetic origins have attracted the attention of scientists. Biotic halogenation can be divided into enzymatic and nonenzymatic pathways, although enzymatic halogenation is believed to be the most common route. Enzymatic halogenation can be grouped into three classes: (1) hypohalite (XO\(^-\)) origin, which is chemically equivalent to a halonium cation (X\(^+\)), (2) halogen radical (X\(^\cdot\)) origin and (3) halide anion (X\(^-\)) origin.

The abundances of halogens vary enormously in nature, for instance, the composition of fluorine (F) : chlorine (Cl) : bromine (Br) : iodine (I) in Earth’s primitive mantle is estimated to be 18:1.4:0.0036:0.001.\(^{127}\) F and Cl are by far the most abundant halogens in nature, and are generally regarded as proxies for the other halogens,\(^{1,127-131}\) Despite fluorine’s high natural abundance, the naturally occurring organofluorides are not common. Since fluoride (F\(^-\)) readily forms hydrogen bonds in water it is difficult to desolvate and generate active F\(^-\) ion in aqueous solution. Fluorine radical (F\(^\cdot\)) is also rare due to its electronegativity. In addition, because of the extremely high redox potential of fluoride (\(E^o[F_2/F^-]: 2.87\) V), which is much higher than that of peroxide (\(E^o[H_2O_2/H_2O]: 1.78\) V), fluoride ion (F\(^-\)) is impossible to be oxidised to the fluoride cation (F\(^+\)) for electrophilic fluorination in nature. These reasons may explain the scarcity of fluorinated natural products with around 30 compounds, even though fluoride is widespread in nature.\(^{32}\)

Due to the high relative natural abundance of chloride and bromide and low natural abundance of iodide, molar ratios of Cl\(^-\) : Br\(^-\) : I\(^-\) is around 500,000 : 1,000 : 1 in ocean water and 100:5:1 in terrestrial soil respectively,\(^{34}\) naturally occurring organochlorine and organobromine compounds represent more than 95% of all organohalogens.\(^{32}\) However, since the redox potentials of Br\(^-\) and I\(^-\) are lower than that of Cl\(^-\) (\(E^o[HOCl/Cl^-]: 1.48\) V versus \(E^o[HOBr/Br^-]: 1.33\) V and \(E^o[HOI/I^-]: 0.99\) V), indicating they are more readily oxidised to Br\(^+\) and I\(^+\) than Cl\(^-\) is to Cl\(^+\), goes some way to explain why organobromides and organoiodides are statistically overrepresented. In the enzymatic oxidative halogenation pathway, an enzyme capable of oxidising chloride can also oxidise bromide and iodide and catalyse the corresponding bromination and iodination reactions. Similarly, an enzyme that can oxidise bromide can also catalyse an iodination
reaction however, an enzyme capable of oxidising iodide is selective and can only catalyse an iodination reaction. In the following discussion of biosynthetic halogenation mechanisms, only chlorination will be described if bromination and iodination follow on the same principle.

5.4.1 Enzymatic Pathways

Enzymatic halogenation is the most common route for halogen incorporation occurring in living organisms and has been being extensively investigated, though a lot of details remain unknown. Structurally and functionally versatile enzymes afford a complex and sophisticated approach to modify metabolites by halogenation, playing a significant role in establishing unique bioactivity. As mentioned above enzymatic pathways are classified into these three groups according to the origins of the halogen and each of these is described below.

5.4.1.1 Halogenation by Halonium Cation, X+

The most common instances of enzymatic halogenation occur by the oxidation of abundant X⁻ to generate the reactive X⁺ species, followed by reaction with electron-rich substrates. Depending on the different oxidants available, this kind of halogenation can be catalysed by two broad classes of enzymes: (i) haloperoxidases (HPO) which are promiscuous utilising peroxide (H₂O₂) and (ii) halogenases which are highly substrate-specific and regioselective utilising molecular oxygen.

5.4.1.1.1 Haloperoxidases (HPOs)

HPOs are widely distributed among terrestrial and marine organisms, especially among fungi of the class hyphomycetes which are common as saprophytes and plant pathogens and marine algae. HPOs are classified as either haem- or vanadate-dependent HPOs in terms of the enzyme metal centres.

For a haem-dependent HPO, the enzyme has an iron (Fe) metal centre. The Fe atom is redox active and changes its oxidation state during the catalytic cycle. Chloroperoxidase (CPO) isolated from fungus Caldariomyces fumago, which produces the chlorinated natural product caldariomycin, is a representative example of a haem-dependent HPO. This enzyme binds an Fe(III) haem-water (H₂O) complex where the catalytic cycle starts and is considered the resting state. Firstly, hydrogen peroxide displaces water and is subsequently deprotonated with assistance from a glutamic acid residue (Glu 183) to
give compound 0 (Scheme 5-1). After that the coordinated and negative charged oxygen of the peroxide is bound to Fe(III), then the peroxide is protonated by Glu 183, then leaves as water after an Fe(IV)-oxo complex, compound I, is formed (Scheme 5-1). The generated Fe(IV)-oxo complex subsequently reacts with chloride (Cl-) by oxidising Cl- to generate Fe(III)-hypohalite (ClO-) complex. Controversially, the generated ClO- is either a ligand coordinated to this Fe(III) haem complex or released to form a free hypochlorous acid (HOCl) which diffuses out of the enzyme through channels. Both of these two hypotheses are supported by corresponding experimental observations, indicating more studies need to be done to address the controversy.\textsuperscript{19,32,36} Finally, either coordinated ClO- or free HOCl reacts with electron-rich organic substrate to yield biotic organochlorine compounds. At the same time the catalytic cycle ends up with Fe(III) haem complex coordinating with H$_2$O (Scheme 5-1).

\textit{Scheme 5-1 Proposed Catalytic Cycle for Haem-dependent CPO}\textsuperscript{19,36}
Another well studied HPO is the vanadate-dependent HPO. Analogous to its haem-dependent counterpart, a vanadate-dependent HPO has a vanadium metal centre while the vanadium atom does not appear to be redox active and maintains its oxidation state during the catalytic cycle. Vanadate-dependent HPOs are responsible for the vast majority of biotic marine organohalogen compounds. Vanadate-dependent bromoperoxidases are widespread among marine seaweeds while chloroperoxidases predominate in terrestrial fungi. The chloroperoxidase from the plant pathogenic fungus Curvularia inaequalis is an intensively studied vanadate-dependent HPO, however, the exact mechanism involving oxygen-bound intermediates is still under debate because of insufficient experimental evidence. Generally, the catalytic cycle is considered to start from the resting state of the enzyme, V(V) with three equatorial vanadate oxygen (O) atoms coordinated with a single axial His 496 ligand and an apical water hydrogen-bonded to a His 404 in a trigonal bipyramidal coordination geometry. The cycle is initiated with hydrogen peroxide displacing water and coordinating with V(V). The His 404 deprotonates hydrogen peroxide and enables H₂O₂ to bind V(V), then a Lys deprotonates the H₂O₂ again and subsequently forms a hydrogen bond with one of the oxygen atoms from the bound peroxide (Scheme 5-2). In the generated peroxo-V(V) complex, the peroxide is equatorially bound in a side-on manner and distorts the geometry to a tetragonal bipyramid (Scheme 5-2), coordinated ligand His 496 and the covalent oxygen atom is equatorial and the oxo group is apical, His 404 is no longer hydrogen-bonded to any oxygen atoms of the cofactor. Then akin to the haem-dependent counterpart, Cl⁻ is oxidised by the bound peroxide and subsequently dioxo-V(V) complex is generated with either coordinated ClO⁻ or free HOCl being formed, which can react with electron-rich organic substrate to yield biotic organochlorine compounds, at the same time the dioxo-V(V) complex coordinates with another water to return the resting state (Scheme 5-2).
In contrast to HPOs using hydrogen peroxide, halogenases utilise dioxygen (O$_2$) as the oxidant during their catalytic halogenation. Flavin-dependent halogenases are the best known halogenases referring to the oxidation of X$^-$ to X$^+$ and exist in numerous organisms, nevertheless, much less is known about their catalytic mechanism compared to the HPOs.$^{18-21,29,32,33,36}$ The tryptophan 7-halogenase (PrnA) isolated from *Pseudomonas fluorescens* BL915 was the first described flavin-dependent halogenase. The mechanism is believed to involve an enzyme bound reduced flavin, FADH$_2$, that reacts with O$_2$ to form the oxidised flavin, FADH-OOH, which subsequently reacts with Cl$^-$ to generate HOCl and FADH-OH. The HOCl produced reacts with a lysine residue to generate a less reactive but chiral chloramine rather than being released to act as a more reactive but unselective chlorinating agent. This can explain why halogenases possess highly substrate-specific and regioselective activities. The chloramine reacts with electron-rich organic substrates to yield biotic organochlorine compounds and returning the amine to re-enter the cycle (Scheme 5-3). The FADH-OH is dehydrated to FAD which is reduced by NADPH with a flavin reductase in order to produce a FADH$_2$. The mechanism is shown in Scheme 5-3.

**Scheme 5-2 Proposed Catalytic Cycle for Vanadate-dependent CPO$_{19,22,35,36,133}$**

5.4.1.1.2 **Halogenases**
5.4.1.2 Halogenation by Halogen Radical, $X^\cdot$

The structures of many halogenated natural products, such as barbamide, dysidenin and hapalindole A, show halogenation occurs at non-activated (not electron-rich) carbon centres, which suggests such halogenations occur via a different mechanism from halonium, $X^+$, mediated halogenations.

Investigations led to the isolation of a class of enzymes which are responsible for halogenations at inactivated carbon centres. Alpha-Ketoglutarate ($\alpha$KG), $O_2$ and the halide, $X^-$, were found to be essential in this highly substrate-specific and regioselective reaction. An Fe(II) metal centre is involved and changes its oxidation state during the
catalytic cycle. This class of enzymes are called non-haem Fe(II)/αKG-dependent halogenases which utilise anionic halide, X⁻, to form X for the generation of halogenated natural products. Syringomycin halogenase (SyrB2) from the bacterium *Pseudomonas syringae* is a well-studied Fe(II)/αKG-dependent chlorogenase. The catalytic cycle starts from the enzyme binding αKG and Cl⁻, a proton from the bonded αKG is transferred to OH resulting in forming H₂O which coordinates with Fe(II) (Scheme 5-4). Molecular oxygen then displaces H₂O and coordinates with Fe(II), an Fe(IV)-superoxo complex is subsequently generated. After that, the dioxygen bond is weakened and broken to form an Fe(IV)-oxo complex with αKG decarboxylation, the generated Fe(IV)-oxo complex then reacts with an organic substrate via hydrogen (H) atom abstraction, leading to a Fe(III)-hydroxy complex and a substrate radical R·. The produced radical R· subsequently reacts with the bound chlorine Cl in Fe(III)-hydroxy complex to furnish a halogenated natural product and an Fe(II)-hydroxy complex is produced, which subsequently binds αKG and Cl⁻ to start another catalytic cycle. The mechanism is described in Scheme 5-4.
Some fluorinated natural products are produced by $F^-$ directly reacting with the electrophilic organic substrate. Some enzymes have been discovered to catalyse this kind of direct fluorination, for example 5'-fluoro-5'-deoxyadenosine synthetase (5'-FDAS) isolated from the bacterium *Streptomyces cattleya*. The mechanism is thought to involve solvated $F^-$ bound to a hydrophobic pocket provided by the enzyme and $F^-$ is desolvated when the protein residues coordinate to $H_2O$. The free $F^-$ generated then attacks the electrophilic 5' carbon of the co-substrate, 5'-deoxyadenosine synthetase (5'-SAM), via a bimolecular nucleophilic substitution (SN2 substitution), and the intermediate 5'-fluoro-5'-deoxyadenosine produced is converted to fluoroacetate and 4-fluorothreonine. The description of this mechanism is shown in Scheme 5-5.
A 5’-FDAS analogue enzyme, identified as chlorinase SalL, isolated from the marine actinomycete Salinispora tropica is regarded as incorporating Cl⁻ into salinosporamide A, the mechanism is similar with 5’-FDAS and displayed in Scheme 5-6.\cite{18,19,21,29}

SAM is also a substrate for a methyltransferase, which transfers the methyl group bonded with sulphur (S) to a halide, a monohalomethane is generated as a result. Methyl chloride transferase was discovered in seaweeds and also in many plants, the mechanism is shown in Scheme 5-7.\cite{19,21}

### 5.4.2 Nonenzymatic Pathways

Remarkably, a few nonenzymatic pathways of biotic halogenations have been discovered as the supplement of enzymatic pathways. Polycyclic chlorinated
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metabolites cyanosporaside A and B in addition to sporolide A and B, isolated from a marine actinomycete genus *Salinispora*, are such examples. They are regarded as originating from nine-membered enediyne precursors, adducting with Cl⁻ is hypothesised to occur after a Bergman cyclization and this is supported by experimental evidence.¹⁸,²⁹,¹³⁴ Therefore, the produced natural products only halogenated at one but never both of two positions of an aromatic ring followed by this pathway. Details are shown in Scheme 5-8.

![Scheme 5-8 Proposed Mechanism of Nonenzymatic Chlorination](image)

5.5 Conclusion

Naturally occurring organohalogen compounds are predominantly processed by chlorine and bromine via halonium cation or halogen radical pathways under the catalysis of various enzymes. The biosynthetic pathway via halide anion, nonenzymatic pathway in particular, is very rare. To date, knowledge about halogenated natural products, especially their biosynthetic mechanisms, is limited even though intensive investigations have been ongoing during the last few decades. Inspiringly, this field has become a research hotspot and the increasing effort may solve numerous “unknowns” about halogenated natural products. As new equipment and technology is applied, discoveries of new species and bioactivities screening become much more efficient, molecular investigations will become much easier, which will lead a more productive trend in this area. Importantly, to dispel public “halogenophobia”, the entire knowledge
of halogenated natural products, especially their beneficial uses, should be exploited and communicated back to our community.

5.6 References

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Chapter 6: Discovery of a Novel Halogenation Reaction

6.1 Introduction

6.1.1 Azaphilones

The word azaphilone signifies the affinity of a compound for nitrogen. The nomenclature is used to describe “a structurally variable family of fungal polyketide metabolites possessing a highly oxygenated pyranoquinone bicyclic core, usually known as isochromene, and a quaternary carbon center.” The azaphilone scaffolds and a representative example citrinin are shown in Figure 6-1.

![Azaphilone Scaffolds and Citrinin Structure](image)

An azaphilone readily reacts with a primary amine to exchange the pyran oxygen with a nitrogen. Many of the azaphilones are orange coloured pigments and in this process a characteristic colour change to a bright red, as a result of generating a vinylogous 4-pyridone, is observed. Some azaphilones are traditionally used as food colorants in East Asian countries, namely pigments produced by fungi in the genus *Monascus*.

In addition, azaphilones are very useful taxonomical markers because of the occurrence of specific and unique azaphilones in some fungal species. Importantly, azaphilones show promising bioactivities, such as enzymatic and molecular inhibitors and regulators, anti-HIV, anticancer, anti-inflammatory, antimicrobial, antiplasmodial, antioxidant, cytotoxic, hypolipidemic and antihypertensive activities. These effects are generally attributed to the reactions of azaphilones with cellular amino groups such as amino acids, proteins and nucleic acids to form nitrogenised azaphilones as shown in Scheme 6-1.
However, these reactions are always nonselective, indiscriminately reacting with amines leading to the main impediment for their development as drug candidates. To date, some reviews of azaphilones are available, in particular two monographs, offering detailed structural commentary and detailing bioactivities. A review published in early 2013 described 373 azaphilones generated by 23 genera from 13 fungal families, with overrepresentation from members of the families Trichocomaceae and Xylariaceae. These azaphilones are classified into 18 different structural categories, and of 373 azaphilones, 90 of them are halogenated, predominantly through chlorination.

