Ethnomycology and Bioprospecting Studies from Papua New Guinea

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

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Author’s Declaration

Except where due acknowledgement has been made, the work presented in this report is my own. A portion of the work presented in Chapter 2: collection of mushrooms (section 2.4) was carried out in the forest area that surrounds the Kiovi, Waefo and Kopanka clans in the Eastern Highlands Province of Papua New Guinea. Collection of specimens from the Kiovi and Waefo was carried out under the leadership of Mr Stewart Wossa and Mr John Nema respectively. In addition, initial extraction (section 2.6.1) was performed within the Science Department, University of Goroka. With this exception, all of the research was carried out within the Research School of Chemistry at The Australian National University during the period of March 2012 and July 2016, under the supervision of Dr Russell A. Barrow. No part of this thesis has been previously submitted for any other degree.

Sequencing experiments for the characterisation of Fulaga dive described in Section 3.3, was carried out in the Research School of Biology at The Australian National University, in collaboration with Dr Peter Solomon. PCR reactions, BLAST search and phylogenetic analysis were performed by Mr Elisha Thynne.

Edwin Moisés Castillo Martínez

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Dedico esta tesis a mi madre, Marta Dolores Martínez, por su amor, trabajo y sacrificio, por sus palabras de aliento, por enseñarme desde niño a luchar para alcanzar y cumplir con mis metas. Con muchísimo cariño, mi más eterna gratitud.
Abstract

Papua New Guineans have medicinal knowledge based on thousands of years of using flora, fauna and fungi as medicines. However, factors such as poor documentation and an increase in the use of allopathic medicine within the island have promoted the rapid loss of such medicinal knowledge. Thus the work presented in this thesis has been directed towards the preservation of first hand traditional knowledge, as it pertains to the use of mushrooms by indigenous inhabitants of Papua New Guinea, in particular those mushrooms with medicinal potential.

A compilation of biologically active secondary metabolites isolated from terrestrial and marine fungi from Papua New Guinea under ethnomycology is presented in chapter one. A brief account of ethnomycology by early Europeans and inhabitants of the new world in pre-Columbian times is also presented.

Chapter two describes the in vitro biological testing of mushrooms used by the Kiovi, Waefo and Kopanka clans. Turbidity-MTT microdilution assay was used to determine the susceptibility of Gram (+) and Gram (-) bacteria against 52 crude mushroom extracts resulting in approximately 31% of the samples tested active against at least one of the organisms used. Ethnomycological comparisons between the Kiovi, Waefo and Kopanka are also presented.

The work described in chapter three includes ethnomycological and taxonomical background of Fulaga dive, common name given to a mushroom member of the Amanitaceae family and used by the Kiovi tribe for its edibility and because it makes them “feel well”. Bioassay guided isolation, purification and structural elucidation of two novel furan fatty acids is detailed.

Chapter four details the synthetic route of the two novel furan fatty acids isolated from Fulaga dive. The synthesis was achieved from commercially available furan. Degradation of this class of compounds is also discussed, where (Z)-9-(5-pentylfuran-2-
yl)-non-8-enoic acid isomerises to the more stable (E)-diastereoisomer followed by olefinic cleavage producing 5-pentyl-2-furaldehyde.

Chapter five reports the synthesis of 26 compounds, that are furan based homologues and thiophene based analogues of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid. Homologues and analogues were assayed for their antibacterial potential against a panel of bacterial human pathogens using the turbidity-MTT assay and their radical scavenging properties using the DPPH assay.

Finally, chapter six includes ethnomycological and taxonomical background of Igura hivi, a mushroom also used by the Kiovi tribe for its edibility and treat stomach complaints. Bioassay guided isolation, purification and structural elucidation of the known antibiotic grifolin is presented. The spectroscopic data obtained for the isolated fungal metabolite matched that reported in the literature.
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<th>Definition</th>
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<tbody>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ANU</td>
<td>The Australian National University</td>
</tr>
<tr>
<td>AS/NZS</td>
<td>Australia/New Zealand Standard™</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BAIB</td>
<td>bis-Acetoxyiodobenzene</td>
</tr>
<tr>
<td>BC</td>
<td>Before Common Era</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Candida albicans (C.P.Robin) Berkhout</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism spectroscopy</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>CNRRRD</td>
<td>Centre for natural resources research and development</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>COSY</td>
<td>homonuclear correlation spectroscopy</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli Castellani and Chalmers</td>
</tr>
<tr>
<td>E. durans</td>
<td>Enterococcus durans Schleifer &amp; Kilpper-Baeiz</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>Enterococcus faecalis (Andrewes and Horder) Schleifer and Kilpper-Baeiz</td>
</tr>
<tr>
<td>E. faecium</td>
<td>Enterococcus faecium (Orla-Jensen) Schleifer &amp; Kilpper-Baeiz</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>Et-</td>
<td>Ethyl-</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>F. graminearum</td>
<td>Fusarium graminearum (Schwein.) Petch</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>F-acid</td>
<td>Furan fatty acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HMPA</td>
<td>Hexamethylphosphoramide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>High resolution</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence spectroscopy</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration of test sample to inhibit 50% of the growth of the organism</td>
</tr>
<tr>
<td>ICBG</td>
<td>International cooperative biodiversity group</td>
</tr>
<tr>
<td>IR</td>
<td>Infra red spectroscopy</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribe spacer</td>
</tr>
<tr>
<td>KAPA</td>
<td>Potassium amino propylamide</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>LR</td>
<td>Low resolution</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>Mycobacterium tuberculosis Zopf</td>
</tr>
<tr>
<td>Me-</td>
<td>Methyl-</td>
</tr>
<tr>
<td>MHA</td>
<td>Mueller Hinton agar</td>
</tr>
<tr>
<td>MHB</td>
<td>Mueller Hinton broth</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant S. aureus</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectroscopy</td>
</tr>
<tr>
<td>MSp</td>
<td>Mushroom sample</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazolyl blue tetrazolium bromide</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAPA</td>
<td>Sodium amino propylamide</td>
</tr>
<tr>
<td>NBS</td>
<td>N-Bromosuccinamide</td>
</tr>
<tr>
<td>n-Bu-</td>
<td>n-Butyl-</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>-OH</td>
<td>Alcohol</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Pseudomonas aeruginosa Migula</td>
</tr>
<tr>
<td>P. betulinus</td>
<td>Piptoporus betulinus (Bull.) P. Karst</td>
</tr>
<tr>
<td>P. citrinum</td>
<td>Penicillium citrinum Thom, C.</td>
</tr>
<tr>
<td>P. microspora</td>
<td>Pestalotiopsis microspore (Spec.) G.C. Zhao &amp; N. Li</td>
</tr>
<tr>
<td>PC</td>
<td>Physical containment</td>
</tr>
</tbody>
</table>
PCR | Polymerase chain reaction
--- | ---
PDC | Pyridinium dichromate
PNG | Papua New Guinea
PU- | Polyunsaturated
ROESY | Rotating frame nuclear Overhouser effect spectroscopy
RSA | Radical scavenging activity
RSC | Research School of Chemistry
*S. aerenicola* | *Salinispora aerenicola*
*S. aureus* | *Staphylococcus aureus* Rosenbach
*S. epidermidis* | *Staphylococcus epidermidis* Evans
*S. scaberrinae* | *Saurauia scaberrinae* Willd.
*S. Typhi* | *Salmonella Typhi* Le Minor & Popoff
SDS | Sodium dodecyl sulphate
*T. morobensis* | *Terminalia morobensis* L.
*T. virens* | *Trichoderma virens* Persoon
T-acid | Thiophene fatty acid
THP | Tetrahydropyran
TIC | Total ion current
UoG | University of Goroka
UV-vis | Ultraviolet-visible spectroscopy
Val | Valine
ZoI | Zone of inhibition
β (-) | Beta lactamase negative
β (+) | Beta lactamase positive
Chapter One

Natural products in drug discovery: an ethnomycological approach


This chapter defines ethnomycology and entails a review of secondary metabolites isolated from fungi collected from the highalnds of Papua New Guinea (PNG).
Chapter 1 : Natural products in drug discovery

1.1 Ethnomycology

Ethnomycology (Gr. ethno = nation + mykes = mushroom + logos = discourse), etymologically, is the study of historical, cultural and sociological uses of fungi by humans.\(^1,2\) As a field of study, ethnomycology began sometimes between the 1950s and ‘60s through the studies of Wasson and Heim in the use of mushrooms for religious purposes in different cultures around the world.\(^3\) However, the degree of popularity ethnomycology has achieved is recent, despite evidence suggesting that mushrooms have been utilised by humans as a source of food, tinder and medicine for thousands of years.\(^1\)

Carbon-14 dating analysis of a painting of 13 objects with mushroom appearance found in a cave closed to Villar del Humo (smoking villages) in Spain, and reports of mycologist Gastón Guzmán, who suggested that the paintings could be of the hallucinogenic fungus *Psilocybe hispanica* Guzmán, were indicative that early Europeans may have used hallucinogenic mushrooms for religious purposes about 6000 years ago.\(^4\) Figure 1.1 shows the mural of a bull and bottom right objects with mushroom appearance.

![Figure 1.1: Earliest evidence for magic mushrooms use in Europe.\(^4\) Photograph retrieved December 2015.](image)
Stories of the interrelationships between fungi and early Europeans do not end in Spain. Ötzi – the iceman (Figure 1.2), a mummy that is on display at the South Tyrol Museum of Archaeology in Bolzano, Italy, is also evidence of the ethnological linkages between humans and fungi. Ötzi was found in the Italian Alps close to the border with Austria. Carbon-14 dating analysis of Ötzi and his belongings revealed that the iceman lived between 3350 and 3100 BC (Before Common Era). Therefore he was alive around 5000 years ago. Amongst his implements, Ötzi carried attached to a string the bracket fungus birch polypore (*Piptoporus betulinus* (Bull.) P. Karst), which is known for its antibiotic potential. It is very likely that Ötzi used the birch polypore as medicine to treat intestinal parasites that he suffered from.\(^5\)

![Figure 1.2: Top left, Ötzi – mummy on display. Bottom left, birch polypore found amongst Ötzi's belongings. Picture on the right, representation of what Ötzi's appearance might have been. Photographs retrieved December 2015 from South Tyrol Museum of Archaeology’s website.\(^5\)](image)
In 2000, Schlegel and co-workers\textsuperscript{6} isolated piptamine (1.1), shown in Figure 1.3, from the fungus birch polypore (\textit{P. betulinus} (Bull.) P. Karst, strain Lu9-1) collected in Thuringia, Germany. Schlegel confirmed that this birch polypore was active against a number of Gram (+) bacteria and fungi, especially against \textit{Staphylococcus aureus} Rosenbach (strain SG511) (MIC 0.78 \(\mu\)g.mL\textsuperscript{-1}) and \textit{Enterococcus faecalis} (Andrewes and Horder) Schleifer and Kilpper-Baeiz (strain 1528) (MIC 1.56 \(\mu\)g.mL\textsuperscript{-1}).\textsuperscript{6}

\centerline{1.1}

\textit{Figure 1.3: Piptamine (1.1) isolated from \textit{P. betulinus} (Bull.) P. Karst (Germany)}

In Mesoamerica several sculptures with mushroom appearance were unearthed in the late 1800s.\textsuperscript{7} However, in early 1960s archaeological investigations were performed on the “enigmatic” objects. Figure 1.4 shows miniature mushroom stones found in Guatemala, which based on their morphology all stones were dated back to pre-Columbian times in a Late Preclassic 1000 – 500 BC.\textsuperscript{8}

\textit{Figure 1.4: Miniature mushroom stones from Guatemala.\textsuperscript{8} Retrieved December 2015}
Since the discovery of such sculptures there was controversy whether Mayans in the south part of Mexico and Guatemala had any cultural or sociological linkages to mushrooms. The controversy was due to the absence of documentation linking the sculptures to the Mayans of that time. However, there was evidence suggesting that Mayans used the hallucinogenic mushroom *Psilocybe cubensis* (Earle) Singer, as part of their religious rituals, hence it was plausible that the sculptures with mushroom appearance found in Mesoamerica were in fact associated with the uses of mushrooms by the Mayans.

Papua New Guinea (PNG) is not the exception. Mushrooms have been an important part of everyday lives of Papua New Guineans for thousands of years for their edibility, medicine and economical livelihood. However, very little has been done to preserve firsthand information as it pertains to the use of mushrooms by Papua New Guineans and therefore, that knowledge is getting rapidly lost. Figure 1.5 shows local villagers in the highlands of PNG selling mushrooms in support of their livelihood.

Figure 1.5: Roadside market Kiovi village, PNG (2012). Men selling mushrooms. Photograph by Anson Barish (UoG). Reproduced with permission.
Ethnomycological studies in PNG have been carried out previously. However, such studies have mostly focused on the use of psychotropic mushrooms in particular the so-called “mushroom madness” excluding hundreds and possibly thousands of mushroom species with medicinal potential that could lead to the discovery of new drugs.\(^3\) In 2001, Hawksworth\(^{10}\) re-estimated the number of fungal species in the world to be of 1.5 million species. Although new evidence indicated that Hawksworth’s estimation was too low, he thought prudent to retain his estimations until a consensus was created to decide whether to increase the number or not.\(^{10}\) The consensus came and new evidence indicated that Hawksworth did underestimate the number of fungal species in the world with 3.5 million species now thought to be conservative.\(^{11}\)

The purpose of this chapter is to present secondary metabolites that have been isolated from macro and/or microfungi from terrestrial and/or marine origins, specifically from PNG. Although there exist several reviews covering secondary metabolites from marine sources,\(^{12,13}\) or fungal metabolites as pharmaceuticals,\(^{14}\) to the best of my knowledge, this is the first attempt at listing natural products isolated from fungi from PNG.

### 1.2 Secondary metabolites isolated from fungi

This review is of the literature from 2002 to present, and it describes 43 compounds from 14 articles and a personal communication. The chemical structures of the 43 natural products isolated from fungi from PNG are shown. Structures of synthetic bioactive homologues or analogues are not shown in this review.

In 2002, Strobel and co-workers\(^{15}\) obtained the endophyte *Pestalotiopsis microspora* (Speg.) G.C. Zhao & N. Li, from the stem of the plant *Terminalia morobensis* L. that grows in the outflowing part of the Sepik river in PNG. Upon bioassay guided purification these authors isolated an isobenzofuranone and with X-ray crystallography for structural elucidation, they named the isolated compound isopestacin (1.2), shown in Figure 1.6. It is worth noting that isopestacin (1.2) was crystallised as two molecules in an asymmetric unit, suggesting its natural existence as a racemic mixture.\(^{15}\)
In vitro biological testings of the isolated isobenzofuranone showed moderate antifungal activity (40 µg.mL⁻¹) against the plant pathogen oomycete Pythium ultimum Trow. In addition, due to structural similarities of the isolated compound to flavonoids, these authors investigated its antioxidant potential. The radical scavenging activity of isopestacin (1.2) was similar to that of ascorbic acid (positive control), as isopestacin (1.2) was able to scavenge hydroxyl free radicals (EC₅₀ = 0.22 mM) based on results by electron spin resonance measurements.¹⁵

The following year, Harper and co-workers,¹⁶ while working with the endophyte P. microspora (Speg.) G.C. Zhao & N. Li extracted from T. morobensis L., isolated a 1,3-dihydro-isobenzofuran, and by X-ray crystallography and NMR spectroscopy analysis, these authors named the isolated compound as pestacin (1.3) (Figure 1.7).¹⁶
Pestacin (1.3) was found to crystallise as one molecule per asymmetric unit, suggesting its natural existence as a mixture of enantiomers. Harper and co-workers\textsuperscript{16} proposed a racemisation mechanism, which proceeded via a cationic intermediate stabilised by seven resonance structures and able to be further stabilised via tautomerisation to the neutral ortho-quinone methide-like structures, as shown in Figure 1.8.

Figure 1.8: A: proposed racemisation mechanism of pestacin (1.3) and B: postulated resonance stabilised structures of cationic intermediate of pestacin (1.3) by Harper and co-workers\textsuperscript{16}
Pestacin (1.3) showed antifungal and antioxidant activities similar to that of isopestacin (1.2). This was not at all surprising because they were related compounds. Pestacin (1.3) exhibited an EC$_{50}$ of 1.7 ± 0.1 mM according to total oxyradical scavenging capacity method, and when compared to trolox (vitamin E derivative) used as a control (EC$_{50}$ 18.8 ± 0.9 mM), pestacin (1.3) appeared to be more effective as an antioxidant.

Pestacin (1.3) was also evaluated for its antimycotic activity. Thus, pestacin (1.3) showed a MIC of 10 µg.mL$^{-1}$ against Pythium ultimum Trow. These results appeared relatively similar to those reported for isopestacin (1.2).$^{16}$

In 2003, Garo and co-workers$^{17}$ isolated the fungus Trichoderma virens Persoon (strains CNL910 and CNK266), identified following morphological and phylogenetic analyses, from the marine ascidian Didemnum molle and the green alga Halimeda sp. J. V. Lamouroux respectively.

The marine invertebrate and alga used for the extraction of T. virens Persoon were collected in the Bismarck Sea off the coast of Madang in PNG.$^{17}$ These authors reported the isolation of dipeptides trichoderamides A and B (1.4 and 1.5) and two known antibiotics gliovirin (1.6) and heptelic acid chlorohydrin (1.7) in small quantities (Figure 1.9).$^{17}$
Trichodermamide A (1.4) was shown to possess (R) stereochemistry at C-8 position by esterification with Mosher’s acid chloride, which resulted in (R)- and (S)-MTPA esters (1.4a and 1.4b) respectively. Analysis of the $^1$H NMR spectra of both esters helped to support those results, as there was evidence for the (R)-MTPA ester (1.4a) olefinic protons at positions C-6 and C-7 were shifted upfield, while the proton at position C-9 moved downfield, while for the (S)-MTPA ester (1.4b) the opposite was observed.

These authors suggested that the total absolute stereochemistry of trichodermamide B (1.4) was 4S, 5R, 8R and 9S. In addition, the absolute stereochemistry of trichodermamide B (1.5) could be determined based on the relative stereochemistry obtained from X-ray analysis and Mosher’s method in the same manner as for trichodermamide A (1.4).
Trichodermamide B (1.5) exhibited in vitro antibacterial activity against amphotericin-resistant *Candida albicans* (C.P.Robin) Berkhout, methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *Enterococcus faecium* (Orla-Jensen) Schleifer & Kilpper-Baeiz (MIC 15 µg.mL\(^{-1}\)). In addition, trichodermamide B (1.5) showed activity against HCT-116 human colon carcinoma (IC\(_{50}\) 0.32 µg.mL\(^{-1}\)).

Trichodermamide A (1.4) did not show any antibacterial or anticancer activity against the organisms tested.\(^{17}\) With regards to gliovirin (1.6) and heptelidic acid chlorohydrin (1.7), these authors did not report any experimental data, as they were known compounds. Chemical structures of gliovirin (1.6) and heptelidic acid chlorohydrin (1.7), shown in this review, were reported by other authors.\(^{18,19}\)

The same year, Amagata and co-workers\(^{20}\) extracted the fungus *Penicillium citrinum* Thom, C. from the sponge, *Axinella sp.* Schmidt from PNG, and reported the discovery of two novel rearranged steroids, isocyclocitrinol A (1.8) and 22-acetylisocyclocitrinol A (1.9) (Figure 1.10).\(^{20}\)

![Figure 1.10: Isocyclocitrinol A (1.8) and 22-acetylisocyclocitrinol A (1.9)](image_url)
It was firstly suggested that isocyclocitrinol A (1.8) was an analogue of the known sesterterpenoid cyclocitrinol (1.8a) shown in Figure 1.11 because of the similarities in the $^{13}$C NMR spectrum. Although extensive NMR experiments were performed, these authors were unable to match the core of cyclocitrinol (1.8a).

The structure of cyclocitrinol (1.8a) was revised by these authors based on comparisons of the $^{13}$C spectra between isocyclocitrinol A (1.8) and cyclocitrinol (1.8a), concluding with the rearranged steroid cyclocitrinol (1.8b), as shown in Figure 1.11.

The chemical structure of isocyclocitrinol A (1.8) was elucidated by X-ray analysis of 22-acetylisocyclocitrinol A (1.9), which consisted of a five, six and two-seven membered ring systems.

X-ray analysis provided guidance for the relative configurations of all asymmetric centres in 22-acetylisocyclocitrinol A (1.9) and the absolute stereochemistry was determined by Mosher’s method. The absolute stereochemistry of isocyclocitrinol A (1.8) and 22-acetylisocyclocitrinol A (1.9) was $3S$, $5S$, $9R$, $14R$, $17S$, $19S$, $20R$ and $22S$ in accordance with comparisons between the spectral data of isocyclocitrinol A (1.8) and Mosher products of 22-acetylisocyclocitrinol A (1.9).
In vitro biological testings indicated weak antibacterial activity of isocyclocitrinol A (1.8) and 22-acetylisocyclocitrinol A (1.9) against *S. epidermidis* and *Enterococcus durans* Schleifer & Kilpper-Baeiz.

In 2006, Amagata and colleagues\(^20\) obtained a fungus (strain 001314c) from a yellow fan-shaped sponge (collection no. 00314, *Ianthella* sp. Pallas) from PNG. Based on DNA sequencing analyses, the closest relative of strain 001314c was *Fusarium graminearum* (Schwein.) Petch. However, due to a genetic distance of 9.75%, these authors were unable to establish the taxonomy of strain 001314c.

Bioassay guided purifications and LC-MS experiments helped Amagata and co-workers\(^20\) in the discovery of three novel cyclic depsipeptides: guangomides A and B (1.10 and 1.11) and homodestcardin (1.12) shown in Figure 1.12.

![Figure 1.12: Guangomides A and B (1.10 and 1.11), homodestcardin (1.12)](image-url)
In general, assignments of the absolute stereochemistry for the three compounds were on the basis of results compiled from HPLC analysis of products derived from Marfey’s acid hydrolysis, NMR experiments, X-ray analysis and observations of optical rotation.

Thus, these authors concluded that guangomide A (1.10) possessed the absolute stereostructure 2S, 9S, 13S, 19S, 24R and 28R. In addition, the absolute stereostructure of guangomide B (1.11) was determined to be the same as that for guangomide A (1.10) according to comparisons between observations of the optical rotations and NMR data for each asymmetric centre.

With regards to the absolute stereostructure of homodestcardin (1.12), this was achieved by comparisons of NMR experimental data and analogy between homodestcardin (1.12) and known compounds homodestruxin B and roseocardin, not shown in this review. In vitro examination of guangomides A and B (1.10 and 1.11) indicated weak antibacterial activity against S. epidermidis and E. durans.21

In 2006, Boot and co-workers22 obtained the fungus Acremonium sp. Link (collection no. 021172cKZ) from cultures of a sponge (Teichaxinella sp. collection no. 02172) collected in Milne Bay, PNG.

The fungus Acremonium sp. Link (collection no. 021172cKZ) was submitted further purification via a bioassay guided fractionation. Upon fractionation, these authors isolated two novel N-methylated linear octapeptides RHM1 and RHM2 (1.13 and 1.14) together with the known efrapeptin G (1.15), shown in Figure 1.13.
Although the analysis of amino acids was not an easy task, these authors used extensive NMR and ESIMS experiments to elucidate the structure of the compounds isolated. Regarding RHM1 (1.13), the configuration was found to be Ac-(R)-Gln-(2S,3S)-Ile-(S)-N-Me-Leu-(2S,3S)-Ile-(S)-N-Me-Val-(2S,3S)-N-Me-Ile-(2S,3S)-N-Me-Val-(2S,3S)-N-Me-Ile-OH, as determined by Marfey’s method.\(^\text{22}\)
With regards to the configuration of RHM2 (1.14), these authors reported: Ac-(R)-Glu-(S)-Val-(S)-N-Me-Leu-(S)-N-Me-Val-(2S,3S)-Ile-(2S,3S)-N-Me-Ile-(2S,3S)-N-Me-Ile-(2S,3S)-N-Me-Ile-OH. This sequence was postulated based on the similarities of biosynthetic origins of RHM1 and RHM2 (1.13 and 1.14).\(^{22}\)

Finally, *in vitro* antibacterial testings indicated that *S. epidermidis* was susceptible to RHM1 (1.13) (MIC 50 µg.mL\(^{-1}\)) and efrapeptin G (1.15) (MIC 80 µg.mL\(^{-1}\)) with RHM2 (1.14) being the least active of the three compounds (MIC > 400 µg.mL\(^{-1}\)). It is worth noting that RHM1 (1.13) and RHM2 (1.14) showed weak cytotoxicity (potency < 16) relative to that shown by efrapeptin G (1.15) (potency 3300) against solid tumour murine cancer cells. It should also be noted that no controls were used in toxicity test.\(^{22}\)

The following year Boot and co-workers\(^{23}\) re-assayed the *Acremonium* sp. Link (strain 021172c) and isolated two novel efrapeptins E\(\alpha\) and H (1.16 and 1.17) as well as the known efrapeptin F (1.18), all shown in Figure 1.14. In addition, the previously reported efrapeptin G (1.15) was also isolated.

Similarly to efrapeptin G (1.15) the elucidation of efrapeptins E\(\alpha\) and H (1.16 and 1.17) was based on NMR and extensive MS data analyses. With regards to the stereostructures of efrapeptins E\(\alpha\) and H (1.16 and 1.17) these authors suggested L stereochemistry for each of their amino acids. *In vitro* biological testings of efrapeptin G, E\(\alpha\) and F (1.15, 1.16 and 1.18) against H125 human lung carcinoma cells exhibited IC\(_{50}\) of 1.3 nM across the three compounds.\(^{23}\)
Boot and colleagues\textsuperscript{23} isolated two novel $N$-methylated linear octapeptides, RHM3 and RHM4 (1.19 and 1.20), from an *Acremonium* sp. Link (strain 021172c). The stereostructures of RHM3 and RHM4 (1.19 and 1.20) shown in Figure 1.15, were relative to that determined for RHM1 (1.13). In addition, two known $N$-methylated scytalediamides A and B (1.21 and 1.22), shown in Figure 1.15, commonly found in *Scytaledium* sp. fungal strains, were also isolated from an *Acremonium* sp. Link (strain 021172c).
These authors did not report any information regarding 1.21 and 1.22 stereostructures, as both were known compounds. Structures of 1.21 and 1.22 shown in Figure 1.15 were reported by other authors.\(^\text{24}\) *In vitro* antibacterial testing for novel RHM3 and RHM4 (1.19 and 1.20) were not performed.\(^\text{23}\)

Figure 1.15: Octapeptides RHM3 and RHM4 (1.19 and 1.20) and scytalidamides A and B (1.21 and 1.22)
Also in 2007, Oh and co-workers\textsuperscript{25} prepared co-cultures of two different microorganisms, as a way to induce the production of novel chemotypes. These authors cultured the marine-derived fungus, \textit{Emericella sp}. (strain CNL-878, isolated from green algae \textit{Halimeda sp}. J. V. Lamouroux Madang Bay, PNG) and the actinomycete, \textit{Salinispora arenicola} Maldonado et al. (strain CNH-665) in a single vessel to replicate microorganism interactions, as it would occur in a natural environment, to induce the production of new natural products. Oh and co-workers\textsuperscript{25} isolated two novel cyclic depsipeptides emericellamides A and B (\textbf{1.23} and \textbf{1.24}) shown in Figure 1.16.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.16}
\caption{Emericellamides A and B (1.23 and 1.24)}
\end{figure}

Relative configurations were assigned for emericellamide A (\textbf{1.23}), as 21\textsuperscript{R*}, 22\textsuperscript{R*} and 23\textsuperscript{S*}, based on \textit{J}-based configurational analyses, using $^3J_{HH}$, $^3J_{CH}$ and NOE correlations.\textsuperscript{25, 26} In addition, the absolute stereochemistry was reported as C-21 \textit{R}, C-22 \textit{R} and C-23 \textit{S} according to Mosher’s derivatisation method.

Oh and co-workers\textsuperscript{25} also found that the amino acid residues in emericellamide A (\textbf{1.23}) possessed \textit{L} stereochemistry, according to comparisons between Marfey standards of \textit{L}- and \textit{D}- Ala, Val and Leu and hydrolysis products of emericellamide A (\textbf{1.23}). The stereostructure of emericellamide B (\textbf{1.24}) was based on comparisons with emericellamide A (\textbf{1.23}) data resulting in 21\textit{R}, 22\textit{R}, 23\textit{S} and 25\textit{S}.\textsuperscript{25}
In vitro antibacterial assessment of the two depsipeptides showed that emericellamide A (1.23) exhibited moderate activity against MRSA (MIC 3.8 µM) and weak cytotoxicity against HCT-116 human colon carcinoma cells (IC\textsubscript{50} 23 µM). In vitro biological activity of emericellamide B (1.24) was weaker than that shown by emericellamide A (1.23) showing an MIC of 6.0 µM against MRSA strains and IC\textsubscript{50} of 40 µM against HCT-116 human colon carcinoma cells.

In 2008 Hoffman and co-workers\textsuperscript{27} isolated the endophyte Phoma sp. Saccardo (strain NG-25) from the lower crown of Saurauia scaberrinae Willd. found near Goroka in the Eastern Highlands Province of PNG. These authors isolated two natural products, usnic acid (1.25) and cercosporamide (1.26), known in the literature for their phytotoxic and antifungal properties.\textsuperscript{27} In addition, Hoffman and co-workers also isolated a new natural product named phomodione (1.27), which is an analogue of usnic acid (1.25). Their structures are shown in Figure 1.17.

![Figure 1.17: Usnic acid (1.25), cercosporamide (1.26) and phomodione (1.27)](image)

Phomodione (1.27) was characterised using HRMS, 1D and 2D NMR experiments and X-ray crystallography analyses. Relative abundances of fragment ions of phomodione (1.27) suggested close relation to usnic acid (1.25). In addition, phomodione (1.27) possess a relative stereochemistry C-4a S\textsuperscript{*} and C-9b R\textsuperscript{*} and it was determined by X-ray crystallography, NMR, IR and Raman spectroscopy.
In vitro antibacterial activity of the three compounds indicated selectivity and similar biological activity against S. aureus according to the diameter of the zone of inhibition (5 mm), as per the disc diffusion assay.

Furthermore, cercosporamide (1.26) and phomodione (1.27) exhibited similar antimycotic activity against the plant pathogens Pythium ultimum Trow (MIC 3 – 5 µg.mL⁻¹), Sclerotinia sclerotiorum (Lib.) de Bary (MIC 3 - 8 µg.mL⁻¹) and Rhizoctonia solani J. G. Kühn (MIC 5 – 10 µg.mL⁻¹), while usnic acid (1.25) was less effective to inhibit the growth of the fungal species tested (MIC > 10 µg.mL⁻¹).²⁷

In 2013 Wijeratne and co-workers²⁸ also isolated phomodione (1.27) from the endophyte Phoma sp. Saccardo (strain NRRL-46751), as Hoffman and colleagues²⁷ did, from the lower crown of the plant Saurauia scaberrinae Willd. in the Eastern Highlands Province in PNG. Phomapyrrolidones A, B and C (1.28, 1.29 and 1.30), shown in Figure 1.18, were isolated in an effort to discover novel antitubercular agents.

Extensive NMR spectroscopy and X-ray crystallography analyses were crucial for the elucidation of the structures of the three alkaloids.

Figure 1.18: Phomapyrrolidones A - C (1.28 – 1.30)
The total relative configuration of phomapyrrolidone A (1.28) was depicted as $1S^*, 4R^*, 7S^*, 8R^*, 10S^*, 12R^*, 13S^*, 14S^*, 15R^*, 16S^*, 1'TS^*$ and $18R^*$ on the basis of comparisons between NMR analyses and relative stereochemistry of related alkaloids embellicines A and B, not shown in this review.

NMR data suggested that phomapyrrolidone B (1.29) was a stereoisomer of phomapyrrolidone A (1.28) with its relative stereochemistry as depicted in Figure 1.18. Phomapyrrolidone C (1.30) was found to be related to phomapyrrolidones A and B (1.28 and 1.29). However, the main differences appeared to be around the succinimide moiety, where resonances such as the imide carbonyl carbon and a methine carbon were absent and replaced by a hemiaminal carbon and oxygenated quaternary carbon. The relative configuration was based on comparisons to phomapyrrolidones A and B (1.28 and 1.29), as depicted in Figure 1.18.28

Phomapyrrolidones B and C (1.29 and 1.30) showed weak in vitro antitubercular activity for replicating cultures of Mycobacterium tuberculosis Zopf (H37Pv, MIC 5.2 – 5.9 µg.mL$^{-1}$) according to microplate Alamar Blue assay method and weak in vitro low oxygen recovery for non-replicating cultures of M. tuberculosis (H37Pv, 13.4 – 15.4 µg.mL$^{-1}$) according to low oxygen recovery assay.

Phomapyrrolidone A (1.28) appeared less active when compared to phomapyrrolidones B and C (1.29 and 1.30) in both assays (MIC 20.1 and 41.1 µg.mL$^{-1}$). The three alkaloids were also tested against vero cells showing very similar IC$_{50}$ (32.4 – 35.7 µM). In addition, all three alkaloids showed no activity against cancer cells tested at concentrations of 5 µM.28
In 2013, Wossa and co-workers\(^2^9\) isolated boleptosins 11 and 12 (1.31 and 1.32), as well as the previously described boleptosins 4 and 7 (1.33 and 1.34) and cycloleucomelone (1.35), from the fruiting bodies of a *Boletopsis* sp. Fayod used traditionally by the Kiovi clan of the Lufa district in the Eastern Highlands Province of PNG for its edibility and to treat gastrointestinal complaints.\(^2^9\) The corresponding chemical structures are shown in Figure 1.19.

\[
\begin{align*}
1.31 \quad R &= O Me \\
1.32 \quad R &= OH \\
1.33 \quad R_1 &= H, \quad R_2 = H, \quad R_3 = Ac, \quad R_4 = H \\
1.34 \quad R_1 &= H, \quad R_2 = H, \quad R_3 = H, \quad R_4 = H \\
1.35 
\end{align*}
\]

*Figure 1.19: Boleptosins 11 and 12 (1.31, 1.32), known boletopsin 4, 7 (1.33, 1.34) and cycloleucomelone (1.35)*

*In vitro* antibacterial testings of the isolated compounds revealed some degree of activity against Gram (+) and Gram (-) bacteria, with all compounds tested being moderately active against *S. epidermidis* IC\(_{50}\) = 242 – 500 µg.mL\(^{-1}\).

In 2014, as part of an international cooperative biodiversity group program, Jadulco and co-workers\(^3^0\) screened extracts of endophytic fungi isolated from terrestrial and marine sources in PNG. Tuberculosis-active pyrrolocin A (1.36) was isolated from a novel fungal strain (NRRL-50135) of a taxonomically unclassified endophyte isolated from the stem of an *Asplenium sp.* L. fern in Watunou, Milne Bay, PNG.\(^3^0\)
NRRL-50135 was subjected to a recombinant expression platform with *Fusarium heterosporus* Link in order to induce the production of more pyrrolocin A (1.36), once NRRL-50135 ceased to produce it naturally. Co-cultures of the two organisms was successful and these authors also isolated two new decalin analogues, pyrrolocin B and C (1.37 and 1.38) shown in Figure 1.20.\(^{30}\)

![Figure 1.20: Pyrrolocin A - C (1.36 – 1.38)]

The desmethyl analogue pyrrolocin B (1.37) having the cis-decalin configuration, according to NMR spectroscopic analysis and absolute configuration of 2\(R\), 3\(S\), 6\(S\), 8\(S\), 11\(R\), 17\(R\) and 5\(^{'R}\), based on Mosher’s approach, was found less active (IC\(_{50}\) 112.9 \(\mu\)M) against *M. tuberculosis* than pyrrolocin A (1.36) (IC\(_{50}\) 26.3 \(\mu\)M).