### 6.1.2 Discovery of an Abiotic Halogenation Reaction among Azaphilones

The chemical investigation of fungus *Penicillium* sp. MINAP 9902 isolated from the fruiting body of the myxomycete *Lycogala epidendrum* led to discovery of some azaphilone compounds, which were isolated from the ethanol/dichloromethane extraction of fungal mycelia. These included the known compound (+)-sclerotiorin (6.1) and a novel non-halogenated compound (+)-deschlorosclerotiorin (6.2). These two compounds were also isolated from a PNG mushroom with the local name Kula avara (Figure 6-2), which belongs to an unidentified Clavarioid species of fungi.
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It is noteworthy that (+)-sclerotiorin is the C-5 chlorinated analogue of (+)-deschlorosclerotiorin and surprising that (+)-deschlorosclerotiorin was cleanly converted to (+)-sclerotiorin after being placed in deuterated chloroform (CDCl$_3$) overnight (Scheme 6-2). We have also observed a similar transformation converting (+)-ochrephilone into (+)-isochromophilone I (Scheme 6-2) in CDCl$_3$. The mechanism which has not been investigated before could conceivably involve a chlorine radical from homolysis of a carbon chlorine bond or a chloride anion from chloroform degradation that results in production of small amounts of hydrochloric acid.

Scheme 6-2 the Abiotic Halogenation Reaction among Isolated Azaphilones
It is now interesting to speculate how many of the 90 halogenated azaphilones, 79 of which are halogenated at the same C-5 position are the results of extracellular modifications. Halogenation reactions observed to be occurring between (+)-deschlorosclerotiorin and ochrephilone may not be occasional but in fact be the norm for such compounds. Hence, it is quite necessary to investigate such a nonenzymatic halogenation reaction.

In order to determine how readily the C-5 position undergoes halogenation, a series of model compounds were proposed. The azaphilone scaffold possessing the pyranoquinone bicyclic core, or more specifically a 6\(H\)-isochromene-6,8(7\(H\))-dione (Figure 6-3) was chosen, varying the nature of the substituent at C-3 in order to investigate the role of the sidechain. As such this sidechain was varied from TMS (6.3), H (6.4), Me (6.5) to 1-propenyl (6.6). In addition azaphilones that possess the analogous isoquinoline-6,8(2\(H\),7\(H\))-dione bicyclic core (6.7-6.10), resulting from reaction of a 6\(H\)-isochromene-6,8(7\(H\))-dione with a primary amine have been considered in this study. (+)-Isochromophilone VI for example\textsuperscript{11,12} could represent the reaction product of (+)-deschlorosclerotiorin (6.2) with 2-aminoethanol with subsequent chlorination or the reaction of (+)-sclerotiorin (6.1) with 2-aminoethanol, presumably formed intracellularly. This study will address the capacity of compounds 6.7 and 6.8 to undergo halogenation.

![Figure 6-3 Structures of Model Compounds](image-url)
In the absence of strongly alkaline conditions chloroform is generally regarded as an inert solvent, however a stoichiometric amount of chlorine radical (Cl·) or chloride (Cl⁻) reasonably exist in the solvent,¹³⁻¹⁷ which might be the origin of chlorine atom in sclerotiorin and isochromophilone I. The model compounds (6.3-6.8) will be exposed to different halogenation conditions in order to ascertain the probable source of the halogen in the halogenated azaphilones.

6.2 Synthesis

6.2.1 Synthetic Strategy

The challenging structures and biological activities associated with the azaphilones have resulted in numerous synthetic efforts being directed toward their synthesis.¹ The first synthesis of sclerotiorin was carried out by Whalley and colleagues in 1969,¹⁸ detailed in 1971,¹⁹ it commenced with 3,5-dihydroxy-4-methylbenzoic acid (6.11) transforming it into racemic sclerotiorin over 12 steps (Scheme 6-3). Notably the chlorine was incorporated relatively early in the synthetic strategy and involved the use of sulfuryl chloride, a source of molecular chlorine, and presumably proceeded by an electrophilic aromatic substitution. Creation of the pyranoquinone heterocycle was achieved through a phosphorous pentoxide mediated cyclisation of a keto-benzaldehyde (6.12) with a subsequent Pb(OAc)₄ oxidation. The yield of the final transformation to afford (±)-sclerotiorin (6.13) was a disappointing 12%.

Scheme 6-3 the First Synthesis of Racemic Sclerotiorin by Whalley and Colleagues¹⁸,¹⁹
More recently, in 2011, Porco and colleagues reported an enantioselective synthesis of (+)-sclerotiorin \(\text{(6.1)}\).\(^{20}\) The ring forming reaction proceeded through a cycloisomerisation of an aryl acetylene \(\text{(6.14)}\) utilising a (+)-sparteine surrogate-Cu\(_2\)O\(_2\) complex to produce the pyranoquinone core \(\text{(6.15)}\) in 76% isolated yield (dr = 12:1). In this approach the chlorination was achieved after the esterification of the tertiary alcohol, using the electrophilic source of chlorine, \(N\)-chlorosuccinimide to transform (+)-deschlorosclerotiorin \(\text{(6.16)}\) into (+)-sclerotiorin \(\text{(6.1)}\), the overall yield of the two steps was 65% (Scheme 6-4).

\[
\begin{align*}
\text{CHO} & \quad \text{OH} & \quad \text{CHO} \\
\text{6.14} & \quad \text{three steps} & \quad \text{6.15} \\
\text{6.16} & \quad \text{NCS} & \quad \text{CH}_3\text{CN} & \quad 65\% \\
& \quad \text{(2 steps)} & \quad \text{Ac}_2\text{O} & \quad \text{DCM} \\
& & & 76\%
\end{align*}
\]

\textit{Scheme 6-4 the Synthesis of (+)-Sclerotiorin by Porco and Colleagues}\(^{20}\)

Other groups have utilised a cycloisomerisation strategy, that utilises a pyrylium salt as a key intermediate, in their efforts to produce the pyranoquinone core.\(^1\) In general a dihydroxy-halobenzaldehyde \(\text{(6.17)}\) is transformed via a Sonogashira coupling with an appropriate alkyne to provide what will become the side chain at the C-6 position. The \(\text{o-}\)-alkynylbenzaldehyde \(\text{(6.18)}\) is cycloisomerised to a 2-benzopyrylium salt \(\text{(6.19)}\) catalysed by transition metal as a Lewis acid, such as Pd (II), Cu (II), Ag (I) or Au (III).\(^{1,21}\) Subsequently, oxidation using a reagent such as \(\text{o-}\)-iodoxybenzoic acid (IBX) or lead tetraacetate are applied to form the final azaphilone core \(\text{(6.20)}\) (Scheme 6-5).\(^1\)
6.2.2 Synthetic Route to the General Precursor (6.27)

Recognising the utility of the chemistry described we devised a synthetic route to achieve 2,4-dihydroxy-6-iodo-3-methylbenzaldehyde (6.27) as a general precursor for Sonogashira couplings (Scheme 6-6).\textsuperscript{20-26} In this direction commercially available 2-methylresorcinol (6.21) was methylated using dimethyl sulfate to furnish 2,6-dimethoxytoluene (6.22) in excellent yield.

Initial attempts to formylate 6.22 using modified Rieche conditions where titanium tetrachloride was replaced with the more easily handled Lewis acidic aluminium trichloride were disappointing returning starting material in addition to low yields of the formylated product, 2-hydroxy-4-methoxy-3-methylbenzaldehyde (6.23), that had been deprotected \textit{ortho} to the site of formylation. Fortunately regioselective installation of the aldehyde was achieved using dichloromethyl methyl ether under standard Rieche conditions to generate 2,4-dimethoxy-3-methylbenzaldehyde (6.24) in excellent (99%) yield.\textsuperscript{26} Its formation was supported by the appearance of a downfield resonance in the \textsuperscript{1}H NMR spectrum at 10.22 ppm and a downfield resonance in the \textsuperscript{13}C NMR spectrum at 189.3 ppm. Protection of the aldehyde by reaction with \textit{N,N'}-dimethylethylenediamine returned the dimethylimidazolidine (6.25) as a colourless oil. Whereas an analogous reaction reported by Ryan and colleagues employed a Dean-Stark trap to remove the water generated,\textsuperscript{24} we found this was unnecessary with the reaction proceeding in refluxing toluene to return the desired product in an excellent (99%) yield. Directed \textit{ortho} metalation (DoM) utilising the influence of the amine nitrogens ensured
deprotonation at -55 °C by t-butyllithium occurred at the C-6 position. The anion generated, upon reaction with 1,2-diiodoethane resulted in clean conversion to an iodinated dimethylimidazolidine which was immediately deprotected by treatment with 2M aqueous hydrochloric acid to afford 6-iodo-2,4-dimethoxy-3-methylbenzaldehyde (6.26). The regioselectivity of the iodination was easily recognised by the correlations in HMBC spectrum. Finally deprotection of the methyl ethers under standard conditions using boron tribromide resulted in the efficient formation of 2,4-dihydroxy-6-iodo-3-methylbenzaldehyde (6.27), the compound to be used as a general precursor for Sonogashira couplings.

Scheme 6-6 the Synthetic Route to Our General Precursor (6.27)

6.2.3 Synthetic Route to 7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.3)

Long provided a convenient approach of Sonogashira coupling by using triethylamine (NEt₃) as both the required base and solvent.²⁵ However, when applying these conditions in our system repeated reactions consistently gave a mixture of the desired alkyne 6.28 along with the desilylated alkyne 6.29 (Scheme 6-7). Since the TMS acetylene is not stable under basic conditions it was apparent that the excessive NEt₃ employed here was causing C-Si bond cleavage resulting in the terminal alkyne 6.29. Jianglong offered another method of Sonogashira coupling by using tetrahydrofuran as solvent while employing only a slight excess of base.²⁷ Ultimately we devised a modified approach combining elements of both Long and Jianglong’s methods, that involved
reacting the general precursor 6.27 with ethynyltrimethylsilane and NEt\textsubscript{3} in dry THF employing bis(triphenylphosphine)palladium(II) dichloride (PdCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2}) and copper(I) iodide (CuI) as catalysts, to deliver an excellent yield of 2,4-dihydroxy-3-methyl-6-((trimethylsilyl)ethynyl)benzaldehyde (6.28) (Scheme 6-7). Cycloisomerisation of 6.28 by gold(III) acetate (Au(OAc)\textsubscript{3}) catalysis proceeded through the presumed benzopyrylium cation (6.30) present as the trfluoroacetate salt. Both silver and gold catalysts were trialed at this stage with the literature supporting their use\textsuperscript{25,27,28} in what is formally a 6-endo-dig cyclisation. We found that Au(OAc)\textsubscript{3} at a catalyst loading of 5 mol\% returned reproducibly good results. No efforts were made to isolate the benzopyrylium salt, instead in-situ oxidation using IBX efficiently gave the racemic pyranoquinone 6.3 in 99% yield over the one pot, two step process (Scheme 6-7). A broad singlet resonance at 4.30 ppm (7-OH), and three diagnostic singlet resonances at 5.48, 6.78 and 8.04 ppm in the \textsuperscript{1}H NMR spectrum in association with two diagnostic ketone resonances at 196.9 and 197.3 ppm in the \textsuperscript{13}C NMR spectrum supported the formation of the product 6.3. Curiously and preempting results to be revealed, tiny amounts of a chlorinated product (m/z 300 and 298), as detected through monitoring of the reaction by GC-MS could always be observed during this cyclisation reaction when a mixture of 1,2-dichloroethane (DCE) and trifluoroacetic acid (TFA) (10:1) was used. To avoid this a mixture of toluene and TFA (10:1) is employed for the cyclisation in the synthesis of 6.3 and all analogous targets in order to eliminate the generation of chlorinated products.
6.2.4 Synthetic Route to 7-hydroxy-7-methyl-6H-isochromene-6,8(7H)-dione (6.4)

Initial efforts for the preparation of 7-hydroxy-7-methyl-6H-isochromene-6,8(7H)-dione (6.4) focussed on deprotection of the silyl protected precursor 6.28. Not surprisingly, the silyl alkyne 6.28 could be deprotected by adding anhydrous K$_2$CO$_3$ in dry methanol to return the terminal alkyne 6.29 (Scheme 6-8) with a yield of 93%.\textsuperscript{25,27} The appearance of a diagnostic alkyne proton resonance at 2.07 ppm in the $^1$H NMR spectrum which was coupled to a resonance at 85.4 ppm in the $^{13}$C NMR spectrum supported the assigned structure. Attempts at the cyclisation of the alkyne 6.29 using the Au(OAc)$_3$ conditions outlined above for the conversion of 6.28 to 6.3 (Scheme 6-7) returned a complex mixture from which the hydrated alkyne, 6-acetyl-2,4-dihydroxy-3-methylbenzaldehyde (6.31) was isolated. Repeated efforts employing a variety of conditions involving both silver(I) nitrate (AgNO$_3$) and Au(OAc)$_3$ were to no avail.\textsuperscript{25,28} Having the pyranoquinone (6.3) in hand our attention turned to efforts to deprotect it as a means to afford the desired compound 6.4. Upon treatment of 6.3 with tetra-$n$-butylammonium fluoride (TBAF) in ethyl acetate (EtOAc) at -78 °C, 6.4 was achieved, albeit in a yield of just 29%
Chapter 6: Discovery of a Novel Halogenation Reaction

(Scheme 6-8). After adding TBAF the orange reaction mixture immediately turns purple and forms an insoluble purple material as the temperature warms up from -78 °C to 0 °C. The isolated compound 6.4 presents as a pale yellow solid that degrades to an insoluble purple complex over time and immediately degrades upon exposure to acidic conditions. These observations indicate 6.4 is very unstable and explain why synthetic efforts employing reactions in an acidic medium (TFA) produced only trace quantities of 6.4.

![Scheme 6-8 Synthetic Route to Compound 6.4](image)

6.2.5 Synthetic Route to 7-hydroxy-3,7-dimethyl-6H-isochromene-6,8(7H)-dione (6.5)

Following the same method, the general precursor 6.27 was reacted with propyne and NEt₃ in dry THF using PdCl₂(PPh₃)₂ and CuI as catalysts to give 2,4-dihydroxy-3-methyl-6-(prop-1-yn-1-yl)benzaldehyde (6.32), which was subsequently subjected to cyclisation by Au(OAc)₃ catalysis and the intermediate benzopyrylium salt was oxidised by IBX to generate the target compound 6.5 in excellent yield (99%) over two steps (Scheme 6-9).

![Scheme 6-9 Synthetic Route to Compound 6.5](image)
6.2.6 Synthetic Route to (\textit{E})-7-hydroxy-7-methyl-3-(prop-1-en-1-yl)-6H-isochromene-6,8(7H)-dione (6.6)

The synthetic route to (\textit{E})-7-hydroxy-7-methyl-3-(prop-1-en-1-yl)-6H-isochromene-6,8(7H)-dione (6.6), proceeds through a Sonogashira coupling between 6-ethynyl-2,4-dihydroxy-3-methylbenzaldehyde (6.29) and (\textit{E})-1-bromo-1-propene (Scheme 6-10).\(^\text{27}\)

The reaction returned the desired (\textit{E})-2,4-dihydroxy-3-methyl-6-(pent-3-en-1-yn-1-yl)benzaldehyde (6.33) as supported by the vicinal coupling constant ($J = 15.8$ Hz) between the olefinic hydrogen resonances, with a modest yield (53%). Cyclisation (Au(OAc)$_3$) and oxidation (IBX) as described previously generated the desired isochromene dione 6.6 in a good yield (62%) with retention of the \textit{trans} disposed double bond as evidenced by the $^1$H NMR data, 6.22 (1H, dq, $J = 15.7$ Hz, 1.6 Hz, 9-H), 6.59 (1H, dq, $J = 15.7$ Hz, 7.0 Hz, 10-H).

![Scheme 6-10 Synthetic Route to Compound 6.6](image)

6.2.7 Synthetic Route to 7-hydroxy-2-(2-hydroxyethyl)-7-methyl-3-(trimethylsilyl)isoquinoline-6,8(2H,7H)-dione (6.7)

With the isochromene dione 6.3 on hand we utilised it to ascertain the conditions required to transform it in to the isoquinoline dione (6.7) desired in our study. Treatment of 6.3 with 2-aminoethanol in dry acetone was observed to result in consumption of the starting material, as assessed by LC-MS analysis after five minutes of reaction. However, at this stage of the reaction the predominant product was observed to be the hemiaminal 6.34 (Scheme 6-11), as assessed by the observation of a protonated molecule ([M+H]$^+$) in the mass spectrum with a $m/z$ of 326. Also evident in the mass spectrum was a trace amount of a compound with $m/z$ of 308 ([M+H]$^+$) presumably corresponding to the desired compound 6.7 (Scheme 6-11). The reaction mechanism (Scheme 6-1) explains these observations. Since the protonation of a hydroxy in the intermediate hemiaminal (6.34 herein) is the slow step in the overall reaction procedure, the immediately generated hemiaminal requires a relative long
reaction time to be finally dehydrated to the nitrogenised azaphilone (compound 6.7 herein).