In addition, pyrrolocin A (1.36) and the desmethyl analogue pyrrolocin C (1.38) (IC\(_{50}\) 56.4 \(\mu\)M), both containing a trans-decalin configuration, was suggestive that the trans configuration and the N-methylation could increase the antimicrobial activity of this class of compounds.\(^{30}\)
In 2015 Beekman and co-workers\textsuperscript{31} isolated boleptosins 13 and 14 (\textbf{1.39} and \textbf{1.40}), shown in Figure 1.21, from the fruiting bodies of a \textit{Boleptosis sp.} collected in the forest that surrounds the Kiovi clan in the Lufa district in the Eastern Highlands Province of PNG\textsuperscript{31}.

![Figure 1.21: Boleptosins 13 and 14 (1.39 and 1.40)](image)

These polybrominated compounds represented the first example naturally produced by a terrestrial macrofungi. In addition, due to insufficient amounts obtained upon isolation from the natural source, these authors used a combination of synthetic work, chromatography and NMR and mass spectrometry.

\textit{In vitro} antibacterial activity of both polybrominated metabolites indicated that boleptosins 13 and 14 (\textbf{1.39} and \textbf{1.40}) exhibited weak antibacterial activity against \textit{E. coli} and \textit{S. epidermidis} (MIC greater than 650 \textmu g.mL\textsuperscript{-1}).\textsuperscript{31}
Lin and co-workers\textsuperscript{32} isolated racemic oxazinin A (\textbf{1.41}), shown in Figure 1.22, from a filamentous fungus (class Eurotiomycetes O. E. Erikss. & Winka, strain 110162) isolated from the ascidian \textit{Lissoclinum patella} from PNG.\textsuperscript{32}

![Oxazinin A (1.41) (racemic)](image)

\textbf{Figure 1.22: Oxazinin A (1.41) (racemic)}

The chemical structure of oxazinin A (\textbf{1.41}) was elucidated following 1D and 2D NMR experiments and its relative configuration was proposed to be 3$S^*$, 11$R^*$, 12$S^*$, 24$S^*$, 3$'R^*$, 24$'R^*$ determined by a combination of ROESY correlation analyses, Chem3D and molecular dynamics simulation modelling tools.\textsuperscript{33}

In addition, oxazinin A (\textbf{1.41}) was found to be present, as a racemic mixture, according to CD spectroscopy analysis. Oxazinin A (\textbf{1.41}) was tested for inhibitory activity against a panel of bacteria showing growth inhibitory activity against \textit{M. tuberculosis} (IC$_{50}$ 2.9 $\mu$M), as well as activity against human CEm-TART T-cell leukaemia line showing a LC$_{50}$ = 4.7 $\mu$M.\textsuperscript{32}
Also in 2015 in a personal communication, Janso J.,\textsuperscript{34} the scientist responsible for screening more than 600 extracts of fungi obtained from terrestrial and marine sources in PNG, isolated three known compounds named: regulotrosin A (1.42), cordypyridone A (1.43) and blennin A (1.44) (Figure 1.23).\textsuperscript{34}

![Regulotrosin A (1.42), Cordypyridone A (1.43), Blennin A (1.44)](image)

Figure 1.23: Regulotrosin A (1.42), Cordypyridone A (1.43), Blennin A (1.44)

Janso J.\textsuperscript{34} communicated that regulotrosin A (1.42) was isolated from the endophyte *Bipolaris sp.* Shoemaker (culture ENDO-0489) and isolated from the roots of a *Rottboellia sp.* L. in Watunou village in PNG. No further characterisation information was provided to us for regulotrosin A (1.42), as it was a known compound. It should be noted that the structure depicted in Figure 1.23 was reported by Stewart and co-workers.\textsuperscript{35}

With regards to cordypyridone A (1.43), this secondary metabolite was isolated from the endophyte *Phomopsis sp.* Sacc. & Roum. (culture ENDO-0539), which was extracted from the roots of an *Areca sp.* L. in Deka Deka island in PNG. No further characterisation information was provided to us for cordypyridone A (1.43), as it was a known compound. It should be noted that the structure depicted in Figure 1.23 was reported by Isaka and co-workers.\textsuperscript{36}
Finally, blennin A (1.44) was isolated from an unidentified basidiomycete (PNG-03S-018), which was isolated from the interior of the sponge Haliclona sp. Grant in Bagabag, PNG. Similar to regulotrosin A (1.42) and cordypyridone A (1.43), no further characterisation was communicated to us, as the chemical structure depicted in Figure 1.23 was reported by De Bernardi and co-workers.37

1.3 Conclusion
A total of 43 secondary metabolites have been isolated from marine and terrestrial fungi from Papua New Guinea (PNG). In addition, approximately 60% (n = 25) of the secondary metabolites isolated were found to be novel compounds exhibiting a wide range of biological activities. Figure 1.24 shows the number of secondary metabolites isolated from fungi from PNG on a yearly basis across a 15-year period.

Figure 1.24: Number of secondary metabolites isolated from marine and terrestrial fungi from Papua New Guinea across a 15-year period from 2000.
A total of 14 fungal species were used in the isolation of secondary metabolites reported in this review. Marine derived fungi have been the most commonly selected fungal types for the discovery of novel compounds (50%, $n = 7$) followed by endophytes (36%, $n = 5$) and macrofungi (14%, $n = 2$).

Marine derived fungi were sourced from sponges (29%, $n = 4$), ascidians (14% $n = 2$) and algae (7%, $n = 1$), while the macrofungi reported herein were collected from the forest that surrounds the Kiovi village in the Eastern Highlands Province of PNG.

Interestingly, of the 25 novel compounds isolated, 15 secondary metabolites were obtained from seven marine derived fungal species resulting in a ratio of approximately 2 new compounds per marine derived fungi isolated. It is also interesting that four novel secondary metabolites were obtained from two species of macrofungi of the same genus, resulting in a ratio of two new compounds per macrofungi tested.

PNG is described in the literature as one of the most biodiverse places in the world.\textsuperscript{38} Considering that out of the 3.5 million fungal species present in the world,\textsuperscript{11} 90,000 are located in PNG,\textsuperscript{29} it can be expected that an enormous number of macrofungi from PNG are waiting to be evaluated for the discovery of new bioactive compounds.
Chapter Two

Mushrooms from Papua New Guinea and their antimicrobial potential

Antimicrobial screening of mushrooms. Pictures from left to right: excerpt of MTT testing (author’s photograph), photograph of a Boletus sp. L. by Anson Barish (UoG) (edited and reproduced with permission) and disc diffusion assay (author’s photograph).

This section examines the mushrooms used by indigenous communities in three provinces of Papua New Guinea (PNG), on the basis of ethnomycology, namely; a) Eastern Highlands Province, b) Simbu Province and c) Jiwaka Province. In vitro antibacterial testing of mushrooms used is also reported.
Chapter 2: Antimicrobial screening of mushrooms

2.1 Introduction

Medicinal plants are the oldest source of biologically active agents and they have for centuries represented an alternative to allopathic medicine or the orthodox system, in which the use of drugs is used for the treatment of various medical conditions. Papua New Guinea (PNG) possesses one of the most biodiverse ecosystems in the world, and the use of plants, as medicines, has been commonly practised.

The government of PNG has worked with an International Cooperative Biodiversity Group (ICBG) to evaluate the use of plants as medicines by exploring their antibacterial activities and to identify chemicals responsible for the biological activities. Papua New Guineans have included the use of mushrooms in their medical traditions, as ethnomycology has been commonly practised for thousands of years. However, the use of mushrooms as food, medicine or for cultural rituals has been poorly documented.

As part of an ongoing study to preserve first-hand knowledge, as it pertains to the use of mushrooms by indigenous tribes in PNG, initial contact was established with one ethnolinguistic group in the Easter Highlands Province. Subsequently, contact was also established with two other groups in the surrounding area.

Therefore, we examined the mushrooms used by the Kiovi tribe from the Eastern Highlands Province, the Waefo tribe from the Simbu Province and the Kopanka clan from the recently created Jiwaka Province (Figure 2.1; numbers on map: 3, 2 and 22 respectively). An emphasis on ethnomycology pertaining to mushrooms' antibacterial potential has been placed.
2.2 Study area and population

Kiovi, Waefo and Kopanka are ethnolinguistic groups living in mountainous regions of PNG. The Kiovi are inhabitants of the Lufa District, which area is at latitude 6°20’57”S and longitude 145°20’31”E in the Eastern Highlands Province (No. 3 on map, Figure 2.1). The 2011 census gave the population of 579,825 inhabitants of Eastern Highlands Province with approximately 61,057 people in Lufa District.44

The Waefo are based in the Siane area in Chuave District, which area is at latitude 6°7’12”S and longitude 145°7’36”E in Simbu Province (No. 2 on map, Figure 2.1). The 2011 population count suggested a total of 376,473 inhabitants of Simbu Province with approximately 39,021 people in Chuave District.44
The Kopanka inhabit the hilltop of Tapiaku village within the Anglimp-South Waghi District, which area is at latitude 5°52’38”S and longitude 144°41’11”E, with a total area of about 1 970 km², in the central highlands region of Papua New Guinea’s newly created Jiwaka Province (No. 22 on map, Figure 2.1). The 2011 population count suggested a total of 343 987 inhabitants of Jiwaka Province with approximately 194 109 people in the Anglimp-South Waghi District. Although no ethnographic studies have been performed on the Kopanka, their village is on the rocky highlands overlooking the Wahgi river and hence the name Tapiaku, which means “on the rocks”.

Similar to other clans in PNG, the Kiovi, Waefo and Kopanka reside in small villages and due to the difficult access to and from the mountainous areas they inhabit, the three ethnolinguistic groups studied herein rely heavily on their land for their livelihood. Therefore, the study areas include the forest that surrounds the Kiovi village, Waefo from the Siane area and Kopanka from the Tapiaku village.

2.3 Ethnomycological data collection

The traditional forest owners and councillors representing the Kiovi, Waefo and Kopanka granted our research team with permission to utilise their local forest for the collection of mushrooms traditionally used by their inhabitants. Formal consent letters signed by the forest owners to conduct research in the area are kept at the University of Goroka (UoG).

Ethnomycological information was gathered using a combination of informal focus groups through semi-structured interviews and forest walks. Focus groups were composed by members of the community 30 years of age or older capable of identifying mushrooms by local names.
Local names and uses of mushrooms were cross-checked with at least two elders and the village chief. Interviews were carried out by Mr Stewart Wossa for the Kiovi and Mr John Nema for the Waefo, as they are both fluent in the Tok Pisin language. Interviews for the Kopanka tribe were carried out by the author of this thesis in English and Mr Stewart Wossa as a translator, also in Tok Pisin language.

### 2.4 Collection of mushroom material

Only mushrooms traditionally used as foods, medicines or in rituals according to the local inhabitants cultural believes, including those that were regarded as potentially poisonous were collected.

Photographic evidence was kept to record habitat and morphological characteristics of the mushrooms and an identity code was assigned on collection. For instance, Kiovi-MSp1; where “Kiovi” represents the name of the village where samples were collected, “M” represents mushroom, “Sp1” indicates sample number one, as the specimen tally from that village. The same is true for the Waefo (e.g. Waefo-MSp1) and Kopanka from Tapiaku (e.g. Tapiaku-MSp1).

For the Kiovi, collections were conducted during the wet season (August to December 2012), as this period presents ideal conditions for wild mushroom harvest. For the Waefo clan, collections were performed during the fruiting season in the area of Siane (November 2012 to February 2013) and finally, for the Kopanka, collections were performed in February 2014. Figure 2.2 shows the collection team from Kopanka clan in Tapiaku village.
Mushroom samples were identified based on morphological comparisons with mushroom specimens published in the literature and voucher samples of all species kept at UoG. Thus far, a total of 79 mushroom samples have been collected amongst the three tribes. There were 26 mushroom samples identified as having ethnological linkages with the Kiovi, 18 mushrooms for the Waefo and 35 mushroom specimens were collected from the Tapiaku under the direction of the custodians of the traditional knowledge and/or landowners of their respective areas. Table 2.1 - Table 2.3 below show an excerpt of the specimens collected from the three tribes. For complete tables showing specimens collected from both groups refer to appendix 1 - 3.
Table 2.1: Mushrooms used by the Kiovi, Eastern Highlands Province, PNG.

<table>
<thead>
<tr>
<th>Specimens collection code</th>
<th>Species (Family)</th>
<th>Local name</th>
<th>Field notes</th>
</tr>
</thead>
</table>
| Kiovi-MSp6                | Lactarius sp. Pers. (Russulaceae) | Dagaidy | - Large fleshy mushroom
- Could be edible if marinated before cooking |
| Kiovi-MSp7                | Russula sp. Pers. (Russulaceae) | Kokai | - Strong red coloured cap and white gills
- Hot in taste
- Regarded as potentially poisonous |
| Kiovi-MSp11               | Ramaria sp. Fr. Ex Bonord. (Ramariaceae) | Dekeja-hava | - Edible mushroom
- Club shape
- Grows on grounds
- Used to treat stomach complaints |
| Kiovi-MSp15               | Amanita sp. Pers. (Amanitaceae) | Fulaga-dive | - Edible mushroom
- Grows on soil
- Used as a food source
- Kiovi people particularly reported that eating this mushroom makes them feel well |
| Kiovi-MSp43               | Albatrellus sp. Gray (Albatrellaceae) | Igura hivi | - Edible mushroom
- Grows on dead trunks
- Kiovi people use this mushroom medicinally to treat stomach upset |

Mushroom photographs by Anson Barish (UoG). Reproduced with permission
## Table 2.2: Mushrooms used by the Waefo, Simbu Province, PNG.

<table>
<thead>
<tr>
<th>Specimens collection code</th>
<th>Species (Family)</th>
<th>Local name</th>
<th>Field notes</th>
</tr>
</thead>
</table>
| Waefo-MSp1                | (Polyporaceae)   | Holipa lua hefole | - Edible mushroom  
- Grows on dead trunks \n- Associated with mosses under moist conditions  
- Cooked in wooden drums before eating |
| Waefo-MSp4                | (Polyporaceae)   | Heleme lua | - Edible mushroom  
- Grows on soil  
- Waefo made emphasis on the good taste of this mushroom |
| Waefo-MSp5                | (Bracket fungus) | He lua | - Edible mushroom  
- Grows on the bark of specific living trees  
- Culturally of great value as it is specially given to pigs to fatten them |
| Waefo-MSp6                | (Polyporaceae)   | Mamona lua homulege | - Edible mushroom  
- Grows on dead trunks  
- Associated with high moisture  
- Raw mushroom induces vomiting  
- Mushroom is cooked before eating |
| Waefo-MSp7                | (Amanitaceae)    | Fiona fululu lua (slippery/waxy) | - Edible mushroom  
- Grows on soil and on dead trunks  
- Mushroom is cooked before eating |

Mushroom photographs by Anson Barish (UoG). Reproduced with permission
Table 2.3: Mushrooms used by the Kopanka, Jiwaka Province, PNG.

<table>
<thead>
<tr>
<th>Specimens collection code</th>
<th>Species (Family)</th>
<th>Local name</th>
<th>Field notes</th>
</tr>
</thead>
</table>
| Tapiaku-MSp9              | *Auricularia* sp. (Dicks.) Pers. (Auriculariaceae) | Koikamuk (rat ears) | • Edible mushroom  
• Eaten cooked or boiled  
• Given to mothers post-partum for womb healing |
| Tapiaku-MSp12             | *Echinoderma* sp. (Pers.) Bon (Agaricaceae) | Nakents (baby faeces) | • Edible mushroom  
• Grows on soil  
• Given to babies as anti-diarrhoeal |
| Tapiaku-MSp18             | *Microporus* sp. P. Beauv. (Polyporaceae) | Willikimik | • Edible mushroom  
• Grows on dead trunks  
• Used for the treatment of pimples and scar removal |
| Tapiaku-MSp21             | *Morchella* sp. (L.) Pers (Morchellaceae) | Kipenembik (devil’s penis) | • Edible mushroom  
• Grows on garden soil  
• Eaten cooked and used as a food source |
| Tapiaku-MSp27             | Missing data | Bongnengs (no English translation) | • Edible mushroom  
• Grows on soil  
• Eaten cooked  
• Given to babies to prevent diseases and to grow healthy |

Mushroom photographs by Anson Barish (UoG). Reproduced with permission
2.5 Mushroom uses amongst the Kiovi, Waefo and Kopanka

Twenty six mushrooms have been collected from the forest that surrounds the Kiovi people under the leadership of Mr Stewart Wossa. Fulaga dive is one of the mushrooms used by the Kiovi for its edibility and because it makes them “feel well”.

The Fulaga dive mushroom (*Amanita sp.* Pers.) is discussed in Chapter 3. Igura hivi (*Albatrellus sp.* Gray) is another mushroom used by the Kiovi to treat stomach complaints, and it is briefly discussed in Chapter 6. Furthermore, 18 mushroom species were identified as having ethnological linkages to the Waefo and 35 mushroom species to the Kopanka. For complete tables outlining ethnomycological data for the three tribes refer to appendices 1 - 3. Figure 2.3 shows ethnomycological comparisons between the three tribes.

![Figure 2.3: Mushroom uses by the Waefo and Kopanka](image-url)
It is not surprising that amongst the three clans, mushrooms are predominantly used as a source of food. Difficult access to provincial areas forces inhabitants of remote communities to live off the forest and hence using mushrooms as foods become common practice. In addition, the three clans utilise mushrooms to feed and fatten pigs. This is not surprising, as pigs are considered highly valuable livestock in PNG. As well as using pigs for food, pigs represent social status. Pigs are mostly used for very important events, such as funerals, weddings, initiation rituals. In addition, the use of pigs is still used as the main dowry in exchange for women.

Mushrooms used to treat pain amongst the Kopanka appeared in second place after those species used mainly as a food source. However, it should be noted that the Kopanka were unable to differentiate amongst Ombnantskim species (sugar cane skin) and hence based on the Kopanka’s communications, mushrooms species used as analgesics would be reduced to one.

Interestingly, the number of mushrooms considered as potentially toxic by the Waefo surpassed even those that were identified as good to eat and it is followed by the Kiovi with five mushrooms species reported as potentially toxic (see Appendix 1 for complete table). Contrastingly, potential toxic mushrooms identified by the Kopanka clan were reduced to two species referred by the common name “Mos” and recorded as Mos-1 and Mos-2 under the codes Tapiaku-MSp33 and Tapiaku-MSp34 respectively (Appendix 3). It should be noted that Mos-2 was reported to be edible. However, the Kopanka reported that gills must be removed before cooking, due to potential toxicity.

Other mushroom species such as Binge bang, Dukdedek and Kalap were reported as inedible species. However, they were used to treat different medical conditions. For complete tables outlining field notes and common names of mushrooms collected amongst the three clans, see Appendices 1 – 3.
It might be plausible that ethnomycology amongst the Kopanka have enable them to identify mushrooms used as stimulants, analgesics, to treat skin conditions or to boost their immune system and prevent diseases, including those with spiritual uses, for example Kombsar (Tapiaku-MSp10, Appendix 3), which means lightning and the Kopanka believes that Kombsar provides them with protection from lightning strikes during the wet season, as it appears after a heavy thunderstorm. Further research should be done to determine why the Kiovi and Waefo people do not possess the same mushroom knowledge when compared to their neighbours the Kopanka.

2.6 Methodology

Previous work within our research group has focused on the antimicrobial screening of ethanolic extracts of mushrooms used by Papua New Guineans employing a paper disc diffusion assay, which relies on the ability of a test sample to diffuse into an agar medium containing a lawn of microorganisms as an indication of biological activity (see section 2.6.2).46

Preliminary testings of mushrooms collected found ‘good’ (>16 mm), ‘moderate’ (10-15 mm) or ‘low’ (<10 mm) levels of activity against microorganisms tested. While the paper disc diffusion assay is a useful tool for the analysis of antimicrobial activity due to its low cost and its ability to be used to test several samples simultaneously, it is limited by the capacity of lipophilic active compounds to diffuse through an aqueous agar medium.

Low levels of activity observed in preliminary testings of pure isolated compounds from extracts of mushrooms of reputed medicinal significance led us to suspect that poor diffusion of the purified compounds was to blame causing uncertainty in the results of preliminary testings.
Such uncertainty prompted us to implement a more reliable and reproducible method for the screening of pure isolated compounds and crude mushroom extracts. Two methods were chosen for this task; a) thiazolyl blue tetrazolium bromide (MTT) assay, for cell proliferation, which relies on a biochemical reaction between blue tetrazolium and living cells of a microorganism to induce a colour change and therefore suggest whether the test sample is active or not (see section 2.6.3);\(^{47,48}\) and b) a turbidity assay, which relies on measuring the optical density (OD) at 600 nm of a bacterial suspension to determine activity of a test sample (see section 2.6.4).\(^{49,50}\)

2.6.1 Preparation of mushroom extracts

Because of Australian Quarantine restrictions in the import of organic materials, the following extraction protocol was performed at UoG prior to shipping to Australia. Specimens were thoroughly washed with water and cut into small pieces. Mushroom material (500 – 1000 g) was macerated in 95\% EtOH for a week in a 1:3 ratio of solid matter and solvent. The resulting fluid extract (approx. 1500 – 3000 mL) was filtered to remove any residues and concentrated under reduced pressure to obtain approximately 50 - 100 mL of concentrated extract. Samples were stored in plastic bottles labelled with the assigned code during collection and shipped to the ANU for further work in the laboratory.

2.6.2 Paper disc diffusion assay

The disc diffusion assay explained herein is based on the antibiotic susceptibility testing by a standardised single disc method or the Kirby-Bauer method.\(^{46}\) In brief, this assay uses 6 mm filter paper discs impregnated with a known concentration of the test sample to be screened (e.g. mushroom extracts, antibiotics, controls) and placed onto a lawn of bacteria in an agar petri dish. The test sample will diffuse out of the paper disk into the media, which was previously covered by a layer of bacteria. Bacteria will be culled or its growth will be interrupted if susceptible to the test sample.
The area with no bacterial growth around the paper disc is referred to as the zone of inhibition (ZoI). The diameter of the ZoI is measured in millimetres (mm) and its size gives an indication whether the bacteria is resistant or susceptible against the test samples. Figure 2.4 shows an example of an active sample with a clear ZoI (right hand side).

![Figure 2.4: Zone of inhibition for active sample (right hand side). Author’s photograph.](image)

A bacterial suspension is prepared from a single colony of the organism to be tested. The suspension is then incubated overnight and diluted to achieve an OD of 0.5 McFarland standard and further diluted 100-fold with a non-specific broth medium. Turbidity could be adjusted visually with the aid of a Wickerham card (Figure 2.5), or by measuring the OD$_{600}$ of the suspension.

The resulting bacterial suspension is subsequently streaked evenly onto the previously prepared agar petri dish with the help of a cotton swab or a glass rod in the shape of a hockey stick.
Paper discs containing a known concentration of the test sample and appropriate controls are placed on the agar with sterile tweezers and gently pressed down to ensure contact with the agar. Petri dishes are subsequently incubated for 18h and the activity of the test samples is determined by measuring the diameter of the ZoI with a ruler.

A measurement of 6 mm indicates the absence of a ZoI and therefore the organism tested could be considered resistant to the test sample. Complete bacterial growth inhibition is determined by the naked eye. However, test sample with a ZoI of equal or more than 16 mm are considered to have ‘good’ inhibition properties, those with a measurement of 10-15 mm are ‘moderate’ and those with a measurement below 10 mm are said to have ‘low’ levels of bacterial growth inhibition.
Although the paper disc assay is very popular due its low cost and its ability to screen several test samples in a time efficient manner, it relies on the ability of the test sample to diffuse from the paper disc into the agar and hence prevent the growth of the microorganism.

This dependence of the test sample to diffuse is a major disadvantage of the paper disc diffusion assay especially when test samples assayed are of high hydrophobicity and hence diffusion into aqueous media could be non-existent resulting in false negatives.\textsuperscript{19} As per the aforementioned reasons, a new test was selected for the antimicrobial screening of mushrooms.

### 2.6.3 MTT colourimetric assay

The modified MTT colourimetric method explained herein is based on a method for drug susceptibility testings as described by Mengatto et al.\textsuperscript{47} and Caviedes et al.\textsuperscript{48} In brief, this method uses a yellow MTT-tetrazolium salt as an indicator for cell proliferation. Yellow MTT-tetrazolium is added to a mixture of bacterial suspension and test sample (e.g. mushroom extracts, antibiotics, and controls) and incubated for 60 min.

The water soluble yellow tetrazolium salt undergoes reduction with hydride from reductases of living cells. This cleavage leads to the development of a blue-purple colour, characteristic of the water-insoluble MTT-formazan. A blue-purple colour suggests that the organism of interest is resistant to the test sample, while a yellow colour is an indication of bacterial susceptibility to the test sample.\textsuperscript{47,48} Figure 2.6 shows reduction of yellow tetrazolium to blue formazan.
Thiazolyl blue tetrazolium bromide or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide is a yellowish water soluble compound that was first developed to test the efficacy of anticancer agents.\textsuperscript{52} MTT-tetrazolium was later adapted to test the efficacy of potential antibacterial compounds.\textsuperscript{47,48}

A bacterial suspension is prepared in the same manner as for the disc diffusion assay, starting from a single colony of the organism to be tested. The suspension is incubated overnight and diluted to achieve an optical density of 0.5 McFarland standard and further diluted 100-folds. The bacterial suspension is subsequently added to a 96 well microtiter plate containing a known concentration of the test sample.

Plates are incubated overnight and yellow MTT-tetrazolium is added to the mixture. After 60 min of incubation the minimum inhibitory concentration (MIC) is assessed visually, as shown in Figure 2.7. MIC refers to the lowest concentration of the test sample capable of producing a yellow colour. Although the reductive mechanism in which yellow MTT-tetrazolium is converted to blue MTT-formazan is not well understood in living bacteria, the MTT assay is widely accepted,\textsuperscript{53} especially as a confirmation technique for the disc diffusion assay.
Figure 2.7: Excerpt of a 96 well plate showing colour change from yellow MTT-tetrazolium (susceptible bacteria) to blue MTT-formazan (resistant bacteria). Photograph adapted from assay performed by the author.

Some advantages of the MTT method include: a) the capability to test multiple samples at different dilutions simultaneously to determine MICs, b) no expensive equipment to measure MICs is required, and c) MICs can be determined visually by a colour change.⁴⁷

Although the MTT assay possesses several advantages when compared to the disc diffusion assay, a major disadvantage is that the MTT assay is unable to visually determine the concentration of test sample capable of inhibiting 50% of the growth of microorganisms (IC₅₀).
In addition, considering that the MTT assay depends on the presence of living cells in order to induce reductive cleavage of yellow MTT-tetrazolium into blue MTT-formazan, it might be plausible that false negatives could be mistakenly reported especially when test samples are unable to inhibit 100% of cell growth. It could also be challenging to the analyst to see a yellow or blue colour especially when dealing with strongly coloured test samples.

2.6.4 Turbidity assay

The turbidity assay explained herein is based on a method for the estimation of the amount of bacteria present in a liquid medium, as described in the literature. In brief, this method requires the use of a spectrophotometer to measure the turbidity of a mixture between bacterial suspension and test sample and subsequently translating the measurements into cell density values.

A bacterial suspension is prepared in the same manner as for the MTT-tetrazolium assay, starting from a single colony of the organism to be tested. OD is measured at 600 nm before and after incubation. A turbid bacterial suspension or high reading in the spectrophotometer suggests that the organism of interest is resistant to the test sample, while a less turbid suspension or low reading in the spectrophotometer is an indication of bacterial growth inhibition and hence susceptibility to the test sample.

Figure 2.8 shows a schematic representation of how the turbidity of a bacterial suspension is measured. In a typical spectrophotometer, a tungsten lamp emits light, which is subsequently dispersed by a diffraction prism. Of the dispersed light a monochromatic light band of 600 nm wavelength passes into the test sample. Light that passes through the solution gets detected and measured electronically providing an OD reading.
In order to achieve an accurate turbidimetric reading, the spectrophotometer must be adjusted to zero percent absorbance. This is done by measuring the OD of the bacterial suspension prior incubation.¹⁸

Some advantages of the turbidity method over the MTT assay and disc diffusion assay are a) that it is possible to determine IC₅₀ based on turbidity measurements of the test samples, b) It is possible to test multiple dilutions simultaneously and c) the turbidity method does not rely on visually inspecting a colour change from yellow to blue to determine bacterial susceptibility, as in the MTT assay.

Although the turbidity method possesses several advantages, it also possesses some disadvantages. For example the use of an expensive instrument (spectrophotometer) to measure the OD of test samples and bacterial suspensions could re-direct scientists to seek for alternative methods. In addition, aggregation and sedimentation of viable or dead cells can interfere with the accuracy of OD measurements. This inaccuracy is due to the inability of the diffracted light in the spectrophotometer to get across the sample test to the detector when the bacterial concentration is too high.⁵⁶
2.6.5 Turbidity-MTT assay

Turbidity-MTT assay provides a better tool for the screening of mushroom extracts and it was chosen for the testings of mushrooms collected from Eastern Highlands Province, Simbu Province and Jiwaka Province from Papua New Guinea. While the turbidity method facilitates calculations of IC$_{50}$ and MIC, the MTT assay serves to visually confirm susceptibility of bacterial strains tested. The technique was prepared as outlined in the literature with minor modifications, and its optimisation proceeded as follows:

a) Preparation of inoculum

Bacterial suspensions were prepared by inoculating a single colony into 10 mL of non-selective Mueller Hinton Broth (MHB) and incubated for 18 - 20h. Inoculum size was adjusted to OD$_{600} = 0.08$ - 0.1 in order to be comparable with the OD of 0.5 McFarland standard. A McFarland standard is prepared by mixing aqueous solutions of barium chloride and sulphuric acid and its OD correlates to the concentration of inoculum of $1.5 \times 10^8$ cfu.mL$^{-1}$ (range $1.0 \times 10^8$ to $2.0 \times 10^8$ cfu.mL$^{-1}$). Figure 2.9 shows individual colony forming units (CFU) (left) and comparison between the turbidity of the inoculum after incubation (right).

Figure 2.9: Single colony forming units (left) to liquid media (right). Author’s photograph.
Following adjustment of inoculum to 0.5 McFarland standard, the concentration of bacterial suspension was further diluted 100-fold to prevent issues observed at earlier stages during the optimisation of the turbidity-MTT method.

Some of these issues included: a) OD outside upper reading limits of spectrophotometer when inoculum size used was equal 0.5 McFarland standard and b) strong colouration characteristic of a number of crude mushroom extracts that were regarded as potential problems especially in the visual evaluation of colour change from yellow to blue in the MTT-assay.

Therefore, decreasing the concentration of the inoculum by 100-fold brought the OD of inoculum within the measuring limits of the spectrophotometer plate reader used. In addition, as well as providing a decrease in the colouration of mushroom extracts, it allowed the control antibiotic to closely reflect the susceptibility data reported by the Clinical and Laboratory Standards Institute (CLSI) guidelines for *E. coli* and *S. aureus*.59

Table 2.4 shows a comparison between experimental susceptibility data of *E. coli* (ATCC 25922) and *S. epidermidis* against kanamycin and that reported by the CLSI.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>*MIC</th>
<th>**CLSI reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> β (-) (ATCC 25922)</td>
<td>5.26 (2.63 – 10.53) (n = 8)</td>
<td>1 – 4 (<em>E.coli</em> ATCC 25922)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> (clinical isolate)</td>
<td>3.95 (1.32 – 10.53) (n = 8)</td>
<td>1 – 4 (S. aureus ATCC 29213)</td>
</tr>
</tbody>
</table>

All units in µg.mL⁻¹. IC₅₀ expressed as median (range). (n) refers to the number of data points. *Experimental MIC for kanamycin. **CLSI reference MIC for kanamycin.* Note: *S. epidermidis* was used experimentally and CLSI reports *S. aureus*.59
b) Bacterial strains used in initial screening

The organisms selected for the initial screening were: a) ATCC 25922 *E. coli* (β-lactamase negative), and b) *S. epidermidis* (clinical isolate).

*E. coli* is a common inhabitant of the large intestine, and while beneficial to humans for its ability to prevent the growth of potentially harmful bacteria, certain strains of *E. coli* are the cause of severe diarrhoea leading to the death of young children.

Based on the current child mortality report published by Liu and co-workers, diarrhoea continues to be the second most common cause of death in children younger than five years of age worldwide, accounting for almost one million deaths per year. In addition, Rangel and colleagues, reported that approximately 74,000 cases of infections such as haemolytic anaemia, thrombocytopenia and renal injury are caused by *E. coli* in the United States per year.

*S. epidermidis* is also part of the normal human flora and despite being considered innocuous, it has become the most important cause of nosocomial infections. Uçkay and co-workers reported that the minimum bactericidal levels of *S. epidermidis* against most antibiotics has increased by 1000-fold. This increase in resistance of *S. epidermidis* is alarming, as it represents a major threat to hospitalised patients.

*E. coli* and *S. epidermidis* are amongst the most commonly isolated pathogens from various clinical conditions. In addition, they are classified as risk level 1 according to the AS/NZS 2243.3:2010 standards suitable for dealing in a physical containment level 1 (PC1) laboratory. *E. coli* and *S. epidermidis* are ideal candidates to use for the initial screening of mushroom extracts for antibacterial activity.
c) **Estimation of percentage of growth inhibition, MIC and IC\(_{50}\)**

Having measured the OD\(_{600}\) of bacterial suspension and test samples before and after the incubation period of 18 - 20h at 37\(^\circ\)C, the growth of the treated cultures was assessed. IC\(_{50}\) were determined based on comparisons with the average OD readings of the untreated control and that of the test sample and calculated as per Equation 1.

\[
IC\(_{50}\) = 10^{\log \left( \frac{A}{B} \right) + \frac{50 - C}{D - C} + \log(B)}
\]

**Equation 1**

where: A: higher concentration of test compound of the two points that brackets 50% inhibition, B: lower concentration of test compound of the two points that brackets 50% inhibition, C: inhibitory activity (%) at the concentration B, D: inhibitory activity (%) at the concentration A.

Inhibitory activity (%) was calculated as per Equation 2.

\[
\%_{\text{Inhibition}} = 100 \times \left[ 1 - \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{growth}} - OD_{\text{blank}}} \right]
\]

**Equation 2**

Where: \(OD_{\text{sample}}\): average OD of sample test or positive control after incubation, \(OD_{\text{blank}}\): average OD of sample test or positive control prior incubation, \(OD_{\text{growth}}\): average OD of bacterial suspension without sample test or positive control.
d) Ratio of test sample to inocula

Mushroom extracts were dissolved in DMSO to produce stock concentrations of 50 mg.mL$^{-1}$ and subsequently diluted to produce working concentrations of 5 mg.mL$^{-1}$. Inoculum size was $1.5 \times 10^8$ cfu.mL$^{-1}$ correlating to 0.5 McFarland standard and further diluted to produce a working inoculum $1.5 \times 10^6$ cfu.mL$^{-1}$.

The volume ratio of test material to bacterial culture once in 96 well plates was approximately 1:5. This allowed a sample test range of $8 - 1052 \, \mu\text{g.mL}^{-1}$ reflecting the ratios used in the MTT method by Steenkamp et al.$^{50}$ In addition, this ratio also allowed a bacterial culture final concentration of $4.0 \times 10^5$ cfu.mL$^{-1}$, providing an appropriate concentration range for the instrument used.

It is worth noting that the concentration of DMSO when preparing test samples was also reduced to 2.1% in 96 well plates reflecting safe concentrations to avoid possible antibacterial effect of such reagent.$^{67}$

e) Conclusions on optimisation

Turbidity-MTT assay for the screening of mushroom extracts appeared advantageous, when compared to disc diffusion assay and turbidity or MTT alone. For instance, the ability of hydrophobic compounds to diffuse out of a paper disc into aqueous agar is known to be a challenge in the Kirby-Bauer assay. However, in the turbidity-MTT this problem is solved.

Test samples are appropriately dissolved to guarantee contact with inoculum. When MTT is used alone, a common issue is the interference between coloured test samples and dye uptake by living microorganisms, and hence not being able to see colour change from yellow to blue, as stipulated in the literature.$^{53}$
Relying on OD measurements has solved this issue.\textsuperscript{54} MTT method has also some advantages against turbidity assay. A possible issue in the turbidity assay is the capability of living and dead cells to segregate or sediment causing erroneous OD measurements. Using MTT as a confirmation tool for the presence of living cells to induce a colour change from yellow to blue could also be considered an advantage.\textsuperscript{56}

Reducing the inoculum size by further diluting its initial concentration from 0.5 McFarland standard, as well as measuring OD\textsubscript{blank} prior incubation, proved to be very useful because readings fell within the limits of detection of the instrument used. Finally, a combination of both MTT (qualitative indicator) and turbidity (quantitative indicator) provides a stronger method for the accuracy of antibacterial testings.

2.6.6 Laboratory testing of mushrooms used by Kiovi, Waefo and Kopanka

A total of 52 mushroom extracts were prepared, from the Kiovi \((n = 26)\), Waefo \((n = 16)\) and Kopanka \((n = 10)\), following the protocol described under methodology in section 2.6.1 and tested for their antibacterial potential. Of the 52 extracts tested, about 29\% \((n = 15)\) were active against one or more of the organisms tested. Of the active species, all showed activity against \(S.\ epidermidis\) with two extracts exhibiting broad spectrum activity.

It is worth noting that most of the active extracts were from mushrooms collected in the forest that surrounds the Kiovi tribe \(23\%, n = 12\)\), followed by the Waefo \(4\%, n = 2\) and Kopanka \(2\%, n = 1\). Table 2.5 shows a summary of extracts with ‘good’ \((IC_{50} < 50 \ \mu g.mL^{-1})\), ‘moderate’ \((IC_{50} < 600 \ \mu g.mL^{-1})\) and ‘low’ \((IC_{50} > 600 \ \mu g.mL^{-1})\) antibacterial activity.
Table 2.5 Mushroom extracts showing antibacterial activity

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Human Pathogenic Bacteria</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. epidermidis (c/i)</td>
<td>E. coli β (-) ATCC 25922</td>
</tr>
<tr>
<td></td>
<td>ZoI (mm)</td>
<td>MIC (µg/mL)</td>
<td>IC50 (µg/mL)</td>
</tr>
<tr>
<td>Kiovi-MSp1</td>
<td>-</td>
<td>-</td>
<td>382 (80) 526</td>
</tr>
<tr>
<td>Kiovi-MSp2</td>
<td>-</td>
<td>-</td>
<td>290 (72) 379</td>
</tr>
<tr>
<td>Kiovi-MSp3</td>
<td>-</td>
<td>-</td>
<td>640 (54) 688</td>
</tr>
<tr>
<td>Kiovi-MSp4</td>
<td>-</td>
<td>7158</td>
<td>2725</td>
</tr>
<tr>
<td>Kiovi-MSp5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kiovi-MSp10</td>
<td>15</td>
<td>1000</td>
<td>500</td>
</tr>
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<td>Kiovi-MSp15</td>
<td>10</td>
<td>519</td>
<td>305</td>
</tr>
<tr>
<td>Kiovi-MSp21</td>
<td>-</td>
<td>23158</td>
<td>7626</td>
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<td>-</td>
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<td>6892</td>
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<tr>
<td>Kiovi-MSp26</td>
<td>-</td>
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<td>2743</td>
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<tr>
<td>Kiovi-MSp29</td>
<td>-</td>
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<tr>
<td>Kiovi-MSp43</td>
<td>15</td>
<td>379</td>
<td>168</td>
</tr>
<tr>
<td>Tapiaku-MSp27</td>
<td>21</td>
<td>295</td>
<td>147</td>
</tr>
<tr>
<td>Waefo-MSp1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Waefo-MSp9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>-</td>
<td>2.63</td>
<td>0.78</td>
</tr>
</tbody>
</table>

ZoI: zone of inhibition as determined by the disc diffusion assay. MIC: minimum inhibitory concentration as determined by turbidity-MTT assay. IC50: concentration of test sample able to inhibit 50% of the growth of microorganisms as determined by turbidity-MTT assay. (%) Inh: percentage of inhibition other than MIC and IC50.

For the Kiovi, Unale kaula (collection no. Kiovi-MSp29) was the only extract that appeared active against S. epidermidis (IC50 49 µg.mL⁻¹) and E. coli β (-) (ATCC 25922) (IC50 975 µg.mL⁻¹). In addition, Fulaga dive (Amanita sp. Pers., collection no. Kiovi-MSp15, IC50 305 µg.mL⁻¹) and Igura hivi (Albatrellus sp. Gray, collection no. Kiovi-MSp43, IC50 168 µg.mL⁻¹), both mushrooms species were used by the Kiovi because it makes them feel well, appeared moderately active against S. epidermidis and investigations on their chemistry are discussed in Chapter 3 and Chapter 6 respectively.

For the Waefo, of the 16 mushroom extracts tested, the edible Holipa lua hefola (collection no. Waefo-MSp1) and inedible Holipa lua namba (collection no. Waefo-MSp9) exhibited low antibacterial activity against S. epidermidis (16 – 30 % inhibition at concentration > 2000 µg.mL⁻¹) according to the turbidity-MTT assay.
Finally, for the Kopanka, of the 10 extracts tested, Bongnengs (collection no. Tapiaku-MSp27) showed broad spectrum activity against the Gram (+) *S. epidermidis* (IC$_{50}$ 147 µg.mL$^{-1}$) and Gram (-) *E. coli* β (-) (ATCC 25922) (IC$_{50}$ 194 µg.mL$^{-1}$) according to the disc diffusion assay and turbidity-MTT assay. Interestingly, the Kopanka reported the use of Bongnengs as a prophylactic agent and hence it is commonly given to babies to prevent diseases and to grow healthy. The antibacterial activity of Bongnengs, as a crude extracts reported herein, clearly contributes to evidence-based traditional use of this mushroom as medicine. The rest of the extracts tested were considered inactive against the bacterial strains tested.

### 2.7 Conclusions

A total of 52 mushroom extracts, from three tribes in PNG (Kiovi, Waefo and Kopanka) were investigated for their antibacterial potential against *S. epidermidis* (clinical isolate) and *E. coli* β (-) ATCC 25922. A total of 15 mushroom extracts (29%, $n = 52$) showed some activity against at least one of the bacterial strains used using the turbidity-MTT assay. Although turbidity-MTT assay has proven to be superior when compared against disc diffusion assay or MTT method alone, *in vitro* antibacterial screenings of mushroom extracts may not be sufficient to correlate effectiveness of such extracts in *in vivo* settings to validate traditional use. However, *in vitro* results provide a starting point for further biological and chemical investigations towards the discovery of new drugs.

### 2.8 Limitation of this study and future directions

Australian Quarantine regulations in the import of organic material presented an issue in the implementation of morphological assessments on mushroom material and genomic DNA extractions for taxonomy studies. There is evidence suggesting that DNA studies have provided more accurate outcome in the investigation of phylogeny of mushrooms species when compared to standard morphological studies. Therefore, for future directions, DNA extractions and phylogenetic analyses should be incorporated for every mushroom species collected from the forest in PNG.
2.9 Experimental

2.9.1 Microorganisms

ATCC strains of \textit{E. coli} \(\beta\) (-) were kindly donated by our collaborators from Macquarie University, Department of Chemistry and Biomolecular Sciences, NSW 2109, and provided in solid media. Clinical isolates of \textit{S. epidermidis} were kindly donated by Department of Microbiology, Canberra Hospital, Garran, ACT 2605 and also provided in solid media. Strains were sub-cultured in liquid media and stored at -30°C in glycerol.

Glycerol stocks of all bacterial cultures were prepared by mixing equal volumes of 80% glycerol and bacterial culture into a labelled glass vial. All media listed below were sourced from Bacto Laboratories Pty Ltd, NSW 2170, Australia and prepared as per the manufacturer’s instructions unless otherwise noted. BBL™ Mueller Hinton broth and DIFCO™ Mueller Hinton agar were used for the growth of the \textit{E. coli} \(\beta\) (-) and \textit{S. epidermidis} strains. The pH of the medium was adjusted to 7.3 ± 0.1 before autoclaving. Microorganisms were stored in slopes on DIFCO™ Mueller Hinton agar.

2.9.2 Controls

Kanamycin sulphate from \textit{Streptomyces kanamyceticus} was used as the antibiotic control for \textit{S. epidermidis} and \textit{E. coli} \(\beta\) (-) and supplied by Sigma Aldrich, Castle Hill, NSW 1765, Australia. Dimethylsulfoxide (DMSO) was used as a negative control and as a solvent for sample preparation.

DMSO was supplied by Thermo Fisher Scientific Australia Pty Ltd., Scoresby, Vic 3179, Australia. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as the dye to determine the viability of the cells and it was sourced from Alfa-Aesar, Heysham, Lancashire, United Kingdom.
2.9.3 Test sample preparation

Ethanolic mushroom extracts were concentrated to dryness under reduced pressure and the resulting dried material re-dissolved in 0.1 mL of DMSO and taken up to 1 mL with MHB to achieve concentrations between 5 to 50 mg.mL$^{-1}$ of test sample.

2.9.4 Equipment

Assays were performed in Nunc 96-well clear flat bottom culture plates (Thermo Fisher Scientific, Roskilde Site, Denmark). All plates were read at 600 nm using the BioTek Epoch microplate spectrophotometer and Gen5™ 2.0 Data Analysis Software (BioTek Instruments, Inc, Winooski, USA). Agar plates were prepared using disposable sterile petri dishes.

The absorbance of the working cultures was read using a UV-Mini 1240 UV-Vis Spectrophotometer (Shimadzu) and incubation was conducted in a laboratory incubator BTC-9090 at 37°C (Thermoline, Wetherill Park, NSW2164, Australia). Sterilisation of media was performed in a Tomy high pressure steam steriliser ES-315 (Quantum Scientific Pty, Ltd).

All rotary evaporation was performed using a Büchi rotary evaporator R-114 with Büchi waterbath B-480. To guarantee sterility, all dealings with microorganisms were carried out under a Gelaire laminar air flow cabinet (Kelly Company, Pty Ltd, Seven Hills, NSW 2147, Australia).

2.9.5 Growth of microorganisms

Mueller Hinton Agar (MHA) was used to make slopes for storage of bacteria. Slopes were stored at 4°C, for up to 3 months, and used to prepare working cultures of the organisms by subculturing on agar plates to isolate single colonies.
A single colony was suspended in 10 mL of MHB and incubated at 37°C at approximately 120 rpm in a shaker incubator for approximately 18 to 20h. The inoculum size used in the assays was estimated using a reference 0.5 McFarland standard, OD<sub>600</sub> = 0.08 – 0.1 for use in turbidity-MTT cell proliferation assays.

2.9.6 Turbidity-MTT biological screening assay

Broth microdilution assay was performed as outlined in the literature<sup>57,50</sup> with minor modifications.<sup>70</sup> Forty microliters of MHB was dispensed into wells 1B–1H of the 96 microtiter well plate. Subsequently, 80 µL of the unfiltered test sample or the appropriate antibiotic (i.e. kanamycin 50 µg.mL<sup>-1</sup> in MHB) was dispensed into well 1A. Forty microliters of the test sample or positive control were removed and serially diluted down the plate. Growth, sterile and negative controls were also included.

150 µL of bacterial suspension diluted to equal the density of 0.5 McFarland standard (OD<sub>600</sub> = 0.08 – 0.1) and further diluted 1:100 in MHB were added to wells 1A–1H. The plate was read at OD<sub>600</sub> to control for pre-existing turbidity of the samples prior to incubation. The plate was incubated at 37°C for 18 - 20h while being agitated on an orbital shaker at 120 rpm. After incubation, the plate was read at OD<sub>600</sub> to assess growth of the treated cultures. MIC (when possible) and IC<sub>50</sub> were determined based on a comparison with the average turbidity readings of the untreated control. For calculations, see Equation 1 and Equation 2 in section 2.6.5-c.

Finally, 10 µL of MTT dye was added to each well in the microtiter plate and incubated for 60 min while being agitated at 120 rpm. After incubation, MIC was inspected visually. Yellow colour indicated bacterial inhibition, while blue colour indicated bacterial growth.
Chapter Three

The Fulaga dive mushroom


This section describes ethnomycological and taxonomical background on Fulaga dive, a Papua New Guinean macrofungi of the genus Amanita. Antimicrobial guided isolation of its biologically active constituents and their structural identification is also reported.
Chapter 3: The Fulaga dive mushroom (Amanitaceae)

3.1 Introduction

Fulaga dive (Figure 3.1) is one of the mushrooms traditionally used, for its edibility, by the Kiovi tribe of the Lufa District, Eastern Highlands Province in Papua New Guinea (PNG). Kiovi particularly reported that after eating this mushroom they experienced a sense of “well-being”. This ethnomycological linkage to the Kiovi gave origin to this work.

![Fulaga dive (Amanita sp. Pers). Author’s photograph.](image)

A crude extract of Fulaga dive (collection no. Kiovi-MSp15) was found active against the Gram (+) bacterium *S. epidermidis* (IC$_{50}$: 305 µg.mL$^{-1}$) according to the turbidity-MTT assay.
Bioassay guided purification led to the isolation of two novel furan fatty acids (F-acids), \((Z\text{-}E)-9-(5\text{-}pentylfuran\text{-}2\text{-}yl)\text{-}non-8\text{-}enoic\text{ acid}\) (3.1 and 3.2) were identified as the components responsible for the observed biological activity. Their chemical structures are depicted in Figure 3.2.

![Figure 3.2: (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) isolated from Fulaga dive](image)

Thus, this chapter entails an investigation of Fulaga dive, bioassay guided isolation and chemical structure elucidation of the compounds responsible for its antibacterial activity.

### 3.2 Furan fatty acids (F-acids)

A saturated 2,5-disubstituted F-acid, \(8\text{-}(5\text{-}hexylfuran\text{-}2\text{-}yl)\text{-}octanoic\text{ acid}\) (3.3) (Figure 3.3), was isolated by Morris et al.\(^{71}\) in 1966 from the oil of *Exocarpus cupressiformis* Labill. seeds.\(^{71}\)

This F-acid natural product represented the first report of its chemical class. Since then, scientists have struggled to determine their precise role in nature. Bioactivity studies on these metabolites suggest that they possess anti-inflammatory and radical scavenging abilities, implying that they may also have the potential of being used as antioxidants to help protect humans against ailments such as arteriosclerosis and cardiovascular disease.\(^{72}\)
In 1974, Glass and colleagues\textsuperscript{73} reported the discovery of eight F-acids isolated from the liver lipids of the northern pike fish (\textit{Esox Lucius} L.) obtained from the lakes of St. Paul, Minnesota, USA.\textsuperscript{73} The discovery of these homologues, shown on Table 3.1, marked the backbone of what is known about the general structure of these types of compounds.

Table 3.1: General structure of the most abundant F-acids

<table>
<thead>
<tr>
<th>Number</th>
<th>-m</th>
<th>-n</th>
<th>-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>2</td>
<td>8</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>4b</td>
<td>4</td>
<td>8</td>
<td>H</td>
</tr>
<tr>
<td>4c</td>
<td>4</td>
<td>8</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>4d</td>
<td>2</td>
<td>10</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>4e</td>
<td>4</td>
<td>10</td>
<td>H</td>
</tr>
<tr>
<td>4f</td>
<td>4</td>
<td>10</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>4g</td>
<td>4</td>
<td>12</td>
<td>H</td>
</tr>
<tr>
<td>4h</td>
<td>4</td>
<td>12</td>
<td>CH$_3$</td>
</tr>
</tbody>
</table>

Furan fatty acids (F-acids) are a class of compounds that have been isolated from a variety of sources, for instance fresh and salt water fish, algae, vegetables, including products commonly found in supermarkets such as butter.\textsuperscript{73-76}

Figure 3.3: Disubstituted furan fatty acid (8-(5-hexylfuran-2-yl)-octanoic acid) from the seed oil of \textit{Exocarpus cupressiformis} Labill.
Although the extent of biological activity of F-acids is not yet known, this type of compounds have gained attention for their role as radical scavengers, their potential as antioxidants, and their value to society for their potential as dietary supplements or functional food. However, scientists confirmed that although the distribution of F-acids was widespread, F-acids were mostly found in small quantities, representing an issue when attempting biosynthetic studies to learn about their occurrence.

Chemically, the most common F-acids reported have been characterised by the presence of either a tri- or tetrarunsubstituted furan ring, differing from one another by the presence or absence of a methyl group on the ring at position 4 and on the length of the chain at positions 2 and 5. In addition, an ‘unusual’ F-acids have been reported in the literature, containing a long unsaturated chain of up to 20 carbons in one of the alpha positions of the furan ring isolated from sea sponges.

Okada and co-workers tested the antioxidant activity of a number of naturally occurring F-acids on the oxidation of linoleic acid, on an effort to determine their biological role. Although these authors found that tetra-substituted F-acids were fundamental in the prevention of the oxidation of linoleic acid, they also found that tri-substituted F-acids were able to delay the oxidation of linoleic acid but their concentrations needed to be adjusted.

Okada and colleagues concluded that there was a correlation between the antioxidant strength of F-acids and the substitution of the furan ring, suggesting that the more substituted the furan ring the better antioxidant activity. Very recently, on another effort to determine the biological role of F-acids, Wakimoto and co-workers tested the anti-inflammatory effect of tetra-substituted F-acids isolated from the New Zealand green-lipped mussel (Perna canaliculus Gmelin). Results of this study revealed the potent anti-inflammatory activity of F-acids in vivo (10 mg.kg\(^{-1}\) of F-acid, 74% reduction of paw swelling in rats).
In late 2012, while screening mushroom extracts used by the Kiovi tribe from PNG, Fulaga dive (collection no. Kiovi-MSp15) appeared active against *S. epidermidis* (IC$_{50}$ 305 µg.mL$^{-1}$). It is worth mentioning that the strain of *S. epidermidis* used for initial screening showed significant resistance against kanamycin, which was used as the positive control for the antibacterial assay. The ratio between IC$_{50}$ of the crude extract and kanamycin was 0.5:1, which indicated that the crude extract tested possessed half the potency of kanamycin against the test organism used. This remarkable finding, prompted us to identify and characterise the compound or compounds responsible for the biological activity shown by the mushroom.

### 3.3 Characterisation of Fulaga dive and purification of bioactive compounds

Fulaga dive (collection no. Kiovi-MSp15) was characterised on the basis of phylogenetic analysis. Genomic DNA was extracted from the stipe of the fungus and subsequently submitted to amplification of the internal transcribed spacer (ITS) by polymerase chain reaction (PCR).$^{82}$

The PCR product was subjected to Sanger sequencing analysis followed by a BLAST search,$^{83}$ which revealed that the three closest relatives of this fungus (*Amanita virginiana* (Murill) Sumst., *Amanita risticii* Tulloss and *Amanita longistriata* S. Imai) were members of the section of edible fungi related to *Amanita caesarea* (Scop.) Pers. It should be noted that Fulaga dive (*Amanita sp.* Pers) lack morphological resemblance to closest relatives.

The differences in appearance could be indicative that Fulaga dive (*Amanita sp.* Pers) is a new species. For detail information about DNA extraction and amplification and sequencing procedures see experimental sections 3.8.8 and section 3.8.9. Voucher samples of this mushroom are kept at Centre for Natural Resources Research and Development (CNRRD) at the University of Goroka (UoG) under Kiovi-MSp15.
After initial extraction, as described in section 2.6.1, ethanol (EtOH) was removed under reduced pressure and the dried extract was reconstituted with methanol (MeOH). The resulting methanolic extract was partitioned with a modified Kupchan, as shown in Figure 3.4. Five new extracts were obtained: hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), n-butanol (n-BuOH) and aqueous.

Kupchan extracts were re-assayed using the turbidity-MTT method described in section 2.9.6. Results from the antimicrobial assay suggested activity across all fractions (Hex, DCM, EtOAc and n-BuOH) except the aqueous fraction. DCM was the highest yielding fraction, with about 1.5 g of material extracted after partition and it was chosen to continue with the isolation of the active metabolites.
The DCM fraction was fractionated on silica gel 60 (230 – 400 mesh) column using petroleum spirit and EtOAc (7:3) as the mobile phase. A total of 45 fractions containing approximately 5 mL of eluent were collected and pooled into four major fractions.

The collection pool was based on indications from TLC, 1D $^1$H-NMR, GC-MS experiments and comparisons with the crude extract of Fulaga Dive. Fraction three contained the major active compounds.

The DCM fraction of Fulaga dive containing the major active compounds was further purified using normal phase HPLC with Pet Sp – EtOAc (7:1 isocratic) to furnish (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) (5 mg) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2) (15 mg).

Below is a snapshot of the GC-MS chromatogram showing two peaks with the same fragmentation pattern with $[M]^+ = 292$ and later identified as two diastereoisomers of a novel F-acid. In addition, another peak with $[M]^+ = 166$ was also later identified as a furaldehyde derivative (3.4) found in Fulaga dive.

Figure 3.5: TIC from a GC-MS displaying major peaks found in Fulaga dive (collection no. Kiovi-MSp15) (Amanita sp. Pers.)
3.4 Elucidation of \((E)-9-(5\text{-pentylfuran}-2\text{-yl})\text{-non-8-enoic acid}\)

Bioassay-guided fractionation led to the isolation of \((E)-9-(5\text{-pentylfuran}-2\text{-yl})\text{-non-8-enoic acid}\) (3.2), which chemical structure is shown in Figure 3.6, as it was the major peak observed in HPLC and GC-MS.

![Figure 3.6: \((E)-9-(5\text{-pentylfuran}-2\text{-yl})\text{-non-8-enoic acid}\) (3.2)](image)

HREIMS suggested a molecular formula of \(C_{18}H_{28}O_3\) through the appearance of a molecular ion \([\text{M}]^{+}\) of \(m/z\) 292.2033 calculated for 292.2038. In addition, this molecular formula implied five degrees of unsaturation, calculated by Equation 3:

\[
\text{DoU} = \frac{2C + 2 + N - X - H}{2}
\]

Equation 3

Where; DoU: degree of unsaturation; C: number of carbon atoms in the molecule; N: number of nitrogen atoms in the molecule; X: number of halogens and H: number of hydrogen atoms in the molecule.

The structure of \((E)-9-(5\text{-pentylfuran}-2\text{-yl})\text{-non-8-enoic acid}\) (3.2) was elucidated using extensive NMR experiments observed in CDCl₃, including \(^1\text{H}, ^1\text{C}, ^1\text{H} – ^1\text{C}\) HSQC, \(^1\text{H} – ^1\text{C}\) HMBC and \(^1\text{H} – ^1\text{H}\) COSY.
The $^1$H NMR spectrum shown in Figure 3.7 displayed 10 distinctive resonances. For example, a typical slightly deformed $A_3M_2$ spin system at 0.90 ppm (J). Such deformation is as a result of virtual coupling between a terminal methyl group with a long hydrocarbon chain (H – I) commonly observed in fatty acids.

Two $A_2M_2$ systems (E, F) and one $A_2M_2XZ$ system (G), all integrating for two protons each, are also visible in the $^1$H NMR spectrum. Finally a complex system of four resonances is also visible in the downfield region (5.88 – 6.18 ppm).

![Figure 3.7: $^1$H (400 MHz) NMR spectrum in CDCl$_3$ of (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2)](image)

On a closer look at the four resonances in the $^1$H NMR spectrum displayed in Figure 3.8, there can be appreciated two distinctive AB spin systems (A and C) and a clear ABX$_2$ spin system (B). A small ABX$_2$ spin system (D) can be also appreciated. However, due to the digital resolution of the instrument used, this spin system was reported as an AB spin system. All four resonances integrated for one proton each.
Mononuclear coupling correlations, $^1$H – $^1$H COSY, allowed the identification of a $E$-alkene consistent with the coupling constants of protons on position 9’’ ($\delta$ 6.13, d, $J = 15.9$ Hz, 1H) and position 8’’ ($\delta$ 6.05, dt, $J = 15.9, 6.4$ Hz, 1H). For chemical structure of 3.2 see Figure 3.6.

The carbon NMR spectrum (not shown herein) displayed 18 discrete resonances and with $^1$H – $^{13}$C HSQC analyses, allowed the confirmation of a number of proton assignments. Finally, $^1$H – $^{13}$C HMBC correlations allowed the elucidation of (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2). A summary of $^1$H and $^{13}$C NMR correlations can be found in Table 3.2.

Figure 3.8: Expansion of region $\delta$ 6.17 – 5.89 ppm of $^1$H (400 MHz) NMR spectrum in CDCl$_3$ of (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2)
Table 3.2: $^1$H and $^{13}$C NMR, HMBC data for (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2)

| Position | $\delta_H$ (ppm) (multiplicity, $|J|$ (Hz)) | $\delta_C$ (ppm) | HMBC ($^1$H $\rightarrow ^{13}$C) |
|----------|--------------------------------------|-----------------|----------------------------------|
| 2        | 151.5                                |                 |                                  |
| 3        | 6.00 (d, 3.1)                        | 106.8           | C-4, C-2, C-9'                   |
| 4        | 5.92 (d, 3.1)                        | 106.1           | C-5, C-3                         |
| 5        | 155.7                                |                 |                                  |
| 1'       | 2.59 (t, 7.5)                        | 28.1            | C-5, C-4, C-2'                   |
| 2'       | 1.76 – 1.24 (m)                      | 27.8            | C-1', C-3'                       |
| 3'       | 1.76 – 1.24 (m)                      | 31.4            | C-4', C-2', C-1'                 |
| 4'       | 1.76 – 1.24 (m)                      | 22.4            | C-5', C-3', C-2'                 |
| 5'       | 0.90 (t, 7.0)                        | 14.0            | C-4', C-3'                       |
| 1''      | 179.4                                |                 |                                  |
| 2''      | 2.35 (t, 7.5)                        | 33.9            | C-1'', C-3''                     |
| 3''      | 1.76 – 1.24 (m)                      | 24.6            |                                  |
| 4''      | 1.76 – 1.24 (m)                      | 28.9            |                                  |
| 5''      | 1.76 – 1.24 (m)                      | 28.8            |                                  |
| 6''      | 1.76 – 1.24 (m)                      | 29.1            |                                  |
| 7''      | 2.15 (ddt, 6.4, 6.4, 7.2 )           | 32.7            | C-9'', C-8'', C-6''              |
| 8''      | 6.05 (dt, 15.9, 6.4)                 | 128.2           | C-9'', C-7''                     |
| 9''      | 6.13 (d, 15.9)                       | 118.8           | C-2, C-8''                       |

3.5 Elucidation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid

Bioassay-guided fractionation also led to the isolation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1), which chemical structure is shown in Figure 3.9.

![Figure 3.9: (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1)](image-url)
HREIMS suggested a molecular formula of $\text{C}_{18}\text{H}_{28}\text{O}_3$ through the appearance of a molecular ion $[\text{M}]^{+} m/z$ 292.2033 calculated for 292.2038, identical to that of $(E)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2). Molecular formula also implied five degrees of unsaturation, as calculated by Equation 3.

The structure of $(Z)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) was also elucidated using extensive NMR experiments observed in C$_6$D$_6$, including $^1$H, $^{13}$C, $^1$H – $^{13}$C HSQC, $^1$H – $^{13}$C HMBC and $^1$H – $^1$H COSY. It should be noted that initial $^1$H NMR experiments were performed in CDCl$_3$. However, due to instability of 3.1 in CDCl$_3$, it was decided to use C$_6$D$_6$ instead. The instability of $(Z)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) is discussed in Chapter 4, section 4.5.8.

Moreover, the $^1$H NMR spectrum also displayed 10 distinctive resonances and it was relatively similar to that for $(E)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2). In addition to the molecular formula and molecular mass calculated for both compounds, $^1$H NMR spectrum was also evidence that $(Z)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and $(E)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2) were related compounds.

On closer inspection of the region between 5.30 – 6.35 ppm, exhibited some remarkable differences, for example the ABX$_2$ spin system (D), shown in Figure 3.10, was drastically shifted upfield (5.40 ppm) when compared to its equivalent ABX$_2$ spin system (B) shown in Figure 3.8 for $(E)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2).

It should be noted that homonuclear $^1$H – $^1$H COSY correlations provided evidence of the presence of a Z-alkene based on coupling constant calculations of protons on position 9’’ ($\delta$ 6.28, d, $J = 11.8$ Hz, 1H) and position 8’’ ($\delta$ 5.40, dt, $J = 11.8$, 7.3 Hz, 1H). For chemical structure of $(Z)$-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) see Figure 3.9.
The upfield shift of the Z-alkene may not be surprising and it could be explained from the location of the E-alkene within the molecule. Because the proton in position 8'' in the E-diastereoisomer (Figure 3.6) is within the paramagnetic region as dictated by the furan core, an increase in its chemical shift is expected in a similar manner to what would be expected for aromatic protons. In the Z-diastereoisomer (Figure 3.9), however, the proton in position 8'' is further away from the paramagnetic region, as dictated by the furan ring. Therefore, it would not be expected that this proton would experience an increase in its chemical shift.

The $^{13}$C NMR spectrum displayed 18 discrete resonances and with $^1$H -- $^{13}$C HSQC analysis, allowed the confirmation of a number of proton assignments. $^1$H -- $^{13}$C HMBC correlations allowed the elucidation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1). A summary of $^1$H and $^{13}$C NMR correlations can be found in Table 3.3.
Table 3.3: $^1$H and $^{13}$C NMR data for (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1)

| Position | $\delta_H$ (ppm) (multiplicity, $|J|$ (Hz)) | $\delta_C$ (ppm) |
|----------|------------------------------------------|------------------|
| 2        |                                          | 152.3            |
| 3        | 6.15 (d, 3.2)                            | 110.2            |
| 4        | 5.92 (d, 3.2)                            | 106.8            |
| 5        |                                          | 156.0            |
| 1'       | 2.55 -2.42 (m)                           | 28.1             |
| 2'       | 1.60 – 1.10 (m)                          | 28.4             |
| 3'       | 1.60 – 1.10 (m)                          | 31.6             |
| 4'       | 1.60 – 1.10 (m)                          | 22.7             |
| 5'       | 0.83 (t, 6.9)                            | 14.1             |
| 1''      |                                          | 178.5            |
| 2''      | 2.04 (t, 7.0)                            | 33.7             |
| 3''      | 1.60 – 1.10 (m)                          | 24.7             |
| 4''      | 1.60 – 1.10 (m)                          | 29.1             |
| 5''      | 1.60 – 1.10 (m)                          | 29.6             |
| 6''      | 1.60 – 1.10 (m)                          | 29.6             |
| 7''      | 2.55 – 2.42 (m)                          | 29.6             |
| 8''      | 5.4 (dt, 11.8, 7.3)                      | 129.6            |
| 9''      | 6.28 (d, 11.8)                           | 118.2            |

3.6 Elucidation of 5-pentylfuran-2-furaldehyde

Bioassay-guided fractionation also led to the isolation of 5-pentyl-2-furaldehyde (3.4), which chemical structure is shown in Figure 3.11.

![Figure 3.11: 5-pentylfuran-2-furaldehyde (3.4)](image-url)
HRESIMS suggested the molecular formula C_{10}H_{15}O_{2} through the appearance of a protonated molecule of $m/z$ 167.1073 calculated for 167.1072. This formula implied four degrees of unsaturation and calculated as per Equation 3.

The structure of 5-pentyl-2-furaldehyde (3.4) was elucidated using extensive NMR experiments observed in CDCl$_3$, including $^1$H, $^{13}$C, $^1$H – $^{13}$C HSQC, $^1$H – $^{13}$C HMBC and $^1$H – $^1$H COSY.

The $^1$H NMR spectrum displayed in Figure 3.12 exhibited seven distinctive resonances, including a slightly deformed A$_3$M$_2$ system at 0.90 ppm (I), due to virtual coupling with a “methylene envelope” (G – H), followed by an A$_2$M$_2$ spin system (F). In addition, two AB spin systems (B, E) belonging to the furan ring and finally a singlet at $\delta$ 9.51 ppm (A), characteristic of an aldehyde are depicted in the $^1$H NMR spectrum.

![Figure 3.12: $^1$H (400 MHz) NMR spectrum in CDCl$_3$ of 5-pentylfuran-2-furaldehyde (3.4)](image-url)
The $^{13}$C NMR spectrum displayed 10 discrete resonances (not shown herein). $^1$H – $^{13}$C HSQC analysis, allowed the assignment of a number of protons and $^1$H – $^{13}$C HMBC correlations allowed the elucidation of 5-pentylfuran-2-furaldehyde (3.4). A summary of $^1$H and $^{13}$C NMR correlations can be found in Table 3.4.

### Table 3.4: $^1$H and $^{13}$C NMR data for 5-pentylfuran-2-furaldehyde (3.4)

| Position | $\delta$H (ppm) (multiplicity, $|J|$ (Hz)) | $\delta$C (ppm) |
|----------|------------------------------------------|-----------------|
| 2        |                                          | 151.9           |
| 3        | 7.16 (d, 3.5)                            | 123.5           |
| 4        | 6.23 (d, 3.5)                            | 108.7           |
| 5        |                                          | 164.3           |
| 1’       | 2.71 (t, 7.7)                            | 28.5            |
| 2’       | 1.86 – 1.55 (m)                          | 27.4            |
| 3’       | 1.49 – 1.21 (m)                          | 31.5            |
| 4’       | 1.49 – 1.21 (m)                          | 22.5            |
| 5’       | 0.90 (t, 7.1)                            | 14.1            |
| 1’’      | 9.51                                     | 177.1           |

5-Pentylfuran-2-furaldehyde (3.4) has been reported in the literature as a natural product isolated from broth cultures of a *Clitoxybe* sp. However, based on observations during the purification of 3.1 and 3.2, there is evidence to believe that 5-pentylfuran-2-furaldehyde (3.4) is an artefact of the novel (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1).

A sample containing a mixture of 3.1, 3.2 and 3.4 was kept at room temperature for 12h. 5-Pentylfuran-2-furaldehyde (3.4) saw an increase in its concentration while the (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) rapidly degraded and hence a decrease in its concentration was observed. F-acids are known for being unstable compounds and it is plausible that 3.1 was transformed to 3.4, making 5-pentylfuran-2-furaldehyde (3.4) a degradation product of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1). Degradation of furan fatty acids (F-acids) is discussed in Chapter 4, section 4.5.8.

3.7 Conclusion

Investigation of the fruiting bodies of Fulaga dive (collection no. Kiovi-MSp15, *Amanita sp.* Pers.) led to the discovery of two novel F-acids, (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2). Demonstrated antibacterial activity of crude extracts of Fulaga dive and isolated compounds has provided insight about the reason for Fulaga dive’s traditional use.

Fulaga dive produced large amounts of the (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2) and enough material of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) to undertake further biological testing. However, the rapid degradation of the latter prompted us to a synthetic approach to produce larger quantities of the two metabolites and test their antibacterial and antioxidant activities. Chapter 4 entails the chemical synthesis of F-acids isolated from Fulaga dive.