After the reaction was allowed to proceed for 48 hours, not surprisingly the major product was observed to be the isoquinoline dione 6.7 with minor amounts of the hemiaminal 6.34 still present (Scheme 6-11). During ESIMS analysis compound 6.7 was found to be unstable in MeOH solutions, converting to the desilylated analogue 6.8.

![Scheme 6-11 Synthetic Route to Compound 6.7]

### 6.2.8 Synthetic Route to 7-hydroxy-2-(2-hydroxyethyl)-7-methylisoquinoline-6,8(2H,7H)-dione (6.8)

Capitalising on the observed instability of 7-hydroxy-2-(2-hydroxyethyl)-7-methyl-3-(trimethylsilyl)isoquinoline-6,8(2H,7H)-dione (6.7), it was exposed to 2-aminoethanol in methanol which effected a rapid conversion to the desired desilylated product 6.8 in 60% yield after chromatography (Scheme 6-12).

![Scheme 6-12 Synthetic Route to Compound 6.8]

With two of the target isoquinolines in hand and with time limitations of the PhD program, the remaining isoquinoline dione targets (6.9 and 6.10) were not further investigated.
6.3 Halogenation Investigation

6.3.1 Halogenation of 7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.3)

6.3.1.1 Chlorination

With pyranoquinone starting materials (6.3-6.6) to investigate the halogenation reaction of azaphilones in hand, we embarked upon a strategy to produce authentic material. Porco, in his synthesis of (+)-sclerotiorin (6.1) utilised an electrophilic chlorination at C-5, employing N-chlorosuccinimide (NCS) as the source of chloronium ion. Mechanistically this is a standard electrophilic chlorination with the regiochemistry explained through the generation of a stabilised pyrylium intermediate (Scheme 6-13).

![Scheme 6-13 Mechanism of NCS Promoted Electrophilic Chlorination at C-5 for (+)-Deschlorosclerotiorin](image)

When 7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.3) was treated with NCS in acetone in the dark, clean C-5 chlorination was observed to produce 5-chloro-7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.35) as a stable orange oil in good (80%) yield (reaction 1, Scheme 6-14). While NCS is sometimes used as a source for chlorine radical in reactions, given the reaction was
performed under dark conditions in the absence of added initiator, this reactivity was
discounted.

In contrast to electrophilic chlorination at C-5 of compound 6.3 by NCS, in reaction 2 in
Scheme 6-14 we observed a nucleophilic chlorination at C-5 of compound 6.3, resulting
in the same product 6.35. In the previously described reaction generating compound 6.3
from 6.28 (Scheme 6-7), we found compound 6.35 was the only product if brine
(saturated aqueous NaCl solution) instead of deionised water was used to wash the
reaction mixture or if solid NaCl was added to the reaction mixture during the work-up
procedure. This nucleophilic chlorination was complete within 0.5 h when a
stoichiometric amount of NaCl was present in the reaction mixture.

For further investigation of this unusual electrophilic chlorination, a solution of
compound 6.3 in EtOAc was treated with NaCl overnight, no matter the presence of TFA
or not and no matter under dark conditions or not, no C-5 chlorinated product 6.35 was
observed as assessed by LC-MS (reaction 3, Scheme 6-14). Curiously, the chlorinated
product 6.35 was produced if the EtOAc solution of 6.3 was treated with NaCl in addition
to Au(OAc)₃, no matter the presence of TFA or not (reaction 4 and 5, Scheme 6-14). It is
worth noting that if TFA was present in the reaction mixture, the reaction to produce
the chlorinated product 6.35 was more rapid.
Unsurprisingly, the exclusive product 5-bromo-7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione \((6.36)\) was generated with an excellent yield (99%) resulting from a nucleophilic bromination, after aqueous sodium bromide (NaBr) solution was used to wash the reaction mixture or if solid NaBr was added to the reaction mixture in the aforementioned reaction designed to generate compound \(6.3\) from \(6.28\) (reaction 1, Scheme 6-15). This C-5 nucleophilic bromination occurred even if only a trace amount of TFA was present in the reaction mixture.
In contrast to the chlorination result (reaction 3, Scheme 6-14), the EtOAc solution of 6.3 was treated with NaBr overnight, under dark conditions or not, produced the brominated product 6.36 when TFA was present as assessed by LC-MS (reaction 2, Scheme 6-15). However, 6.36 was not observed in the absence of TFA under the same reaction conditions (reaction 3, Scheme 6-15). In the presence of Au(OAc)$_3$, 6.3 was readily brominated at C-5 by NaBr, TFA was not required to present (reaction 4 and 5, Scheme 6-15), nevertheless the presence of TFA contributed to a higher yield of the brominated product 6.36.

In an experiment designed to assess the competitiveness of chlorination versus bromination a reaction (reaction 6, Scheme 6-15) was performed and at the workup phase a mixture of excess solid NaCl and NaBr (1:1 by weight) was added generating compound 6.36 from 6.28 as the exclusive product, as observed by LC-MS. This is all the more noteworthy given the molar excess of NaCl exceeded that of NaBr.
Scheme 6-15 Bromination of Compound 6.3

6.3.1.3 Iodination

We observed through LC-MS that a small amount of a suspicious iodinated product 7-hydroxy-5-iodo-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.37) was always present in the reaction mixture when generating compound 6.3 from 6.28.
Without adding exogenous iodine, we attributed the iodination to the reagent IBX or its degradation products containing iodine. When an aqueous solution of sodium iodide (NaI) was used to wash the reaction mixture or if solid NaI was added to the reaction mixture, an increasing, but still small amount of the aforementioned iodinated product (6.37) was observed (reaction 1, Scheme 6-16). In contrast to the chlorination and bromination, the iodinated product wasn’t able to be isolated due to its low yield. Repeated efforts employing a variety of conditions involving different concentrations of aqueous NaI solution, under an argon atmosphere and dark conditions were to no avail.

In order to acquire the authentic material of the C-5 iodinated product 6.37, compound 6.3 was treated with N-iodosuccinimide (NIS) in acetone in the dark, whereon clean C-5 electrophilic iodination was observed to produce 6.37 in good (85%) yield (reaction 2, Scheme 6-16). As assessed by LC-MS, the suspected iodinated product generated in reaction 1 was confirmed to be 6.37 through co-elution and identical mass spectra.

In order to investigate the nucleophilic iodination of compound 6.3, the EtOAc solution of it was treated with NaI under an argon atmosphere and dark conditions. Regardless of the presence or absence of TFA, 10 min later only trace amounts of C-5 nucleophilic iodination product 6.37 was observed as assessed by LC-MS (reaction 3, Scheme 6-16). It is worth noting that when TFA was present, the reaction mixture immediately turned dark brown colour after NaI was added. While the reaction mixture changed to pale brown colour after NaI was added without the presence of TFA. Unfortunately, no increase in the amount of 6.37 was observed with the reaction going overnight. The iodinated product 6.37 was also generated in a trace amount if an EtOAc solution of 6.3 was treated with NaI in addition to Au(OAc)₃, no matter the presence of TFA or not (reaction 4, Scheme 6-16).
6.3.1.4 Fluorination

As discussed in the introduction of chapter five (Section 5.4), fluoride readily forms hydrogen bond to water, resulting in fluoride being extremely difficult to be desolvated making a nucleophilic fluorination is challenging. Unsurprisingly, no fluorinated products such as 5-fluoro-7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.38) were observed as assessed by LC-MS after an aqueous solution of sodium fluoride (NaF) was used to wash the reaction mixture or if solid NaF was added to the mixture in the reaction designed to generate compound 6.3 from 6.28 (reaction 1, Scheme 6-17). Repeated efforts employing a variety of conditions were to no avail (reactions 1-4, Scheme 6-17).
Scheme 6-17 Fluorination of Compound 6.3

Based on the halogenation investigation of compound 6.3, the exclusive C-5 chlorination, bromination and iodination were observed by both electrophilic and nucleophilic substitution mechanisms, while no fluorination was observed. As mentioned in the chapter five section 5.4.1.3, a nucleophilic halogenation is uncommon in nature, especially via a nonenzymatic pathway, where so far only one example of nucleophilic addition to an 4,7-indane diradical by chloride was discovered (chapter five section 5.4.2). In this work we have discovered the first nonenzymatic nucleophilic halogen substitution reaction to occur in nature. Inspired by advantages of the nucleophilic halogenation reaction over its electrophilic counterpart, such as an excellent yield (up to 99%), an ultra-high reaction ratio (only one shake in the separating funnel), clean (no side product succinimide was generated which can be challenging for purification) and an ultra-simple work-up, the utility of this nucleophilic halogenation reaction was further investigated on the other synthetic analogues 6.4-6.6.
Among three feasible nucleophilic halogenations, chlorination and bromination were much more facile than iodination. Therefore, the following halogenation investigations of substrates $6.4$-$6.6$ focused on chlorination and bromination.

### 6.3.2 Halogenation of 7-hydroxy-7-methyl-6H-isochromene-6,8(7H)-dione ($6.4$)

#### 6.3.2.1 Chlorination

As mentioned in section 6.2.4, the molecule bearing a hydrogen at C-3, compound $6.4$, is very unstable and its C-5 chlorinated analogue $6.39$ can only be obtained in quantity from the deprotection of $6.35$ rather than direct chlorination from $6.4$ (reaction 1, Scheme 6-18). Despite compound $6.4$ being unstable in aqueous or acidic conditions, pure $6.4$ was treated with solid NaCl in EtOAc and small amounts of $6.39$ were observed after 0.5 h, as assessed by LC-MS (reaction 2, Scheme 6-18). The chlorinated product $6.39$ was significantly more stable than its non-chlorinated precursor $6.4$.

![Scheme 6-18 Synthesis of Compound 6.39](image)

#### 6.3.2.2 Bromination

Similarly, compound $6.4$ C-5 brominated product $6.40$ was obtained from deprotection of $6.36$ (reaction 1, Scheme 6-19). However, small quantities of $6.40$ were observed after 0.5 h when pure $6.4$ was treated with solid NaBr in EtOAc (reaction 2, Scheme 6-19). Once again the halogenated, in this case brominated, product $6.40$ was more stable than the dehalogenated compound $6.4$. 
6.3.3  Halogenation of 7-hydroxy-3,7-dimethyl-6H-isochromene-6,8(7H)-dione (6.5)

6.3.3.1 Chlorination

7-hydroxy-3,7-dimethyl-6H-isochromene-6,8(7H)-dione (6.5) was treated with NCS in acetone overnight, with the resultant C-5 chlorination product 6.41 being generated with a modest yield 55% (reaction 1, Scheme 6-20). As we would predict, when reaction mixture designed to produce 6.5 from 6.32 was washed with a brine solution or if solid NaCl was added during the normal workup procedure compound 6.41 instead of compound 6.5 was exclusively observed as assessed by LC-MS (reaction 2, Scheme 6-20). It is worth noting that a mixture of the chlorinated product 6.41 and compound 6.5 was observed if TFA concentration in the reaction mixture was low. When pure compound 6.5 dissolved in EtOAc and treated with NaCl overnight, regardless of the presence of TFA or not, a small amount of 6.41 was observed as assessed by LC-MS (reaction 3, Scheme 6-20). Compound 6.5 was also found unstable in aqueous or acidic conditions while, like its hydrogen analogue 6.39, the C-5 chlorinated product 6.41 was more stable.
6.3.3.2 Bromination

When compound 6.5 was treated with NBS in acetone for 10 min, C-5 brominated product 6.42 was generated with an excellent yield 89% (reaction 1, Scheme 6-21). Compound 6.42 was also generated, with a yield 67%, after a saturated aqueous NaBr solution was used to wash the reaction mixture in the workup phase of the reaction designed to generate compound 6.5 from 6.32 (reaction 2, Scheme 6-21). As assessed by LC-MS, compound 6.42 was the only product identified even if TFA was absent in the reaction mixture. When pure 6.5 was dissolved in EtOAc and treated with NaBr overnight, regardless of the presence of TFA or not, a small amount of 6.42 was observed as assessed by LC-MS (reaction 3, Scheme 6-21). Curiously, much more 6.42 was generated with the presence of TFA than without TFA. The C-5 brominated product 6.42 was found to be significantly more stable than the deshalogenated compound 6.5.
6.3.4 Halogenation of \((E)\)-7-hydroxy-7-methyl-3-(prop-1-en-1-yl)-6\(H\)-isochromene-6,8(7\(H\))-dione (6.6)

6.3.4.1 Chlorination

The chlorinated product 6.43 was generated with a 68% yield after NaCl brine was used to wash the reaction mixture in the reaction designed to generate compound 6.6 from 6.33 (reaction 1, Scheme 6-22). When pure compound 6.6 was dissolved in EtOAc and treated with NaCl overnight, regardless of the presence of TFA or not, a small amount of 6.43 was observed as assessed by LC-MS (reaction 2, Scheme 6-22). Similarly, much more 6.43 was generated with the presence of TFA than without TFA. In the presence of Au(OAc)\(_3\), a higher yield of product 6.43 was observed (reaction 3, Scheme 6-22). Compound 6.6 was found to decompose in aqueous or acidic conditions while the chlorinated product 6.43 was found to be more stable than the deshalogenated counterpart, 6.6.
6.3.4.2 Bromination

The brominated product 6.44 was generated with a modest yield of 46% after saturated aqueous NaBr solution was used to wash the reaction mixture in the reaction designed to generate compound 6.6 from 6.33 (reaction 1, Scheme 6-23). When a pure solution of compound 6.6 in EtOAc was washed in a separating funnel with aqueous saturated NaBr solution, a small amount of 6.44 was observed as assessed by LC-MS (reaction 2, Scheme 6-23). The C-5 brominated product 6.44 was found to be more stable than compound 6.6.
6.3.5 Halogenation of 7-hydroxy-2-(2-hydroxyethyl)-7-methyl-3-(trimethylsilyl)isoquinoline-6,8(2H,7H)-dione (6.7)

6.3.5.1 Chlorination

Having unambiguously established the capacity of the isochromene-6,8(7H)-diones (6.3-6.6) to undergo nucleophilic halogenation under mild conditions, the question now arose as to whether the isoquinoline-6,8(2H,7H)-diones underwent a similar transformation. An authentic sample of the chlorinated product of the isoquinoline-6,8(2H,7H)-dione (6.7) was prepared by treating the chlorinated isochromene 6.35 with 2-aminoethanol in anhydrous acetone (reaction 1, Scheme 6-24). Within 10 min a colour change from orange to red had occurred and TLC indicated the presence of a more polar compound. Upon workup a red oil was obtained in excellent yield (93%) with spectral data, including the downfield resonance in the $^1$H NMR spectrum at 8.15 (1H, s, 1-H), supporting the formation of the isoquinoline 6.45. With authentic material in hand a pure sample of compound 6.7 was dissolved in MeOH and treated with solid NaCl overnight (reaction 2, Scheme 6-24). A chlorinated product with identical retention time and mass spectrum to the authentic material 6.45 was observed as assessed by LC-MS. As mentioned in section 6.2.7, compound 6.7 was found unstable in MeOH solution readily undergoing TMS deprotection. Compound 6.45 was also found to decompose slowly in MeOH solution although it was more stable than compound 6.7.