3.8 Experimental

3.8.1 NMR spectroscopy

Unless otherwise stated, all spectra were recorded on a Bruker Biospin GmbH NMR instrument at 300 K with probe 5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0258. $^1$H and $^{13}$C NMR experiments were undertaken at 400 MHz and 100 MHz respectively. For the elucidation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1), $^1$H and $^{13}$C spectra were recorded at 800 MHz and 200 MHz on an Ascend Bruker-800 instrument, at 298 K with probe 5 mm CPTCI 1H-13C/ 15N/ D Z-GRD Z44909/ 0022.

Chemical shifts ($\delta$) are reported in parts per million (ppm). $^1$H NMR spectra are referenced to the signal from CDCl$_3$ at 7.26 ppm or C$_6$D$_6$ at 7.16 ppm. $^{13}$C NMR spectra are referenced to the central peak in the signal from CDCl$_3$ at 77.0 ppm or C$_6$D$_6$ at 128.0 ppm. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: $^1$H, $^{13}$C, $^1$H-$^1$H COSY, $^1$H-$^{13}$C HSQC and $^1$H-$^{13}$C HMBC.
3.8.2 Mass spectrometry

Low resolution EI mass spectra (LREIMS) were recorded on a Finningan Polaris Q ion trap mass spectrometer using positive electron ionisation (EI+) mode at 70 eV. High resolution EI mass spectra (HREIMS) positive electron ionisation were recorded on a Waters VG autospec premier spectrometer at 70 eV.

Low resolution ESI mass spectra (LRESIMS) were recorded on a ZMD micromass spectrometer with Waters Alliance 2690 HPLC. High resolution ESI mass spectra (HRESIMS) were recorded on a Waters LCT premier XE time-of-flight (ToF) mass spectrometer.

Gas chromatography – mass spectrometry (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] 350°C and split 2:1 using Helium as carrier gas (1 mL/min) by using positive ion detection.

Oven temperature program started at 80 °C for 2 min, increasing 10°C/min up to 250°C, holding that temperature for 10 min. Mass/charge ratios (m/z) are reported and the relative abundance of fragments is shown as percentage of the base peak intensity.

3.8.3 IR spectroscopy

IR spectra were recorded on a Bruker Alpha-P spectrophotometer. Samples were free of solvent and diagnostic peaks are reported in wavenumbers (cm⁻¹).
3.8.4 UV measurements
UV spectra were recorded on a UV-Mini 1240 UV-Vis Spectrophotometer (Shimadzu); using 1.00 cm quartz cells in DCM. Absorption maxima are recorded in nm (log ε).

3.8.5 Chromatography
Thin layer chromatography (TLC) was performed with Merck aluminium backed plates, pre-coated with silica gel 60 F254 (0.2 mm). TLC plates were observed under UV light (254 nm) and/or by developing in ceric phosphomolybdic acid (100 mL water, 5 g phosphomolybdic acid, 0.6 g Ce(SO$_4$)$_2$, 6 mL concentrated H$_2$SO$_4$) dip, followed by heating.

Flash chromatography was performed under pressure using normal phase silica gel (230-400 mesh Scharlau 60) as a stationary phase and reagent grade solvents as eluent. Semi-preparative HPLC was performed using an Agilent 1100 system with diode array detector, using a Phenomenex Luna 5µ Silica (2) 100 A, 250 x 10.00 mm 5 micron column and pre-column Phenomenex part no. AJO-7220.

Purification of (Z- & E)-diastereoisomers (3.1 and 3.2); elution was carried out under gradient starting at 1:9 EtOAc and Pet Sp. for 40 min then changing to 100% EtOAc up to 65 min run. Flow rate was setup at 2 mL/min and detection at λ 254 nm and 290 nm.

For biological testings, 96 microtiter plates were read using a BioTek Epoch microplate spectrophotometer at 600 nm. Gen5™ 2.0 Data Analysis Software (BioTek Instruments, Inc, Winooski, USA) was used for data analysis. Agar plates were prepared using disposable sterile petri dishes. The absorbance of the working cultures was read using a UV-Mini 1240 UV-Vis Spectrophotometer (Shimadzu).
3.8.6 Solvents, culture media and controls

In general, all organic solvents were reagent grade solvents for extractions and chromatography and used without further purification unless otherwise stated. Pet Sp. (60 – 80 °C) and EtOAc were distilled prior to use. Purification of other solvents was carried out using typical procedures or as stipulated under Perrin & Armarego, Purification of Laboratory Chemicals, 3rd Edition.86

All media, BBL™ Mueller Hinton broth and DIFCO™ Mueller Hinton agar were sourced from Bacto Laboratories Pty Ltd, Australia and prepared as per the manufacturer’s instruction unless otherwise noted. The pH of the medium was adjusted to 7.3 ± 0.1 before autoclaving.

Kanamycin sulphate from Streptomyces kanamyceticus was used as the antibiotic control for the sensitive strains of S. epidermidis and E. coli β (-) and supplied by Sigma Aldrich, St Louis, USA. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used as the dye to determine the viability of the cells and it was sourced from Alfa-Aesar, Heysham, England.

3.8.7 Turbidity-MTT assay

Turbidity-MTT microdilution assay for Kupchan extracts and isolated compounds from Fulaga dive (Amanita sp. Pers.) were performed as outlined in 0 section 2.9.6

3.8.8 DNA extraction

Approximately 50 mg of grand stipe were collected and digested at 70°C for 10 min with 400 μl digestion TES lysis buffer [10 mM Tris (pH 8), 1 mM EDTA (pH 8) and 1% sodium dodecyl sulphate (SDS)]. Following neutralisation with 400 μl 2.8 M KOAc mixture was spin at 4°C at 2500 rpm for 10 min. Supernatant was transferred to a new microfuge tube and DNA was purified by adding approximately 800 μl of isopropanol followed by spinning for 15 min at 2500 rpm.
Solvent was removed and genomic DNA (gDNA) was rinsed five times with 800 µl of 95% EtOH followed by 800 µl of 70% EtOH. gDNA was allowed to dry and reconstituted with TE buffer [10 mM Tris and 1 mM EDTA (both at pH 8)] for long term storage.82

3.8.9 Amplification and sequencing.
Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, California, USA), in a volume of 10 µl containing 0.5 µM of each of ITS primers,83 0.08 µl of Takara Ex Taq and, 1 µl of Ex Taq buffer (Takara Bio, Otsu-shi, Japan), and 1 µl of gDNA. The thermal cycler program used was 2 mins at 95°C and 35 cycles of 30 sec at 95°C, 30 sec at 56°C, and 40 sec at 72°C.

The amplification products were purified with the QIAquick PCR purification kit (QIAGEN, Basel, Switzerland). The sequencing reaction of the amplification product was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, California, USA) using the ITS4, reverse primer and BigDye® kit (Applied Biosystems, Rotkreuz, Switzerland). The thermal cycler program used was 5 mins at 94°C and 30 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. Sequence product was analysed with a 96 capillary 3730 DNA Analyser (Applied Biosystems, California, USA).83

3.8.10 Metabolite data
(Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2): pale yellow solid; UV (DCM) λ\text{max} (log ε) 373 (1.69), 276 (3.23) nm; IR (neat) ν\text{max} 3400 – 2400 (br) 2924 (s), 1703 (s), 778 (m) cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\), 400 MHZ); and \(^{13}\)C NMR (CDCl\(_3\), 100 MHZ) see table 3.2 and 3.3. EIMS \text{m/z} 292 [M\text{+}]\(^{+}\) (74), 235 (22), 177 (100), 151 (21), 121 (26), 107 (84) 95 (24), 55 (26); HREIMS \text{m/z} 292.2033 (calculated for C\(_{18}\)H\(_{28}\)O\(_3\), 292.2038).
5-pentylfuran-2-furaldehyde (3.4): pale yellow oil; $^1$H NMR (CDCl$_3$, 400 MHZ) and $^{13}$C NMR (CDCl$_3$, 100 MHZ) see table 3.4. EIMS $m/z$ 166 [M]$^+$ (50), 109 (100), 81 (57), 53 (31); HREIMS $m/z$ 167.1073[M+H]$^+$ (calculated for C$_{10}$H$_{15}$O$_2$, 167.1072).
Chapter Four

Synthesis of novel furan fatty acids:
(Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid

This section is primarily concerned with the synthesis of two novel naturally occurring furan fatty acids (F-acids), (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), isolated from Fulaga dive (Amanita sp. Pers.) native to the Eastern Highlands Province, Papua New Guinea (PNG). In addition, this section also provides background on previous synthetic routes to F-acids and the biosynthetic origin of this class of compounds.
Chapter 4 : Synthesis of furan fatty acids (F-acids)

4.1 Introduction

Furan fatty acids (F-acids) are a class of compounds that have been isolated from a variety of sources, including fresh and salt water fish, algae and vegetables. Glass and co-workers characterised eight F-acids homologues by the presence of either a tri- or tetrasubstituted furan ring. F-acids differed from one another by the presence or absence of a methyl group on the ring at carbon 4 and on the length of the chain at carbon 2 and 5. It is worth noting that two ‘unusual’ F-acids have been reported in the literature; a) a disubstituted F-acid isolated from the seed oil of *Exocarpus cupressiformis* Labill. and b) a F-acid containing a long unsaturated chain of up to 20 carbons in one of the alpha positions of the furan ring isolated from sea sponges. 

Although the extent of biological activity of F-acids is not yet known, this type of compounds have gained attention for their role as radical scavengers and their potential as antioxidants. Batna and Spiteller performed a series of experiments using a synthetic C-18 disubstituted F-acid, 9-(5-pentylfuran-2-yl)-nonanoic acid (4.1), to study its oxidative stability via conversion to (Z)-10,13-dioxo-octadec-11-enoic acid (4.2). Figure 4.1 shows a schematic representation of the oxidation of the C-18 synthetic disubstituted F-acid.

When 4.1 was stirred under pure oxygen in the dark or under light irradiation, no spontaneous oxidation was observed. In addition, when the enzyme soy bean...
lipoxigenase-1 (L-O') was added to the reaction and the experiment repeated under the same conditions, 4.1 remained unchanged. The same was true when 4.1 was in contact with linoleic acid; no dioxoenoic acid (4.2) was formed. However, when linoleic acid and L-O' were present in the mixture, the oxidation of 4.1 occurred and (Z)-10,13-dioxo-octadec-11-enoic acid (4.2) was formed in small amounts as the degradation product.  

Batna and Spiteller provided a mechanistic explanation for the conversion of 4.1 to dioxoenoic acid (4.2). These authors suggested that the first step involved the oxidation of linoleic acid (4.3) by L-O' in the presence of oxygen to form (9Z, 11E)-9-hydroperoxyoctadecadienoic acid (4.4), as shown in Figure 4.2. Subsequently, 4.4 was cleaved to form alkoxyl radicals (R-O') or peroxyl radicals (R-OO') following reaction with divalent metal ions (e.g. Fe^{2+}) available from the enzyme soy bean lipoxigenase-1 (L-O'), similar to a Fenton reaction. These authors also suggest that a peroxyl radical (R-OO') is an intermediate product of the oxidation of linoleic acid (4.3) by L-O'.

![Figure 4.2: Oxidation of linoleic acid (4.3) by soybean-lipoxigenase-1 (L-O') to alkoxyl radicals (R-O') or peroxyl radicals (R-OO')]
Batna and Spiteller\textsuperscript{87} presumed that one of the radicals formed after initial oxidation of linoleic acid (4.3) could then attack positions 2 or 5 of the furan ring of their synthetic 9-(5-pentylfuran-2-yl)-nonanoic acid (4.1) forming a furanoid radical intermediate, which rapidly undergoes ring opening to give a stable mesomeric radical. The stability of this intermediate radical allows it to trap a second alkoxyl radical (R-O’) forming a ketal, which undergoes hydrolysis to give the corresponding \((Z)\)-10,13-dioxo-octadec-11-enoic acid (4.2). The diagram shown in Figure 4.3, illustrates that a single F-acid can scavenge two alkoxyl radicals.

Figure 4.3: Oxidation of 9-(5-pentylfuran-2-yl)-nonanoic acid (4.1) by alkoxyl radicals.

Batna and Spiteller\textsuperscript{87} also suggested that only small amounts of disubstituted F-acid (4.1) was oxidised to its corresponding dioxenoic acid (4.2).\textsuperscript{87} Such observation was consistent with the work of Okada and co-workers\textsuperscript{80} on the reactivity of F-acids. Okada and co-workers\textsuperscript{80} suggested that the reactivity of F-acids was dependant on the number
4.2 **Biosynthesis of F-acids**

F-acids are biosynthesised from acetic acid units in a similar manner to their straight-chain fatty acids counterpart.\(^{88}\) Scheinkoenig and Spiteller\(^ {88}\) demonstrated through their feeding experiments of \(^{13}\)C labelled acetate on suspension cultures of a *Saccharum sp.*, that the basic carbon chain skeleton of F-acids was formed following the fatty acid pathway. Thus, in order to understand the biosynthetic origin of F-acids it is important to understand the general process of how fatty acids are formed.

4.2.1 **Formation of acetyl coenzyme A**

Coenzyme A (CoA), a heat stable cofactor, was found to be required in many enzyme-catalysed acetylations: CoA consists of an adenine nucleotide at one end of the molecule. This nucleotide is joined to a pantothenate unit by pyrophosphate. CoA also consists of a \(\beta\)-mercaptoethylamine unit with a terminal sulphydryl group, the latter being the reactive site in this molecule.\(^ {89}\) Figure 4.4 shows the structure of CoA.

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**Figure 4.4: Coenzyme A (CoA)**
CoA can be converted to acetyl-CoA by oxidative decarboxylation of pyruvate with nicotinamide adenine dinucleotide (NAD$^+$). This reaction is catalysed by the enzyme pyruvate dehydrogenase (Figure 4.5).\cite{89}

![Oxidative decarboxylation of pyruvate to acetyl CoA](image)

**Figure 4.5: Oxidative decarboxylation of pyruvate to acetyl CoA**

### 4.2.2 Formation of malonyl coenzyme A

The fatty acid synthesis starts with the irreversible carboxylation of acetyl-CoA to malonyl-CoA, which is catalysed by the enzyme acetyl-CoA carboxylase.\cite{89} Figure 4.6 shows the formation of malonyl-CoA.

![Generation of malonyl CoA](image)

**Figure 4.6: Generation of malonyl CoA**

The enzyme acetyl-CoA carboxylase contains a biotin prosthetic group, composed of two fused five-membered heterocyclic rings. One of the five-membered rings is a cyclic sulphide with a long saturated hydrocarbon chain ending with a carboxyl group. The second five-membered ring is a cyclic urea moiety. The carboxyl group of biotin is covalently attached to the ε-amino group of a lysine residue. The biotin prosthetic group reacts with bicarbonate to produce a carboxybiotin intermediate. In the next step, deprotonation occurs causing formation of carbon dioxide, which subsequently acts as
an electrophile in the reaction with the acetyl-CoA anion to create malonyl-CoA (Figure 4.7).  

4.2.3 The synthesis of fatty acids  
The synthesis of fatty acids continues with the attachment of acetyl-CoA and malonyl-CoA to an acyl carrier protein (ACP). These reactions are catalysed by acetyl- and malonyl transacylase enzymes.
The next step in the elongation phase of fatty acid synthesis is the reaction between acetyl-ACP and malonyl-ACP in a Claisen-like condensation where acyl-malonyl-ACP is the condensing enzyme. The product of this reaction is the four carbon unit acetoacetyl-ACP and CO$_2$ is released (Figure 4.8).$^{89}$

The next three steps in the synthesis of fatty acids involve a series of enzymatically controlled reactions firstly reducing the keto group at carbon-3 of acetoacetyl-ACP to 3-hydroxybutyryl-ACP using NADPH as the reducing agent. 3-Hydroxybutyryl-ACP subsequently undergoes a dehydration reaction via an E1cB mechanism producing crotonyl-ACP and finally reduction of the double bond of crotonyl-ACP leads to butyryl-ACP. In the second round of the elongation phase of fatty acid synthesis, butyryl-ACP condenses with malonyl-ACP to form a six-carbon unit and the process of elongation is ready to be repeated.$^{89}$

![Fatty acid pathway](image)

**Figure 4.8: Fatty acid pathway**

### 4.2.4 The synthesis of F-acids

After the discovery of eight F-acids from the northern pike fish by Glass and co-workers,$^{73}$ scientists speculated whether fish synthesised this type of compounds on their own or if they acquired them from their diet. Scientists agreed that depending on the season and the type of fish the concentration of F-acids changed dramatically.$^{73}$
Sand and co-workers\textsuperscript{90} performed \textit{in vivo} experiments on interconversions of F-acids in fish by administering $^{14}$C-labelled acetate.\textsuperscript{90} These authors concluded that fish are unable to biosynthesise F-acids on their own, though fish were able to metabolise this class of compounds in a similar manner to polyunsaturated fatty acids.

In addition, Sand and co-workers\textsuperscript{90} also concluded that the acetate is incorporated into the carboxylic side chain by chain elongation. However, acetate is not incorporated into the alkyl chain and furan ring, suggesting that fish obtained F-acids from their diet.\textsuperscript{90}

Glass and co-workers,\textsuperscript{78} while characterising F-acids from fish lipids, speculated that this class of compounds might be biosynthesised from linoleic acid (4.3).\textsuperscript{78} Subsequently, Gorst-Allman and co-workers,\textsuperscript{91} while investigating the occurrence of F-acids in plants, confirmed that compounds of this class containing a pentyl side chain in one of the alpha positions of the furan ring do come from linoleic acid (4.3).\textsuperscript{91}

Peroxidation of linoleic acid (4.3) is still speculated to be initiated by the enzyme lipoxygenase (L-O·) and oxygen from air. This lipoxygenase type reaction with linoleic acid (4.3) produces a peroxyl radical (R-OO'). Initial peroxidation is followed by ring closure and bond rearrangement producing a disubstituted F-acid.

Finally, the methyl groups in the $\beta$-position of the furan ring are incorporated via a methylation reaction with methionine forming 9-(3,4-dimethyl-5-pentylfuran-2-yl)-nonanoic acid (4.5).\textsuperscript{91} Figure 4.9 shows a schematic representation of peroxidation of linoleic acid (4.3) to 4.5.
Chapter four: Synthesis of furan fatty acids (F-acids)

4.3 Examples on previous chemical synthetic pathways

F-acids are a class of compounds that have been characterised as either tri- or tetrasubstituted furan derivatives. However, a disubstituted F-acid, 8-(5-hexylfuran-2yl)-octanoic acid (3.3), isolated from the seed oil of *Exocarpus cupressiformis* Labill. has been reported. The synthesis of 3.3 was first reported by Elix and Sargent and the reaction scheme is shown in Figure 4.10.

Reaction sequence started from 2-furoic acid, which was converted to 2-furoyl chloride (4.6) with thionyl chloride. Subsequent reaction of 2-furoyl chloride (4.6) with dipentyl cadmium afforded 2-hexanoylfuran (4.7).

A modified Wolff-Kishner reaction was performed on 2-hexanoylfuran (4.7) reducing the ketone to give 2-hexylfuran (4.8). The synthesis continued with acylation of 2-hexylfuran (4.8) with suberic anhydride and boron trifluoride diethyl etherate to give 8-(5-hexyl-2-furyl)-8-oxo-oxtanoic acid (4.9).
Finally reduction of the ketoacid in 4.9 with a modified Wolff-Kishner reaction afforded 8-(5-hexylfuran-2yl)-octanoic acid (3.3).

Glass and co-workers\textsuperscript{73} reported that 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (4.10) was the most abundant F-acid extracted from fish lipids and Rahn and co-workers\textsuperscript{93} reported its total synthesis. The reaction sequence is shown in Figure 4.11.

The first step involved a condensation reaction between furan-3,4-diylbis-(methylenediacetate (4.11) and pentanoic acid anhydride in the presence of boron trifluoride diethyl etherate to produce (2-pentanoylfuran-3,4-diyl)-bis-(methylenediacetate (4.12). Subsequent reduction of all carbynols under Wolff-Kishner conditions gave the 5-pentylfuran-dimethanol derivative (4.13).
Figure 4.11: Synthesis of 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (4.10)

The hydroxyl groups of 4.13 were exchanged by chlorine with phosgene and the resulting dichloro compound (4.14) underwent reduction reaction with LiAlH₄ to give 3,4-dimethyl-2-pentylfuran (4.15). Insertion of the fourth substituent on the furan ring to produce 2-(10-chlorodecyl)-3,4-dimethyl-5-pentylfuran (4.16) was achieved by metalation with n-BuLi and subsequent reaction of the lithiated derivative with 1-chloro-10-iododecane.

Finally, 4.16 underwent lithium-halogen exchange and carbonation to produce the desire tetrasubstituted F-acid 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (4.10).
Rahn and co-workers\textsuperscript{94} also reported the synthesis of a radioactive F-acid to study their biological conversions, following an alternative route to that shown on Figure 4.11. 3,4-Dimethyl-5-pentylfuran (4.15) was formylated under Vilsmeier’s conditions producing 3,4-dimethyl-5-pentyl-2-furaldehyde (4.17).

Wittig olefination between 4.17 and the ylid prepared from (6-carbomethoxyhexyl)-triphenylphosphonium iodide gave 4.18. Lindlar’s hydrogenation gave a tetra substituted F-acid methylester (4.19) and subsequent Arndt-Eistert homologation reaction with $^{14}$CH$_2$N$_2$ introduced the additional labeled carbon producing the F-acid derivative (4.20). Figure 4.12 shows the synthetic scheme.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{synthesis_scheme.png}
\caption{Synthesis of labelled F-acid derivative}
\end{figure}

On an attempt to synthesise the second most abundant F-acid 11-(3-methyl-5-pentylfuran-2-yl)-undecanoic acid (4.21) reported by Glass and co-workers,\textsuperscript{73} Bach and Krueger\textsuperscript{95} took advantage of the reactivity of bromine as a substituent at the furan ring to synthesise 4.21.
Dibromo-furaldehyde (4.22) was used as starting material and it was cross-coupled with 10-undecynoic acid benzyl ester, under Sonogashira conditions, which selectivity at position 2 of the furan ring furnished 4.23. A Wittig olefination was subsequently carried out, achieving in this manner 4.24.

Finally, Negishi coupling allowed the exchanged of bromine at the β-position on the furan moiety in 4.24 by a methyl group and subsequent hydrogenation afforded the trisubstituted F-acid, 11-(3-methyl-5-pentylfuran-2-yl)-undecanoic acid (4.21). Figure 4.13 shows the reaction scheme.
4.4 Synthesis of (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid

Previous synthetic approaches served as precedent for a synthetic route towards the preparation of F-acids (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) isolated from Fulaga dive (Amanita sp. Pers.). Considering the availability of potential starting materials in our laboratory a retrosynthetic analysis was performed in order to determine starting points for the forward synthesis of such F-acids. Figure 4.14 shows a schematic representation of interconversions and disconnections leading to candidates for the forward synthesis of 3.1 and 3.2.

![Figure 4.14: Retrosynthetic analysis for the synthesis of F-acids (3.1 and 3.2) (X = halogen)](image-url)
Thus, it seemed reasonable to achieve target acids \((Z & E)-9-(5\text{-pentylfuran-2-yl})\text{-non-8-enoic acid (3.1 and 3.2) through functional group interconversion and selective reduction of a furan alkynol derivative similar to that used by Bach and Kruger}^{95}\) (Figure 4.13) but taking care to stop at the alkene without over-reduction to the alkane. In addition, through disconnection of the alkynol chain from the furan core, we would afford a terminal acetylenic alcohol and a halogenated alkyl furan derivative.

Finally, the halogenated alkylfuran could be achieved by disconnection of the halogen substituent and the pentyl side chain from the furan core, giving the convenient and commercially available starting materials: furan and a five-carbon haloalkane. Similarly, the long chain terminal acetylenic alcohol could be prepared from the alkylation of propargyl alcohol and subsequent migration of the triple bond to the end of the chain.

### 4.5 Results and discussion

#### 4.5.1 Alkylation of furan

The first step in the forward synthesis towards the two naturally occurring F-acids, \((Z & E)-9-(5\text{-pentylfuran-2-yl})\text{-non-8-enoic acid (3.1 and 3.2) was the ortho-lithiation and subsequent alkylation of furan (4.25) with }n\text{-butyllithium (}n\text{-BuLi}) and 1\text{-bromopentane to form 5-pentylfuran (4.26) as shown of Figure 4.15.}

![Figure 4.15: Alkylation of furan (4.25), 95% yield.](image)
The use of alkyllithium reagents to induce deprotonation at one of the α-positions of the furan ring prior to addition of an electrophile has been previously explored. As shown in Figure 4.16, mechanistic evidence of this type of reactions suggest that lithium coordinates with the oxygen of furan (4.25) while the alkyl portion of n-BuLi removes a proton producing butane. The furan-lithium intermediate undergoes an SN2 reaction with 1-bromopentane to yield 5-pentylfuran (4.26) and lithium bromide, which is easily removed during aqueous work-up.

![Figure 4.16: Mechanistic representation of the alkylation of furan (4.25)](image)

Although mechanistic evidence was suggestive of a straightforward transformation, first attempts were challenging and optimisation became important. First, with general directions from Koldobskii and co-workers, n-BuLi was added dropwise to a solution of furan (4.25) in dried THF at 0°C. The resulting mixture was stirred for 30 min before 1-bromopentane was added at -78°C. The resulting 1:1:1 ratio mixture was allowed to stir for an extra 30 min before work-up.

This attempt proved to be inefficient, as mostly starting material was recovered. The poor yield obtained (approx. 13%) prompted us to modify the reaction conditions in an attempt to improve the yield of the reaction.
Citron and co-workers\textsuperscript{98} provided ideas to modify the reaction. The temperature of addition of the alkyl lithium was modified from 0°C to -78°C. It was assumed that by decreasing the temperature, the deprotonation process could be sufficiently slow to guarantee complete deprotonation of furan (4.25) avoiding side reactions between the alkyl lithium and the electrophile alone.

After addition of the electrophile, the 1:1:1 mixture was allowed to stir for three hours, allowing to reach room temperature, to make sure the reaction could go to completion. Unfortunately this second attempt also proved to be inefficient, as mostly unreacted starting material was present at the end of the reaction. However, in a parallel experiment where a slightly higher concentration of the alkyl lithium was used, the results were more promising, as the yield of the reaction increased. Uncertainties about the true concentration of the alkyl lithium used in the first two attempts prompted us to re-standardise \textit{n}-BuLi against \textit{N}-benzylbenzamide under the exact conditions as described by Burchat and colleagues.\textsuperscript{99}

Finally, the work of Rhan and co-workers\textsuperscript{93} on the synthesis of F-acids, the work of Thomas and co-workers\textsuperscript{100} on the synthesis of thiophenes, and the experience gained from failed experiments provided successful directions for the synthesis of 5-pentylfuran (4.26).

By adding two equivalents of furan (4.25) to a solution of \textit{n}-BuLi in dried THF at -20°C, deprotonation of furan (4.25) was achieved in a controlled manner and prevented side reactions between the alkyl lithium and the electrophile once added. The resulting mixture was brought to 0°C and stirred for 2.5h at which time 1-bromopentane was added and the mixture was stirred overnight at room temperature, producing 5-pentylfuran (4.26) in high yields (95%).
Evidence of a successful reaction was obtained from NMR analyses. For example, the appearance of two doublets of doublets at 7.30 ppm (A) and 6.29 ppm (B) in the aromatic region of the $^1$H NMR spectrum, as well as a doublet of doublet of triplets at 5.98 ppm (C) suggesting substitution in one of the alpha positions of the furan ring. Figure 4.17 shows downfield region of the $^1$H NMR spectrum of 5-pentylfuran (4.26).

![NMR spectrum](image)

**Figure 4.17:** Downfield region of $^1$H (400 MHz) NMR spectrum in CDCl$_3$ of 5-pentylfuran (4.26)

### 4.5.2 Halogenation of 5-pentylfuran

The second step in the synthesis of (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) was the halogenation of 5-pentylfuran (4.26). Bach and Kruegar$^{95}$ took advantage of the reactivity of bromine in the synthesis of F-acids.
Therefore, the use of \(N\)-bromosuccinamide (NBS) to incorporate bromine seemed reasonable. Figure 4.18 shows the reaction scheme of the bromination of \(4.26\).

![Figure 4.18: Bromination of 5-pentylfuran (4.26)](image)

Although the use of NBS in halogenation reactions has been well documented,\(^{101-102}\) most attempts at this reaction proved challenging, as mostly complex mixture of resonances were observed in the \(^1\)H NMR spectrum.

Looking at the mechanisms of this type of transformations, light and heat play the role of radical initiators in the homolysis of NBS to release one bromine atom and produce hydrobromic acid (HBr), as a by-product, upon reaction with alkylfuran. HBr subsequently undergoes homolytic cleavage and interacts with another NBS producing bromine, which becomes the brominating agent in the transformation to 2-bromo-5-pentylfuran \((4.27)\).\(^{96}\)

Based on the mechanism shown in Figure 4.19, there is no reason to think that NBS could also remove one hydrogen (-H\(_1\)) from the side chain at position C-1, indicating that bromination can also occur at the allylic carbon. However, that still does not explain the complex mixture of resonances observed in the \(^1\)H NMR spectrum.
Considering the photosensitivity of bromination reactions, with general instructions from Raheem and co-workers, the reaction mixture was protected from light. In addition, NBS was added at 0°C and the mixture was allowed to slowly reach 25°C. By reducing the temperature of addition and protecting the reaction mixture from light, the halogenation could be slowed down to avoid side reactions, as on previous attempts.

Evidence of bromination in the alpha position of the furan ring was confirmed by the disappearance of a doublet of doublet of triplets in the $^1$H NMR spectrum and its conversion to a slightly deshielded AB system belonging to 2-bromo-5-pentylfuran (4.27). Although the reaction seemed to have worked, according to the $^1$H NMR spectrum, it rapidly decomposed to a complex mixture of products. This issue prompted me to seek other strategies to make a more stable halogenated derivative.
Gidron and co-workers in their experiments with α-oligofurans, as semiconductors, provided general directions in the use of elemental iodine as the halogenating agent. Figure 4.20 shows the general reaction scheme.

![Reaction Scheme](image)

**Figure 4.20: Iodination of 5-pentylfuran (4.26), 72% yield.**

5-Pentylfuran (4.26) was added to a solution of \(n\)-BuLi in dried THF at -20°C and subsequently stirred for 2h at 0°C. A solution of iodine in dried THF was added and the resulting mixture was stirred overnight.

As shown in Figure 4.21, the downfield region of the \(^1\)H NMR spectrum in black exhibits the disappearance of an ABC spin system (7.30 ppm, labelled as A) and its conversion into a slightly more deshielded AB spin system, supported by the \(^1\)H NMR spectrum in red (6.40 ppm, labelled as A).

The downfield movement of the AB spin system is expected due to the incorporation of an electron donating group such as iodine in the \(\alpha\)-position of the furan ring. Thus, switching to iodine as the halogenating agent rendered 2-iodo-5-pentylfuran (4.28) with few by-products.
**4.5.3 Protection, alkylation and deprotection of propargyl alcohol**

The third step towards the synthesis 3.1 and 3.2 was to create a 9-carbon acetylenic alcohol to serve as a substrate in the subsequent cross-coupling reaction with the halo-heteroaryl compound 4.28. Thus, propargyl alcohol (4.29) and 1-bromohexane were the obvious reactants to carry out the synthesis of such acetylenic alcohol.

There is precedent indicating that propargyl alcohol (4.29) could be deprotonated twice when a strong base such as $n$-BuLi is used.\(^6\) The deprotonation process indicates that an alkoxyde would be firstly formed, due to a pKa 16 of the hydroxyl proton.
In addition, a dianion would be subsequently created, as the proton attached to the sp hybridised carbon (pKa 25) would be also removed.

According to literature, if an electrophile reacts with an anion, the electrophile would preferentially react at the alkynyl anion and not the alkoxide. However, there is no evidence suggesting that an electrophile would not react at the alkoxide too.

Thus, propargyl alcohol (4.29) underwent a tetrahydropyranilation reaction with 3,4-dihydro-2H-pyran (DHP), using pyridinium p-toluenesulfonate (PPTS) as a catalyst to protect the hydroxyl group from deprotonation and in that manner guarantee alkylation in the desired position. Figure 4.22 shows the reaction scheme for the protection of propargyl alcohol.

![Reaction Scheme](image)

**Figure 4.22: Protection of propargyl alcohol (4.29), 90% yield.**

In this reaction, the π-bond, chimerically assisted by the oxygen of DHP, would react with the acidic proton of PPTS and subsequently 4.29 acting as a nucleophile would approach the oxonium ion formed. The conjugate base of PPTS would regain a proton forming 2-(prop-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.30) as shown in Figure 4.23.
Upon protection of propargyl alcohol (4.29), the \( sp \) hybridised carbon was deprotonated by \( n\text{-BuLi} \) at \(-78^\circ\text{C} \) in dried THF. The lithiated intermediate subsequently underwent an SN2 reaction with 1-bromohexane forming 2-(non-2-yn-1-yloxy)-tetrahydro-2\( H \)-pyran (4.31). Hexamethylphosphoramide (HMPA) was added to the reaction mixture, to solvate lithium cations to generate more naked anions, in order to accelerate the otherwise slow SN2 reaction between the lithiated intermediate and alkylhalide. 2-(Non-2-yn-1-yloxy)-tetrahydro-2\( H \)-pyran (4.31) was used in the next step without further purification and/or characterisation.
The final step before transitioning to cross-coupling reactions was the deprotection of 2-(non-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.31). The THP group being unstable under acid conditions, provided clues of how to remove it from 4.31.\(^6\) In the presence of acid, a lone pair of electrons from the acyclic oxygen atom of 4.31 accepts a proton from the acid causing electron movement from the THP group to reform the alcohol. In addition, an oxonium ion is created and it is subsequently decomposed to 2-methoxytetrahydro-2H-pyran, as shown on Figure 4.25.

![Figure 4.25: Acid hydrolysis of acetal in MeOH](image)

Experimentally, 2-(non-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.31) was deprotected with p-toluensulfonic acid in MeOH producing non-2-yn-1-ol (4.32). As shown in Figure 4.26, deprotection of the alkylated alcohol was evident by the disappearance of all resonances in the \(^1\)H NMR spectrum characteristic of a tetrahydropyran. In addition, typical resonances of a long chain internal acetylenic alcohol were also evident. For example a terminal methyl group (D) followed by a methylene “envelop” (C), as well as, an \(A_2M_2X_2\) spin system at 2.21 ppm (B) and an \(A_2X_2\) spin system (A) deshielded due to inductive effect by the hydroxyl group of the alcohol.
Figure 4.26: $^1$H (400 MHz) NMR spectrum in CDCl$_3$ of non-2-yn-1-ol (4.32), 90\% yield.

4.5.4 Alkyne “zipper” isomerisation

The internal alkyne of non-2-yn-1-ol (4.32) must be converted into a terminal alkyne in order to perform cross-coupling reactions with 2-iodo-5-pentylfuran (4.28). Initial directions were given by Brown and Yamashita$^{104}$ from their work on the isomerisation of long chain alkynes.

The original reaction used 2-hexyne as the test compound and potassium 3-aminopropylamide (KAPA), classified as a “superbase” of high reactivity created \textit{in situ} and key reagent in the isomerisation reaction. KAPA was prepared from the reaction between potassium hydride (KH) and aminopropylamine (APA). It should be noted that at this point, it was considered to replace KH for sodium hydride (NaH), as a more manageable reagent.
As shown in Figure 4.27, mechanistically, KAPA allows a series of protonations and deprotonations via an allene intermediate until the internal triple bond migrates to the terminal position of the chain. The final proton is transferred to the carbanion during acid work-up to form the terminal alkyne.

![Figure 4.27: “Zipper” isomerisation reaction mechanism](image)

Sodium hydride (NaH) was used instead of KH to produce a solution of NAPA and key reagent for this reaction. Precedent for the use of NaH was provided by Goh and co-workers.\textsuperscript{105} It is worth noting that early attempts at the “zipper” reaction using the protected acetylenic alcohol (4.31) as a test sample were unsuccessful. Success was not achieved even when NAPA was replaced for the original KAPA.
It seemed plausible that for the “zipper” reaction to take place, the most acidic proton in 4.31 must have been abstracted by NAPA or KAPA. However, the presence of a THP protecting group having electron withdrawing effect from the neighbouring acetal would make the proton 1’-H, shown in Figure 4.28, more acidic and the reaction would not take place.

As described in section 4.5.3, 2-(non-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.31) was deprotected under acidic conditions forming non-2-yn-1-ol (4.32) and re-submitted to isomerisation reaction under the conditions used by Goh and colleagues. With 4.32, as the new substrate, it would be expected that an excess of NAPA would deprotonate the most acidic proton (pKa 15) forming an alkoxide. The formation of an alkoxide as an electron source around 1’-H will decrease the acidity of this proton, favouring 4’-H proton to be abstracted by NAPA and form the allene intermediate until non-8-yn-1-ol (4.33) is formed. Figure 4.29 shows the reaction scheme.

Figure 4.28: “Zipper” reaction on 2-(non-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.31)

Figure 4.29: “Zipper” reaction on non-2-yn-1-ol (4.32), 70% yield.
The completion of the alkyne isomerisation reaction was confirmed by the presence of the acetylenic methine resonance as a triplet (1.93 ppm, $J = 2.7$ Hz) in the $^1$H proton NMR spectrum. In addition, the disappearance of a singlet quaternary carbon, at 86.6 ppm in the $^{13}$C NMR spectrum and appearance of a protonated carbon resonance at 68.1 ppm supported acetylene migration to the terminal position and hence the formation of non-8-yn-1-ol (4.33).

4.5.5 Cross-coupling reaction

The fifth step towards the synthesis of (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) was a Sonogashira cross-coupling reaction between 2-iodo-5-pentylfuran (4.28) and non-8-yn-1-ol (4.33) to produce 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34). Figure 4.30 shows the reaction scheme.

Sonogashira reactions between heteroaryliodide compounds and terminal alkynols, using different catalysts and bases, have been previously reported in the literature.\textsuperscript{106-108} In addition, Bach and Krueger\textsuperscript{95} also used Sonogashira cross-coupling reactions in their synthesis of the second most abundant F-acid reported by Glass and co-workers,\textsuperscript{78} 11-(3-methyl-5-pentylfuran-2-yl)-undecanoic acid (4.21).

As presented in Figure 4.31, a Sonogashira cross-coupling reaction begins with the palladium catalytic cycle, which forms a complex due to oxidative addition of an alkylhalide to the palladium catalyst (Pd\textsuperscript{0}).

Figure 4.30: Sonogashira cross-coupling reaction, 44% yield.
This complex increases the oxidation state of Pd\(^0\) to Pd\(^{\text{II}}\). The palladium catalytic cycle continues with transmetalation with an organocopper reagent, which is simultaneously formed through a reaction between a terminal alkynol and a copper catalyst in the presence of a base such as pyrrolidine.

The alkynol is deprotonated by the base and the resulting alkynyl anion replaces iodide in the Pd\(^{\text{II}}\) complex, regenerating the copper iodide catalyst. Finally, reductive elimination gives the new cross-coupled product and the palladium catalyst is regenerated.

Experimentally, Alami and co-workers\(^{110}\) provided directions for the synthesis of 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34). Thus, 2-iodo-5-pentylfuran (4.28) was cross-coupled with non-8-yn-1-ol (4.33) using tetrakis-(triphenylphosphine)-palladium and copper iodide (CuI) as catalyst and co-catalyst respectively.
Pyrrolidine was used as the solvent and base and the reaction mixture was allowed to stir overnight at room temperature, producing 4.34 after purification. The disappearance of the acetylenic proton at 1.93 ppm in the $^1$H NMR spectrum and the appearance of a new quaternary carbon in the $^{13}$C NMR spectrum at 71.4 ppm as a result of the new sigma carbon-carbon bond formed between 4.28 and the alkynol 4.33 served as confirmation that 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34) was successfully synthesised.

**4.5.6 Oxidation of 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol**

The retrosynthetic analysis described in section 4.4 provided general guidelines for the synthesis of (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2). Thus, in the final stages of the synthesis of F-acids isolated from Fulaga dive, it seemed reasonable to begin by selectively reducing the alkyne to the corresponding Z- and E-alkenes, followed by functional group interconversion to obtain the target acids.

After the initial attempts at reducing 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34), the resulting Z-furan alcohol derivative appeared to be unstable showing a very particular degradation pathway, which is discussed in more detail in section 4.5.8. Because of the instability of the Z-furan alcohol, it was decided that functional group interconversion was going to be explored followed by selective reduction of the alkyne.

There is precedence available on how to convert primary alcohols to their corresponding carboxylic acids. A common method is the use of potassium permanganate (KMnO$_4$) in basic solutions. However, being a strong oxidiser, KMnO$_4$ is capable of oxidising double and triple bonds before oxidising the intended alcohol. Therefore, this method was found unsuitable, as oxidative cleavage of the triple bond and the π-bonds of furan in 4.34 were likely to occur.
Another common method is the use of Jones reagent for the interconversions of primary alcohols to their corresponding carboxylic acids.\textsuperscript{111} Jones reagent uses a solution of chromium (VI) in aqueous sulfuric acid (H\textsubscript{2}SO\textsubscript{4}), which forms a red solution of chromic acid. The resulting red solution turns green by the building up of chromium (IV), which is an indication that the reaction went to completion. Considering the well-known acid sensitivity of furan derivatives, the use of Jones reagent was also found unsuitable for our purpose.

Finally, Corey and Schmidt\textsuperscript{112} reported the use of pyridinium dichromate (PDC) in dimethylformamide (DMF) to convert primary alcohols to carboxylic acids at neutral conditions. The neutral conditions were found to be appealing for our purpose. As shown in the proposed mechanism on Figure 4.34, the primary alcohol would react with a chromium (VI) complex, which subsequently could also react intra or intermolecularly to produce an aldehyde intermediate and its corresponding chromium (IV), as by-product. The aldehyde could presumably further react with any excess of chromium (VI) present in the mixture to produce the corresponding carboxylic acid.

![Figure 4.32: Proposed mechanistic approach of Corey and Schmidt oxidation.\textsuperscript{113,114}](image-url)
Experimentally, 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34) was added to a solution of PDC in DMF at 0°C and the reaction mixture was stirred overnight allowing to reach room temperature. Partial oxidation of 4.34 to its corresponding aldehyde intermediate was confirmed by the appearance of an aldehydic proton at around 9.75 ppm in the $^1$H NMR spectrum. However, the formation of carboxylic acid was uncertain, as several unidentified proton resonances were also visible.

In light of the uncertainty of whether the single step reaction was in fact successful, GC-MS analysis was performed. GC-MS clearly suggested the formation of the aldehyde derivative by the appearance of a molecular ion [M]$^+$ at m/z 274 though no evidence of a carboxylic acid was detected. It was then decided to explore other oxidation methods to obtain the carboxylic acid in two steps.

a) Oxidation of primary alcohol to aldehyde

With general directions obtained from Zúñiga and co-workers,\textsuperscript{115} a mixture of catalytic amounts of TEMPO radical and a slight excess of bis-acetoxy-iodobenzene (BAIB) in DCM were used to promote the transformation of 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34) to the corresponding aldehyde 4.35, as shown in Figure 4.33.

The oxidation of the primary alcohol to aldehyde was performed successfully and it was used in the next step without further purification.

![Figure 4.33: Epp and Widlanski oxidation of primary alcohols.\textsuperscript{116}](image-url)
Although many efforts have been done to determine the mechanism of the oxidation of an alcohol to its corresponding aldehyde using oxoammonium salts as primary oxidant, the mechanism is not very well understood. However, experimental studies have been reported on the mechanism of this reaction suggesting that TEMPO alone is not the primary oxidant.\textsuperscript{117}

Figure 4.34 shows a schematic representation of acid catalysed “bismutation”, where TEMPO undergoes two different transformations. For example, TEMPO can be reduced to form hydroxylamine or oxidised to form its corresponding oxoammonium salt. The resulting oxoammonium salt becomes the primary oxidant.

Furthermore, Epp and Widlanski\textsuperscript{116} suggested that the first molecule of acid responsible for the catalysis of TEMPO to its oxoammonium salt could be generated by ligand exchange between BAIB and the primary alcohol.

An interaction of this sort would release acetic acid capable of initiating the “bismutation” of TEMPO radical. The resulting oxoammonium salt would react with the primary alcohol to form the desired aldehyde and hydroxylamine as a by-product.
Hydroxylamine could be further oxidised by BAIB back to TEMPO, also generating another molecule of acetic acid, initiating the catalytic cycle. Figure 4.35 shows a schematic representation of the oxidation of a primary alcohol as per Epp and Widlanski.¹¹⁶

![Figure 4.35: Oxidation of primary alcohols to aldehydes by Epp and Widlanski]¹¹⁶

b) Oxidation of aldehyde to carboxylic acid

Lindgren and Nilsson¹¹⁸ reported the preparation of carboxylic acids from aldehydes using chlorite as the oxidant. Almost a decade later, Pinnick and co-workers¹¹⁹ reported very similar work to Lindgren and Nilsson’s in the oxidation of aldehydes with some variations in their methodology.

Thus, hereafter the conversion of aldehydes to carboxylic acids under sodium chlorite (NaClO₂) in aqueous tertiary butanol (t-BuOH) would be referred to as Pinnick-Lindgren oxidation.¹¹⁸-¹¹⁹
Chapter four: Synthesis of furan fatty acids (F-acids)

9-(5-Pentylfuran-2-yl)-non-8-ynal (4.35) was oxidised under Pinnick-Lindgren\textsuperscript{118-119} conditions to form 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) and the reaction scheme is shown in Figure 4.36

\[ \text{Figure 4.36: Pinnick-Lingren oxidation of aldehyde to carboxylic acid, 80\% yield.} \]

Mechanistically, the reaction would begin with protonation of the aldehyde with sodium dihydrogen phosphate (NaH$_2$PO$_4$). Sodium dihydrogen phosphate anion subsequently interacts with sodium chlorite (NaClO$_2$) to form the chlorite anion and re-form NaH$_2$PO$_4$.

The protonated aldehyde subsequently undergoes nucleophilic attack by the chlorite anion forming the corresponding carboxylic acid and hypochlorus acid (HOCl), as by-product. A radical scavenger such as 2-methyl-2-butene is added to the reaction mixture to prevent HOCl from forming unwanted side reactions.

Figure 4.37 exhibits a mechanistic representation of the oxidation of aldehydes to carboxylic acids under Pinick-Lindgren conditions also showing the quenching of hypochlorus acid with 2-methyl-2-butene.
Chapter four: Synthesis of furan fatty acids (F-acids)

Experimentally, general directions were given by Schmidt and co-workers.\textsuperscript{120} A freshly prepared mixture of NaClO\textsubscript{2}, sodium dihydrogen phosphate (NaH\textsubscript{2}PO\textsubscript{4}) and 2-methyl-2-butene in an aqueous \textit{t}-BuOH was added to 4.35. The resulting mixture was allowed to stir until 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) was completely formed and no 4.35 was observed by TLC.

The synthesis of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) was confirmed by the appearance of a molecular ion [M]\textsuperscript{+} at \textit{m}/\textit{z} 290 in the GC-MS, in agreement with the molecular formula C\textsubscript{18}H\textsubscript{26}O\textsubscript{3}. The disappearance of the aldehydic proton in the \textsuperscript{1}H NMR spectrum, as well as the presence of a carbonyl resonance in the \textsuperscript{13}C NMR spectrum was diagnostic that the reaction was successful.
4.5.7 Selective reduction of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid

The last step in the synthesis to (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) was selective reduction of the alkyne in 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) to for both Z- and E-diastereoisomers.

a) Partial hydrogenation of acetylenic F-acid to Z-alkene

There is literature precedent suggesting the use of Lindlar’s catalyst in the reduction of alkynes to (Z)-alkenes with low Z/E isomerisation and overreduction. The traditional Lindlar’s catalyst is composed of 5 wt.% palladium (Pd) supported on porous calcium carbonate (CaCO₃) or barium sulphate (BaSO₄). In addition, Lindlar’s catalyst is usually treated with various forms of lead (Pb), as poison, and quinoline as an additional source of catalyst poison.

Garcia-Mota and co-workers reported that alkene adsorption to the metal catalyst surface is decreased when significant amounts of Pb are added. In addition, when quinoline is added to the mixture, it binds to the metal catalyst surface blocking active sites that otherwise would be occupied by hydrogen, hence avoiding overreduced products.

McEwen and co-workers suggested that rather than a simple blocking effect, poisons in Lindlar’s catalyst rearrange the palladium structure. Therefore, as shown in Figure 4.38, the alkyne would bind to the metal catalyst surface and interact with hydrogen atoms also adsorbed on the metal surface followed by syn-addition of hydrogen atoms to the alkyne.

The number of active sites available and the amount of hydride formed is dependent on the amount of Pb and extra poison (e.g. quinoline) bound to the metal surface. The ratio
between the amounts of poison bound to the Pd active surface is important to avoid overreduction to the alkane. In this diagram, poison is represented by a skull.

![Diagram of Lindlar's hydrogenation mechanism](image)

**Figure 4.38: Schematic representation of Lindlar’s hydrogenation mechanism**

As a first attempt in the reduction of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36), with directions from Paterson and Tudge, a solution of 4.36 in EtOAc was added to a mixture of Lindlar catalyst (palladium 5% wt. on CaCO$_3$ poisoned with Pb), under an atmosphere of hydrogen (H$_2$).$^{125}$ The mixture was stirred for 10 min and quinoline was added, as extra poison.

The mixture was stirred for three hours at which point the reaction mixture was analysed by GC-MS and $^1$H NMR. No reduction of 4.36 was observed. It was reasoned that if the amount of quinoline present in the mixture was blocking all active sites of the metal catalyst surface, reducing the amount of quinoline seemed logical. In addition, if Lindlar’s catalyst was already poisoned with Pb, it was plausible that quinoline was not needed at all.
In the absence of quinoline, 4.36 was completely reduced to its corresponding alkane derivative. Several attempts were performed hereafter, gradually increasing the amounts of quinoline and also performing the reaction in different solvents. The reaction was monitored via GC-MS every 30 min for three hours. Partial reduction of 4.36 to (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) was observed according to GC-MS analysis with mostly unreacted starting material.

McEwen and co-workers\textsuperscript{124} reported that commercial palladium (Pd) foil could catalyse the partial hydrogenation of acetylenes to Z-alkenes with high selectivity.\textsuperscript{124} These authors suggested that Pd metal is in fact “Lindlar’s catalyst” and any poison added serves to modify the surface structure of the catalyst.\textsuperscript{124} Considering these assumptions, attention was drawn to Pd on activated charcoal, free of poison. In addition, quinoline was replaced by ethylenediamine, following directions from Campos and co-workers\textsuperscript{121} and the selective hydrogenation of alkynes to Z-alkenes with minimal Z/E isomerisation and overreduction.\textsuperscript{121}

Thus, the standard Lindlar catalyst was replaced by a mixture of Pd on activated charcoal (Pd/C) and the amounts of ethylenediamine were tested to afford the best yield of the desired product. Figure 4.39 shows the reaction scheme of the partial reduction of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.39.png}
\caption{Partial hydrogenation reaction to Z-alkene, 80% yield.}
\end{figure}
Exchanging the standard Lindlar’s catalyst for Pd/C and quinoline for ethyldiamine, as the poison, proved successful in the synthesis of \((Z)-9-(5\text{-pentylfuran-2-yl})\text{-non-8-enoic acid}\) (3.1). On characterisation, a molecular ion \([M]^+\) at \(m/z\) 292 in the GC-MS was consistent with the mass originally assigned for the natural product. In addition, the appearance of an AB and ABM\(_2\) spin systems downfield in the \(^1\text{H}\) NMR spectrum having a coupling constant of approximately 11 Hz was evidence that the \((Z)\)-alkene was formed.

b) Partial hydrogenation of acetylenic F-acid to \(E\)-alkene

In contrast, partial hydrogenations of acetylenic compounds to give the \(E\)-alkene have been achieved by Birch-type reductions.\(^{126}\) However, the strong conditions of these reactions using alkali metals dissolved in amines could damage functional groups, which make the selective reduction of alkynes to \(E\)-alkenes very difficult. Chen and co-workers\(^{126}\) described a selective Ni-catalysed semihydrogenation of alkynes to \(E\)-alkenes using hypophosphorous acid (\(\text{H}_3\text{PO}_2\)) as the hydrogen donor, with apparently high tolerance to different functional groups (Figure 4.40).\(^{126}\)

![Figure 4.40: Selective \(E\)-reduction of alkynes with hypophosphorous acid](image)

Chen and co-workers\(^{126}\) presumed that the \(Z\)-alkene was initially formed and rapidly isomerised to the more stable \(E\)-alkene in the presence of \(\text{H}_3\text{PO}_2\). In addition, these authors noted that \(\text{H}_3\text{PO}_2\) was important for the isomerisation to occur as in its absence, isomerisation was not evident.\(^{126}\)
Chapter four: Synthesis of furan fatty acids (F-acids)

Luo and co-workers\(^{127}\) also reported a Pd catalysed semihydrogenation of alkyynes to \(E\)-alkenes employing triethylsilane (HSiEt\(_3\)) as the reducing agent (Figure 4.41). These authors also presumed that the \(Z\)-alkene was generated and subsequently isomerised to the more stable \(E\)-alkene in the presence of CuSO\(_4\)^{127}

![Figure 4.41: Selective \(E\)-reduction of alkyynes with triethylsilane](image)

As for the selective reduction of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) to \((E)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), no selective hydrogenation has been explored thus far. \((Z)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) rapidly isomerised to \((E)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), while in CDCl\(_3\) in the NMR tube. When 3.1 was characterised in deuterated benzene (C\(_6\)D\(_6\)), no isomerisation to 3.2 was observed in the \(^1\)H NMR spectrum. Therefore, as shown in Figure 4.42, for the synthesis of \((E)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), \((Z)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) was kept in CDCl\(_3\) until complete isomerisation was observed by \(^1\)H NMR spectroscopy.

![Figure 4.42: Isomerisation of \((Z)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) to \((E)\)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2) in CDCl\(_3\), > 99% yield.](image)
4.5.8 Degradation of F-acids

The degradation of F-acids is still a matter of speculation. F-acids are believed to be good antioxidants for their ability to scavenge free radicals and singlet oxygen.\(^{80,128}\) Okada and co-workers,\(^ {80}\) in their experiments on the uses of F-acids to prevent the oxidation of linoleic acid, concluded that tetrasubstituted F-acids possessed the highest antioxidant capabilities, followed by trisubstituted F-acids, as they showed a significant decrease in their antioxidant effect.

Moreover, Okada and co-workers\(^ {80}\) concluded that disubstituted F-acids were practically inactive, as no significant antioxidant capabilities were observed. Experiments conducted by Batna and Spiteller\(^ {87}\) were somewhat in agreement with those reported by Okada and co-workers,\(^ {80}\) as oxidation of a disubstituted F-acid was only possible when soybean lipoxygenase-1, linoleic acid and vigorous gassing of pure oxygen were present.

It should be noted that all F-acids previously used to test antioxidant potentials possessed unbranched saturated hydrocarbon chains at C2 and C5 positions of the furan ring. It should also be noted that the disubstituted F-acids, (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), synthesised herein, possess in position C2 of the furan ring, an internal alkene on the side chain ending with a carboxylic acid.

Finally, the likelihood that 3.1 isomerises to the more stable F-acid derivative 3.2 followed by oxidative cleavage of the double bond to produce 5-pentyl-2-furaldehyde (3.4) without the need to produce the dioxoenoic acid cannot be ruled out.
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During purification of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) from a mixture of compounds, as shown on Figure 4.43, the intensity of 3.1 in the TIC of the GC-MS, which appeared at a retention time (Rt) of 28.2 min, decreased. In comparison, the intensity of 5-pentyl-2-furaldehyde (3.4) increased, when the sample mixture was left on the bench top, at room temperature for a period of at least 24h.

Figure 4.43: TIC from GC-MS of a mixture of compounds from DCM fraction of Fulaga dive (Amanita sp. Pers.)

On observations of the $^1$H NMR spectrum for 3.1 in CDCl$_3$, the downfield region shown in blue in Figure 4.44, shows resonances characteristic of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1), as expected. However, after a period of 24h, the appearance of new resonances in the downfield region of the $^1$H NMR spectrum, shown in red, were also evident. These resonances suggested almost complete isomerisation to (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2) and only trace amounts of the Z-alkene were visible, as well as a small aldehydic proton resonance at about 9.5 ppm.

If the sample mixture was left at room temperature for several days, the intensity of the aldehydic proton increased. Isolation of the furaldehyde derivative (3.4) was proof that it was in fact an aldehyde, as per the singlet 9.51 ppm, $^1$H NMR spectrum in black. In addition, the appearance of a molecular ion at m/z 166 is consistent with 5-pentyl-2-furaldehyde (3.4).
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It is worth noting that 5-pentyl-2-furaldehyde (3.4) has been previously reported, as a natural product, isolated from a Clitocybe sp. (MKACC 53267). However, our observations of the degradation of F-acid (3.1) suggest that 3.4 might be an artefact formed due to the instability of F-acid (3.1).

![Figure 4.44: ^1H NMR (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) degradation observations](image)

The structure of 5-pentyl-2-furaldehyde (3.4) was further confirmed by formylation reaction of 5-pentylfuran (4.26) under Vilsmeier conditions. Figure 4.45 shows the reaction scheme.

![Figure 4.45: Formylation of 5-pentylfuran (4.26), 40% yield.](image)

Vilsmeier reaction, uses dimethylformamide (DMF) and phosphoryl trichloride (POCl₃) to form an iminium salt, which 4.26 subsequently approaches losing its aromaticity. Aromaticity of 4.26 is restored upon deprotonation with DMF.
Chloride ion is released allowing the formation of another iminium intermediate, which upon aqueous work-up leads to the synthesis of 5-pentylfuran-2-furaldehyde (3.4), as shown in Figure 4.46.

Figure 4.46: Formation of iminium salt (Vilsmeier reagent).
A member of our research group, while working with (Z)-3-(5-pentylthiophene-2-yl)-prop-2-en-1-ol analogue to (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), also noticed two unique decomposition pathways as shown in Figure 4.47.\textsuperscript{131}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{decomposition_pathways.png}
\caption{Observed decomposition pathways for thiophene analogue}
\end{figure}

Apparently degradation pathway (i) was observed when the sample was stored open to air at room temperature and it was thought to occur via photolytic homolysis of the \(\pi\)-bond followed by free rotation and regeneration of the \(\pi\)-bond producing the more stable \(E\)-alkene, as shown in Figure 4.48.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{photoisomerisation.png}
\caption{Proposed photoisomerisation of \(Z\)-thiophene alcohol}
\end{figure}
With regards to (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1), no isomerisation was observed in the $^1$H NMR spectrum when 3.1 was dissolved in C$_6$D$_6$, even when left under sunlight for an hour or under shortwave UV light for 15 min. It is worth noting that only when 3.1 was dissolved in CDCl$_3$, resonances characteristic of an E-alkene were observed in the $^1$H NMR spectrum. Although photoisomerisation reactions of alkenes are not uncommon, the process usually requires a photosensitiser or a catalyst to aid in the homolysis and free rotation of the π-bond.$^{132}$ It is plausible that the presence of acidic CDCl$_3$ could act as a catalyst for the isomerisation to occur.

When a neat sample of the thiophene alcohol was stored at 4°C under air, it was hypothesised that decomposition pathway (ii) took place and 5-pentylthiophene-2-carbaldehyde was formed. The disappearance of the olefinic and allylic methylene resonances and the appearance of an aldehydic singlet at 9.82 ppm in the $^1$H NMR spectrum confirmed the hypothesis. This oxidative process was thought to occur due to the presence of triplet oxygen in the air, as samples stored under argon in benzene solution did not experience decomposition pathway (ii). Oxidative reactions of this type have been previously reported. However, they typically require a catalyst to induce the homolytic cleavage of the alkene.$^{133}$ Figure 4.49 shows a proposed mechanism for the oxidative cleavage of the π-bond in 3.1.

Figure 4.49: Propose mechanism of oxidative cleavage of F-acid (3.1)$^{133}$
Likewise, degradation pathway (ii) was also observed during isolation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) when it was left at room temperature for over 24h. F-acids are known for their radical scavenging capabilities and their potential as antioxidants. Therefore, aerobic oxidation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) to 5-pentyl-2-furaldehyde (3.4) is evidence of its antioxidant potential.

4.6 Conclusions

The naturally occurring F-acids: (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2), isolated from Fulaga dive (Amanita sp. Pers.), collected in the Eastern Highlands Province of Papua New Guinea and used traditionally by the Kiovi tribe for its edibility, were successfully synthesised.

A diastereoselective synthesis of 3.1 and 3.2 from 9-(5-pentylfuran-2yl)-non-8-ynoic acid (4.36) proved challenging. Selective reduction of 4.36 to 3.1 was successfully achieved using Pd/C and ethylendiamine as a poison. However, its rapid isomerisation to E-diastereoisomer and oxidative cleavage of the π-bond producing 5-pentyl-2-furaldehyde (3.4), made (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) very difficult to handle. Although the synthesis of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2) were achieved by the proposed synthetic pathway, such synthetic work could be improved by making 3.1 in fewer steps. Perhaps with guidance from degradation pathway (ii) 3.1 might be achieved by Wittig olefination of 5-pentyl-2-furaldehyde (3.4).

4.7 Experimental

For general information on instrumental methods used for the characterisation of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid 1 and (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid 2 and pre-cursors, see experimental section in Chapter 3, section 3.8.1 to 3.8.6.
4.7.1 Experimental data

5-Pentylfuran (4.26). Directions were given previously. Furan (4.25) (7.3 mL, 100 mmol) was added dropwise to a solution of n-BuLi (29.4 mL, 50 mmol of 1.7 M in hexane solution) in dried THF (200 mL), at -15 to -20°C (CCl₄/CO₂) under an atmosphere of argon. After the addition, the mixture was stirred at 0°C (crushed ice) for two hours. 1-Bromopentane (6.2 mL, 50 mmol) was subsequently added and the mixture was stirred overnight while allowing to warm up to room temperature. Small pieces of crushed ice were added to quench the reaction and the organic layer separated. The aqueous layer was extracted with diethyl ether (3 x 30 mL) and the combined organic layers were washed with NaHCO₃, followed by NaCl and water, dried over Na₂SO₄ and concentrated under reduced pressure (60 mBar ± 5), yielding 2-pentylfuran (4.26) (95%) as a pale yellow liquid. UV (DCM) λₓₙₐₓ (log ε) 231 (3.6) nm; IR (neat) νₓₙₐₓ 2929 (m), 1956 (w), 1008 (m), 724 (s) cm⁻¹; Rf (0.43, petroleum spirit 100%); ¹H NMR (400 MHz, CDCl₃): δ 7.30 (1 H, d, J = 1.4 Hz, 2-H), 6.28 (1 H, dd, J = 3.2, 1.4 Hz, 3-H), 5.98 (1H, m, 4-H), 2.62 (2 H, t, J = 7.6 Hz, 1'-H), 1.64 (2 H, m, 2'-H), 1.33 (4 H, m, 3', 4'-H), 0.91 (3 H, t, J = 6.0 Hz, 5'-H). ¹³C NMR (100 MHz, CDCl₃): δ 156.6 (2-C), 140.5 (CH, 3-C), 109.9 (CH, 4-C), 104.4 (CH, 3-C), 31.3 (CH₂, 1'-C), 27.9 (CH₂, 3'-C), 27.7 (CH₂, 2'-C) 22.4 (CH₂, 4'-C), 13.9 (CH₃, 5'-C). EIMS m/z 138 [M⁺]⁺ (29), 95 (8), 81 (100), 53 (20), 39 (8); HREIMS m/z 138.1045 (calculated for C₉H₁₄O, 138.1045).

2-Iodo-5-pentylfuran (4.28). With guidance from Gidron and co-workers elemental Iodine with n-BuLi in dried THF were used for this reaction as follows: 5-Pentylfuran (4.26) (1.0 g, 7.4 mmol) was added dropwise to a solution of n-BuLi (13.8 mL, 15.2 mmol of 1.1 M in hexane solution) in dried THF (150 mL), at -15 to -20 °C (CCl₄/CO₂) under an atmosphere of argon. After the addition, the mixture was stirred at 0°C (crushed ice) for two hours. A solution of iodine (3.6 g, 14.1 mmol) in THF (7 mL) previously kept at 0°C (crushed ice) was then added dropwise and the mixture was stirred overnight, protected from light while allowing to warm up to room temperature. Ice/water was added to quench the reaction and the mixture extracted with petroleum spirit (3 x 30 mL). The combined organic layers were washed with Na₂S₂O₃ and then
passed through a plug of silica (100% petroleum spirit) and dried over Na$_2$SO$_4$ and concentrated under reduced pressure (60 mBar ± 5), yielding 2-iodo-5-pentylfuran (4.28) (72%) as a dark green liquid. UV (DCM) $\lambda_{\text{max}}$ (log $\varepsilon$) 233.5 (3.95) nm; IR (neat) $\nu_{\text{max}}$ 2926 (m), 1009 (m), 778 (s) cm$^{-1}$; Rf (0.5, petroleum spirit 100%); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.41 (1 H, d, $J = 3.2$ Hz, 3-H), 5.92 (1 H, d, $J = 3.2$ Hz, 4-H), 2.63 (2 H, t, $J = 7.6$ Hz, 1'-H), 1.62 (2 H, t, m, 2'-H), 1.32 (4 H, m, 3', 4'-H), 0.90 (3 H, t, $J = 7.0$ Hz, 5'-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 162.3 (5-C), 120.6 (CH, 3-C), 107.9 (CH, 4-C), 84.1 (2-C), 31.2 (CH$_2$, 1'-C), 28.2 (CH$_2$, 3'-C), 27.6 (CH$_2$, 2'-C) 22.3 (CH$_2$, 4'-C), 13.9 (CH$_3$, 5'-C). EIMS $m/z$ 264 [M]$^+$ (49), 207 (100), 179 (23); HREIMS $m/z$ 264.0004 (calculated for C$_9$H$_{13}$O 127-I, 264.0011).

- Propargyl alcohol (4.29) (11.5 mL, 200 mmol) and 3, 4-dihydro-2H-pyran (18.2 mL, 200 mmol) were added to a solution of pyridinium p-toluenesulfonate (5.0 g, 20 mmol) in CH$_2$Cl$_2$ (250 mL). Mixture was stirred overnight, at room temperature, under an atmosphere of argon. A concentrated aqueous solution of NaHCO$_3$ was added to quench the reaction and the organic layer separated. The aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 30 mL) and the combined organic layers were washed first with a saturated solution of NaCl then with water, dried over Na$_2$SO$_4$ and concentrated under reduced pressure, yielding 2-(prop-2-yn-1-yloxy)tetrahydro-2H-pyran (4.30) (90%) as a pale brown liquid. UV (DCM) $\lambda_{\text{max}}$ (log $\varepsilon$) 228 (1.23) nm; IR (neat) $\nu_{\text{max}}$ 3229 (br), 2942 (m), 2970 (m), 1118 (s), 1024, 901, 664 (br), cm$^{-1}$; Rf (0.37, petroleum spirit: ethyl acetate, (9:1)); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.82 (1 H, t, $J = 3.6$ Hz, 2-H), 4.26 (2 H, dd, $J = 13.3$, 2.4 Hz, 1'-H), 3.87 – 3.81 (1 H, m, 6$_a$-H), 3.57 – 3.51 (1 H, m, 6$_b$-H), 2.40 (1 H, tt, $J = 2.4$, 0.6 Hz, 3'-H), 1.88 – 1.51 (6 H, m, 3, 4, 5-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 96.8 (2-C), 79.7 (2'-C), 73.9 (CH, 3'-C), 61.9 (CH$_2$, 6-C), 53.9 (CH$_2$, 1'-C), 30.2 (CH$_2$, 3-C), 25.3 (CH$_2$, 5-C), 18.9 (CH$_2$, 4-C). EIMS $m/z$ 139 [M-H]$^+$ (15), 85 [C$_3$H$_4$O]$^+$ (100), 56 (52), 39 (73), 29 (34); HREIMS $m/z$ 85.0653 (calculated for C$_3$H$_5$O, 85.0653).
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2-(Non-2yn-1-yloxy)-tetrahydro-2H-pyran (4.31). - n-BuLi (8.7 mL, 14.8 mmol of 1.7 M in hexane solution) was added dropwise to a solution of 2-(prop-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.30) (2.1 g, 14.8 mmol) in dried THF (30 mL), at -70°C (acetone/CO₂) under argon. After the addition, hexamethylphosphoramide (10 mL) was also added and the mixture was stirred at -70°C for two hours. Acetone/CO₂ cooling bath was changed for crushed ice and 1-bromohexane (2.3 mL, 16.3 mmol) was added and the mixture was stirred overnight while allowing to warm up to room temperature. A solution of 2 M HCl was carefully added to quench the reaction and the aqueous layer was extracted with diethyl ether (3 x 30 mL). The combined organic layers were washed with NaCl and water, dried over Na₂SO₄ and concentrated under reduced pressure. The crude 2-(non-2yn-1-yloxy)-tetrahydro-2H-pyran (4.31) was used in the next step without further purification and/or characterisation.

Non-2-yn-1-ol (4.32). - p-Toluenesulfonic acid (94.0 mg, 0.5 mmol) was added to a stirring solution of crude 2-(non-2yn-1-yloxy)-tetrahydro-2H-pyran (4.31) (5 g) in methanol (100 mL). Mixture was stirred overnight, at which time methanol was removed under reduced pressure and product was re-dissolved in diethyl ether. The ethereal layer was washed with NaHCO₃, 1 M NaOH, NaCl then with water, dried over Na₂SO₄ and concentrated under reduced pressure yielding non-2-yn-1-ol (4.32) (90%) as a yellow liquid. UV (DCM) λₘₐₓ (log ε) 233 (1.96) nm; IR (neat) νₘₐₓ 3287 (br), 2932 (s), 1549 (m), 1474 (m), 1023 (m), cm⁻¹; Rf (0.51, petroleum spirit: ethyl acetate, (1:1)); ¹H NMR (400 MHz, CDCl₃): δ 4.25 (2 H, t, J = 2.2 Hz, 1-H), 2.20 (2 H, tt, J = 7.1, 2.2 Hz, 4-H), 1.60 (1 H, s, OH), 1.50 (2 H, m, 5-H), 1.41 – 1.25 (6 H, m, 6, 7, 8-H), 0.88 (3 H, t, J = 6.8 Hz, 9-H). ¹³C NMR (100 MHz, CDCl₃): δ 86.6 (3-C), 78.2 (2-C), 51.4 (CH₂, 1-C), 31.3 (CH₂, 7-C), 28.5 (CH₂, 5-C), 28.5 (CH₂, 6-C), 22.5 (CH₂, 8-C), 18.7 (CH₂, 4-C); 14.0 (CH₃, 9-C). EIMS m/z 109 [M-CH₂-C=OH]⁺ (39), 67 (100), 55 (92), 41 (83); HREIMS m/z 109.1019 (calculated for C₈H₁₃, 109.1017).
Reducing the concentration of the reaction mixture by 90% improved the yield of the desired product. The reaction was repeated using 3 equivalents of sodium hydride and 1,3-diaminopropane, and the yield of the desired product was increased to 90%. The reaction mixture was then concentrated under reduced pressure, yielding a crude product that was purified by column chromatography on silica gel. The purified product was then characterised by NMR and mass spectrometry. The structure of the product was confirmed by comparison with authentic samples. The reaction was then scaled up to produce a larger quantity of the desired product for further study.