Scheme 6-24 Synthesis of Compound 6.45
6.3.5.2 Bromination

In an analogue manner to the method employed for the production of the authentic chlorinated material (6.45), an authentic sample of the brominated product 6.46 was obtained after compound 6.36 was treated with 2-aminoethanol in anhydrous acetone (reaction 1, Scheme 6-25). Compound 6.46 was isolated as a bright red oil in excellent yield (99%) following chromatography. The compound now possessed poor solubility in acetone while good solubility in MeOH and displayed resonances in the $^1$H NMR consistent with an isoquinoline, specifically two singlet resonances at 7.29 and 8.13 ppm assigned to the 4-H and 1-H hydrogens respectively. The mass spectrum also displayed the characteristic ions associated with a brominated product, in this case ions at $m/z$ 387 (19) and 385 (18). When a sample of the pure compound 6.7 was dissolved in MeOH and stirred in the presence of solid NaBr overnight (reaction 2, Scheme 6-25), 6.46 was readily observed as assessed by LC-MS. Similarly to previous observations, 6.46 was found to be significantly more stable than 6.7.

![Scheme 6-25 Synthesis of Compound 6.46](image)

6.3.6 Halogenation of 7-hydroxy-2-(2-hydroxyethyl)-7-methylisoquinoline-6,8(2H,7H)-dione (6.8)

6.3.6.1 Chlorination

While the anticipated halogenation reactions proceeded smoothly with the isoquinoline 6.7, we chose to demonstrate it was also a general reaction by exposing 7-hydroxy-2-(2-hydroxyethyl)-7-methylisoquinoline-6,8(2H,7H)-dione (6.8) to the same reaction conditions.
Authentic material was obtained by recognising the TMS group of compound 6.45 is deprotected in MeOH, thus compound 6.45 was dissolved in MeOH and subjected to 2-aminoethanol to return compound 6.47 with an excellent isolated yield (90%) (reaction 1, Scheme 6-26). Compound 6.47 presents as an extremely polar red solid, is sparingly soluble in most organic solvents but has a good solubility in DMSO. When a sample of pure compound 6.8 was dissolved in MeOH and stirred with the addition of solid NaCl, compound 6.47 was observed after 0.5 h as assessed by LC-MS (reaction 2, Scheme 6-26). In the presence of Au(OAc)₃, after 0.5 h compound 6.47 was observed to be generated much more rapidly as assessed by LC-MS (reaction 3, Scheme 6-26).

Scheme 6-26 Chlorination of Compound 6.8

6.3.6.2 Bromination

In a manner analogous to previous reactions the authentic C-5 brominated product 6.48 was achieved by placing the solution of compound 6.46 in MeOH and exposing it to 2-aminoethanol (reaction 1, Scheme 6-27) which returned the desired compound in excellent yield (99%) as a red solid. Compound 6.48 only has a good solubility in DMSO but is poorly soluble in other solvents. When a sample of pure compound 6.8 was dissolved in MeOH and treated with solid NaBr, compound 6.48 was observed after 0.5 h as assessed by LC-MS (reaction 2, Scheme 6-27). In the presence of Au(OAc)₃, after 0.5
h the brominated compound 6.48 was observed much more rapidly as assessed by LC-MS (reaction 3, Scheme 6-27).

**Scheme 6-27 Synthesis of Compound 6.48**

6.3.7 Attempted Conversions between Chlorination and Bromination Products

All the synthetic chlorinated products produced in this study were treated with NaBr solution in EtOAc and solid NaBr in order to ascertain if a dynamic equilibrium allowed for the exchange of a halogen. Not unexpectedly brominated products were not observed for any of the reactions as assessed by LC-MS, regardless of the presence of Au(OAc)₃ and/or TFA or not.

Conversely all the synthetic brominated products were treated with NaCl solution in EtOAc and solid NaCl. Once again no chlorinated products were observed as assessed by LC-MS, regardless of the presence of Au(OAc)₃ and/or TFA or not.

6.4 Discussion and Conclusion

6.4.1 Discovery of Nonenzymatic Nucleophilic Halogenation Reaction

Our initial observation recognised an unprecedented chlorination reaction taking place in the azaphilones when dissolved in deuterated chloroform. (+)-Deschlorosclerotiorin (6.2) and (+)-ochrephilone were transformed into (+)-sclerotiorin (6.1) and (+)-
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isochromophilone I respectively (Scheme 6-2), prompting us to investigate the reaction in detail. We have observed that isochromene-6,8(7H)-diones (6.3-6.6) and isoquinoline-6,8(2H,7H)-diones (6.7-6.8) (Figure 6-3) in the presence of nucleophilic sources of halide readily undergo substitution at C-5. All the more remarkable is that this site is also halogenated under electrophilic conditions using the reagents NCS, NBS or NIS. The fact that identical products are yielded when electrophilic reagents are replaced by the corresponding sodium halides confirms our discovery of a nonenzymatic halogenation through a nucleophilic substitution reaction. These enzyme-free halogenation reactions among the azaphilones and their analogues represent a completely novel discovery.

The fact that 79 of the 90 known halogen containing azaphilones are halogenated at C-5 raises the question as to whether these compounds are actually natural products. Indeed it raises the issue of what constitutes a natural product? Is a natural product a compound produced inside a cell by metabolic processes or do they also include compounds produced extracellularly? Is (+)-5-bromoochrephilone (Figure 6-4), isolated from a Penicillium species fermented in a medium containing potassium bromide, a natural product or an artefact of the process used to generate the precursor compound? We take the position that a natural product includes all compounds produced in nature regardless of whether they are generated inside or outside the cell. We accept that what some researchers call artefacts we call natural but see these compounds as expressions of the diversity of nature.

![Figure 6-4 Structures of Brominated and Chlorinated Ochrephilones](image)

*Figure 6-4 Structures of Brominated and Chlorinated Ochrephilones*

Among the halides, as mentioned in chapter five (Section 5.4), chloride is predominant in nature explaining why the halogenated azaphilones are almost exclusively chlorinated. It is reasonable to consider this novel halogenation reaction exists widely in nature. That
means our discovery of a mild, rapid, nucleophilic, non-enzymatic halogenation will expand the knowledge of biosynthetic pathways of natural products.

6.4.2 Features of the Novel Halogenation Reaction

6.4.2.1 Reactive Site

Our investigation using the model compounds (6.3-6.8) confirms the nucleophilic halogenations occur exclusively at the C-5 position among three available positions in the bicyclic core C-1, C-4 and C-5 (Scheme 6-28). It is noteworthy that C-5 is the most electron rich of the three available sites for substitution with the highest electron density, as indicated by the $^{13}$C NMR chemical shifts ranging from a low of 97.4 ppm (6.7) through to 107.1 ppm (6.4). This observation can explain why only C-5 substituted products are produced when NCS, NBS and NIS, regarded as the sources of electrophilic halonium cations are used but seems contrary to a mechanism supporting nucleophilic substitution. As such it suggests the novel halogenation reaction does not proceed by a direct reaction of a halide anion at the C-5 position, but instead progresses through a complex transformation of the bicyclic core involving a pyrylium or pyridinium cation.

![Scheme 6-28 the Nucleophilic Halogenations of Model Compounds](image)

Once the C-5 position has been substituted by one halogen atom, experimental results show the product is not able to be substituted by further halogen atoms.

6.4.2.2 Product Stability

The stability of the isochromene/isoquinoline dione products with various C-3 side chains were observed to increase in the following order; H< methyl< propenyl< TMS. That is the stability increased along with the increasing steric bulk of the C-3 side chain. This might be explained by the decreasing ability of the molecule to act as a Michael
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acceptor. Products were also observed to be much more stable after C-5 halogen substitution, with brominated products being more stable than the corresponding chlorinated products, following this order (at C-5) H < Cl < Br. For instance, model compound 6.4, with H in C-3 position, is vulnerable to acid and very mild base and degrades rapidly when exposed in air, however, its halogenated products are readily handled under these conditions.

6.4.2.3 Reaction Rates

Among halogen anions, rates of substitution in the novel halogenation reaction follow the order I⁻ > Br⁻ > Cl⁻, which matches their order of nucleophilicity. This order can be observed from the reactions of the model compound 6.3 with NaI, NaBr and NaCl. Iodination can occur rapidly (10 min) without any catalysts (see Section 6.4.2.4), bromination proceeds with TFA as catalyst while chlorination requires both TFA and gold catalysts. In addition, during the cyclization and oxidation step to generate the model compounds 6.3-6.6, the occurrence of small amounts of C-5 iodinated products indicates the facile nature and rapid rate of the iodination reaction. However, contrary to expectation, only trace amounts of iodinated products were observed among reactions conducted with NaI. Since I⁻ is sensitive to oxygen and light, oxidising to I₂, the generated product of a reaction between Au(OAc)₃ and NaI would be AuI₃, which would immediately degrade to AuI and I₂. The active catalyst Au(III) (detailed in the following section 6.4.2.4) becomes the inactive Au(I), and the I₂ produced readily reacts with unsaturated bonds in substrate molecules.

As a very weak nucleophile, not surprisingly, F⁻ was not observed to react at C-5 either in direct fluorination reaction attempts with azaphilones and their synthetic analogues or in TMS group deprotection reactions.

6.4.2.4 Catalysts

Acids including both the Lewis acid, Au(OAc)₃, and the Brønsted acid, TFA, are catalysts used in this novel halogenation reaction. Take the example of model compound 6.3, here the chlorination only occurs at an appreciable rate with the presence of both Au(OAc)₃ and TFA while bromination is able to occur with only the presence of TFA. Essentially, Au(III) is the active oxidation state of the catalyst, and the gold will maintain the same active, Au(III), oxidation state after generating AuBr₃ or AuCl₃, resulting from a reaction between Au(OAc)₃ and NaBr or NaCl respectively. However, as discussed in
the previous section, gold changes from the active oxidation state Au(III) to inactive Au(I) after a reaction with NaI. Au(III) is regarded as a Lewis acid coordinating to the C=C double bonds which enables the nucleophilic attack at C-5 by a halide anion. In a similar manner if the Brønsted acid (TFA) is regarded as coordinating to the electron rich double bond an analogous mechanism can proceed. This does not discount the complex equilibria which would also be in motion surrounding protonating the two carbonyl oxygen atoms in the pyranoquinone bicyclic core.

6.4.2.5 Structural Requirements

Based on our previous and current observation, the halogenation occurs regardless of the substitution at C-7 with free hydroxy (6.3-6.8) or ester groups (ochrephilone or deschlorosclerotiorin, see Scheme 6-2) readily transforming. It was also observed that the nature of the C-3 side chain was inconsequential to the progress of the reaction but did impact the stability of the molecule, with the more sterically demanding C-3 substituents rendering substrates more stable. Finally the nature of the bicyclic ring tolerates both isochromene-6,8(7H)-diones (e.g. 6.3-6.6) and isoquinoline-6,8(2H,7H)-diones (e.g. 6.7-6.8).

6.4.3 Proposed Reaction Mechanism

Based on these observations, we have proposed a mechanism for the nucleophilic halogenation reaction as described below (Scheme 6-29). We suggest that when Au(OAc)₃ is present (pathway a), the Lewis acidic Au(III) coordinates the electron rich carbon-carbon double bond between C-5 and C-4a, resulting in the increased electrophilicity of this carbon-carbon double bond. Halide (X⁻) then attacks at the less hindered C-5 position and H⁺ is taken at the C-4a position, consistent with the observed reaction outcome. After this the oxygen (or nitrogen in isoquinoline examples) donates a lone pair of electrons expelling hydride as a leaving group and generating an aromatic pyrylium (or pyridinium) cation in the process. Loss of a proton, perhaps concomitantly with the hydride loss effectively producing dihydrogen (H₂) generates the C-5 and C-4a carbon-carbon double bond quenching the cation and thus a halogenated substrate is returned. If Lewis acid Au(OAc)₃ is not present, the Brønsted acid (H⁺) coordinates with the carbon-carbon double bond between C-5 and C-4a, effectively following the same procedure.
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Scheme 6-29 Proposed Mechanism of Nucleophilic Halogenation Reaction

This proposed mechanism explains why the similar halogenation reactions occur in solvents like chloroform (which may contain acid HCl) and why acids are catalysts. Additionally, it can explain the exclusive halogenation at position C-5. If halide reacted at the more sterically congested C-4a, subsequent aromatisation to generate the pyrylium cation would expel halide and subsequent loss of a proton (effective loss of HX) would regenerate the starting material. The increased stability of the halogenated products observed over their non-halogenated counterparts suggests thermodynamic stability selects for these compounds in the complex equilibria. During the reaction, the generation of hydride seems rare, but not unknown with the example of generation being observed in the Cannizzaro Reaction.31

6.4.4 Essential Structure

Based on the proposed mechanism, the key intermediate of this halogenation reaction is the capacity to form a pyrylium (or pyridinium) ion. Thus the bicyclic core 7,8-dihydro-
6H-isochromene or 2,6,7,8-tetrahydroisoquinoline (Figure 6-5) represents the essential structural elements which enable this halogenation reaction. It is worth noting that the 78 azaphilones halogenated at C-5 position possess one of these essential structures (Figure 6-5).

![Figure 6-5 Essential Structure for the Halogenation Reaction](image)

### 6.4.5 Heat Induced Transformation

An interesting rearrangement/degradation was observed for molecules possessing the a 2,2-disubstituted cyclohex-4-ene-1,3-dione such as are present in the azaphilones studied here. While compound purity was readily ascertained from analysis of the $^1$H NMR spectrum (Figure 6-6), upon injection (inlet temperature 250 °C) to the gas chromatogram two peaks in the TIC displaying near identical mass spectra were always observed (Figure 6-7).

![Figure 6-6 $^1$H NMR Spectrum of Compound 6.5 (400 MHz, (CD$_3$)$_2$CO)](image)
The ratio between the two peaks observed in the TIC varied as a function of the inlet temperature indicating the changes were thermally induced. For instance, pure compound **6.5** generates two significant peaks (retention times of 14.21 min and 15.60 min) when the inlet temperature was set to 250 °C (Figure 6-7), however, a single peak (retention time 15.60 min) was observed when the inlet temperature was set to 125 °C. In the mass spectra fragments of [M-42]⁺ and [M-43]⁺ can be observed for each peak but with different ratios (Figure 6-7). It is reasonable that an isomer is generated upon heating to high temperatures, however, all efforts to generate such an isomer and elucidate its structure by heating pure sclerotiorin or its analogues in NMR tubes under argon protection failed. A proposed mechanism of the thermally induced transformation is described below (Scheme 6-30) which can conceivably follow one of two pathways, given a single additional peak is observed at elevated inlet temperatures.

![Gas Chromatogram (TIC) and EI-MS of Compound 6.5 (Inlet 250 °C)](image)

**Scheme 6-30 Proposal Mechanism of Thermo Transformation**

Either pathway produces a molecule that can be rationalised as generating fragments ions of [M-42]⁺ and [M-43]⁺. The HREIMS results confirm that fragments of [M-42]⁺ and
[M-43]$^+$ are [M-C$_2$H$_2$O]$^+$ and [M-C$_2$H$_3$O]$^+$ with a plausible mechanism shown in (Scheme 6-31).

\[
\text{Scheme 6-31 Probable Fragmentations Leading to [M-42]$^+$ and [M-43]$^+$}
\]

6.5 Experimental

6.5.1 General Procedure of Halogenation Reaction via Sodium Halides

A EtOAc solution (5 mL) of pure compound (6.3-6.8, 10 mg) was added to either solid sodium halide (50 mg, excess) or a saturated aqueous solution of the sodium halide (5 mL) at room temperature, then the reaction mixture was stirred for 12 h.

The reaction mixture was ready for GC-MS and/or LC-MS analysis. The production of halogenated product was confirmed by the comparison of its retention time and mass spectrum with that of the reference halogenated product which was previously synthesised.

6.5.2 Equipment and Chemical Reagents

For General Experimental conditions see chapter two experimental section.

6.5.3 Experimental Data

\((+)-\text{sclerotiorin (6.1). A bright orange solid, } [\alpha]_{D}^{20} = + 565 \text{ (c = 0.034, EtOH). } \)

$^1$H NMR (500 MHz, CDCl$_3$):

\[
\delta 0.85 (3H, dd, J = 7.4, 7.4 \text{ Hz, 15-H}), 1.00 (3H, d, J = 6.7 \text{ Hz, 16-H}), 1.10 (3H, ddq, J = 13.2, 7.4, 5.8 \text{ Hz, 14-Hb}), 1.43 (1H, ddq, J = 13.2, 7.4, 5.8 Hz, 14-Ha), 1.56 (3H, s, 7-Me), 1.83 (3H, d, J = 1.1 \text{ Hz, 17-H}), 2.16 (3H, s, 7-\text{COOC}_2\text{H}_3), 2.48 (1H, m, 13-H), 5.70 (1H, brd, J = 9.7 \text{ Hz, 12-H}), 6.07 (1H, d, J = 15.7 \text{ Hz, 9-H}), 6.63 (1H, s, 4-H), 7.05 (1H, d, J = 15.7 \text{ Hz, 10-H}), 7.93 (1H, s, 1-H).$

$^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 12.1 (15-C), 12.5 (17-C), 20.2 (7-\text{COOC}_2\text{H}_3), 20.3 (16-C), 22.6
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(7-Me), 30.2 (14-C), 35.2 (13-C), 84.7 (7-C), 106.5 (4-C), 110.8 (5-C), 114.6 (8a-C), 115.8 (9-C), 132.1 (11-C), 138.9 (4a-C), 143.0 (10-C), 149.0 (12-C), 152.8 (1-C), 158.2 (3-C), 170.2 (7-COOCH3), 186.1 (6-C), 191.9 (8-C). ESIMS: m/z [M+2+H]+ 393 (35), [M+H]+ 391 (100), 351 (15), 349 (46), 305 (17), 303 (48). HREIMS: m/z 390.1242 (calculated for C21H23O535Cl 390.1234), 392.1217 (calculated for C21H23O537Cl 392.1205).

(+) -deschlorosclerotiorin (6.2). A bright orange solid, [α]D10 = + 136 (c = 0.014, EtOH). 1H NMR (500 MHz, C6D6): δ 0.77 (3H, dd, J = 7.5, 7.5 Hz, 15-H), 0.88 (3H, d, J = 6.7 Hz, 16-H), 1.16 (1H, m, 14-Ha), 1.25 (1H, m, 14-Ha), 1.50 (3H, s, 7-Me), 1.57 (3H, s, 17-H), 1.82 (3H, s, 7-COOCH3), 2.24 (1H, m, 13-H), 5.18 (1H, s, 9.8 Hz, 12-H), 5.45 (1H, d, J = 15.8 Hz, 9-H), 5.60 (1H, s, 5-H), 6.68 (1H, d, J = 15.8 Hz, 10-H), 7.36 (1H, s, 1-H). 13C NMR (125 MHz, C6D6): δ 12.1 (15-C), 12.3 (17-C), 20.4 (7-COOCH3), 26.1 (16-C), 29.8 (7-Me), 30.4 (14-C), 35.2 (13-C), 87.5 (7-C), 106.5 (5-C), 108.5 (4-C), 110.8 (8a-C), 116.2 (9-C), 123.4 (11-C), 132.2 (4a-C), 141.2 (10-C), 147.2 (12-C), 152.7 (1-C), 165.3 (3-C), 168.4 (7-COOCH3), 189.3 (6-C), 194.2 (8-C). ESIMS: m/z [M+Na]+ 379 (100), [M+H]+ 357 (70), 315 (37), 273 (18). HREIMS: m/z 356.1627 (calculated for C21H24O5 356.1624). Note: these compounds were acquired in deuterated benzene to prevent chlorination at C-5.

1,3-dimethoxy-2-methylbenzene (6.22). A solution of compound 6.21 (5.0 g, 40.3 mmol) in anhydrous acetone (240 mL) was added to a oven-dried round bottom flask. Then anhydrous K2CO3 (2.0 eq., 11.1 g, 80.6 mmol) and Me2SO4 (2.0 eq., 7.64 mL, 80.6 mmol) were added and the reaction mixture was stirred at refluxed overnight under an argon atmosphere. The reaction mixture was filtered by Buchner funnel, the filtrate was concentrated in vacuo, the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as a colourless oil (99%, 6.1 g, 40.2 mmol). 1H NMR (400 MHz, CDCl3): δ 2.12 (3H, s, 2-Me), 3.84 (6H, s, 1,3-OMe), 6.56 (2H, d, J = 8.4 Hz, 4, 6-H), 7.13 (1H, t, J = 8.4 Hz, 5-H). 13C NMR (100 MHz, CDCl3): δ 8.3 (2-Me), 55.9 (1,3-OMe), 103.7 (4,6-C), 114.7 (2-C), 126.3 (5-C), 158.5 (1,3-C). EIMS: m/z [M]+ 152 (100), 137 (27), 121 (30). HREIMS: m/z 152.0837 (calculated for C9H12O2 152.0837).
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2-hydroxy-4-methoxy-3-methylbenzaldehyde (6.23). A solution of compound 6.22 (0.1 g, 0.7 mmol) in anhydrous DCM (10 mL) was added in a oven-dried 2-neck round bottom flask. Then Cl₂CHOCH₃ (1.25 eq., 75 μL, 0.8 mmol) and AlCl₃ (2.5 eq., 220 mg, 1.7 mmol) were added dropwise to the flask 0 °C under an argon atmosphere, and the mixture was warmed up to room temperature and stirred for 3 h. The reaction mixture was poured onto crushed ice and stirred for 0.5 h, then extracted with DCM three times. The combined organic layer was washed with 1M HCl, then washed with brine and dried over Na₂SO₄ before concentration in vacuo. Then the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as colourless crystals (68%, 74 mg, 0.5 mmol). ¹H NMR (400 MHz, CDCl₃): δ 2.09 (3H, s, 3-Me), 3.91 (3H, s, 4-OMe), 6.55 (1H, d, J= 8.7 Hz, 5-H), 7.36 (1H, d, J= 8.7 Hz, 6-H), 9.71 (1H, s, 1-CHO), 11.44 (1H, s, 2-OH). ¹³C NMR (100 MHz, CDCl₃): δ 7.4 (3-Me), 56.0 (4-OMe), 102.9 (5-C), 113.5 (3-C), 115.6 (1-C), 133.4 (6-C), 161.2 (2-C), 164.5 (4-C), 194.9 (1-CHO). EIMS: m/z [M]+ 166 (100), 148 (26), 136 (21), 77 (19). HREIMS: m/z 166.0631 (calculated for C₉H₁₀O₃ 166.0630).

2,4-dimethoxy-3-methylbenzaldehyde (6.24). A solution of compound 6.22 (0.4 g, 2.7 mmol) in anhydrous DCM (30 mL) was added in a oven-dried 2-neck round bottom flask. Then Cl₂CHOCH₃ (1.25 eq., 310 μL, 3.4 mmol) and TiCl₄ (2.5 eq., 754 μL, 6.8 mmol) were added dropwise to the flask at 0 °C under an argon atmosphere, and the mixture stirred for 1 h. The reaction mixture was poured onto crushed ice and stirred for 0.5 h, then extracted with DCM three times. The combined organic layer was washed with 1M HCl, then washed with brine and dried over Na₂SO₄ before concentration in vacuo. Then the residue was subjected to flash column chromatography on silica using 25% EtOAc in petroleum ether as eluent to return the title compound as colourless crystals (99%, 486 mg, 2.7 mmol). ¹H NMR (400 MHz, CDCl₃): δ 2.15 (3H, s, 3-Me), 3.85 (3H, s, 4-OMe), 3.89 (3H, s, 2-OMe), 6.73 (1H, d, J= 8.8 Hz, 5-H), 7.73 (1H, d, J= 8.8 Hz, 6-H), 10.22 (1H, s, 1-CHO). ¹³C NMR (100 MHz, CDCl₃): δ 8.6 (3-Me), 56.0 (4-OMe), 63.3 (2-OMe), 106.7 (5-C), 120.2 (3-C), 122.9 (1-C), 128.1 (6-C), 162.7 (2-C), 164.1 (4-C), 189.3 (1-CHO). EIMS: m/z [M]+ 180 (100), 163 (88), 135 (55), 77 (47). HREIMS: m/z 180.0786 (calculated for C₁₀H₁₂O₃ 180.0786).
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2-(2,4-dimethoxy-3-methylphenyl)-1,3-dimethylimidazolidine (6.25). A solution of compound 6.23 (2.0 g, 10.9 mmol) in anhydrous toluene (20 mL) was added to an oven-dried round bottom flask. Then N,N'-dimethylethylendiamine (1.2 eq., 1.40 mL, 13.0 mmol) was added to the flask and the reaction mixture refluxed under an argon atmosphere until TLC indicated the starting material was consumed. The reaction mixture was dried over Na₂SO₄ and concentrated in vacuo to return the title compound as a colourless oil (99%, 2.7 g, 10.9 mmol). \(^1\)H NMR (400 MHz, CDCl₃): δ 2.16 (3H, s, 3-Me), 2.19 (6H, s, 1',3'-Me), 2.55-2.597 (2H, m, 4',5'-Ha), 3.35-3.39 (2H, m, 4',5'-Hb), 3.71 (3H, s, 2 or 4-OMe), 3.76 (1H, s, 2'-H), 3.82 (3H, s, 2 or 4-OMe), 6.71 (1H, d, \(J = 8.7\) Hz, 5-H), 7.46 (1H, d, \(J = 8.7\) Hz, 6-H). \(^13\)C NMR (100 MHz, CDCl₃): δ 9.2 (3-Me), 39.7 (1',3'-Me), 53.4 (2,4-OMe), 55.6 (4' or 5'-C), 61.4 (4' or 5'-C), 107.0 (5-C), 118.5 (1 or 3-C), 124.2 (1 or 3-C), 127.1 (6-C), 158.5 (2 or 4-C), 159.1 (2 or 4-C). EIMS: \(m/z \) [M]+ 249 (27), 206 (29), 163 (21), 99 (100). HREIMS: \(m/z\) 249.1603 (calculated for C₁₄H₂₁N₂O₂ 249.1603).

6-iodo-2,4-dimethoxy-3-methylbenzaldehyde (6.26). A solution of compound 6.25 (2.7 g, 10.9 mmol) in anhydrous diethyl ether (50 mL) was added to a oven-dried 2-neck round bottom flask. Then t-BuLi solution (1.3 M in hexane, 1.1 eq., 9.22 mL, 12.0 mmol) was added dropwise to the flask at -55 °C, the mixture was allowed to warm to 0 °C and stirred under an argon atmosphere for 1 h. After that the reaction mixture was cooled down to -78 °C, a solution of 1,2-diiodoethane (1.2 eq., 3.7 g, 13.1 mmol) in anhydrous diethyl ether (28 mL) was added and the reaction mixture warmed to room temperature and stirred for 0.5 h. The reaction mixture was quenched with 2 N HCl (50 mL) and stirred for 0.5 h, then extracted with DCM three times. The combined organic layer was washed with saturated aqueous NH₄Cl and dried over Na₂SO₄ before concentration in vacuo. The residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a colourless solid (87%, 2.9 g, 9.5 mmol). \(^1\)H NMR (400 MHz, CDCl₃): δ 2.09 (3H, s, 3-Me), 3.80 (3H, s, 2-OMe), 3.88 (3H, s, 4-OMe), 7.24 (1H, s, 5-H), 10.05 (1H, s, 1-CHO). \(^13\)C NMR (100 MHz, CDCl₃): δ 8.7 (3-Me), 56.3 (4-OMe), 62.9 (2-OMe), 95.2 (6-C), 119.9 (5-C), 121.9 (3-C), 122.5 (1-C), 162.1 (2-C), 162.9 (4-C), 191.6 (1-CHO). EIMS: \(m/z \) [M]+ 306 (100), 289 (68), 260 (22), 162 (57), 117 (60), 75 (64). HREIMS: \(m/z\) 305.9745 (calculated for C₁₀H₁₁I₂O₂ 305.9753).
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**2,4-dihydroxy-6-iodo-3-methylbenzaldehyde (6.27).** A solution of compound 6.26 (0.4 g, 1.3 mmol) in anhydrous DCM (10 mL) was added to a oven-dried 2-neck round bottom flask. Then BBr₃ solution (1 M in DCM, 4.0 eq., 5.24 mL, 5.2 mmol) was added dropwise to the flask at -78 °C, the reaction mixture was warmed to room temperature and stirred under an argon atmosphere overnight. The reaction mixture was then cooled to 0 °C, quenched with water and stirred for 0.5 h, then extracted with DCM three times. The combined organic layer was washed with brine and dried over Na₂SO₄ before concentration in vacuo. The residue was subjected to flash column chromatography on silica using 30% EtOAc in petroleum ether as eluent to return the title compound as a colourless amorphous solid (86%, 375 mg, 1.4 mmol). ¹H NMR (400 MHz, (CD₃)₂CO): δ 2.02 (3H, s, 3-Me), 7.14 (1H, s, 5-H), 9.81 (1H, s, 1-CHO), 12.80 (1H, s, 2-OH). ¹³C NMR (100 MHz, (CD₃)₂CO): δ 7.4 (3-Me), 100.4 (6-C), 113.1 (1-C), 113.9 (3-C), 121.0 (5-C), 163.8 (4-C), 164.4 (2-C), 201.0 (1-CHO). EIMS: m/z [M⁺] 278 (100), 150 (23), 122 (17), 83 (21). HREIMS: m/z 277.9442 (calculated for C₈H₇O₃I 277.9440).

**2,4-dihydroxy-3-methyl-6-((trimethylsilyl)ethynyl)benzaldehyde (6.28).** A solution of compound 6.27 (35 mg, 0.12 mmol) in anhydrous THF (3 mL) was added to an oven-dried and air-free 2-neck round bottom flask. Then PdCl₂(PPh₃)₂ (5 mol%, 4 mg, 0.006 mmol), Cul (5 mol%, 1 mg, 0.006 mmol), trimethylsilylacetylene (2.0 eq., 36 μL, 0.24 mmol) and triethylamine (1.3 eq., 22 μL, 0.24 mmol) was added to the flask and the reaction mixture was allowed to stir at room temperature under an argon atmosphere for 4 h. The reaction mixture was diluted by 40 mL water, then immediately neutralised by 0.1 M HCl and extracted with EtOAc three times. The combined organic layer was washed with brine and dried over Na₂SO₄ before concentration in vacuo. The residue was subjected to flash column chromatography on silica using 15% EtOAc in petroleum ether as eluent to return the title compound as a yellow solid (99%, 31 mg, 0.12 mmol). ¹H NMR (400 MHz, (CD₃)₂CO): δ 0.26 (9H, s, 2'-TMS), 2.06 (3H, s, 3-Me), 6.69 (1H, s, 5-H), 10.18 (1H, s, 1-CHO), 12.36 (1H, s, 2-OH). ¹³C NMR (100 MHz, (CD₃)₂CO): δ -0.2 (2'-TMS), 7.6 (3-Me), 100.7 (1'-C), 101.4 (2'-C), 113.5 (5-C), 113.8 (3-C), 114.4 (1-C), 126.5 (6-C), 163.5 (4-C), 163.8 (2-C), 195.2 (1-CHO). EIMS: m/z [M⁺] 248 (14), 233 (100).
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6-ethynyl-2,4-dihydroxy-3-methylbenzaldehyde (6.29). A solution of compound 6.28 (31 mg, 0.12 mmol) in anhydrous MeOH (3 mL) was added to a oven-dried round bottom flask. Then anhydrous K₂CO₃ (1.5 eq., 26 mg, 0.18 mmol) was added to the flask and the mixture was stirred at room temperature under an argon atmosphere for 1 h. The reaction mixture was diluted by 30 mL water, then neutralised by 0.5 M HCl and immediately extracted with EtOAc three times. The combined organic layer was washed with brine and dried over Na₂SO₄ before concentration in vacuo. The residue was subjected to flash column chromatography on silica using 35% EtOAc in petroleum ether as eluent to return the title compound as a yellow solid (93%, 21 mg, 0.1 mmol).¹H NMR (400 MHz, (CD₃)₂CO): δ 2.07 (3H, s, 3-Me), 4.06 (1H, s, 2'-H), 6.73 (1H, s, 5-H), 10.19 (1H, s, 1-CHO), 12.39 (1H, s, 2-OH).¹³C NMR (100 MHz, (CD₃)₂CO): δ 7.5 (3-Me), 79.4 (1'-C), 85.4 (2'-C), 113.9 (5-C), 113.9 (3-C), 114.7 (1-C), 125.8 (6-C), 163.2 (4-C), 163.9 (2-C), 195.2 (1-CHO). EIMS: m/z [M]⁺ 176 (100), 148 (30), 91 (30). HREIMS: m/z 176.0473 (calculated for C₁₀H₈O₃ 176.0473).