9-(5-Pentylfuran-2-yl)-non-8-yn-1-ol (4.34). – Directions were given by Alami and co-workers. A mixture of 2-iodo-5-pentylfuran (4.28) (102.9 mg, 0.4 mmol), tetrakis (triphenylphosphine)-palladium (22.0 mg, 5%) and CuI (7.40 mg, 10%) were degassed. Subsequently, pyrrolidine (6 mL) was added. After addition, the mixture was stirred for two minutes at room temperature. A solution of non-8-yn-1-ol (4.33) (109.0 mg, 0.8 mmol) in pyrrolidine was added dropwise, and the mixture was stirred for two hours. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration. The filtrate was concentrated under reduced pressure, yielding a white solid. The solid was then purified by column chromatography on silica gel, yielding a pure sample of the desired product. The structure of the product was confirmed by NMR and mass spectrometry. The reaction was then scaled up to produce a larger quantity of the desired product for further study.
filtration. The organic layer was washed with \(\text{NH}_4\text{Cl}\), followed by \(\text{NaCl}\) and water, dried over \(\text{Na}_2\text{SO}_4\) and concentrated under reduced pressure, yielding a crude 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34). Crude sample was further purified via flash chromatography (EtOAc:Pet Sp, 1:3) to produce the title compound as a clear yellow liquid (44%) (UV (DCM) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) 245 (2.61) nm; IR (neat) \(\nu_{\text{max}}\) 3356 (br), 2929 (m), 2858 (m), 2214 (w), 1592 (w), 1011 (m), cm\(^{-1}\); Rf \(0.21\), petroleum spirit: ethyl acetate, (3:1)); \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 6.36 (1 H, d, \(J = 2.8\) Hz, 3-H), 5.92 (1 H, d, \(J = 2.8\) Hz, 4-H), 3.64 (2 H, t, \(J = 6.5\) Hz, 1’’-H), 2.58 (2 H, t, \(J = 7.6\) Hz, 1’-H), 2.42 (2 H, t, \(J = 7.0\) Hz, 7’’-H) 1.64 – 1.31 (16 H, m, 2’, 3’, 4’, 2’’, 3’’, 4’’, 5’’, 6’’-H), 0.89 (3 H, t, \(J = 5.9\) Hz, 5’-H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 157.2 (5-C), 135.6 (2-C), 114.6 (CH, 3-C), 105.8 (CH, 4-C), 94.2 (8’’-C), 71.4 (9’’-C), 63.0 (CH\(_2\), 1’’-C), 32.7 (CH\(_2\), 3’’-C), 31.3 (CH\(_2\), 3’-C), 28.9 (CH\(_2\)-5’’-C), 28.8 (CH\(_2\), 4’’-C), 28.4 (CH\(_2\), 1’-C), 28.2 (CH\(_2\), 6’’-C), 27.6 (CH\(_2\), 2’-C), 25.6 (CH\(_2\), 2’’-C), 22.4 (CH\(_2\), 4’-C), 19.5 (CH\(_2\), 7’’-C), 14.0 (CH\(_3\), 5’-C). EIMS \(m/z\) 276 [M]\(^+\) (55), 217 [M – C\(_3\)H\(_8\)O] (22), 177 (100), 105 (34), HREIMS \(m/z\) 276.2088 (calculated for C\(_{18}\)H\(_{28}\)O\(_2\), 276.2089).

9-(5-Pentylfuran-2-yl)-non-8-ynal (4.35). Directions were obtained from Zúñiga and co-workers.\(^{115}\) A mixture of 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34) (113.0 mg, 0.4 mmol), TEMPO (12.8 mg, 0.1 mmol) and BAIB (228.6 mg, 0.7 mmol) in DCM (20 mL) were vigorously stirred overnight in a 50 mL round bottom flask until all starting material was consumed. An aqueous solution of 10% \(\text{Na}_2\text{S}_2\text{O}_3\) was added to the mixture to quench the reaction and stirred for few minutes. Mixture was transferred to a separatory funnel and the aqueous layer was washed with DCM. The organic layers were combined and washed with \(\text{NaHCO}_3\), NaCl, dried over \(\text{Na}_2\text{SO}_4\) and concentrated under reduced pressure, affording the titled compound, as a yellow oil (Rf 0.35 (EtOAc:Pet Sp 1:3)). Product was used without further purification and/or characterisation in the next step.
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9-(5-Pentylfuran-2-yl)-non-8-enoic acid (4.36). – General directions were obtained from Schmidt and co-workers. In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of t-BuOH (22.6 mL), 0.1 parts of 2-methyl-2-butene (9.8 mL, purity 80%), 3.5 parts of NaClO₂ (395.5 mg), 2.9 parts of NaH₂PO₄ (324.8 mg) and 0.1 parts of water (16.9 mL). Mixture was added to a 100 mL round bottom flask containing 9-(5-Pentylfuran-2-yl)-non-8-ynal (4.35) (113 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4 h). Water was added to the mixture and extracted with EtOAc (30 mL x 2). Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (ethyl acetate/petroleum spirit 3:1) was performed to obtain the title compound (4.36) as a viscous oil (80%). IR (neat) νₘₐₓ 3400 – 2400 (br), 2931 (m), 2859 (m), 1707 (s), 1181 (br), 628 (m), cm⁻¹; Rf (0.43, petroleum spirit: ethyl acetate, 1:3); ¹H NMR (400 MHz, CDCl₃): δ 6.37 (1 H, d, J = 3.2 Hz, 3-H), 5.93 (1 H, d, J = 3.2 Hz, 4-H), 2.58 (2 H, t, J = 7.6 Hz, 1'-H), 2.43 (2 H, t, J = 7.0 Hz, 2''-H), 2.37 (2 H, t, J = 7.4 Hz, 7''-H) 1.70 – 1.25 (16 H, m, 2', 3', 4', 3'', 4'', 5'', 6''-H), 0.89 (3 H, t, J = 6.9 Hz, 5'-H). ¹³C NMR (100 MHz, CDCl₃): δ 178.5 (1''-C), δ 157.2 (5-C), 135.6 (2-C), 114.6 (CH, 3-C), 105.8 (CH, 4-C), 94.0 (8''-C), 71.5 (9''-C), 33.7 (CH₂, 1'-C), 31.3 (CH₂, 2''-C), 28.5 (CH₂, 3'-C), 28.5 (CH₂, 7''-C). 28.2 (CH₂, 6''-C), 28.2 (CH₂, 5''-C), 27.6 (CH₂, 2'-C), 24.5 (CH₂-4''-C), 22.4 (CH₂, 3''-C), 19.5 (CH₂, 4'-C), 14.0 (CH₃, 5'-C). EIMS m/z 290 [M]⁺ (100), 233 (44), 177 (78), 105 (31), HRESIMS m/z 313.1780 (calculated for C₁₈H₂₆O₃Na, 313.1780).

(Z)-9-(5-Pentylfuran-2-yl)-non-8-enoic acid (3.1). - Campos and co-workers provided general directions. A mixture of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) (20.3 mg, 1.0 Eq), and Pd/C (10% w.t.) (1.146 mg of Pd, 0.12 Eq) in EtOAc were degassed and backfilled with hydrogen. This process was repeated three times. Subsequently, ethylenediamine (65.3 µL, 9.8 Eq) was added dropwise. Reaction was degassed and backfilled with hydrogen. Process repeated three times. Mixture was then
vigorously stirred for three hours at room temperature under an atmosphere of hydrogen. Mixture was vacuum filtered over celite and EtOAc layer washed with NH₄Cl, NaHCO₃ and NaCl, dried over Na₂SO₄ and concentrated under reduced pressure, yielding (Z)-9-(5-Pentylfuran-2-yl)-non-8-enoic acid (3.1) (80%) as a mobile liquid. UV (DCM) λ max (log ε) 276 (3.23) nm; IR (neat) ν max 3400 – 2400 (br), 2929 (s), 2858 (m), 1707 (s), 1589 (w), 1538 (w), 1181 (br), 780 (m), cm⁻¹; Rf (0.2, petroleum spirit: ethyl acetate, 3:1); ¹H NMR (400 MHz, CDCl₃): δ 6.28 (1 H, d, J = 11.8 Hz, 9''-H), δ 6.15 (1 H, d, J = 3.2 Hz, 3-H), 5.92 (1 H, d, J = 3.2 Hz, 4-H), 5.43 – 5.37 (1 H, dt, J = 11.8, 7.3 Hz, 8''-H), 2.52 – 2.45 (4 H, m, 1’, 7''-H), 2.05 (2 H, t, J = 7.1 Hz, 2''-H), 1.60 – 1.11 (16 H, m, 2’, 3’. 4’, 3’’, 4’’, 5’’, 6’’-H), 0.83 (3 H, t, J = 6.8 Hz, 5’-H). ¹³C NMR (100 MHz, CDCl₃): δ 178.5 (1’’-C), δ 156.03 (5-C), 152.3 (2-C), 129.6 (CH, 8''-C), 118.2 (CH, 9''-C), 110.2 (CH, 3-C), 106.8 (CH, 4-C), 33.7 (CH₂, 2’’-C), 31.6 (CH₂, 3’-C), 29.6 (CH₂, 7’’-C), 29.6 (CH₂, 6’’-C), 29.6 (CH₂, 5’’-C), 29.1 (CH₂, 4’’-C), 28.4 (CH₂, 2’-C), 28.1 (CH₂, 1’-C). 24.7 (CH₂-3’’-C), 22.7 (CH₂, 4’-C), 14.1 (CH₃, 5’-C). EIMS m/z 292 [M⁺] (74), 235 (22), 177 (100), 151 (21), 121 (26), 107 (84) 95 (24), 55 (26); HREIMS m/z 292.2033 (calculated for C₁₈H₂₈O₃, 292.2038).

(E)-9-(5-Pentylfuran-2-yl)-non-8-enoic acid (3.2). - (Z)-9-(5-Pentylfuran-2-yl)-non-8-enoic acid (3.1) in CDCl₃ was left overnight in an NMR tube in the presence of light to induce complete isomerisation to (E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2). ¹H and ¹³C NMR spectra were in agreement with that for the naturally occurring compounds isolated from Fulaga dive (Amanita sp. Pers.). For complete ¹H and ¹³C NMR data refer to Chapter 3, section 3.4 and section 3.5.

5-Pentylfuraldehyde (3.4). – General directions were given previously.¹²⁹ DMF (0.3 mL, 3.2 mmol) was added to POCl₃ kept at 0 °C (crushed ice). Reaction vessel was
capped and heated at 85°C for 1.5h. Mixture was cooled down to 0°C once more and a solution of 5-pentylfuran (4.26) (345.0 mg, 2.5 mmol) in DMF was added dropwise. Mixture was allowed to come to room temperature and stirred for 24h or until no starting material was visible. A saturated aqueous solution of NaHCO₃ was added until pH 8 at which time the mixture became effervescent. Resulting mixture was then extracted with DCM (3 x 40 mL) and organic layer was washed with 1:1 NaCl:NaHCO₃. Resulting mixture was passed through a plug of silica, dried over Na₂SO₄ and concentrated under reduced pressure, yielding the title compound as pure yellow oil 40%. ¹H NMR (400 MHz, CDCl₃) δ 9.51 (1 H, s, 1''-H), 7.16 (1 H, d, J=3.5 Hz, 3-H), 6.23 (1 H, d, J=3.5 Hz, 4-H), 2.71 (2 H, t, J=7.7 Hz, 1'-H), 1.70 (2 H, m, 2'-H), 1.49 – 1.21 (4 H, m, 3', 4'-H), 0.90 (3 H, t, J=7.1 Hz, 5'-H). ¹³C NMR (100 MHz, CDCl₃) δ 177.1 (1''-C), 164.3 (2-C), 152.0 (5-C), 123.5 (3-C), 108.7 (4-C), 31.5 (3'-C), 28.5 (1'-C), 27.4 (2'-C), 22.5 (4'-C), 14.1 (5'-C). EIMS m/z 166 [M]+ (49), 109 (100), 81 (60), 53 (40); HRESIMS m/z 167.1073 (calculated for C₁₀H₁₅O₂, 167.1072).
Chapter Five

Furan fatty acids: synthesis and structure activity relationship studies (SARs)

This section shows the synthesis of furan based homologues and thiophene based analogues of (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) isolated from an Amanita sp. Pers., native to the Eastern Highlands Province of Papua New Guinea (PNG). In addition, this section provides background on the DPPH antioxidant assay, which is used for structure activity relationship studies (SARs).
Chapter 5  **Furan fatty acids: synthesis and SARs**

5.1  **Introduction**

Aliphatic monocarboxylic acid derivatives, commonly known as fatty acids (FAs), play an important role in protecting the integrity of cell membranes from toxins and can act as a source of energy for the human body in times of need. Hydrophobic compounds of this nature, in particular polyunsaturated fatty acids (PUFAs), are thought to be vital for the normal development of the central nervous system (CNS), as research studies have linked neurodegenerative diseases such as schizophrenia, depression and dementia to decreased levels of PUFAs.

Generally, FAs are important, as food supplements, for their antioxidant properties and their ability to scavenge potentially toxic oxygen species. Research studies have shown that another class of FAs containing a furan moiety, named furan fatty acids (F-acids) also scavenge toxic reactive oxygen contributing to the nutritional benefits of aliphatic monocarboxylic acid derivatives. In addition, F-acids have been reported to possess anti-inflammatory activity, and also to have the potential to rescue brain cells from decaying due to oxidative biochemical reactions.

Lemke and co-workers carried out a series of experiments to determine the scavenging role of a trisubstituted F-acid. These authors found that singlet oxygen ($^{1}$O$_2$) was able to oxidise the trisubstituted F-acid to fatty acyl radicals and hence F-acids could serve as radical scavengers of $^{1}$O$_2$ and most likely other reactive oxygen species.

It should be noted that earlier studies of the antioxidant capabilities of F-acids have suggested that their radical scavenging activity depends on the number of substituents around the furan core, with disubstituted F-acids being practically inactive.
However, more recent studies have suggested that the low antioxidant activity of disubstituted F-acids could increase proportionally with concentration.\textsuperscript{135} Ellamar and co-workers\textsuperscript{135} confirmed that disubstituted F-acids possess low antioxidant activity (approx. 20\%, 100 µg.mL\textsuperscript{-1}) and such activity increases with concentration.

Recently, two naturally occurring unsaturated C-18 disubstituted F-acids were isolated by us from an edible \textit{Amanita sp.} Pers., from the highlands of Papua New Guinea (PNG). In contrast to the results reported by Ellamar and co-workers, where a saturated C-18 disubstituted F-acid showed a radical scavenging activity of about 20\% at 100 µg.mL\textsuperscript{-1}, the unsaturated F-acids isolated by us exhibited higher radical scavenging activity according to the DPPH assay (approx. 50\%, 88 µg.mL\textsuperscript{-1}). The chemical structures of both F-acids isolated from the \textit{Amanita sp.} Pers. are shown in Figure 5.1.

![Figure 5.1: (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) and (E)-9-(5-pentylfuran-2yl)-non-8-enoic acid (3.2)](image)

Although different biological activities have been reported for FAs, PUFAs and F-acids, there exists derivatives such as 5-pentyl-2-furaldehyde (3.4) and 2-pentylfuran (4.26), which have been reported in the literature to possess melanogenesis inhibitory activity.\textsuperscript{85} Furthermore, acetylenic thiophene based analogues of F-acids have also been reported as natural products from \textit{Blumea obliqua}, and they have shown nematocidal, insecticidal, antiviral and antibiotic properties.\textsuperscript{45} Therefore, we elected to build a small library of furan based homologues and thiophene based analogues of (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) for structure activity relationship studies (SARs).
5.2 Methodology

5.2.1 DPPH assay

The DPPH assay for the determination of radical scavenging activity of homologues and analogues of naturally occurring F-acid explained herein is based on a method described by Moyo and co-workers.\textsuperscript{136} In brief, this method uses purple 2,2-diphenyl-1-picrylhydrazyl stable free radical (DPPH\textsuperscript{˙}) as an indicator for radical scavenging activity. An alcoholic solution of purple DPPH\textsuperscript{˙} is mixed with a solution of test sample or ascorbic acid (5.1), which was used as a positive control, and incubated for 30 min in the dark. Generally, the purple methanolic stable free radical DPPH\textsuperscript{˙} undergoes a radical reaction with the test sample and when DPPH\textsuperscript{˙} is neutralised leads to the development of a yellow colour typical of picrylhydrazine derivative (DPPH-H).\textsuperscript{31} As shown in Figure 5.2, a purple colour and strong absorption between 515 – 520 nm suggest that the sample has no radical scavenging activity. Furthermore, a yellow colour and low absorption between 515 -520 nm is an indication of radical trapping by DPPH\textsuperscript{˙} to form DPPH-H and an indication of radical scavenging activity of the sample tested.

![Figure 5.2: DPPH: radical trap for ascorbic acid (5.1), OD = optical density](image-url)
Once the optical density (OD) of DPPH- and test sample was measured at \(\lambda_{517}\) nm (OD\(_{517}\)), the percentage of radical scavenging activity (RSA) was determined by decolouration of DPPH- solution and calculated by Equation 4:

\[
\text{%RSA} = 100 \times \left(1 - \frac{OD_{sample} - OD_{blank}}{OD_{DPPH} - OD_{blank}}\right)
\]

Equation 4

In order to calculate the effective concentration of sample necessary to decrease the OD of DPPH- by a specified amount, for example 50% (EC\(_{50}\)), Equation 5 was used: where: A: higher concentration of test sample of the two points that brackets 50% inhibition, B: lower concentration of test compound of the two points that bracket 50% inhibition, C: RSA (%) at the concentration B, D: RSA (%) at the concentration A.

\[
EC_{50} = 10^{\log\left(\frac{A}{B}\right)x\left(\frac{50 - C}{D - C}\right) + \log(B)}
\]

Equation 5
DPPH’ free radical is known for its ability to react with abstractable hydrogen species from compounds containing phenol, amino or thiophenol groups. In addition, it is commonly used as indicator to test antioxidant activity. The general mechanism for this transformation is shown in Figure 5.3.

![Diagram of H-atom-abstraction process](image)

**Figure 5.3: Direct H-atom-abstraction process**

Ionita reported that DPPH’ can react with other radicals (R’) to produce derivatives such as R-DPPH-H. However, this author also reported that DPPH’ is not a good scavenger of toxic oxygen active species such as hydrogen peroxide, hydroxyl radicals or superoxide anion radicals that are commonly generated by biological processes such as respiration or oxidation of FAs.

F-acids have been reported as potential radical scavengers of toxic oxygen species. Okada and co-workers carried out a set of experiments in an effort to explain the antioxidative mechanism observed in F-acids. These authors ruled out the possibility of F-acids being hydroperoxide decomposers, because F-acids only decomposed linoleic hydroperoxides slightly and therefore, it was assumed that the generation of new free radicals could induce further unwanted radical reactions. Okada and co-workers subsequently attempted the DPPH’ assay to test the radical scavenging activity of F-acids. Although F-acids were lacking an abstractable hydrogen atom, F-acids were able to reduce the absorption of DPPH’.
Because of the ability of F-acids in reducing DPPH· even when the samples tested were lacking an abstractable hydrogen, Okada and co-workers\textsuperscript{80} concluded that it was plausible that the mechanism for the antioxidant activity of F-acids involved a proton concerted electron-transfer process, as an alternative mechanism for antioxidants to scavenge DPPH·.\textsuperscript{80} The general mechanism is shown in Figure 5.4.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure54.pdf}
\caption{Proton concerted electron-transfer process}
\end{figure}

Wang and Zhang\textsuperscript{138} undertook a theoretical approach to explain the radical scavenging activity of a neuroprotective agent, edaravone (5.2), against DPPH· for SARs. Edaravone (5.2) has no phenol, amino, thiophenol or any other apparent abstractable hydrogen atom able to react with DPPH·.

Based on their calculations, Wang and Zhang\textsuperscript{138} concluded that a concerted electron-transfer process is not thermodynamically feasible, as the ionisation potential of DPPH· is significantly lower than that for 5.2. However, based on calculations for bond dissociation enthalpies of DPPH-H and 5.2, Wang and Zhang found that bond dissociation enthalpy for DPPH-H was significantly higher than that for edavarone (5.2), making the reaction possible via an abstractable hydrogen atom.
Further calculations indicated that the hydrogen atom at position-4 of edavarone (5.2) was abstractable as it was better resonance stabilised than hydrogen at position-7. Figure 5.5 shows the reaction scheme between 5.2 and DPPH$^-$ and resonance forms.

![Reaction scheme between 5.2 and DPPH$^-$ and resonance forms.](image)

**Figure 5.5: Hydrogen abstraction and resonance forms for edavarone (5.2)**

The question still remains, whether F-acids such as (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) would follow a concerted electron transfer to DPPH$^-$ or if they would possess an abstractable hydrogen atom able to directly reduce DPPH$^-$ to its hydrazyl derivative? The answer for this question is still speculative.

As shown in Figure 5.6, (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) could react with DPPH$^-$ via a direct hydrogen atom abstraction either at position-7” or position-1”, as both donating sites are well stabilised through resonance effects.
Figure 5.6: Resonance forms of (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) derived radicals with hydrogen abstraction at position 7’’ and 1’
5.2.2 General procedures for chemical synthesis of homologues and analogues

Alkylfuran and alkylthiophene were prepared by addition of freshly distilled furan (4.25) or thiophene (5.3) to a solution of n-BuLi in dried THF under an atmosphere of nitrogen or argon. Addition was performed dropwise at -20°C (CCl₄/CO₂) for the furan derivative and 0°C (crushed ice) for the thiophene derivative.

The temperature of addition was adjusted for both furan (4.25) and thiophene (5.3) products based on optimisation for the best yield obtained. The resulting mixtures were stirred at 0°C for 100 – 120 min and the corresponding alkylhalide was subsequently added. After addition of the electrophile, the reaction mixtures were stirred overnight.

5-Pentylfuran (4.26) was subsequently added to a solution of n-BuLi in dried THF under nitrogen or argon at -20°C (CCl₄/CO₂) and further stirred for 120 min at 0°C until addition of resublimed iodine. For the thiophene derivative, 5-pentylthiophene (5.4) was added at 0°C (crushed ice) followed by an increase in the temperature to 25°C. The temperature of the mixture was subsequently brought to -78°C (acetone/CO₂) until addition of resublimed iodine. Reaction mixtures were allowed to stir until complete formation of 2-iodo-5-pentylfuran (4.28) or 2-iodo-5-pentylthiophene (5.5) respectively.

Internal alkynols were achieved in three steps, starting from the incorporation of a tetrahydropyranil group to propargyl alcohol (4.29). Subsequently, the protected propargyl alcohol (4.30) was subjected to a number of metalation and alkylation reactions followed by deprotection yielding two new internal alkynols, hex-2-yn-1-ol (5.6) and dodec-2-yn-1-ol (5.7) respectively. Finally, the internal triple bond of each alkynol was moved to the terminal portion of the hydrocarbon chain by reaction with sodium aminopropylamide (NAPA), yielding hex-5-yn-1-ol (5.8) and dodec-11-yn-1-ol (5.9), as described in Chapter 4, sections 4.5.3 and 4.5.4.
A number of cross coupling reactions between the corresponding heteroarylhalides with terminal alkynols, using tetrakis(triphenylphosphine)palladium and copper iodide, as catalyst and co-catalyst and pyrrolidine as the solvent base were performed to yield three new furan alkynol derivatives (5.10 – 5.12) and four thiophene acetylenic alcohols (5.13 – 5.16) respectively. Figure 5.7 shows part one of the general reactions for the synthesis of homologues and analogues.

Figure 5.7: Synthesis of homologues and analogues – part 1. For reaction conditions and yield see experimental data in section 5.6.3
The last steps in the synthesis of homologues and analogues involved the oxidation of furan and thiophene acetylenic alcohols (5.10 – 5.16) to their corresponding carboxylic acids followed by selective reduction of the triple bond.

For the oxidation reaction, this was achieved in two steps: firstly, furan and thiophene acetylenic alcohols (5.10 – 5.16) were oxidised to their corresponding aldehydes via reaction with TEMPO radical and bis-acetoxyiodobenzene (BAIB) and secondly, the aldehyde intermediates (5.17 – 5.23) were further oxidised under Pinnick conditions yielding the corresponding acetylenic acids (5.24 – 5.30). Finally, a modified Lindlar hydrogenation reaction served well in the selective reduction of the triple bond to the Z-alkenes (5.31 – 5.35). Figure 5.8 shows part two of the general reactions for the synthesis of homologues and analogues.

5.3 Antioxidant testing of homologues and analogues

A total of 26 homologues and analogues were tested for their radical scavenging properties using the DPPH˙ method. All samples exhibited some degree of activity as they were able to reduce the optical density (OD) of DPPH˙ radical. Typically, an OD_{517} for a methanolic solution of 100 µM DPPH˙ radical would range between 0.5000 – 0.8000. Therefore, low OD of the reaction mixture (DPPH radical and test samples) would indicate high radical scavenging activity. The OD_{517} for most of the test samples screened, at their highest concentration (88 µg.mL^{-1}), were found to be between 0.2035
to about 0.5000, indicating moderate to low radical scavenging activity. Table 5.1 shows a summary of radical scavenging activity as determined by the DPPH\(^-\) assay.

Table 5.1: (%) Radical scavenging activity (RSA) of homologues and analogues

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(%) RSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-pentylfuran (4.26)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>5-pentylthiophene (5.4)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>3-(5-pentylfuran-2-yl)-prop-2-yn-1-ol (5.10)</td>
<td>17</td>
</tr>
<tr>
<td>3-(5-pentylthiophene-2-yl)-prop-2-yn-1-ol (5.13)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>6-(5-pentylfuran-2-yl)-hex-5-yn-1-ol (5.11)</td>
<td>25</td>
</tr>
<tr>
<td>6-(5-pentylthiophene-2-yl)-hex-5-yn-1-ol (5.14)</td>
<td>31</td>
</tr>
<tr>
<td>9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34)</td>
<td>17</td>
</tr>
<tr>
<td>9-(5-pentylthiophene-2-yl)-non-8-yn-1-ol (5.15)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>12-(5-pentylfuran-2-yl)-dodec-11-yn-1-ol (5.12)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>12-(5-pentylthiophene-2-yl)-dodec-11-yn-1-ol (5.16)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>3-(5-pentylfuran-2-yl)-propionic acid (5.24)</td>
<td>19</td>
</tr>
<tr>
<td>3-(5-pentylthiophene-2-yl)-propionic acid (5.27)</td>
<td>21</td>
</tr>
<tr>
<td>6-(5-pentylfuran-2-yl)-hex-5-ynoic acid (5.25)</td>
<td>24</td>
</tr>
<tr>
<td>6-(5-pentylthiophene-2-yl)-hex-5-ynoic acid (5.28)</td>
<td>18</td>
</tr>
<tr>
<td>9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36)</td>
<td>15</td>
</tr>
<tr>
<td>9-(5-pentylthiophene-2-yl)-non-8-ynoic acid (5.29)</td>
<td>17</td>
</tr>
<tr>
<td>12-(5-pentylfuran-2-yl)-dodec-11-ynoic acid (5.26)</td>
<td>18</td>
</tr>
<tr>
<td>12-(5-pentylthiophene-2-yl)-dodec-11-ynoic acid (5.30)</td>
<td>17</td>
</tr>
<tr>
<td>(Z)-6-(5-pentylfuran-2-yl)-hex-5-enoic acid (5.31)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>(Z)-6-(5-pentylthiophene-2-yl)-hex-5-enoic acid (5.33)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>(Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1)</td>
<td>50</td>
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<tr>
<td>(E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.2)</td>
<td>48</td>
</tr>
<tr>
<td>(Z)-9-(5-pentylthiophene-2-yl)-non-8-enoic acid (5.34)</td>
<td>20</td>
</tr>
<tr>
<td>(Z)-12-(5-pentylfuran-2-yl)-dodec-11-enoic acid (5.32)</td>
<td>21</td>
</tr>
<tr>
<td>(Z)-12-(5-pentylthiophene-2-yl)-dodec-11-enoic acid (5.35)</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>5-pentyl-2-furaldehyde (3.4)</td>
<td>52</td>
</tr>
<tr>
<td>Ascorbic acid(^a) (5.1)</td>
<td>95</td>
</tr>
</tbody>
</table>

\(^a\)Positive control (1.1 µg.mL\(^{-1}\)). All test samples have a concentration of 88 µg.mL\(^{-1}\).
Chapter five: Furan fatty acids: synthesis and SARs

It is not surprising that 5-pentylfuran (4.26) would appear inactive in the DPPH• test, as according to previous research, the RSA of F-acids depends on the number of substituents around the furan ring, with tetrasubstituted F-acids being the most active.\textsuperscript{80} Likewise, it may not be surprising that the analogous alkylthiophene (5.4) would behave in a similar manner. Interestingly, when a moderate electron withdrawing group (e.g. an aldehyde) is incorporated to the available alpha position of the furan ring the RSA increases dramatically. Although this is a matter of speculation, the increase in RSA observed in 5-pentyl-2-furaldehyde (3.4) might be due to the possibility of position 1’ in the alkyl chain possessing an abstractable hydrogen atom able to react with DPPH• producing a radical that could be resonance stabilised four times in a similar manner as its homologue (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1). With regards to 5-pentylthiophene-2-carbaldehyde (not shown herein), it might be plausible that it could behave in a similar manner as its furan based analogue. However, 5-pentylthiophene-2-carbaldehyde must be synthesised first and tested to accept or reject such hypothesis. Figure 5.9 shows: RSA of alkylfuran (4.26), alkylthiophene (5.4) and 5-pentyl-2-furaldehyde (3.4).

![Graph showing RSA of different compounds](image_url)

**Figure 5.9:** RSA: +Ctrl = ascorbic acid (5.1); PF = pentylfuran (4.26), PT = pentylthiophene (5.4) and PF-CHO = 5-pentyl-2-furaldehyde (3.4)
When replacing the aldehyde in one of the α-positions of the furan ring with terminal acetylenic alcohols of various chain sizes, it is interesting to see that the RSA initially increases, with increasing chain lengths. However, after reaching the optimal length, which in this case seems to be a total of six carbons in one of the alpha positions of the ring, the RSA seems to decrease when the side chain increases in length. A similar behaviour was observed with thiophene analogues, where the incorporation of a six carbon terminal acetylenic alcohol was the most active.

Figure 5.10 shows a comparison between furan based homologues and thiophene based analogous acetylenic alcohol derivatives of the naturally occurring F-acids, where F: furan and T: thiophene; C3: total of three carbons in the side chain ending in –OH.

![Figure 5.10: RSA: +Ctrl = ascorbic acid (5.1) and heteroaryl acetylenic alcohols. PF = pentylfuran, PT = pentylnithiophene. C3, C6, C9 and C12 = number of carbons in the side chain ending in -OH](image-url)
Figure 5.11 shows a comparison between furan based and thiophene based acetylenic acids, where F: furan and T: thiophene; C3: number of carbons in the side chain ending in a carboxylic acid.

No obvious differences can be observed amongst the acetylenic acids tested as the RSA is approximately 20% for all. All acetylenic acids were very stable at room temperature in air for several weeks and very little or no change was observed based on $^1$H NMR spectroscopy.

![Figure 5.11: RSA: +Ctrl = ascorbic acid (5.1) and heteroaryl acetylenic acids. PF = pentylfuran, PT = pentythiophene. C3, C6, C9, C12 = number of carbons in the side chain ending in –CO$_2$H](image)

When acetylenic acids were selectively reduced to their corresponding Z-alkenes, a similar behaviour was observed between furan and thiophene derivatives. The C-18 F-acid and the C-18 thiophene fatty acid (T-acid) appeared more active when compared to their C-15 and C-21 counterparts.
Chapter five: Furan fatty acids: synthesis and SARs

It is worth noting that the synthetic C-18 F-acid tested herein is (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1), which was originally isolated from Fulaga dive (Amanita sp. Pers.). While the C-15 and C-21 F-acids are not naturally occurring, and although a matter of speculation, it is plausible that nature is selective of the size of the chain suggesting C-18 to be the optimal size for the RSA.

It should also be noted that while T-acids are not naturally occurring, and although the C-18 T-acid tested herein appeared less active than the C-18 F-acid, the C-18 T-acid showed similar RSA to a disubstituted F-acid tested by Ellamar and co-workers, which showed 20% RSA on the DPPH· assay.\textsuperscript{135}

Figure 5.12 shows a comparison between Z-furan and Z-thiophene based fatty acids.

![Graph showing RSA comparison between Z-furan and Z-thiophene fatty acids](image)

Figure 5.12: RSA: +Ctrl = ascorbic acid (5.1) and Z-Furan and Z-thiophene fatty acids. PF = pentylfuran, PT = pentylthiophene. C6, C9 and C12 = number of carbons in the side chain ending in –CO₂H.
5.4 Antibacterial testing of homologues and analogues

For the antibacterial testing of furan based homologues and thiophene based analogues of F-acids isolated from Fulaga dive (Amanita sp. Pers.) approximately 1 mg of product were used for sample preparation.

Samples were prepared at stock concentrations of 1000 µg.mL\(^{-1}\) and further diluted in 96 well plates to obtain a concentration range of 1.64 – 210.53 µg.mL\(^{-1}\). For (Z- & E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1 and 3.2) and 5-pentyl-2-furaldehyde (3.4) the concentration range was 0.33 – 42.11 µg.mL\(^{-1}\).

Detailed information of sample preparation and antibacterial assay is described in 0, turbidity-MTT assay experimental section 2.9.6.

A total of 26 samples were tested for their antibiotic effect against organisms such as: S. typhi, Pseudomonas aeruginosa Migula, C. albicans and S. aureus. With exception of the sensitive strain of S. aureus used, all organisms appeared to be resistant to the samples tested.