7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.3). A solution of compound 6.28 (20 mg, 0.08 mmol) in anhydrous toluene (2 mL) was added to a oven-dried 2-neck round bottom flask. Then Au(OAc)₃ (5 mol%, 1 mg, 0.004 mmol) and TFA (200 μL) were added to the flask and the mixture was stirred at room temperature under an argon atmosphere for 2 h. After that IBX (1.5 eq., 34 mg, 0.12 mmol) was added to the flask and the reaction mixture was stirred for 0.5 h. The reaction mixture was diluted by 20 mL EtOAc and washed with deionised water three times, the organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 70% EtOAc in petroleum ether as eluent to return the title compound as a viscous bright yellow oil (99%, 22 mg, 0.08 mmol).¹H NMR (400 MHz, (CD₃)₂CO): δ 0.30 (9H, s, 3-TMS), 1.43 (3H, s, 7-Me), 4.30 (1H, brs, 7-OH), 5.48 (1H, s, 5-H), 6.78 (1H, s, 4-H), 8.04 (1H, s, 1-H).¹³C NMR (100 MHz, (CD₃)₂CO): δ -3.0 (3-TMS), 28.2 (7-Me), 84.3 (7-C), 106.2 (5-C), 116.7 (8a-C), 120.8 (4-C), 142.1 (4a-C), 154.8 (1-C), 170.2 (3-C), 196.9 (8-C), 197.3 (6-C). EIMS: m/z [M]⁺ 264 (32), 222 (100), 193 (44), 73 (84). HREIMS: m/z 264.0818 (calculated for C₁₃H₁₆O₄Si 264.0818).
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6-acetyl-2,4-dihydroxy-3-methylbenzaldehyde (6.31). A solution of compound 6.29 (10 mg, 0.06 mmol) in anhydrous toluene (2 mL) was added to a oven-dried 2-neck round bottom flask. Then Au(OAc)₃ (5 mol%, 1 mg, 0.003 mmol) and TFA (200 μL) were added to the flask and reaction mixture was stirred at room temperature under an argon atmosphere until the starting material was consumed as indicated by TLC. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 40% EtOAc in petroleum ether as eluent to return the title compound as a pale yellow solid (30%, 3 mg, 0.02 mmol). ¹H NMR (400 MHz, (CD₃)₂CO): δ 2.10 (3H, s, 3-Me), 2.59 (3H, s, 2’-H), 7.02 (1H, s, 5-H), 10.03 (1H, s, 1-CHO), 12.86 (1H, s, 2-OH). ¹³C NMR (100 MHz, (CD₃)₂CO): δ 7.8 (3-Me), 29.4 (2’-C), 110.4 (5-C), 111.8 (1-C), 115.4 (3-C), 142.7 (6-C), 162.2 (4-C), 164.8 (2-C), 196.6 (1-CHO). EIMS: m/z [M⁺] 194 (94), 151 (100), 123 (26). HREIMS: m/z 194.0580 (calculated for C₁₀H₁₀O₄).

7-hydroxy-7-methyl-6H-isochromene-6,8(7H)-dione (6.4). A solution of compound 6.3 (13 mg, 0.05 mmol) in EtOAc (6 mL) was added to a round bottom flask. Then TBAF solution (1M in THF, 1.1 eq., 52 μL, 0.05 mmol) was added dropwise to the flask at -78 °C before the mixture was warmed to 0 °C and stirred for 0.5 h. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using 70% EtOAc in acetone as eluent to return the title compound as a pale yellow solid (29%, 3 mg, 0.01 mmol). ¹H NMR (400 MHz, (CD₃)₂CO): δ 1.44 (3H, s, 7-Me), 4.34 (1H, brs, 7-OH), 5.53 (1H, s, 5-H), 6.58 (1H, d, J = 5.8 Hz, 4-H), 7.41 (1H, d, J = 5.8 Hz, 3-H), 7.96 (1H, s, 1-H). ¹³C NMR (100 MHz, (CD₃)₂CO): δ 28.1 (7-Me), 84.4 (7-C), 107.1 (5-C), 113.0 (4-C), 117.6 (8a-C), 143.0 (4a-C), 149.9 (3-C), 153.5 (1-C), 196.6 (8-C), 196.9 (6-C). EIMS: m/z [M⁺] 192 (29), 150 (100), 121 (43), 92 (37), 63 (36). HREIMS: m/z 192.0428 (calculated for C₁₀H₈O₄). HRESIMS: m/z 193.0499 (calculated for C₁₀H₉O₄Na).

2,4-dihydroxy-3-methyl-6-(prop-1'-yn-1'-yl)benzaldehyde (6.32). A solution of compound 6.27 (50 mg, 0.2 mmol) in anhydrous THF (3 mL) was added to a oven-dried and air-free 2-neck round bottom flask, PdCl₂(PPh₃)₂ (5 mol%, 6 mg, 0.009 mmol) and Cul (5 mol%, 2 mg, 0.009 mmol) were added to the flask. Then propyne (5.0 eq., 68 μL, 0.9
mmol) and triethylamine (1.5 eq., 38 μL, 0.3 mmol) were added to the flask at -78 °C, after that the reaction mixture was warmed to room temperature and stirred under an argon atmosphere for 2.5 h. The reaction mixture was diluted by 30 mL water, then neutralised by 0.1 M HCl and extracted with EtOAc three times. The combined organic layer was washed with brine and dried over Na₂SO₄ before concentration in vacuo. The residue was subjected to flash column chromatography on silica using 40% EtOAc in petroleum ether as eluent to return the title compound as a yellow solid (99%, 37 mg, 0.2 mmol). ¹H NMR (400 MHz, (CD₃)₂CO): δ 2.04 (3H, s, 3-Me), 2.10 (3H, s, 3’-H), 6.60 (1H, s, 5-H), 10.16 (1H, s, 1-CHO), 12.40 (1H, s, 2-OH). ¹³C NMR (100 MHz, (CD₃)₂CO): δ 4.1 (3’-C), 7.4 (3-Me), 75.6 (1’-C), 93.5 (2’-C), 112.5 (3-C), 112.9 (5-C), 114.7 (1-C), 128.0 (6-C), 163.3 (4-C), 163.8 (2-C), 195.7 (1-CHO). EIMS: m/z [M]+ 190 (100), 161 (20), 147 (30), 115 (20). HREIMS: m/z 190.0630 (calculated for C₁₁H₁₀O₃ 190.0630).

7-hydroxy-3,7-dimethyl-6H-isochromene-6,8(7H)-dione (6.5). A solution of compound 6.32 (30 mg, 0.16 mmol) in anhydrous toluene (2 mL) was added to a oven-dried 2-neck round bottom flask. Then Au(OAc)₃ (5 mol%, 3 mg, 0.008 mmol) and TFA (200 μL) were added to the flask and the reaction mixture was stirred at room temperature under an argon atmosphere until the starting material was consumed. After that IBX (1.1 eq., 50 mg, 0.18 mmol) was added to the flask, the reaction mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed with deionised water three times, the organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was subjected to flash column chromatography on Sephadex-LH20 using acetone as eluent to return the title compound as a viscous bright orange oil (99%, 33 mg, 0.16 mmol). ¹H NMR (400 MHz, (CD₃)₂CO): δ 1.42 (3H, s, 7-Me), 2.20 (3H, s, 3-Me), 5.44 (1H, s, 5-H), 6.37 (1H, s, 4-H), 7.96 (1H, s, 1-H). ¹³C NMR (100 MHz, (CD₃)₂CO): δ 19.2 (3-Me), 28.3 (7-Me), 84.1 (7-C), 105.7 (5-C), 109.3 (4-C), 116.8 (8a-C), 144.7 (4a-C), 153.7 (1-C), 160.2 (3-C), 196.6 (8-C), 196.8 (6-C). EIMS: m/z [M]+ 206 (27), 164 (100), 135 (39). HREIMS: m/z 206.0581 (calculated for C₁₁H₁₀O₄ 206.0579). HRESIMS: m/z 207.0661 (calculated for C₁₁H₁₁O₄ 207.0657), 229.0478 (calculated for C₁₁H₁₀O₄Na 229.0477).
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(E)-2,4-dihydroxy-3-methyl-6-(pent-3'-en-1'-yn-1'-yl)benzaldehyde (6.33). A solution of compound 6.29 (33 mg, 0.2 mmol) in anhydrous THF (3 mL) was added to a oven-dried and air-free 2-neck round bottom flask. Then PdCl$_2$(PPh$_3$)$_2$ (5 mol%, 7 mg, 0.009 mmol), Cul (5 mol%, 2 mg, 0.009 mmol) and (E)-1-bromo-1-propene (2.0 eq., 32 μL, 0.4 mmol) and triethylamine (1.3 eq., 32 μL, 0.2 mmol) were added to the flask and the reaction mixture was stirred at room temperature under an argon atmosphere for 1 h. After this it was diluted by 30 mL water, then neutralised by 0.1 M HCl and extracted with EtOAc three times. The combined organic layer was washed with brine and dried over Na$_2$SO$_4$ before concentration in vacuo. The residue was subjected to flash column chromatography on silica using 40% EtOAc in petroleum ether as eluent to return the title compound as a yellow solid (53%, 21 mg, 0.1 mmol).

$^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.85 (3H, dd, J= 6.9, 1.8 Hz, 5’-H), 2.05 (3H, s, 3-Me), 5.82 (1H, dq, J= 15.8, 1.8 Hz, 3’-H), 6.37 (1H, dq, J= 15.8, 6.9 Hz, 4’-H), 6.63 (1H, s, 5-H), 10.16 (1H, s, 1-CHO), 12.40 (1H, s, 2-OH). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 7.5 (3-Me), 18.9 (5’-Me), 83.4 (1’-C), 95.1 (2’-C), 110.9 (3’-C), 112.7 (5-C), 113.0 (3-C), 114.3 (1-C), 127.4 (6-C), 142.8 (4’-C), 163.2 (4-C), 163.9 (2-C), 195.4 (1-CHO).

(E)-7-hydroxy-7-methyl-3-(prop-1-en-1-yl)-6H-isochromene-6,8(7H)-dione (6.6). A solution of compound 6.33 (12 mg, 0.06 mmol) in anhydrous toluene (2 mL) was added to an oven-dried 2-neck round bottom flask. Then Au(OAc)$_3$ (5 mol%, 1 mg, 0.003 mmol) and TFA (200 μL) were added to the flask and the reaction mixture was stirred at room temperature under an argon atmosphere until the starting material was consumed. After that IBX (1.5 eq., 24 mg, 0.08 mmol) was added to the flask and the reaction mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed with deionised water three times, the organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 80% EtOAc in acetone as eluent to return the title compound as a viscous bright yellow oil (62%, 8 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.44 (3H, s, 7-Me), 1.92 (3H, dd, J= 7.0, 1.6 Hz, 11-H), 4.30 (1H, brs, 7-OH), 5.52 (1H, s, 5-H), 6.22 (1H, dq, J= 15.7, 1.6 Hz, 9-H), 6.42 (1H, s, 4-H), 6.59 (1H, dq, J= 15.7, 7.0 Hz, 10-H), 7.98 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 18.5 (11-C), 28.3 (7-Me), 84.1 (7-C), 107.0 (5-C), 109.0 (4-C), 116.6 (8a-C), 123.6 (9-C), 135.6 (10-C), 144.4 (4a-C), 153.2 (1-C), 156.3 (3-C), 196.4 (8-C), 196.4 (8-C).
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196.7 (6-C). EIMS: $m/z$ [M]$^+$ 232 (33), 190 (100), 161 (40), 149 (14). HREIMS: $m/z$ 232.0736 (calculated for C$_{13}$H$_{12}$O$_4$ 232.0736). HRESIMS: $m/z$ 255.0628 (calculated for C$_{13}$H$_{12}$O$_4$Na 255.0633).

7-hydroxy-2-(2-hydroxyethyl)-7-methyl-3-(trimethylsilyl)isoquinoline-6,8(2H,7H)-dione (6.7). A solution of compound 6.3 (10 mg, 0.04 mmol) in anhydrous acetone (3 mL) was added to a oven-dried 2-neck round bottom flask. Then 2-aminoethanol (1.0 eq., 2 μL, 0.04 mmol) was added to the flask and the mixture was stirred at room temperature under an argon atmosphere for 48 h before it was concentrated in vacuo. The residue was subjected to flash column chromatography on silica using acetone as eluent to return the title compound as a bright red oil (82%, 10 mg, 0.03 mmol). $^1$H NMR (400 MHz, CD$_3$OD): δ 0.43 (9H, s, 3-TMS), 1.46 (3H, s, 7-Me), 3.82 (2H, m, 2’-H), 4.08 (2H, m, 1’-H), 5.21 (1H, s, 5-H), 6.81 (1H, s, 4-H), 8.08 (1H, s, 1-H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ -0.21 (3-TMS), 29.1 (7-Me), 59.6 (1’-C), 62.0 (2’-C), 83.8 (7-C), 97.4 (5-C), 116.7 (8a-C), 127.6 (4-C), 144.2 (1-C), 151.4 (4a-C), 156.1 (3-C), 197.3 (6-C), 201.4 (8-C). EIMS: $m/z$ [M]$^+$ 307 (32), 264 (60), 236 (18), 73 (100). HREIMS: $m/z$ 307.1241 (calculated for C$_{15}$H$_{21}$NO$_4$Si 307.1240). HRESIMS: $m/z$ 308.1317 (calculated for C$_{15}$H$_{22}$NO$_4$Si 308.1318).

7-hydroxy-2-(2-hydroxyethyl)-7-methylisoquinoline-6,8(2H,7H)-dione (6.8). A solution of compound 6.7 (12 mg, 0.04 mmol) in MeOH (3 mL) was added to a round bottom flask. Then 2-aminoethanol (1.0 eq., 2 μL, 0.04 mmol) was added to the flask and the mixture was stirred at room temperature until the starting material was consumed. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using acetone as eluent to return the title compound as a bright red oil (60%, 6 mg, 0.02 mmol). $^1$H NMR (400 MHz, CD$_3$OD): δ 1.47 (3H, s, 7-Me), 3.82 (2H, t, $J$ = 4.8 Hz, 2’-H), 3.98 (2H, t, $J$ = 4.8 Hz, 1’-H), 5.22 (1H, s, 5-H), 6.62 (1H, d, $J$ = 7.3 Hz, 4-H), 7.33 (1H, d, $J$ = 7.3 Hz, 3-H), 8.02 (1H, s, 1-H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 29.1 (7-Me), 60.3 (1’-C), 61.6 (2’-C), 83.8 (7-C), 97.5 (5-C), 117.4 (8a-C), 118.2 (4-C), 140.2 (3-C), 142.7 (1-C), 153.1 (4a-C), 196.8 (6-C), 201.2 (8-C). EIMS: $m/z$ [M]$^+$ 235 (72), 192 (100), 164 (32), 148 (86), 87 (58). HREIMS: $m/z$ 235.0847 (calculated for C$_{12}$H$_{13}$NO$_4$ 235.0845). HRESIMS: $m/z$ 236.0921 (calculated for C$_{12}$H$_{14}$NO$_4$ 236.0923), 258.0741 (calculated for C$_{12}$H$_{13}$NO$_4$Na 258.0742).
5-chloro-7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.35).

Method One (electrophilic substitution): A solution of compound 6.3 (15 mg, 0.06 mmol) in acetone (3 mL) was added to a round bottom flask wrapped with aluminium foil to keep the contents protected from light. Then NCS (1.1 eq., 9 mg, 0.07 mmol) was added to the flask and the reaction mixture was stirred at room temperature under dark conditions overnight. The reaction mixture was concentrated in vacuo, the residue was subjected to flash column chromatography on silica using 55% EtOAc in petroleum ether as eluent to return the title compound as a viscous bright yellow oil (80%, 14 mg, 0.05 mmol).

Method Two (nucleophilic substitution): A solution of compound 6.28 (109 mg, 0.44 mmol) in toluene (2 mL) was added to a round bottom flask. Then Au(OAc)$_3$ (5 mol%, 8 mg, 0.022 mmol) and TFA (200 μL) were added to the flask and the mixture was stirred at room temperature until the starting material was consumed. Then IBX (1.5 eq., 185 mg, 0.66 mmol) was added to the flask and the reaction mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed with brine three times, the organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 55% EtOAc in petroleum ether as eluent to return the title compound as a viscous bright yellow oil (80%, 93 mg, 0.3 mmol).

The data sets for both compounds were identical. $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 0.35 (9H, s, 3-TMS), 1.47 (3H, s, 7-Me), 6.95 (1H, s, 4-H), 8.12 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ -3.0 (3-TMS), 27.6 (7-Me), 84.8 (7-C), 109.6 (5-C), 116.4 (8a-C), 116.8 (4-C), 137.3 (4a-C), 154.1 (1-C), 172.9 (3-C), 190.8 (6-C), 195.5 (8-C). EIMS: $m/z$ [M+2]$^+$ 300 (6), [M]$^+$ 298 (16), 258 (40), 256 (100), 73 (43). HREIMS: $m/z$ 300.0400 (calculated for C$_{13}$H$_{15}$O$_4$Si$_{37}$Cl 300.0399), 298.0420 (calculated for C$_{13}$H$_{15}$O$_4$Si$_{35}$Cl 298.0428).

5-bromo-7-hydroxy-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.36). A solution of compound 6.28 (20 mg, 0.08 mmol) in toluene (2 mL) was added to a round bottom flask. Then Au(OAc)$_3$ (5 mol%, 2 mg, 0.004 mmol) and TFA (200 μL) was added to the flask, reaction mixture was stirred at room temperature until the starting material was consumed. Then IBX (1.5 eq., 34 mg, 0.12 mmol) was added to the flask and the mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed...
with saturated aqueous NaBr solution three times, the organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using 85% EtOAc in petroleum ether as eluent to return the title compound as a viscous bright yellow oil (99%, 28 mg, 0.08 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): $\delta$ 0.35 (9H, s, 3-TMS), 1.47 (3H, s, 7-Me), 6.97 (1H, s, 4-H), 8.10 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): $\delta$ -3.0 (3-TMS), 27.8 (7-Me), 84.9 (7-C), 101.8 (5-C), 117.2 (8a-C), 119.4 (4-C), 139.7 (4a-C), 154.2 (1-C), 173.2 (3-C), 191.1 (6-C), 195.6 (8-C). EIMS: $m/z$ [M+2]$^+$ 344 (6), [M]$^+$ 342 (6), 302 (38), 300 (38), 248 (100), 231 (68), 203 (19), 73 (39). HREIMS: $m/z$ 343.9904 (calculated for C$_{13}$H$_{15}$O$_4$Si$_{81}$Br 343.9903), 341.9921 (calculated for C$_{13}$H$_{15}$O$_4$Si$_{79}$Br 341.9923).

7-hydroxy-5-iodo-7-methyl-3-(trimethylsilyl)-6H-isochromene-6,8(7H)-dione (6.37). A solution of compound 6.3 (12 mg, 0.05 mmol) in acetone (3 mL) was added to a round bottom flask wrapped with aluminium foil. Then NIS (1.2 eq., 12 mg, 0.054 mmol) was added to the flask and the mixture was stirred at room temperature under dark conditions for 0.5 h. The reaction mixture was then concentrated in vacuo, the residue was subjected to flash column chromatography on silica using 90% EtOAc in petroleum ether as eluent to return the title compound as a viscous bright yellow oil (85%, 15 mg, 0.04 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): $\delta$ 0.36 (9H, s, 3-TMS), 1.46 (3H, s, 7-Me), 4.61 (1H, s, 7-OH), 6.97 (1H, s, 4-H), 7.98 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): $\delta$ -3.0 (3-TMS), 28.0 (7-Me), 81.5 (5-C), 84.2 (7-C), 118.1 (8a-C), 124.5 (4-C), 143.8 (4a-C), 154.1 (1-C), 173.5 (3-C), 192.8 (6-C), 196.0 (8-C). EIMS: $m/z$ [M]$^+$ 390 (24), 348 (100), 263 (34), 73 (54). HREIMS: $m/z$ 389.9782 (calculated for C$_{13}$H$_{15}$O$_4$Si$_{127}$I 389.9784). HRESIMS: $m/z$ 390.9857 (calculated for C$_{13}$H$_{15}$O$_4$Si$_{127}$I 390.9863).

5-chloro-7-hydroxy-7-methyl-6H-isochromene-6,8(7H)-dione (6.39). A solution of compound 6.35 (9 mg, 0.03 mmol) in EtOAc (2 mL) was added to a round bottom flask, TBAF solution (1.0 M in THF, 1.2 eq., 34 μL, 0.03 mmol) was added dropwise and the mixture was stirred at room temperature for 10 min. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (99%, 8 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): $\delta$ 1.49 (3H, s, 7-Me), 4.69 (1H, s, 7-OH), 6.84 (1H, d, J= 5.8
Hz, 4-H), 7.65 (1H, d, J= 5.8 Hz, 3-H), 8.05 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 27.5 (7-Me), 84.9 (7-C), 109.7 (4-C), 110.5 (5-C), 117.3 (8a-C), 138.6 (4a-C), 151.9 (3-C), 152.8 (1-C), 190.5 (6-C), 195.3 (8-C). EIMS: m/z [M+2]$^+$ 228 (11), [M]$^+$ 226 (20), 186 (33), 184 (100), 149 (34). HREIMS: m/z 228.0014 (calculated for C$_{10}$H$_7$O$_4$Cl 228.0003), 226.0038 (calculated for C$_{10}$H$_7$O$_4$Cl 226.0033). HRESIMS: m/z 250.9904 (calculated for C$_{10}$H$_7$O$_4$NaCl 250.9901), 248.9932 (calculated for C$_{10}$H$_7$O$_4$NaCl 248.9931).

5-bromo-7-hydroxy-7-methyl-6H-isochromene-6,8(7H)-dione (6.40).

A solution of compound 6.35 (10 mg, 0.03 mmol) in EtOAc (2 mL) was added to a round bottom flask, TBAF solution (1.0 M in THF, 1.2 eq., 36 μL, 0.04 mmol) was added dropwise and the mixture was stirred at room temperature for 10 min. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (99%, 8 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.49 (3H, s, 7-Me), 4.69 (1H, s, 7-OH), 6.87 (1H, d, J= 5.8 Hz, 4-H), 7.67 (1H, d, J= 5.8 Hz, 3-H), 8.04 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 27.6 (7-Me), 84.9 (7-C), 102.5 (5-C), 112.3 (4-C), 118.0 (8a-C), 141.0 (4a-C), 152.2 (3-C), 152.9 (1-C), 190.8 (6-C), 195.4 (8-C). EIMS: m/z [M+2]$^+$ 272 (9), [M]$^+$ 270 (9), 248 (100), 230 (36), 228 (38), 203 (14), 149 (19). HREIMS: m/z 271.9508 (calculated for C$_{10}$H$_7$O$_4$Br 271.9507), 269.9529 (calculated for C$_{10}$H$_7$O$_4$Br 269.9528). HRESIMS: m/z 294.9406 (calculated for C$_{10}$H$_7$O$_4$NaBr 294.9405), 292.9423 (calculated for C$_{10}$H$_7$O$_4$NaBr 292.9425).

5-chloro-7-hydroxy-3,7-dimethyl-6H-isochromene-6,8(7H)-dione (6.41).

A solution of compound 6.5 (11 mg, 0.06 mmol) in acetone (3 mL) was added to a round bottom flask wrapped with aluminium foil. NCS (1.5 eq., 11 mg, 0.08 mmol) was added to the flask and the mixture was stirred at room temperature under dark conditions overnight before it was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (55%, 7 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.47 (3H, s, 7-Me), 2.33 (3H, s, 3-Me), 4.65 (1H, s, 7-OH), 6.67 (1H, s, 4-H), 8.04 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 19.5 (3-Me), 27.7 (7-Me), 84.6 (7-C), 106.2 (4-C), 109.2 (5-C), 116.4 (8a-C), 140.2 (4a-C), 153.1 (1-C), 162.6 (3-C), 190.3 (6-C), 195.3 (8-C). EIMS: m/z [M+2]$^+$ 242 (6),
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[M]+ 240 (16), 200 (33), 198 (100). HREIMS: m/z 242.0159 (calculated for C_{11}H_{9}O_{4}{^{37}}Cl 242.0160), 240.0187 (calculated for C_{11}H_{9}O_{4}{^{35}}Cl 240.0189). HRESIMS: m/z 265.0058 (calculated for C_{11}H_{9}O_{4}Na{^{37}}Cl 265.0058), 263.0087 (calculated for C_{11}H_{9}O_{4}Na{^{35}}Cl 263.0087).

5-bromo-7-hydroxy-3,7-dimethyl-6H-isochromene-6,8(7H)-dione (6.42).

Method One: A solution of compound 6.5 (12 mg, 0.06 mmol) in acetone (3 mL) was added to a round bottom flask, NBS (1.2 eq., 12 mg, 0.07 mmol) was added and the mixture was stirred at room temperature for 10 min before it was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (89%, 15 mg, 0.05 mmol).

Method Two: A solution of compound 6.32 (9 mg, 0.05 mmol) in toluene (2 mL) was added to a round bottom flask, then Au(OAc)$_3$ (5 mol%, 1 mg, 0.002 mmol) and TFA (200 μL) was added to the flask and the mixture was stirred at room temperature until the starting material was consumed. IBX (1.5 eq., 29 mg, 0.11 mmol) was added and the reaction mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed with saturated aqueous NaBr solution three times, the organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (67%, 9 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.47 (3H, s, 7-Me), 2.33 (3H, s, 3-Me), 6.69 (1H, s, 4-H), 8.02 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 19.6 (3-Me), 27.8 (7-Me), 84.7 (7-C), 101.1 (5-C), 108.8 (4-C), 117.1 (8a-C), 142.5 (4a-C), 153.1 (1-C), 163.0 (3-C), 190.6 (6-C), 195.4 (8-C). EIMS: m/z [M+2]$^+$ 286 (16), [M]$^+$ 284 (16), 244 (98), 242 (100), 99 (40). HREIMS: m/z 285.9665 (calculated for C$_{11}$H$_9$O$_4${$^{81}}$Br 285.9664), 283.9685 (calculated for C$_{11}$H$_9$O$_4${$^{79}}$Br 283.9684). HRESIMS: m/z 308.9565 (calculated for C$_{11}$H$_9$O$_4$Na{81}Br 308.9561), 306.9583 (calculated for C$_{11}$H$_9$O$_4$Na{79}Br 306.9582).

(E)-5-chloro-7-hydroxy-7-methyl-3-(prop-1-en-1-yl)-6H-isochromene-6,8(7H)-dione (6.43). A solution of compound 6.33 (10 mg, 0.05 mmol) in toluene (2 mL) was added to a
round bottom flask, Au(OAc)$_3$ (5 mol%, 1 mg, 0.002 mmol) and TFA (200 μL) was added and the mixture was stirred at room temperature until the starting material was consumed. IBX (1.5 eq., 19 mg, 0.07 mmol) was added and the mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed with brine three times. The organic layer was dried over Na$_2$SO$_4$ then concentrated in vacuo and the residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (68%, 8 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.48 (3H, s, 7-Me), 1.95 (3H, dd, J= 7.0, 1.5 Hz, 11-H), 4.65 (1H, brs, 7-OH), 6.39-6.43 (1H, m, 9-H), 6.68 (1H, s, 4-H), 6.69-6.75 (1H, m, 10-H), 8.06 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 18.7 (11-C), 27.7 (7-Me), 84.6 (7-C), 105.8 (4-C), 110.4 (5-C), 116.2 (8a-C), 123.8 (9-C), 137.2 (10-C), 140.0 (4a-C), 152.6 (1-C), 158.1 (3-C), 190.2 (6-C), 195.1 (8-C). EIMS: m/z [M+2]* 268 (13), [M]+ 266 (19), 226 (34), 224 (100). HREIMS: m/z 268.0308 (calculated for C$_{13}$H$_{11}$O$_4$Cl 268.0316), 266.0333 (calculated for C$_{13}$H$_{11}$O$_4$Cl 266.0346). HRESIMS: m/z 291.0227 (calculated for C$_{13}$H$_{11}$O$_4$Na$^{37}$Cl 291.0214), 289.0243 (calculated for C$_{13}$H$_{11}$O$_4$Na$^{35}$Cl 289.0244).

![Chemical Structure](E)-5-bromo-7-hydroxy-7-methyl-3-(prop-1-en-1-yl)-6H-isochromene-6,8(7H)-dione (6.44). A solution of compound 6.33 (10 mg, 0.05 mmol) in toluene (2 mL) was added to a round bottom flask, Au(OAc)$_3$ (5 mol%, 1 mg, 0.002 mmol) and TFA (200 μL) were added and the mixture was stirred at room temperature until the starting material was consumed. Then IBX (1.5 eq., 19 mg, 0.07 mmol) was added and the mixture was stirred for 0.5 h before it was diluted by 20 mL EtOAc and washed with saturated aqueous NaBr solution three times. The organic layer was collected, dried over Na$_2$SO$_4$ and concentrated in vacuo and the residue was subjected to flash column chromatography on silica using EtOAc as eluent to return the title compound as a viscous bright yellow oil (46%, 7 mg, 0.02 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO): δ 1.48 (3H, s, 7-Me), 1.95 (3H, dd, J= 7.0, 1.6 Hz, 11-H), 4.64 (1H, brs, 7-OH), 6.43 (1H, dq, J= 15.6, 1.6 Hz, 9-H), 6.70 (1H, s, 4-H), 6.71 (1H, dq, J= 15.6, 7.0 Hz, 10-H), 8.04 (1H, s, 1-H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO): δ 18.7 (11-C), 27.9 (7-Me), 84.7 (7-C), 102.5 (5-C), 108.4 (4-C), 117.0 (8a-C), 123.7 (9-C), 137.3 (10-C), 142.4 (4a-C), 152.6 (1-C), 158.4 (3-C), 190.5 (6-C), 195.2 (8-C). EIMS: m/z [M+2]* 312 (20), [M]+ 310 (20), 270 (96), 268 (100). HREIMS: m/z 311.9831 (calculated for C$_{13}$H$_{11}$O$_4^{81}$Br 311.9820), 309.9846 (calculated for C$_{13}$H$_{11}$O$_4^{79}$Br
309.9841). HRESIMS: m/z 334.9718 (calculated for C_{13}H_{11}O_{4}Na^{81}Br 334.9718), 332.9732 (calculated for C_{13}H_{11}O_{4}Na^{79}Br 332.9738).

5-chloro-7-hydroxy-2-(2-hydroxyethyl)-7-methyl-3-(trimethylsilyl)isoquinoline-6,8(2H,7H)-dione (6.45). A solution of compound 6.35 (20 mg, 0.08 mmol) in anhydrous acetone (3 mL) was added to a oven-dried 2-neck round bottom flask. 2-Aminoethanol (1.0 eq., 5 μL, 0.08 mmol) was added and the mixture was stirred at room temperature under an argon atmosphere for 10 min. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using acetone as eluent to return the title compound as a bright red oil (93%, 22 mg, 0.06 mmol). \(^1\)H NMR (400 MHz, CD_{3}OD): δ 0.47 (9H, s, 3-TMS), 1.47 (3H, s, 7-Me), 3.85 (2H, m, 2'-H), 4.16 (2H, J= 5.2 Hz, 1'-H), 7.22 (1H, s, 4-H), 8.15 (1H, s, 1-H). \(^{13}\)C NMR (100 MHz, CD_{3}OD): δ -0.23 (3-TMS), 28.8 (7-Me), 59.7 (1'-C), 62.0 (2'-C), 84.6 (7-C), 100.6 (5-C), 116.9 (8a-C), 123.6 (4-C), 143.3 (1-C), 146.2 (4a-C), 158.3 (3-C), 189.9 (6-C), 199.7 (8-C). EIMS: m/z [M+2]^{+} 343 (14), [M]^{+} 341 (35), 301 (27), 299 (75), 271 (13), 269 (32), 75 (30), 73 (100). HREIMS: m/z 343.0823 (calculated for C_{15}H_{20}NO_{4}Si^{37}Cl 343.0821), 341.0849 (calculated for C_{15}H_{20}NO_{4}Si^{35}Cl 341.0850). HRESIMS: m/z 344.0895 (calculated for C_{15}H_{21}NO_{4}Si^{37}Cl 344.0899), 342.0926 (calculated for C_{15}H_{21}NO_{4}Si^{35}Cl 342.0928).