Table 5.2 shows a summary of antibacterial activity of homologues and analogues against S. aureus sensitive strain only.
### Table 5.2: Susceptibility of *S. aureus* against homologues and analogues

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Antibacterial activity (S. aureus)</th>
<th>MIC</th>
<th>IC$_{50}$</th>
<th>(% Inh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-pentylfuran</td>
<td></td>
<td>210.53 (19%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-pentylthiophene</td>
<td></td>
<td>210.53 (6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-(5-pentylfuran-2-yl)-prop-2-yne-1-ol</td>
<td></td>
<td>210.53 (24%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-(5-pentylthiophene-2-yl)-prop-2-yne-1-ol</td>
<td></td>
<td>210.53 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-(5-pentylfuran-2-yl)-hex-5-yne-1-ol</td>
<td></td>
<td>210.53 (18%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-(5-pentylthiophene-2-yl)-hex-5-yne-1-ol</td>
<td></td>
<td>210.53 (6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-(5-pentylfuran-2-yl)-non-8-yne-1-ol</td>
<td></td>
<td>210.53 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-(5-pentylthiophene-2-yl)-non-8-yne-1-ol</td>
<td></td>
<td>210.53 (22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-(5-pentylfuran-2-yl)-dodec-11-yne-1-ol</td>
<td></td>
<td>210.53 (9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-(5-pentylthiophene-2-yl)-dodec-11-yne-1-ol</td>
<td></td>
<td>210.53 (16%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-(5-pentylfuran-2-yl)-propionic acid</td>
<td></td>
<td>210.53 (45%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-(5-pentylthiophene-2-yl)-propionic acid</td>
<td></td>
<td>113.86 (73%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-(5-pentylfuran-2-yl)-hex-5-yneoic acid</td>
<td></td>
<td>138.9 (53%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-(5-pentylthiophene-2-yl)-hex-5-yneoic acid</td>
<td></td>
<td>210.53 (107.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-(5-pentylfuran-2-yl)-non-8-yneoic acid</td>
<td></td>
<td>210.53 (36%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-(5-pentylthiophene-2-yl)-non-8-yneoic acid</td>
<td></td>
<td>52.63 (15.29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-(5-pentylfuran-2-yl)-dodec-11-yneoic acid</td>
<td></td>
<td>210.53 (24.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-(5-pentylthiophene-2-yl)-dodec-11-yneoic acid</td>
<td></td>
<td>210.53 (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-6-(5-pentylfuran-2-yl)-hex-5-enoic acid</td>
<td></td>
<td>129.21 (81%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-6-(5-pentylthiophene-2-yl)-hex-5-enoic acid</td>
<td></td>
<td>210.53 (130.09)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid</td>
<td></td>
<td>20.01 (67%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-9-(5-pentylfuran-2-yl)-non-8-enoic acid</td>
<td></td>
<td>29.63 (66%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-9-(5-pentylthiophene-2-yl)-non-8-enoic acid</td>
<td></td>
<td>88.21 (72%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-12-(5-pentylfuran-2-yl)-dodec-11-enoic acid</td>
<td></td>
<td>116.86 (82%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-12-(5-pentylthiophene-2-yl)-dodec-11-enoic acid</td>
<td></td>
<td>210.53 (34%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-pentyl-2-furaldehyde</td>
<td></td>
<td>42.11 (28%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin$^a$</td>
<td></td>
<td>1.32 (0.50)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Positive control (50µg.mL$^{-1}$), all samples (1000 µg.mL$^{-1}$). Numerical values for minimum inhibitory concentration (MIC), inhibition concentration at 50% (IC$_{50}$) and inhibition (Inh)
concentration are also given in µg.mL⁻¹

*S. aureus* growth inhibition was compared against a single concentration of all samples tested, which was chosen from the range 1.64 – 210.53 µg.mL⁻¹. Monosubstituted furans and thiophenes, as well as their disubstituted alcohol derivatives showed low levels of inhibition of bacterial growth (52.63 µg.mL⁻¹, < 20 %) suggesting that at the concentration tested *S. aureus* was resistant to the compounds tested.

Figure 5.13 compares monosubstituted furan and thiophene and their alcohol derivatives against kanamycin, as positive control.

![Graph](image_url)

**Figure 5.13**: Bacterium growth inhibition: +Ctrl = kanamycin, F = furan, T = thiophene, P = pentyl. C3, C6, C9, C12 = number of carbons in the side chain ending in -OH
When comparing disubstituted furan and thiophene acetylenic acid derivatives, the story was different from that of furan and thiophene alcohols. The percentage of inhibition increased in all acetylenic acids when compared to their alcohol derivatives, especially for 9-(5-pentylthiophen-2-yl)-non-8-ynoic acid (5.29) (PT-C9CO₂H). In order to understand why this happened, we need to understand how this class of compounds might work in vivo. Until then, it is very difficult to speculate without knowing the mechanism of action of these class of compounds.

To the best of my knowledge, this is the first time furan and thiophene based fatty acids have been evaluated for their antibacterial potential. Figure 5.14 shows a comparison between furan and thiophene acetylenic acids.

Figure 5.14: Bacterium growth inhibition: +Ctrl = kanamycin, F = furan, T = thiophene. C3, C6, C9, C12 = number of carbons in the side chain ending in -CO₂H.
Interestingly, when comparing F-acids and T-acids, with Z-geometry the story changed again. Although the percentage of bacterium growth inhibition increased in all samples tested when compared to the antibacterial activity of their alcohol derivatives, *S. aureus* showed higher susceptibility to synthetic (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid (3.1) (ZFC9) (> 80%, 52.63 µg.mL<sup>-1</sup>). In addition, *S. aureus* showed higher susceptibility to a mixture of two diastereoisomers from the naturally occurring F-acids (3.1 and 3.2), (ZE-FC9-Nat) (> 95%, 10.52 µg.mL<sup>-1</sup>).

Although a matter of speculation, the increase in activity exhibited by a mixture of 3.1 and 3.2 against the bacteria tested, when compared to the activity shown by 3.1 alone, might be indicative that synergism could play a role in such increase in activity. Figure 5.15 shows a comparison between Z-F-acids and Z-T-acids antibacterial activity against *S. aureus*.

![Figure 5.15: Bacterium growth inhibition: +Ctrl = kanamycin, F = furan, T = thiophene, Nat = natural. C6, C9, C12 = number of carbons in the side chain ending in -CO₂H](image-url)
Chapter five: Furan fatty acids: synthesis and SARs

5.5 Conclusion

A total of 26 compounds have been investigated for their antibiotic potential against *S. aureus* (clinical isolate), *S. typhi*, *P. aeruginosa* and *C. albicans*. *E. coli* was not included in the final screening because crude extracts and F-acids isolated from Fulaga dive (*Amanita sp.* Pers.) did not show any growth inhibition and therefore it was concluded that *E. coli* was resistant against the F-acid tested. The test samples screened showed low to moderate activity against the Gram (+) *S. aureus*. Although *in vitro* antibacterial screenings of these compounds may not be sufficient to correlate effectiveness *in vivo* to validate traditional use, these results do provide a starting point for further biological and chemical investigation towards the discovery of new drugs.

As for furan based homologues and thiophene analogues to the naturally occurring F-acids isolated from Fulaga dive (*Amanita sp.* Pers.) to the best of my knowledge, this is the first time they have been screened for their antibacterial potential. Although some of the results reported herein show good bacterium growth inhibition, the samples tested are no match for the antibiotic strength of kanamycin. Finally I can conclude that *S. aureus* showed more susceptibility against (Z)-9-(5-pentylfuran-2-yl)-non-8-enoic acid and 9-(5-pentylthiophene-2-yl)-non-8-ynoic acid.

5.6 Experimental

For general information on instrumental methods used for the characterisation of F-acids, T-acids and pre-cursors, see experimental section in Chapter 3, section 3.8.1 to 3.8.6.

5.6.1 Culture media and controls

All media, BBL™ Mueller Hinton broth and DIFCOTM Mueller Hinton agar were sourced from Bacto Laboratories Pty Ltd, 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 before autoclaving.
Kanamycin sulphate from Streptomyces kanamyceticus supplied by Sigma Aldrich, Castle Hill, NSW 1765, Australia, was used as the antibiotic control for the sensitive strains of *S. aureus*, multidrug resistant *S. aureus*, *S. typhi* and *P. aeruginosa*. Amphotericin B was used as the positive control for *C. albicans* and also supplied by Sigma Aldrich. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used as the dye to determine the viability of the cells, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used for the antioxidant assay with ascorbic acid as the positive control. MTT, DPPH and ascorbic acid were sourced from Alfa-esar, Heysham, England.

5.6.2 Bioassays

For the DPPH˙ assay, sample tests and positive control were prepared at the same concentrations. First a stock solution of the appropriate positive control was prepared in MeOH to achieve a concentration of 10 mM. Stock solution was further diluted to a working solution of 1 mM.

A methanolic stock solution of DPPH was also prepared at a concentration of 1000 µM. DPPH stock solution was further diluted to a working concentration of 200 µM. Radical scavenging reaction was carried out in 96 well plates and therefore, 100µL of MeOH were added to wells 1b to 1h followed by 200µL of test sample or positive control to well 1a. Samples and/or positive control were serially diluted (1:2). Finally 100µL of a methanolic solution of DPPH was added to the wells and incubated for 30 min in the dark. Serial concentration range of positive control and samples after addition of DPPH was 0.7 µg.mL⁻¹ to 88 µg.mL⁻¹. Upon incubation, OD₅₁₇ was read to assess radical scavenging activity of test samples.

Turbidity-MTT microdilution assay was performed as outlined in Chapter 2, section 2.9.6
5.6.3 Experimental data

5-Pentylthiophene (5.4). A two-necked round bottom flask was charged with 200 mL of dried THF at 0 ºC. Subsequently, n-BuLi (37.0 mL, 44.0 mmol of 1.2 M in hexane solution) was added dropwise, followed by freshly distilled thiophene (5.3) (4.8 mL, 60.0 mmol). The resulting mixture was stirred for 100 min at which point 1-bromopentane (5.8 mL, 46.2 mmol) was added, and the reaction mixture was allowed to stir overnight. Water was added to quench the reaction and the resulting mixture was extracted with diethyl ether (2 x 30 mL). The organic layer was washed with saturated NH₄Cl solution (3 x 50 mL), saturated NaHCO₃ solution (2 x 50 mL) and dried with Na₂SO₄, filtered and reduced in vacuo yielding the title compound, as a mobile yellow oil (95%). Rf (0.71, Pet. Spirit 100%). UV (CH₂Cl₂) λmax (log ε) 234.5 (3.57). IR νmax 2925 (m), 1459 (w), 1035 (m), 687 (s) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δH 7.11 (1 H, d, J = 5.1, 1.2 Hz, 2'H), 6.92 (1 H, dd, J = 3.5 Hz, 3'H), 6.79 (1 H, d, J = 3.5 Hz, 4'H), 2.83 (2 H, t, J = 7.8, 7.4 Hz, 1'-H), 1.69 (2 H, m, 2'-H), 1.37 (4 H, m, 3'H & 4'H), 0.91 (3 H, t, J = 7.1 Hz, 5'-H). ¹³C NMR (CDCl₃, 100 MHz) δC 145.9 (C, 2'-C), 126.6 (CH, 3'-C), 123.9 (CH, 4'-C), 122.7 (CH, 5'-C), 31.5 (CH₂, 1'-C), 31.3 (CH₂, 2'-C), 29.9 (CH₂, 3'-C), 22.4 (CH₂, 4'-C), 14.0 (CH₃, 5'-C). GC-MS (+ve ion) m/z: [M]⁺ 154 (40), 111 (8), 97 (100), 84 (5), 45 (9). HREIMS m/z 154.0816 (calculated for C₉H₁₄S 154.0816).

2-Iodo-5-pentylthiophene (5.5). A two-necked round bottom flask was charged with a solution of 5-pentylthiophene (5.4) (3.0 g, 19.8 mmol) in dried THF (90 mL) 0ºC. Subsequently, n-BuLi (16.5 mL, 19.5 mmol of 1.2 M in hexane solution) was added dropwise, resulting in a dark brown solution. The resulting mixture was allowed to reach room temperature (RT), and subsequently cooled to -78ºC (acetone/CO₂), at which time a solution of iodine (5.0 g, 19.6 mmol) in dried THF (12 mL) was added, generating a dark red solution. The resulting mixture was allowed to reach RT and it was stirred for an extra 120 min. The reaction mixture was poured into DCM (240 mL), and the organic layer washed with saturated NaHCO₃ solution (2 x 30 mL) and 1:1 NaCl:Na₂S₂O₃ solution (4 x 30mL). The resulting solution was dried with Na₂SO₄, filtered and reduced in vacuo, affording the title compound, as a dark red oil (4.34g,
80\%). UV (CH₂Cl₂) λ\text{max} (log ε) 255.0 (3.74), IR ν\text{max} 3426 (b), 2926 (m), 1455 (m), 739 (s) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δH 7.04 (1 H, d, J = 3.5, 3'-H), 6.47 (1 H, d, J = 3.5, 4'-H), 2.79 (2 H, t, J = 7.6, 1'-H), 1.64 (2 H, m, 2'-H), 1.33 (4 H, m, 3',4'-H), 0.90 (3 H, m, 5'-H). ¹³C NMR (CDCl₃, 100MHz) δC 152.2 (C, 5-C), 136.5 (CH, 3-C), 125.8 (CH, 4-C), 69.3 (C, 2-C), 31.2 (CH₂, 1'-C), 31.1 (CH₂, 2'-C), 30.2 (CH₂, 3'-C), 22.4 (CH₂, 4'-C), 14.0 (CH₃, 5'-C). GC-MS (+ve ion) m/z: [M⁺] 280 (27), 223 (100), 207 (9), 167 (14), 97 (21). HREIMS m/z 279.9778 (calculated for C₉H₁₃S 127 I).

2-(Hex-2-yn-1-loxy)-tetrahydro-2H-pyran (5.6a). - n-BuLi (8.3 mL, 14.1 mmol of 1.7 M in hexane solution) was added dropwise via syringe to a solution of 2-(prop-2-yn-1-loxy)-tetrahydro-2H-pyran (4.30) (2.0 g, 14.1 mmol) in dried THF (30 mL), at -70°C (acetone/CO₂) under an atmosphere of argon. Subsequently, hexamethylphosphoramide (10 mL) was also added and the mixture was stirred at -70°C for two hours. Acetone/CO₂ cooling bath was changed for crushed ice and 1-bromopropane (1.4 mL, 15.5 mmol) was added and the mixture was stirred overnight while allowing to reach RT. A solution of 2 M HCl was carefully added to quench the reaction and the aqueous layer was extracted with diethyl ether (3 x 30 mL). The combined organic layers were washed with NaCl and water, dried over Na₂SO₄ and concentrated under reduced pressure. The crude mixture was used in the next step without further purification and/or characterisation.

2-(Dodec-2-yn-1-loxy)-tetrahydro-2H-pyran (5.7a). - n-BuLi (8.4 mL, 14.3 mmol of 1.7 M in hexane solution) was added dropwise to a solution of 2-(prop-2-yn-1-loxy)-tetrahydro-2H-pyran (4.30) (2.0 g, 14.3 mmol) in dried THF (30 mL), at -70°C (acetone/CO₂) under an atmosphere of argon. Subsequently, hexamethylphosphoramide (10 mL) was added and the mixture was stirred at -70 °C for two hours. Acetone/CO₂ cooling bath was changed for crushed ice and 1-bromononane (2.5 mL, 15.7 mmol) was added and the mixture was stirred overnight while allowing to
reach RT. A solution of 2 M HCl was carefully added to quench the reaction and the aqueous layer was extracted with diethyl ether (3 x 30 mL). The combined organic layers were washed with NaCl and water, dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The crude mixture was used in the next step without further purification and/or characterisation.

**Hex-2-yn-1-ol (5.6).** $p$-Toluenesulfonic acid (95.6 mg, 0.6 mmol) was added to a stirring solution of crude 2-(hex-2-yn-1-yloxy)-tetrahydro-2H-pyran (5.6a) (3.8 g) in methanol (100 mL). Mixture was stirred overnight, at which time methanol was removed under reduced pressure and product was re-dissolved in ether. The ethereal layer was washed with NaHCO$_3$, 1 M NaOH, NaCl then with water, dried over Na$_2$SO$_4$ and concentrated under reduced pressure yielding hex-2-yn-1-ol (5.6) (77%) as a yellow liquid. UV (DCM) $\lambda_{\text{max}}$ (log $\varepsilon$) 239 (1.68) nm; IR (neat) $\nu_{\text{max}}$ 3356, 2933, 1485, 1309, 1009, cm$^{-1}$; Rf (0.43, petroleum spirit: ethyl acetate, 1:1); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.25 (2 H, t, $J = 2.2$ Hz, 1-H), 2.19 (2 H, tt, $J = 7.2$, 2.2 Hz, 4-H), 1.68 (1 H, s, OH), 1.53 (2 H, dq, $J = 7.2$, 7.2, 5-H), 0.97 (3 H, t, $J = 7.2$, 6-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 86.4 (3-C), 78.4 (2-C), 51.4 (CH$_2$, 1-C), 21.9 (CH$_2$, 5-C), 20.7 (CH$_2$, 4-C), 14.4 (CH$_3$, 6-C). EIMS $m/z$ 97 [M-H$^+$] (18), 83 (100), 69 (56), 55 (91), 41 (75); HREIMS $m/z$ 97.0650 (calculated for C$_6$H$_9$O, 97.0653).

**Dodec-2-yn-1-ol (5.7).** $p$-Toluenesulfonic acid (92.3 mg, 0.5 mmol) was added to a stirring solution of 2-(dodec-2-yn-1-yloxy)-tetrahydro-2H-pyran (5.7a) (4.9 g) in methanol (100 mL). Mixture was stirred overnight, at which time methanol was removed under reduced pressure and product was re-dissolved in ether. The ethereal layer was washed with NaHCO$_3$, 1 M NaOH, NaCl then with water, dried over Na$_2$SO$_4$ and concentrated under reduced pressure yielding dodec-2-yn-1-ol (5.7) (85%) as a yellow liquid. UV (DCM) $\lambda_{\text{max}}$ (log $\varepsilon$) 229 (1.77) nm; IR (neat) $\nu_{\text{max}}$ 3301, 2922, 2853, 1002, cm$^{-1}$; Rf (0.57, petroleum spirit: ethyl acetate, 1:1); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.24 (2 H, m, 1-H),
2.20 (2 H, tt, J = 7.1, 2.16 Hz, 4-H), 1.69 (1 H, s-broad, OH), 1.50 (2 H, m, 5-H), 1.26 (12 H, m, 6, 7, 8, 9, 10, 11-H), 0.87 (3 H, t, J = 6.6, 12-H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 86.6 (3-C), 78.3 (2-C), 51.4 (CH$_2$, 1-C), 31.9 (CH$_2$, 10-C), 29.5 (CH$_2$, 5-C), 29.3 (CH$_2$, 6-C), 29.1 (CH$_2$, 7-C), 28.9 (CH$_2$, 8-C), 28.6 (CH$_2$, 9-C), 22.6 (CH$_2$, 11-C), 18.7 (CH$_2$, 4-C); 14.1 (CH$_3$, 12-C).

EIMS m/z 151 [M - H$_2$C=OH]$^+$ (4), 111 (26), 67 (90), 55 (100), 41 (3493); HREIMS m/z 151.1488 (calculated for C$_{11}$H$_{19}$, 151.1487).

**Hex-5-yn-1-ol (5.8).** Sodium hydride (562.8 mg, 23.5 mmol) was dissolved in 14 mL of anhydrous 1,3-diaminopropane (APA) under argon at RT, to give a light grey, effervescent solution of NAPA. Mixture was then heated at 70°C for 40 min, at which time colour changed to clear brown. Hex-2-yn-1-ol (5.6) (766.0 mg, 7.8 mmol) was added at RT. After addition, mixture became thicker and a strong brown colour and fine effervescence were observed. Iced water and saturated NH$_4$Cl were carefully added to quench the reaction, followed by extraction with diethyl ether (3 x 30 mL). The combined organic layers were further washed with 2 M HCl, NaHCO$_3$, NaCl, water, dried over Na$_2$SO$_4$ and concentrated under reduced pressure, yielding hex-5-yn-1-ol (5.8) (42%) as a clear orange liquid. UV (DCM) $\lambda_{\text{max}}$ (log ε) 230 (2.59) nm; IR (neat) $\nu_{\text{max}}$ 3293, 2938, 2867, 1059, 628, cm$^{-1}$; Rf (0.36, petroleum spirit: ethyl acetate, 1:1);

$^1$H NMR (400 MHz, CDCl$_3$): δ 3.67 (2 H, t, J = 6.1 Hz, 1-H), 2.23 (2 H, td, J = 6.9, 2.6 Hz, 4-H), 1.95 (1 H, t, J = 2.6 Hz, 6-H), 1.73 – 1.58 (4 H, m, 2, 3-H), 1.53 (1 H, s, OH).

$^{13}$C NMR (100 MHz, CDCl$_3$): δ 84.2 (5-C), 68.5 (CH, 6-C), 62.3 (CH$_2$, 1-C), 31.7 (CH$_2$, 2-C), 24.7 (CH$_2$, 3-C), 18.2 (CH$_2$, 4-C). EIMS m/z 98 [M]$^+$ (26), 97 [M – H] (6), 67 (100), 53 (60), 39 (93); HREIMS m/z 97.0654 (calculated for C$_6$H$_9$O, 97.0653).

**Dodec-11-yn-1-ol (5.9).** Sodium hydride (562.8 mg, 23.5 mmol) was dissolved in 14 mL of anhydrous 1,3-diaminopropane (APA) under argon at RT, to give a light grey, effervescent solution of NAPA. Mixture was then heated at 70°C for 40 min, at which time colour changed to clear brown. Dodec-2-yn-1-ol (5.7) (766 mg, 7.8 mmol) was added at RT. After addition, mixture became thicker
and a strong brown colour and fine effervescence were observed. Iced water and saturated NH₄Cl were carefully added to quench the reaction, followed by extraction with diethyl ether (3 x 30 mL). The combined organic layers were further washed with 2 M HCl, NaHCO₃, NaCl, water, dried over Na₂SO₄ and concentrated under reduced pressure, yielding dodec-11-yn-1-ol (5.9) (67%) as a clear orange liquid. UV (DCM) \(\lambda_{\text{max}} (\log \varepsilon)\) 244 (2.29) nm; IR (neat) \(\nu_{\text{max}}\) 3311, 2924, 2854, 1052, 627, cm\(^{-1}\); Rf (0.42, petroleum spirit: ethyl acetate, (1:1)); \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) 3.63 (2 H, t, \(J = 6.6\) Hz, 1-H), 2.17 (2 H, td, \(J = 7.0, 2.6\) Hz, 10-H), 1.93 (1 H, t, \(J = 2.6\) Hz, 12-H), 1.59 – 1.45 (6 H, m, 2, 3, 9-H), 1.28 (10 H, m, 4, 5, 6, 7, 8-H). \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta\) 84.8 (11-C), 68.0 (CH, 12-C), 63.0 (CH₂, 1-C), 32.8 (CH₂, 2-C), 29.5 (CH₂, 4-C), 29.4 (CH₂, 5-C), 29.4 (CH₂, 6-C), 29.1 (CH₂, 7-C); 28.7 (CH₂, 9-C), 28.5 (CH₂, 8-C), 25.7 (CH₂, 3-C) 18.3 (CH₂, 10-C). EIMS \(m/z\) 182 [M]⁺ (4), 81 (82), 67 (100), 55 (70), 41 (63); HREIMS \(m/z\) 67.0548 [M – (H₂C)_6H₂C=OH]⁺ (calculated for C₃H₇, 67.0548).

- In a two-necked round bottom flask, 2-iodo-5-pentylfuran (4.28) (100.0 mg, 0.4 mmol), Pd(PPh₃)₄ (23.0 mg, 5%), CuI (7.6 mg, 10%), propargyl alcohol (4.29) (42.5 mg, 0.8 mmol), pyrrolidine (3 mL) was added. The reaction was degassed several times and back filled with argon. Reaction mixture was stirred at RT overnight covered in aluminium foil. The reaction was cooled to 0°C at which time 15 mL of saturated NH₄Cl solution was slowly added. The reaction was extracted with 3 portions of Et₂O (15 mL) and the combined organic layers were washed with saturated NH₄Cl solution (3 x 20 mL) followed by H₂O (20 mL), and concentrated under reduced pressure. The dark orange crude was subjected to flash column chromatography (silica, eluent pet. spirit: Et₂O = 1:1), affording the title compound as a light yellow oil (60%). UV (MeOH) \(\lambda_{\text{max}} (\log \varepsilon)\) 260 (4.03) nm; IR (neat) \(\nu_{\text{max}}\) 3390 (br), 2928 (m), 2220 (w), 1014 (m), 786 (m), cm\(^{-1}\); Rf (0.3, petroleum spirit: ethyl acetate, (3:1)); \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) 6.50 (1 H, d, \(J = 3.3\) Hz, 3-H), 5.97 (1 H, dt, \(J = 3.3, 0.8\) Hz, 4-H), 4.50 (2 H, s, 1''-H), 2.59 (2 H, t, \(J = 7.6\) Hz, 1’-H), 1.67 – 1.60 (2 H, m, 2’-H), 1.33 – 1.29 (4 H, m, 3’,4’-H), 0.89 (3 H, t, \(J = 6.9\) Hz, 5’-H). \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta\) 158.4 (5-C), 134.4 (2-C), 116.8 (3-C), 106.2 (4-C), 91.4 (3’’-C), 51.6 (1’’-C), 28.7 (14-C).
31.3 (1'-C), 28.2, 27.5, 22.3, 14.0 (5'-C). EI-MS m/z (%): [M]+ 192 (90), 135 (100), 107 (57). HREIMS m/z 192.1148 (calculated for C_{12}H_{16}O_{2}, 192.1150).

3-(5-pentylthiophene-2-yl)-prop-2-yn-1-ol (5.13).

- 2-Iodo-5-pentylthiophene (5.5) (152.3 mg, 0.5 mmol), Pd(PPh\textsubscript{3})\textsubscript{4} (31.4 mg, 5%) and CuI (10.3 mg, 10%) were degassed several times and backfilled with argon. Pyrrolidine (10 mL) was subsequently added. After addition, the mixture was stirred for two minutes at RT. A solution of propargyl alcohol (60.9 mg, 1.1 mmol) in pyrrolidine was then added dropwise, and the mixture was stirred overnight. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration on silica using a mixture of EtOAc and petroleum spirit (1:1). Organic layer was washed with NH\textsubscript{4}Cl, followed by NaCl and water, dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated under reduced pressure, yielding 3-(5-pentylthiophene-2-yl)-prop-2-yn-1-ol (5.13) (47%) as a clear orange liquid. Rf (0.5, EtOAc:Pet. spirit 1:1) IR ν\textsubscript{max} 3318 (w, br), 2956 (m), 2927 (s), 2856 (m), 2219 (w), 1537 (m), 1354 (m), 1185 (m), 1012 (vs), 799 (vs). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δH 7.03 (1 H, d, J = 3.6, 3'-H), 6.63 (1 H, d, J = 3.6, 4'-H), 4.49 (2 H, s, 1''-H), 2.76, (2 H, t, J = 7.6, 1'-H), 1.66 (3 H, m, 2'-H), 1.30 (4 H, m, 3', 4'-H), 0.89 (3 H, t, J = 7.0, 5'-H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 100 MHz) δC 148.6 (C, 5-C), 132.5 (CH, 3-C), 124.0 (CH, 4-C), 119.6 (C, 2-C), 90.3 (C, 2''-C), 79.6 (C, 3''-C), 51.8 (CH\textsubscript{2}, 1''-C), 31.2 (CH\textsubscript{2}, 1'-C), 31.2, 30.1, 22.3, 14.0 (CH\textsubscript{3}, 5'-C) EIMS m/z: [M]+ 208 (78), 151 (100), 123 (53), 79 (11). HREIMS m/z 208.0921 (calculated for C\textsubscript{12}H\textsubscript{16}OS 208.0922)

6-(5-pentylfuran-2-yl)-hex-5-yn-1-ol (5.11).

- 2-Iodo-5-pentylfuran (4.28) (99.0 mg, 0.4 mmol), Pd(PPh\textsubscript{3})\textsubscript{4} (23.0 mg, 5%) and CuI (7.6 mg, 10%) were degassed several times and backfilled with argon. Pyrrolidine (10 mL) was subsequently added. After addition, the mixture was stirred for two minutes at RT. A solution of hex-5-yn-1-ol (5.8) (130.2 mg, 1.3 mmol) in pyrrolidine was then added dropwise, and the mixture was stirred for two hours. Petroleum ether was added
to the resulting mixture and the precipitate was removed by filtration. Organic layer was washed with NH₄Cl, followed by NaCl and water, dried over Na₂SO₄ and concentrated under reduced pressure, yielding the title compound (61%) as a mobile liquid. UV (MeOH) λₘₐₓ (log ε) 256 (4.05) nm; IR (neat) νₘₐₓ 3371 (br), 2929 (s), 2861 (m), 2214 (w), 1649 (m), 784 (sh) cm⁻¹; Rf (0.17, petroleum spirit: ethyl acetate, 3:1); ¹H NMR (400 MHz, CDCl₃): δ 6.37 (1 H, d, J = 3.2 Hz, 3-H), 5.93 (1 H, d, J = 3.2 Hz, 4-H), 3.70 (2 H, t, J = 6.0 Hz, 1''-H), 2.58 (2 H, t, J = 7.6 Hz, 4''-H), 2.48 (2 H, t, J = 6.7 Hz, 1'-H) 1.75 – 1.61 (6 H, m, 2', 2'', 3''-H), 1.53 (1H, s, OH). 13C NMR (100 MHz, CDCl₃): δ 157.3 (5-C), 135.5 (2-C), 114.7 (CH, 3-C), 105.8 (CH, 4-C), 93.7 (5''-C), 71.4 (6''-C), 62.4 (CH₂, 1''-C), 31.8 (CH₂, 2''-C), 31.3 (CH₂, 2'-C), 28.2 (CH₂, 1'-C), 24.7 (CH₂, 3'-C), 22.4 (CH₂, 4'-C), 19.3 (CH₂, 1'-C), 14.0 (CH₃, 5'-C). EIMS m/z [M⁺] 234 (37), 177 (86), 159 (100), 149 (17), 133 (33); HREIMS m/z 234.1617 (calculated for C₁₅H₂₂O₂, 234.1620).

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6-(5-pentyliophiene-2-yl)-hex-5-yn-1-ol (5.14). 2-Iodo-5-pentyliophiene (5.5) (149.5 mg, 0.5 mmol), Pd(PPh₃)₄ (34.6 mg, 5%) and CuI (9.5 mg, 10%) were degassed several times and backfilled with argon. Pyrrolidine (10 mL) was subsequently added. After addition, the mixture was stirred for two minutes at RT. A solution of hex-5-yn-1-ol (5.8) (104.6 mg, 1.1 mmol) in pyrrolidine was then added dropwise, and the mixture was stirred overnight. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration on silica using a mixture of EtOAc and petroleum spirit (1:1). Organic layer was washed with NH₄Cl, followed by NaCl and water, dried over Na₂SO₄ and concentrated under reduced pressure, yielding the title compound (56%) as a clear orange liquid. Rf (0.45, EtOAc; petroleum spirit 1:1) IR νₘₐₓ 3370 (w, br), 2927 (s), 2219 (w), 780 (sh) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 6.92 (1 H, d, J = 3.5, 3-H), 6.59 (1 H, d, J = 3.5, 4-H), 3.70 (2 H, t, J = 6.0, 1''-H), 2.74, (2 H, t, J = 7.6, 1'-H), 2.46 (2 H, t, J = 6.6 Hz, 4''-H), 1.77 – 1.59 (6 H, m), 1.38 – 1.28 (3 H, m), 0.89 (3 H, t, J = 7.0, 5'-H). ¹³C NMR (CDCl₃, 100 MHz) δ 147.0 (5-C), 131.1 (CH, 3-C), 123.9 (CH, 4-C), 121.3 (2-C), 93.1 (5''-C), 74.7 (6''-C), 62.6 (CH₂, 1''-C), 32.1 (CH₂, 2''-C), 31.4 (CH₂, 1'-C), 31.3 (CH₂, 3'-C), 27.6 (CH₂, 3'-C), 24.7 (CH₂, 3'-C), 22.4 (CH₂, 4'-C), 19.3 (CH₂, 1'-C), 14.0 (CH₃, 5'-C).
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3'-C), 30.2 (CH$_2$, 2'-C), 25.1 (CH$_2$, 3''-C), 22.5 (CH$_2$, 4'-C), 29.6 (CH$_2$, 4''-C), 14.1 (CH$_3$, 5'-C). HREIMS m/z 250.1382 (calculated for C$_{13}$H$_{22}$OS 250.1391)

HREIMS m/z 292.1858 (calculated for C$_{18}$H$_{28}$OS 292.1861)

mg, 0.5 mmol), Pd(PPh$_3$)$_4$ (34.6 mg, 5%) and CuI (9.5 mg, 10%) were degassed several times and backfilled with argon. Pyrrolidine (10 mL) was subsequently added. After addition, the mixture was stirred for two minutes at RT. A solution of non-8-yn-1-ol (4.33) (151.1 mg, 1.1 mmol) in pyrrolidine was added dropwise, and the mixture was stirred overnight. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration on silica using a mixture of EtOAc and petroleum spirit (1:1).

Organic layer was washed with NH$_4$Cl, followed by NaCl and water, dried over Na$_2$SO$_4$ and concentrated under reduced pressure, yielding 9-(5-pentylthiophene-2-yl)-non-8-yn-1-ol (5.15) (87%) as a clear orange liquid. Rf (0.48, EtOAc:petroleum spirit 1:1) IR $\nu_{\text{max}}$ 3355 (br), 2927 (s), 2856 (m), 2219 (w), 1590 (w), 780 (m).

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.92 (1 H, d, $J = 3.5$, 3'-H), 6.58 (1 H, d, $J = 3.5$, 4'-H), 3.65 (2 H, t, $J = 6.6$, 1''-H), 2.74 (2 H, t, $J = 7.6$, 1'-H), 2.40 (2 H, t, $J = 7.0$, 7''-H), 1.62-1.31 (12 H, m, 2', 6'', 5'', 4'', 3'', 2''-H), 0.89 (3 H, t, $J = 4.8$, 5'-H)

$^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$

146.7 (5-C), 130.8 (CH, 3-C), 123.8 (CH, 4-C), 93.5 (2-C), 63.0 (8''-C), 53.4 (9''-C), 32.7 (CH$_2$, 1''-C), 31.2, 31.1, 30.1, 28.9, 28.8, 28.5, 25.6 (CH$_2$, 1'-C), 25.6, 22.4, 19.7, 14.0 (CH$_3$, 5'-C). EIMS m/z: [M]+ 292 (53), 235 (27), 193 (100), 163 (27), 135 (33), 91 (15). HREIMS m/z 292.1858 (calculated for C$_{18}$H$_{28}$OS 292.1861)

HREIMS m/z: 240.5 mg, 1.3 mmol, in pyrrolidine was added.
dropwise, and the mixture was stirred overnight. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration on silica using a mixture of EtOAc and petroleum spirit (1:1). Organic layer was washed with NH₄Cl, followed by Na₂SO₄ and dried over Na₂SO₄ and concentrated under reduced pressure, yielding 12-(5-Pentylfuran-2-yl)-dodec-11-yn-1-ol (5.12) (89%) as a clear orange liquid. UV (MeOH) λmax (log ε) 254 (3.89) nm; IR (neat) νmax 3335 (br), 2924 (sh), 2854 (m), 1592 (w), 1537 (w) 781 (m) cm⁻¹. A solution of dodecynol (5.12) in pyrrolidine was added dropwise, and the mixture was stirred overnight. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration on silica using a mixture of EtOAc and petroleum spirit (1:1). After addition, the mixture was degassed several times and backfilled with argon. Pyrrolidine (10 mL) was subsequently added. After addition, the mixture was stirred for two minutes at RT. A solution of dodec-11-yn-1-ol (5.9) (219.3 mg, 1.2 mmol) in pyrrolidine was added dropwise, and the mixture was stirred overnight. Petroleum ether was added to the resulting mixture and the precipitate was removed by filtration on silica using a mixture of EtOAc and petroleum spirit (1:1). Organic layer was washed with NH₄Cl, followed by NaCl and water, dried over Na₂SO₄ and concentrated under reduced pressure, yielding 12-(5-pentylthiophene-2-yl)-dodec-11-yn-1-ol (5.16) (81%) as a clear orange liquid. 