5-bromo-7-hydroxy-2-(2-hydroxyethyl)-7-methyl-3-(trimethylsilyl)isoquinoline-6,8(2H,7H)-dione (6.46). A solution of compound 6.36 (14 mg, 0.04 mmol) in anhydrous acetone (3 mL) was added to a oven-dried 2-neck round bottom flask. 2-aminoethanol (1.0 eq., 3 μL, 0.04 mmol) was added and the mixture was stirred at room temperature under an argon atmosphere for 10 min. The reaction mixture was concentrated in vacuo and the residue was subjected to flash column chromatography on silica using acetone as eluent to return the title compound as a bright red oil (99%, 17 mg, 0.04 mmol). \(^1\)H NMR (400 MHz, CD_{3}OD): δ 0.48 (9H, s, 3-TMS), 1.47 (3H, s, 7-Me), 3.85 (2H, J= 5.0 Hz, 2'-H), 4.16 (2H, J= 5.0 Hz, 1'-H), 7.29 (1H, s, 4-H), 8.13 (1H, s, 1-H). \(^{13}\)C NMR (100 MHz, CD_{3}OD): δ -0.23 (3-TMS), 28.9 (7-Me), 59.7 (1'-C), 62.0 (2'-C), 84.7 (7-C), 91.6 (5-C), 117.6 (8a-C), 126.3 (4-C), 143.2 (1-C), 147.8 (4a-C), 158.3 (3-C), 190.3 (6-C), 199.9 (8-C). EIMS: m/z [M+2]^{+} 387 (19), [M]^{+} 385 (18), 345 (31), 343 (35), 306 (48), 75 (31), 73 (100). HREIMS: m/z 387.0326 (calculated for C_{15}H_{21}NO_{4}Si^{37}Cl 387.0325), 385.0348 (calculated for C_{15}H_{21}NO_{4}Si^{35}Cl 385.0350).
C_{15}H_{20}NO_4Si^{81}Br 387.0325, 385.0335 (calculated for C_{15}H_{20}NO_4Si^{79}Br 385.0345). HRESIMS: m/z 388.0408 (calculated for C_{15}H_{21}NO_4Si^{81}Br 388.0403), 386.0427 (calculated for C_{15}H_{21}NO_4Si^{79}Br 386.0423).

5-chloro-7-hydroxy-2-(2-hydroxyethyl)-7-methylisoquinoline-6,8(2H,7H)-dione (6.47). A solution of compound 6.45 (22 mg, 0.06 mmol) in MeOH (3 mL) was added to a round bottom flask. 2-aminoethanol (1.0 eq., 4 μL, 0.06 mmol) was added to the flask and the mixture was stirred at room temperature for 10 min. The reaction mixture was concentrated in vacuo and the residue was washed with petroleum ether twice, followed by EtOAc twice and finally by MeOH three times to return the title compound as a bright red solid (90%, 16 mg, 0.06 mmol). \(^1\)H NMR (400 MHz, (CD$_3$)$_2$SO): \(\delta\) 1.30 (3H, s, 7-Me), 3.65-3.68 (2H, m, 2'-H), 4.00 (2H, t, \(J = 5.0\) Hz, 1'-H), 5.39 (1H, s, 7-OH), 6.79 (1H, d, \(J = 7.4\) Hz, 4-H), 7.60 (1H, d, \(J = 7.4\) Hz, 3-H), 8.04 (1H, s, 1-H). \(^{13}\)C NMR (100 MHz, (CD$_3$)$_2$SO): \(\delta\) 27.6 (7-Me), 58.4 (1'-C), 59.9 (2'-C), 82.7 (7-C), 98.9 (5-C), 112.2 (4-C), 115.2 (8a-C), 140.5 (1-C), 140.8 (3-C), 144.9 (4a-C), 186.9 (6-C), 197.8 (8-C). EIMS: m/z \([M+2]^+\) 271 (24), \([M]^+\) 269 (74), 229 (34), 227 (100), 184 (24), 182 (55). HREIMS: m/z 271.0430 (calculated for C$_{12}$H$_{12}$NO$_4^{37}$Cl 271.0425), 269.0445 (calculated for C$_{12}$H$_{12}$NO$_4^{35}$Cl 269.0455). HRESIMS: m/z 272.0511 (calculated for C$_{12}$H$_{13}$NO$_4^{37}$Cl 272.0504), 270.0537 (calculated for C$_{12}$H$_{13}$NO$_4^{35}$Cl 270.0533).

5-bromo-7-hydroxy-2-(2-hydroxyethyl)-7-methylisoquinoline-6,8(2H,7H)-dione (6.48). A solution of compound 6.46 (15 mg, 0.04 mmol) in MeOH (3 mL) was added to a round bottom flask. 2-aminoethanol (1.0 eq., 3 μL, 0.20 mmol) was added and the mixture was stirred at room temperature for 10 min. The reaction mixture was concentrated in vacuo and the residue was washed with petroleum ether twice, followed by EtOAc twice and finally by MeOH three times to return the title compound as a bright red solid (99%, 13 mg, 0.04 mmol). \(^1\)H NMR (400 MHz, (CD$_3$)$_2$SO): \(\delta\) 1.30 (3H, s, 7-Me), 3.65-3.68 (2H, m, 2'-H), 4.00 (2H, t, \(J = 4.8\) Hz, 1'-H), 5.39 (1H, s, 7-OH), 6.83 (1H, d, \(J = 7.4\) Hz, 4-H), 7.60 (1H, d, \(J = 7.4\) Hz, 3-H), 8.02 (1H, s, 1-H). \(^{13}\)C NMR (100 MHz, (CD$_3$)$_2$SO): \(\delta\) 27.7 (7-Me), 58.4 (1'-C), 59.9 (2'-C), 82.8 (7-C), 90.6 (5-C), 114.8 (4-C), 116.0 (8a-C), 140.5 (1-C), 141.0 (3-C), 146.6 (4a-C), 187.3 (6-C), 197.9 (8-C). EIMS: m/z \([M+2]^+\) 315 (24), \([M]^+\) 313 (19), 273 (23), 271 (24), 69 (100). HREIMS: m/z 314.9938 (calculated...
for C\textsubscript{12}H\textsubscript{12}NO\textsubscript{4}\textsuperscript{81}Br 314.9929, 312.9950 (calculated for C\textsubscript{12}H\textsubscript{12}NO\textsubscript{4}\textsuperscript{79}Br 312.9950).

HRESIMS: \textit{m/z} 337.9836 (calculated for C\textsubscript{12}H\textsubscript{12}NO\textsubscript{4}Na\textsuperscript{81}Br 337.9827), 335.9842 (calculated for C\textsubscript{12}H\textsubscript{12}NO\textsubscript{4}Na\textsuperscript{79}Br 335.9847).

6.6 References

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6.7 Appendix NMR Spectra of Novel Compounds

Figure 6-8 $^1$H NMR Spectrum of Compound 6.1 (500 MHz, CDCl$_3$)

Figure 6-9 $^{13}$C NMR Spectrum of Compound 6.1 (125 MHz, CDCl$_3$)

Figure 6-10 $^1$H NMR Spectrum of Compound 6.2 (500 MHz, C$_6$D$_6$)

Figure 6-11 $^{13}$C NMR Spectrum of Compound 6.2 (125 MHz, C$_6$D$_6$)

Figure 6-12 $^1$H NMR Spectrum of Compound 6.27 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-13 $^{13}$C NMR Spectrum of Compound 6.27 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-14 $^1$H NMR Spectrum of Compound 6.3 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-15 $^{13}$C NMR Spectrum of Compound 6.3 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-16 $^1$H NMR Spectrum of Compound 6.31 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-17 $^{13}$C NMR Spectrum of Compound 6.31 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-18 $^1$H NMR Spectrum of Compound 6.4 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-19 $^{13}$C NMR Spectrum of Compound 6.4 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-20 $^1$H NMR Spectrum of Compound 6.32 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-21 $^{13}$C NMR Spectrum of Compound 6.32 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-22 $^1$H NMR Spectrum of Compound 6.5 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-23 $^{13}$C NMR Spectrum of Compound 6.5 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-24 $^1$H NMR Spectrum of Compound 6.6 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-25 $^{13}$C NMR Spectrum of Compound 6.6 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-26 $^1$H NMR Spectrum of Compound 6.7 (400 MHz, CD$_3$OD)

Figure 6-27 $^{13}$C NMR Spectrum of Compound 6.7 (100 MHz, CD$_3$OD)

Figure 6-28 $^1$H NMR Spectrum of Compound 6.8 (400 MHz, CD$_3$OD)

Figure 6-29 $^{13}$C NMR Spectrum of Compound 6.8 (100 MHz, CD$_3$OD)

Figure 6-30 $^1$H NMR Spectrum of Compound 6.35 (400 MHz, (CD$_3$)$_2$CO)
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Figure 6-31 $^{13}$C NMR Spectrum of Compound 6.35 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-32 $^1$H NMR Spectrum of Compound 6.36 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-33 $^{13}$C NMR Spectrum of Compound 6.36 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-34 $^1$H NMR Spectrum of Compound 6.37 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-35 $^{13}$C NMR Spectrum of Compound 6.37 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-36 $^1$H NMR Spectrum of Compound 6.39 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-37 $^{13}$C NMR Spectrum of Compound 6.39 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-38 $^1$H NMR Spectrum of Compound 6.40 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-39 $^{13}$C NMR Spectrum of Compound 6.40 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-40 $^1$H NMR Spectrum of Compound 6.41 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-41 $^{13}$C NMR Spectrum of Compound 6.41 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-42 $^1$H NMR Spectrum of Compound 6.42 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-43 $^{13}$C NMR Spectrum of Compound 6.42 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-44 $^1$H NMR Spectrum of Compound 6.43 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-45 $^{13}$C NMR Spectrum of Compound 6.43 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-46 $^1$H NMR Spectrum of Compound 6.44 (400 MHz, (CD$_3$)$_2$CO)

Figure 6-47 $^{13}$C NMR Spectrum of Compound 6.44 (100 MHz, (CD$_3$)$_2$CO)

Figure 6-48 $^1$H NMR Spectrum of Compound 6.45 (400 MHz, CD$_3$OD)

Figure 6-49 $^{13}$C NMR Spectrum of Compound 6.45 (100 MHz, CD$_3$OD)

Figure 6-50 $^1$H NMR Spectrum of Compound 6.46 (400 MHz, CD$_3$OD)

Figure 6-51 $^{13}$C NMR Spectrum of Compound 6.46 (100 MHz, CD$_3$OD)

Figure 6-52 $^1$H NMR Spectrum of Compound 6.47 (400 MHz, (CD$_3$)$_2$SO)

Figure 6-53 $^{13}$C NMR Spectrum of Compound 6.47 (100 MHz, (CD$_3$)$_2$SO)

Figure 6-54 $^1$H NMR Spectrum of Compound 6.48 (400 MHz, (CD$_3$)$_2$SO)

Figure 6-55 $^{13}$C NMR Spectrum of Compound 6.48 (100 MHz, (CD$_3$)$_2$SO)
**Figure 6-8** $^1$H NMR Spectrum of Compound 6.1 (500 MHz, CDCl$_3$)
Figure 6-9 $^{13}$C NMR Spectrum of Compound 6.1 (125 MHz, CDCl$_3$)
Figure 6-10 $^1$H NMR Spectrum of Compound 6.2 (500 MHz, $C_6D_6$)
Figure 6-11 $^{13}$C NMR Spectrum of Compound 6.2 (125 MHz, C$_6$D$_6$)
Figure 6-12 $^1$H NMR Spectrum of Compound 6.27 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-13 $^{13}$C NMR Spectrum of Compound 6.27 (100 MHz, $(CD_3)_2CO$)
**Figure 6-14** $^1$H NMR Spectrum of Compound 6.3 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-15: $^{13}$C NMR Spectrum of Compound 6.3 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-16 $^1$H NMR Spectrum of Compound 6.31 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-17: $^{13}$C NMR Spectrum of Compound 6.31 (100 MHz, (CD$_3$)$_2$CO)
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Figure 6-18 $^1$H NMR Spectrum of Compound 6.4 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-19 $^{13}$C NMR Spectrum of Compound 6.4 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-20 $^1$H NMR Spectrum of Compound 6.32 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-21 $^{13}$C NMR Spectrum of Compound 6.32 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-22 $^1$H NMR Spectrum of Compound 6.5 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-23 $^{13}$C NMR Spectrum of Compound 6.5 (100 MHz, $(CD_3)_2CO$)
Figure 6-24 $^1H$ NMR Spectrum of Compound 6.6 (400 MHz, $(CD_3)_2CO$)
Figure 6-25 $\textsuperscript{13}$C NMR Spectrum of Compound 6.6 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-26 $^1$H NMR Spectrum of Compound 6.7 (400 MHz, CD$_3$OD)
Figure 6-27 $^{13}$C NMR Spectrum of Compound 6.7 (100 MHz, CD$_3$OD)
Figure 6-28 $^1$H NMR Spectrum of Compound 6.8 (400 MHz, CD$_3$OD)
Figure 6.29 $^{13}$C NMR Spectrum of Compound 6.8 (100 MHz, CD$_3$OD)
Figure 6-30 $^1$H NMR Spectrum of Compound 6.35 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-31 $^{13}$C NMR Spectrum of Compound 6.35 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-32 $^1$H NMR Spectrum of Compound 6.36 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-33 $^{13}$C NMR Spectrum of Compound 6.36 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-34 $^1$H NMR Spectrum of Compound 6.37 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-35 $^{13}$C NMR Spectrum of Compound 6.37 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-36 $^1$H NMR Spectrum of Compound 6.39 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-37 $^{13}$C NMR Spectrum of Compound 6.39 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-38 $^1$H NMR Spectrum of Compound 6.40 (400 MHz, (CD$_3$)$_2$CO)
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Figure 6-39 $^{13}$C NMR Spectrum of Compound 6.40 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-40 $^1$H NMR Spectrum of Compound 6.41 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-41 $^{13}$C NMR Spectrum of Compound 6.41 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-42 $^1$H NMR Spectrum of Compound 6.42 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-43 $^{13}$C NMR Spectrum of Compound 6.42 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-44 $^1$H NMR Spectrum of Compound 6.43 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-45 $^{13}$C NMR Spectrum of Compound 6.43 (100 MHz, (CD$_3$)$_2$CO)
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Figure 6-46 $^1$H NMR Spectrum of Compound 6.44 (400 MHz, (CD$_3$)$_2$CO)
Figure 6-47 $^{13}$C NMR Spectrum of Compound 6.44 (100 MHz, (CD$_3$)$_2$CO)
Figure 6-48 $^1$H NMR Spectrum of Compound 6.45 (400 MHz, CD$_3$OD)
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Figure 6-49 $^{13}$C NMR Spectrum of Compound 6.45 (100 MHz, CD$_3$OD)
Figure 6-50 $^1$H NMR Spectrum of Compound 6.46 (400 MHz, CD$_3$OD)
Figure 6-51 $^{13}$C NMR Spectrum of Compound 6.46 (100 MHz, CD$_3$OD)
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Figure 6-52 $^1$H NMR Spectrum of Compound 6.47 (400 MHz, (CD$_3$)$_2$SO)
Figure 6-53 $^{13}$C NMR Spectrum of Compound 6.47 (100 MHz, (CD$_3$)$_2$SO)
Figure 6-54 $^1$H NMR Spectrum of Compound 6.48 (400 MHz, (CD$_3$)$_2$SO)
Figure 6-55 $^{13}$C NMR Spectrum of Compound 6.48 (100 MHz, (CD$_3$)$_2$SO)