RF (0.3, EtOAc:petroleum spirit 1:3) IR ν 3334 (br), 2927 (sh), 2856 (m), 1537 (w), 780 (m). ¹H NMR (400 MHz, CDCl₃): δ 6.92 (1 H, d, J = 3.5, 3-H), 6.58 (1 H, d, J = 3.5, 4-H), 3.64 (2 H, t, J = 7.0 Hz, 1'-H), 2.41 (2 H, t, J = 7.08 Hz, 10''-H), 1.66 – 1.52 (8 H, m, 3', 4', 2'', 7''-H), 1.46 – 1.39 (2 H, m, 8''-H), 1.29 (12 H, m, 2', 3'', 4'', 5'', 6'', 9''-H), 0.88 (3 H, t, J = 6.9 Hz, 5''-H). ¹³C NMR (100 MHz, CDCl₃): δ 157.1 (C), 114.6 (CH, 3-C), 105.8 (CH, 4-C), 94.2 (11''-C), 71.3 (12''-C), 63.0 (CH₂, 1''-C), 32.8 (CH₂, 2''-C), 31.3 (CH₂, 2'-C), 29.5 (CH₂, 6''-C), 29.4 (CH₂, 5''-C). 29.1 (CH₂, 4''-C), 28.9 (CH₂, 9''-C), 28.8 (CH₂, 8''-C), 28.4 (CH₂, 7''-C), 28.2 (CH₂, 1'-C), 27.6 (CH₂, 3'-C), 25.7 (CH₂, 3''-C), 22.3 (CH₂, 4'-C), 19.5 (CH₂, 10''-C), 13.9 (CH₃, 5'-C). EIMS m/z 318 [M]+ (47), 218 (54), 177 (100), 161 (46), 147 (32); HREIMS m/z 318.2559 (calculated for C₂₁H₃₃O₂, 318.2559).
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1''-H), 2.70 (2 H, t, J = 7.6, 1'-H), 2.40 (2 H, t, J = 7.1, 7''-H), 1.66 - 1.30 (22 H, m, 2''-4''-H & 2''-9''-H), 0.89 (3 H, t, J = 6.9, 5'-H) \( ^{13} \)C NMR (CDCl\(_3\), 100 MHz) \( \delta \) 146.7 (5-C), 130.8 (CH, 3-C), 123.7 (CH, 4-C), 121.4 (2-C), 93.6 (8''-C), 74.1 (9''-C), 63.1 (CH\(_2\), 1''-C), 32.8, 31.2, 31.2, 30.1, 29.4, 29.5, 29.4, 29.1, 28.9, 28.6 (CH\(_2\), 1'-C), 28.3, 25.7, 22.4, 19.7, 14.0 (CH\(_3\), 5'-C). EIMS \( m/z \) \( [M]^{+} \) 334 (37), 234 (60), 193 (100), 177 (43), 163 (33), 134 (25), 121 (16), 97 (13), 41 (12). HREIMS \( m/z \) 334.2336 (calculated for C\(_{21}\)H\(_{34}\)O\(_3\) 334.2330).

3-(5-Pentylfuran-2-y1)-prop-2-yunal (5.17) – A mixture of 3-(5-pentylfuran-2-y1)-prop-2-yn-1-ol (5.10) (113.0 mg, 0.4 mmol), TEMPO (12.8 mg, 0.1 mmol) and BAIB (228.6 mg, 0.7 mmol) were vigorously stirred in DCM (20 mL) overnight until all starting material was consumed. An aqueous solution of 10% sodium thiosulphate was added to the mixture to quench the reaction and stirred for few minutes. Mixture was transferred to a separatory funnel and the aqueous layer was washed once with DCM. The organic layers were combined and washed with sodium bicarbonate, NaCl, dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure, affording the titled compound, as a yellow oil (Rf 0.35, EtOAc:Pet Sp, 1:3). Product was used without further purification and/or characterisation in the next step.

3-(5-Pentythiophene-2-y1)-prop-2-ynal (5.20) – A mixture of 3-(5-pentylthiophene-2-y1)-prop-2-yn-1-ol (5.13) (128.0 mg, 0.6 mmol), TEMPO (11.3 mg, 0.1 mmol) and BAIB (211.0 mg, 0.7 mmol) were vigorously stirred in DCM (25 mL) overnight. 10% sodium thiosulphate (10 mL) was used to quench the reaction, which was then transferred to a separatory funnel. The aqueous layer was washed with DCM (2 x 20 mL), and the combined organic layers washed with saturated NaHCO\(_3\) solution (2 x 20 mL), NaCl solution (2 x 20 mL), then dried with Na\(_2\)SO\(_4\). After being reduced in vacuo, the title product was afforded as a clear yellow oil. Rf (0.58, DCM 100%) EIMS \( m/z \) \([M]^{+}\) 206 (63), 149 (100), 121 (49),
77 (12), 69 (8). Product was used without further purification and/or characterisation in the next step.

6-(5-Pentylfuran-2-yl)-hex-5-ynal (5.18). - A mixture of 6-(5-pentylfuran-2-yl)-hex-5-yn-1-ol (5.11) (113.0 mg, 0.4 mmol), TEMPO (12.8 mg, 0.1 mmol) and BAIB (228.6 mg, 0.7 mmol) were vigorously stirred in DCM (20 mL) overnight until all starting material was consumed. An aqueous solution of 10% sodium thiosulphate was added to the mixture to quench the reaction and stirred for few minutes. Mixture was transferred to a separatory funnel and the aqueous layer was washed once with DCM. The organic layers were combined and washed with sodium bicarbonate, NaCl, dried over Na₂SO₄ and concentrated under reduced pressure, affording the titled compound, as a yellow oil (Rf 0.35, EtOAc:Pet sp, 1:3). Product was used without further purification and/or characterisation in the next step.

6-(5-Pentylthiophene-2-yl)-hex-5-ynal (5.21). - A mixture of 6-(5-pentylthiophene-2-yl)-hex-5-yn-1-ol (5.14) (74.5 mg, 0.3 mmol), TEMPO (9.3 mg, 0.1 mmol) and BAIB (166.0 mg, 0.5 mmol) were vigorously stirred in DCM (25 mL) overnight. 10% sodium thiosulphate (10 mL) was used to quench the reaction, which was then transferred to a separating funnel. The aqueous layer was washed with DCM (2 x 20 mL), and the combined organic layers washed with saturated NaHCO₃ solution (2 x 20 mL), NaCl solution (2 x 20 mL), then dried with Na₂SO₄. After being reduced in vacuo, the title product was afforded as a clear yellow oil. Rf (0.47, EtOAc:Pet sp 1:3). Product was used in the next step without further purification or characterisation.
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9-(5-pentylthiophene-2-yl)-non-8-ynal (5.22). A mixture of 9-(5-pentylthiophene-3-yl)-non-8-yn-1-ol (5.15) (137.2 mg, 0.47 mmol, 1 Eq), TEMPO (14.6 mg, 0.092 mmol, 0.2 Eq) and BAIB (262 mg, 0.81 mmol, 1.73 Eq) were vigorously stirred in DCM (25 mL) overnight. 10% sodium thiosulphate (10 mL) was used to quench the reaction, which was then transferred to a separating funnel. The aqueous layer was washed with DCM (2 x 20 mL), and the combined organic layers washed with saturated NaHCO₃ solution (2 x 20 mL), NaCl solution (2 x 20 mL), then dried with Na₂SO₄. After being reduced in vacuo, the title product was afforded as a clear yellow oil. Rf (0.50, EtOAc:Pet sp 1:3). Product was used in the next step without further purification or characterisation.

12-(5-Pentylfuran-2-yl)-dodec-11-ynal (5.19). - A mixture of 12-(5-pentylfuran-2-yl)-dodec-11-yn-1-ol (5.12) (113.0 mg, 0.4 mmol), TEMPO (12.8 mg, 0.1 mmol) and BAIB (228.6 mg, 0.7 mmol) were vigorously stirred in DCM (20 mL) overnight until all starting material was consumed. An aqueous solution of 10% sodium thiosulphate was added to the mixture to quench the reaction and stirred for few minutes. Mixture was transferred to a separatory funnel and the aqueous layer was washed once with DCM. The organic layers were combined and washed with sodium bicarbonate, NaCl, dried over Na₂SO₄ and concentrated under reduced pressure, affording the titled compound, as a yellow oil (Rf 0.35 (EtOAc:Pet Sp 1:3)). Product was used without further purification and/or characterisation in the next step.

12-(5-pentylthiophene-2-yl)-dodec-11-ynal (5.23). - A mixture of 12-(5-pentylthiophene-2-yl)-dodec-11-yn-1-ol (5.16) (162.7 mg, 0.5 mmol), TEMPO (15.2 mg, 0.1 mmol) and
BAIB (271.3 mg, 0.8 mmol) were vigorously stirred in DCM (25 mL) overnight. 10% sodium thiosulphate (10 mL) was used to quench the reaction, which was then transferred to a separating funnel. The aqueous layer was washed with DCM (2 x 20 mL), and the combined organic layers washed with saturated NaHCO₃ solution (2 x 20 mL), NaCl solution (2 x 20 mL), then dried with Na₂SO₄. After being reduced in vacuo, the title product was afforded as a clear yellow oil. Rf (0.52, EtOAc:Pet sp 1:3). Product was used in the next step without further purification or characterisation.

3-(5-Pentylfuran-2-yl)-prop-2-ynoic acid (5.24). - In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of tert-butanol (22.6 mL), 0.1 parts of 2-methyl-2-butene (9.8 mL, purity 80%), 3.5 parts of sodium chlorite (395.5 mg), 2.9 parts of sodium dihydrogen phosphate (324.8 mg) and 0.1 parts of water (16.9 mL). Mixture was added to a 100 mL round bottom flask containing 3-(5-pentylfuran-2-yl)-prop-2-ynal (5.17) (113.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc (30 mL x 2). Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (25% petroleum spirit in ethyl acetate/ethanol 3:1) was performed to obtain the title compound as a viscous oil (64%). UV (MeOH) ) λ max (log ε) 283 (3.81) nm; IR (neat) ν max 3401 (br), 2926 (m), 2190 (w), 1587 (m), 1378 (m), cm⁻¹; Rf (0.13, 25% petroleum spirit in ethyl acetate/ethanol, 3:1); ¹H NMR (400 MHz, CDCl₃): δ 6.73 (1 H, d, J = 3.3 Hz, 3-H), 6.11 (1 H, d, J = 3.3 Hz, 4-H), 2.63 (2 H, t, J = 7.5 Hz, 1’-H), 1.65 (2 H, tt, J = 7.5, 2'-H), 1.38 – 1.29 (4 H, m, 3’,4’-H), 0.91 (3 H, J = 6.7 Hz, 5’-H). ¹³C NMR (100 MHz, CDCl₃): δ 161.0 (5-C), 160.1 (1’’-C), 135.5 (2-C), 120.8 (CH, 3-C), 117.7 (3’’-C), 107.9 (CH, 4-C), 72.5 (2’’-C), 32.4 (CH₂, 3’-C), 29.1 (CH₂, 1’-C), 28.6 (CH₂, 2’-C), 23.4 (CH₂, 4’-C), 14.3 (CH₃, 5’-C). HRESI-MS m/z 229.0839 (calculated for C₁₂H₁₄O₃Na, 229.0841).
3-(5-pentylthiophene-2-yl)-prop-2-ynoic acid (5.27) – In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of tert-butanol (10.4 mL), 0.1 parts of 2-methyl-2-butene (4.5 mL of 2 M in THF solution), 3.5 parts of sodium chlorite (182.2 mg), 2.9 parts of sodium dihydrogen phosphate (149.4 mg) and 0.1 parts of water (7.3 mL). Mixture was added to a 100 mL round bottom flask containing 3-(5-pentylthiophene-2-yl)-prop-2-ynal (5.20) (52.1 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc (30 mL x 2). Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (25% EtOH in EtOAc/pet spirit 3:1) was performed to obtain the title compound as a viscous oil (65%). IR (neat) νmax 3398 (br), 2924 (m), 2187 (w), 1587 (m), 1378 (m), cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 7.36 (1 H, d, J = 3.7 Hz, 3-H), 6.74 (1 H, d, J = 3.7 Hz, 4-H), 2.82 (2 H, t, J = 7.6 Hz, 1’-H), 1.68 (2 H, tt, J = 7.6, 2’-H), 1.37 – 1.30 (4 H, m, 3’,4’-H), 0.90 (3 H, J = 6.9 Hz, 5’-H). ¹³C NMR (100 MHz, CDCl₃): δ 156.0 (1’’-C), 153.8 (5-C), 137.8 (3-C), 133.3 (CH, 4-C), 125.1 (2-C), 124.1 (2’’-C), 80.5 (3’’-C), 31.3 (CH₂, 1’-C), 31.2 (CH₂, 3’-C), 30.5 (CH₂, 2’-C), 22.5 (CH₂, 4’-C), 14.1 (CH₃, 5’-C). HRESI-MS m/z 245.0622 (calculated for C₁₂H₁₄O₂Na₂S, 245.0612).

6-(5-Pentylfuran-2-yl)-hex-5-ynoic acid (5.25) – Following the work of Schmidt and co-workers the oxidation of aldehyde to carboxylic acid was as follows:¹²⁰ In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of tert-butanol (22.6 mL), 0.1 parts of 2-methyl-2-butene (9.8 mL, purity 80%), 3.5 parts of sodium chlorite (395.5 mg), 2.9 parts of sodium dihydrogen phosphate (324.8 mg) and 0.1 parts of water (16.9 mL). Mixture was added to a 100 mL round bottom flask containing 6-(5-pentylfuran-2-yl)-hex-5-ynal (5.18) (113 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc (30 mL x 2). Combined organic layers were washed
with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (ethyl acetate:petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (70%). UV (MeOH) λ_max (log ε) 254 (3.98) nm; IR (neat) ν_max 2956 (m), 2931 (m), 1861 (m), 1712 (s), cm⁻¹; Rf (0.25, petroleum spirit: ethyl acetate, (1:1)); ¹H NMR (400 MHz, CDCl₃): δ 6.39 (1 H, d, J = 3.2 Hz, 3-H), 5.94 (1 H, d, J = 3.2 Hz, 4-H), 2.60 – 2.52 (6 H, m, 1’, 2”, 4”-H), 1.93 (2 H, tt, J = 7.1, 3’’-H), 1.63 (2 H, tt, J = 7.1, 2’-H), 1.33 – 1.29 (4 H, m, 3’, 4’-H) 0.89 (3 H, t, J = 6.8 Hz, 5’-H). ¹³C NMR (100 MHz, CDCl₃): δ 177.3 (1’-C), 157.4 (5-C), 135.3 (2-C), 115.0 (CH, 3-C), 105.8 (CH, 4-C), 92.5 (5’’-C), 72.4 (6’’-C), 32.4 (CH₂, 4”-C), 31.3 (CH₂, 3’-C), 28.2 (CH₂, 1’-C), 27.6 (CH₂, 2’-C). 23.3 (CH₂, 3’’-C), 22.4 (CH₂, 4’-C), 18.9 (CH₂, 2’’-C), 13.9 (CH₃, 5’-C). HRESIMS m/z 271.1311 (calculated for C₁₅H₂₀O₃ 23Na 271.1310).

6-(5-pentylthiophene-2-yl)-hex-5-ynoic acid (5.28). - In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of tert-butanol (14.8 mL), 0.1 parts of 2-methyl-2-butene (6.4 mL of 2 M in THF solution), 3.5 parts of sodium chlorite (258.6 mg), 2.9 parts of sodium dihydrogen phosphate (212.1 mg) and 0.1 parts of water (10.3 mL). Mixture was added to a 100 mL round bottom flask containing 6-(5-pentylthiophene-2-yl)-hex-5-ynal (5.21) (73.9 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc (30 mL x 2). Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (ethyl acetate:petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (75%) IR (neat) ν_max 2954 (m), 2929 (m), 1858 (m), 1710 (s), cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 6.93 (1 H, d, J = 3.6 Hz, 3-H), 6.59 (1 H, d, J = 3.6 Hz, 4-H), 2.74 (2 H, t, J = 7.6 Hz, 1’-H), 2.60 – 2.52 (4 H, m, 1’’, 4’’-H), 1.92 – 1.30 (10 H, m, 2’’, 3’’’, 2’, 3’, 4’-H), 0.90 (3 H, t, J = 6.8 Hz, 5’-H). ¹³C NMR (100 MHz, CDCl₃): δ 177.2 (1’’-C), 147.3 (5-C), 131.3 (3-C), 124.0 (CH, 4-C), 121.1 (2-C), 91.9 (5”-C), 75.3 (6’’-C), 23.6 (CH₂, 2’’’-C), 31.4 (CH₂, 1’-C), 31.3 (CH₂, 3’-C), 22.4 (CH₂, 4’-C), 18.9 (CH₂, 2’’-C), 13.9 (CH₃, 5’-C).
30.2, 23.7, 22.5, 19.2 (CH$_2$, 4’’-C), 14.1 (CH$_3$, 5’-C). HREIMS m/z 287.1092 (calculated for C$_{15}$H$_{20}$O$_2$23NaS 287.1082).

9-(5-pentylthiophene-2-yl)-non-8-ynoic acid (5.29). In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of tert-butanol (27.2 mL), 0.1 parts of 2-methyl-2-butene (11.8 mL of 2 M in THF solution), 3.5 parts of sodium chlorite (476.8 mg), 2.9 parts of sodium dihydrogen phosphate (390.9 mg) and 0.1 parts of water (19.1 mL). Mixture was added to a 100 mL round bottom flask containing 9-(5-pentylthiophene-2-yl)-non-8-ynal (5.22) (136.9 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc (30 mL x 2). Combined organic layers were washed with NaHCO$_3$, NaCl, dried over Na$_2$SO$_4$ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/pet spirit 3:1) was performed to obtain the title compound as a viscous oil (80%). IR (neat) $\nu$$_{max}$ 2930 (m), 1708 (s), 1180 (br) 630 (m), cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.92 (1H, d, $J$ = 3.6 Hz, 3-H), 6.59 (1H, d, $J$ = 3.6 Hz, 4-H), 2.74 (2H, t, $J$ = 7.6 Hz, 1’-H), 2.43 – 2.34 (4H, m, 2’, 7’’-H), 1.70 – 1.29 (14H, m, 2’, 3’, 4’, 3’’, 4’’, 5’’, 6’’-H), 0.89 (3H, t, $J$ = 6.9 Hz, 5’-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 178.4 (1’’-C), $\delta$ 145.0 (5-C), 131.0 (CH, 3-C), 123.9 (CH, 4-C), 121.5 (2-C), 93.4 (8’’-C), 74.4 (9’’-C), 33.8 (CH$_2$, 2’’-C), 31.4 (CH$_2$, 1’-C), 31.3, 30.3, 28.7, 28.6, 28.5, 24.7, 22.5, 19.8 (CH$_2$, 7’’-C), 14.1 (CH$_3$, 5’-C). HREIMS m/z 329.1560 (calculated for C$_{18}$H$_{26}$O$_2$23NaS, 329.1551).

12-(5-Pentylfuran-2-yl)-dodec-11-ynoic acid (5.26). In a test tube with stopper were vigorously stirred until well mixed 0.2 parts of tert-butanol (22.6 mL), 0.1 parts of 2-methyl-2-butene (9.8 mL, purity 80%), 3.5 parts of sodium chloride (395.5 mg), 2.9 parts of sodium dihydrogen phosphate (324.8 mg) and 0.1 parts of water (16.9 mL). Mixture was added to a 100 mL
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round bottom flask containing 12-(5-pentylfuran-2-yl)-dodec-11-ynal (5.19) (113.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃): 13.0 mg, 1.0 part) and stirred vigorously until no starting material was visible by TLC (3-4h). Water was added to the mixture and extracted with EtOAc 30 mL x 2. Combined organic layers were washed with NaHCO₃, NaCl, dried over Na₂SO₄ and evaporated under reduced pressure. Purification by flash chromatography (EtOAc/petroleum spirit 3:1) was performed to obtain the title compound as a viscous oil (85%). UV (MeOH) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 255 (3.68) nm; IR (neat) \( \nu_{\text{max}} \) 2928 (sh), 2855 (m), 2213 (w). 1H NMR (500 MHz, CDCl₃):
(400 MHz, CDCl3): δ 6.92 (1 H, d, J = 3.6 Hz, 3-H), 6.58 (1 H, d, J = 3.6 Hz, 4-H), 2.74 (2 H, t, J = 7.6 Hz, 1'-H), 2.40 (2 H, t, J = 7.6 Hz, 2''-H), 2.35 (2 H, t, J = 7.5 Hz, 10''-H), 1.68 – 1.54 (2 H, m, 2''-H), 1.36 – 1.28 (18 H, m, 3’, 4’, 3”, 4”, 5”, 6”, 7”, 8”, 9”-H), 0.89 (3 H, t, J = 6.9 Hz, 5’-H). 13C NMR (100 MHz, CDCl3): δ 178.0 (1”-C), 146.9 (5-C), 130.9 (CH, 3-C), 123.9 (CH, 4-C), 121.6 (2-C), 93.7 (11”-C), 74.2 (12”-C), 33.8 (CH2, 2’”-C), 31.4 (CH2, 3’”-C), 31.3 (CH2, 8’”-C), 30.3 (CH2, 7”-C). 29.4 (CH2, 6’”-C), 29.3 (CH2, 5’”-C), 29.2 (CH2, 4’”-C), 29.2 (CH2, 1’”-C), 29.0 (CH2, 9’”-C), 28.8 (CH2, 2’-C), 24.8 (CH2, 3’-C), 22.5 (CH2, 4’-C), 19.8 (CH2, 10’-C), 14.1 (CH3, 5’-C). EIMS m/z [M]+ 348 (40), 291 (30), 234 (55), 193 (100), 177 (48). HRESIMS m/z 371.2030 (calculated for C21H35O23NaS 371.2021).

(Z)-6-(5-Pentylfuran-2-yl)-hex-5-enoic acid

(5.31) - A mixture of 6-(5-pentylfuran-2-yl)-hex-5-ynoic acid (5.25) (58.2 mg, 1.0 Eq), and Pd/C (10% w.t.) (2.98 mg of Pd, 0.12 Eq) in EtOAc were degassed and backfilled with hydrogen. This process was repeated three times. Then, ethylenediamine (153 μL, 9.8 Eq) was added dropwise. Reaction was once more degassed and backfilled with hydrogen. Process repeated three times. Mixture was then vigorously stirred for three hours at room temperature under an atmosphere of hydrogen. Mixture was vacuum filtered over celite and EtOAc layer washed with ammonium chloride, sodium bicarbonate and sodium chloride, dried over Na2SO4 and concentrated under reduced pressure, yielding the title compound (47%) as a mobile liquid. 1H NMR (400 MHz, C6D6): δ 6.19 (1 H, dt, J = 11.7, 1.7 Hz, 6’’-H), δ 6.10 (1 H, d, J = 3.2 Hz, 3-H), 5.90 (1 H, d, J = 3.2 Hz, 4-H), 5.22 (1 H, dt, J = 11.7, 7.4 Hz, 5’’-H), 2.53 – 2.38 (4 H, m, 1’, 4’-H), 2.13 (2 H, t, J = 7.5 Hz, 2’-H), 1.69 – 1.15 (8 H, m, 2’, 3’, 4’, 3’-H), 0.83 (3 H, t, J = 6.84 Hz, 5’-H). 13C NMR (100 MHz, C6D6): δ 179.8 (1”-C), δ 156.0 (5-C), 152.0 (2-C), 139.3 (CH, 5”-C), 118.9 (CH, 6’-C), 110.6 (CH, 3-C), 106.9 (CH, 4-C), 31.6 (CH2, 2’’-C), 30.2 (CH2, 3’-C), 28.8 (CH2-4’’-C), 28.4 (CH2, 2’-C), 28.1 (CH2, 1’-C). 24.8 (CH2-3’’-C), 22.8 (CH2, 4’-C), 14.2 (CH3, 5’-C). EIMS m/z 250 [M]+ (97), 193 (73), 177 (100), 151 (35), 133 (55); HREIMS m/z 250.1569 (calculated for C13H22O3, 250.1569).
**Chapter five: Furan fatty acids: synthesis and SARs**

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*(Z)-6-(5-Pentylthiophene-2-yl)-hex-5-enoic acid (5.33).* A mixture of 6-(5-pentylthiophene-2-yl)-hex-5-enoic acid (5.28) (24.8 mg, 1.0 Eq), and Pd/C (10% w.t.) (1.2 mg of Pd, 0.12 Eq) in EtOAc were degassed and backfilled with hydrogen. This process was repeated three times. Then, ethylenediamine (61.3 µL, 9.8 Eq) was added dropwise. Reaction was once more degassed and backfilled with hydrogen. Process repeated three times. Mixture was then vigorously stirred for three hours at room temperature under an atmosphere of hydrogen. Mixture was vacuum filtered over celite and EtOAc layer washed with ammonium chloride, sodium bicarbonate and sodium chloride, dried over Na$_2$SO$_4$ and concentrated under reduced pressure, yielding the title compound (45%) as a mobile liquid. $^1$H NMR (400 MHz, C$_6$D$_6$): $\delta$ 6.72 (1 H, d, $J = 3.6$ Hz, 3-H), 6.56 (1 H, d, $J = 3.6$ Hz, 4-H), 6.44 (1 H, dt, $J = 11.5$, 1.2 Hz, 6’’-H), $\delta$ 5.23 (1 H, dt, $J = 11.5$, 7.2 Hz, 5’’-H), 2.60 (2 H, t, $J = 7.6$ Hz, 1’-H), 2.38 – 2.33 (2 H, m, 2’’-H), 2.11 – 2.06 (2 H, m, 4’’-H), 1.58 – 1.13 (8 H, m, 2’, 3’, 4’, 3’’-H), 0.89 (3 H, t, $J = 7.1$ Hz, 5’-H). $^{13}$C NMR (100 MHz, C$_6$D$_6$): $\delta$ 179.2 (1’’-C), $\delta$ 146.5 (5-C), 143.9 (2-C), 143.2 (CH, 5’’-C), 138.7 (CH, 6’’-C), 124.2 (CH, 3-C), 123.5 (CH, 4-C), 32.0 (CH$_2$, 2’’-C), 31.7 (CH$_2$, 3’’-C), 31.6 (CH$_2$-4’’-C), 30.4 (CH$_2$, 2’-C), 23.1 (CH$_2$, 1’-C), 22.8 (CH$_2$-3’’-C), 14.4 (CH$_2$, 4’- C), 14.2 (CH$_3$, 5’-C). EIMS $m/z$ 266 [M$^+$] (48), 207 (100), 165 (70); HREIMS $m/z$ 266.1341 (calculated for C$_{15}$H$_{22}$O$_2$S, 266.1341).

*(Z)-9-(5-Pentylthiophene-2-yl)-non-8-enoic acid (5.34).* A mixture of 9-(5-pentylthiophene-2-yl)-non-8-ynoic acid (5.29) (43.6 mg, 1.0 Eq), and Pd/C (10% w.t.) (1.81 mg of Pd, 0.12 Eq) in EtOAc were degassed and backfilled with hydrogen. This process was repeated three times. Then, ethylenediamine (93 µL, 9.8 Eq) was added dropwise. Reaction was once more degassed and backfilled with hydrogen. Process repeated three times. Mixture was then vigorously stirred for three hours at room temperature under an atmosphere of hydrogen. Mixture was vacuum filtered over
celite and EtOAc layer washed with ammonium chloride, sodium bicarbonate and sodium chloride, dried over Na₂SO₄ and concentrated under reduced pressure, yielding the title compound (75%) as a mobile liquid. ¹H NMR (400 MHz, C₆D₆): δ 6.77 (1 H, d, J = 3.5 Hz, 3-H), δ 6.58 (1 H, d, J = 3.5 Hz, 4-H), 6.52 (1 H, dt, J = 11.3, 1.8 Hz, 9′′-H), 5.42 (1 H, dt, J = 11.3, 7.2 Hz, 8′′-H), 2.62 (2 H, t, J = 7.5 Hz, 1′-H), 2.44 (2 H, m, 7′′-H), 2.03 (2 H, t, J = 7.5 Hz, 2′′-H), 1.61 – 1.04 (16 H, m, 2′, 3′, 4′, 3″, 4″, 5″, 6″-H), 0.86 (3 H, t, J = 6.8 Hz, 5′-H). ¹³C NMR (100 MHz, C₆D₆): δ 180.1 (1′′′-C), δ 146.0 (5′-C), 139.1 (2′-C), 129.7 (CH, 8′′-C), 127.6 (CH, 9′′-C), 124.2 (CH, 3′-C), 122.9 (CH, 4′-C), 34.1 (CH₂, 2′′-C), 31.7 (CH₂, 3′′-C), 31.6 (CH₂, 7′′-C), 30.4 (CH₂, 6′′-C), 29.7 (CH₂, 5′′-C), 29.6 (CH₂, 4′-C), 29.3 (CH₂, 2′-C), 29.1 (CH₂, 1′-C), 24.9 (CH₂-3′′-C), 22.8 (CH₂, 4′-C), 14.2 (CH₃, 5′-C). EIMS m/z 308 [M]+ (55), 193 (100), 149 (70); HREIMS m/z 308.1811 (calculated for C₁₈H₂₈O₃S 308.1810).

(Z)-12-(5-Pentylfuran-2-yl)-dodec-11-enoic acid (5.32) - A mixture of 12-(5-pentylfuran-2-yl)-dodec-11-ynoic acid (5.26) (38 mg, 1.0 Eq), and Pd/C (10% w.t.) (1.45 mg of Pd, 0.12 Eq) in EtOAc were degassed and backfilled with hydrogen. This process was repeated three times. Then, ethylenediamine (75 µL, 9.8 Eq) was added dropwise. Reaction was once more degassed and backfilled with hydrogen. Process repeated three times. Mixture was then vigorously stirred for three hours at room temperature under an atmosphere of hydrogen. Mixture was vacuum filtered over celite and EtOAc layer washed with ammonium chloride, sodium bicarbonate and sodium chloride, dried over Na₂SO₄ and concentrated under reduced pressure, yielding the title compound (43%) as a mobile liquid. ¹H NMR (400 MHz, C₆D₆): δ 6.30 (1 H, dd, J = 11.7, 1.8 Hz, 12′′′-H), δ 6.17 (1 H, d, J = 3.2 Hz, 3-H), 5.93 (1 H, d, J = 3.2 Hz, 4-H), 5.50 (1 H, dt, J = 11.7, 7.3 Hz, 11′-H), 2.61 – 2.46 (4 H, m, 1′, 10′′′-H), 2.10 (2 H, t, J = 7.4 Hz, 2′-H), 1.60 – 1.10 (18 H, m, 2′, 3′, 4′, 3″, 4″, 5″, 6″, 7″, 8′′-H), 0.83 (3 H, t, J = 6.7 Hz, 5′-H). ¹³C NMR (100 MHz, C₆D₆): δ 178.3 (1′′′-C), δ 155.8 (5′-C), 152.4 (2′-C), 129.8 (CH, 11′′-C), 118.2 (CH, 12′′-C), 110.3 (CH, 3′-C), 106.9 (CH, 4′-C), 33.9 (CH₂, 2′′′-C), 31.7, 30.1, 29.9, 29.84, 29.81, 29.60, 29.34, 28.5, 25.04, 22.8 (CH₂, 4′-C), 33.9 (CH₂, 2′′′-C).
14.2 (CH₃, 5'-C). EIMS m/z 334 [M]⁺ (50), 277 (20), 177 (100), 107 (45); HREIMS m/z 334.2508 (calculated for C₁₈H₂₈O₃, 334.2508).

(Z)-12-(5-Pentylthiophene-2-yl)-dodec-11-enoic acid (5.35). - A mixture of 12-(5-pentylthiophene-2-yl)-dodec-11-ynoic acid (5.30) (60.4 mg, 1.0 Eq), and Pd/C (10% w.t.) (2.20 mg of Pd, 0.12 Eq) in EtOAc were degassed and backfilled with hydrogen. This process was repeated three times. Then, ethylenediamine (113.4 µL, 9.8 Eq) was added dropwise. Reaction was once more degassed and backfilled with hydrogen. Process repeated three times. Mixture was then vigorously stirred for three hours at room temperature under an atmosphere of hydrogen. Mixture was vacuum filtered over celite and EtOAc layer washed with ammonium chloride, sodium bicarbonate and sodium chloride, dried over Na₂SO₄ and concentrated under reduced pressure, yielding the title compound (50%) as a crude mobile liquid. 

1H NMR (400 MHz, C₆D₆): δ 6.77 (1 H, d, J = 3.5 Hz, 3-H), δ 6.58 (1 H, d, J = 3.5 Hz, 4-H), 6.54 (1 H, dt, J = 11.4, 1.9 Hz, 12''-H), 5.48 (1 H, dt, J = 11.4, 7.3 Hz, 11''-H), 2.55 – 2.49 (2 H, m, 10''-H), 2.45 (2 H, t, J = 7.6 Hz, 1'-H), 2.12 – 2.07 (2 H, m, 2''-H), 1.52 – 1.05 (18 H, m, 2', 3', 4', 3'', 4'', 5'', 6'', 7'', 8''-H), 0.83 (3 H, t, J = 6.7 Hz, 5'-H). 

13C NMR (100 MHz, C₆D₆): δ 180.4 (1''-C), 146.8 (5-C), 143.4 (2-C), 129.9 (CH, 11''-C), 122.9 (CH, 12''-C), 94.0 (CH, 3-C), 75.2 (CH, 4-C), 31.9 (CH₂, 2''-C), 31.7 , 30.0 , 29.9 , 29.9 , 29.7 , 29.6, 29.2, 29.0 , 25.0 , 20.0 (CH₂, 4''-C), 14.2 (CH₃, 5''-C). EIMS m/z 350 [M]⁺ (52), 193 (100), 167 (62), 123 (48); HREIMS m/z 350.2284 (calculated for C₂₁H₃₄O₂S, 350.2280).
Chapter Six

The Igura Hivi Mushroom

Photograph of an Albatrellus sp. Gray by Anson Barish (UoG) (reproduced with permission)

This section describes ethnomycological and taxonomical background on Igura hivi, a Papua New Guinean macrofungi of the genus Albatrellus Gray. Antimicrobial guided isolation of its biological active constituents and their structure identification is also provided.
Chapter 6  The Igura hivi mushroom (*Albatrellus sp. Gray*)

6.1  Introduction

Igura hivi (Figure 6.1) is one of the mushrooms traditionally used by the Kiovi tribe of Lufa District, Eastern Highlands Province in Papua New Guinea (PNG) for its edibility and medicinal properties. Igura hivi in the local language of the Kiovi means bitter mushroom.

![Figure 6.1: Igura hivi (Albatrellacea). Photograph by Anson Barish (UoG) (reproduced with permission)](image)

Traditionally, this mushroom is boiled before consumption and while the water residue is drunk to treat stomach upsets, according to Kiovi elders, eating one of these mushrooms could sustain a person to go without food for a minimum of two days. Furthermore, elders have reported that Igura hivi was the mushroom of choice during periods of food scarcity brought about by tribal wars and drought. Ethnomycological linkages to the Kiovi reported herein gave origin to this work.
Biological testings performed on crude extracts of Igura hivi revealed that it was active against Gram (+) bacteria such as *Staphylococcus aureus* Rosenbach [zone of inhibition (ZOI) 15 mm diameter] and *Staphylococcus epidermidis* Evans (IC$_{50}$ = 168 µg mL$^{-1}$). In addition, results from the anticancer tests performed at the Queensland Institute of Medical Research (QIMR) also showed strong anticancer activities through growth inhibitory activities against melanoma (MM96L), breast cancer (MCF7), leukaemia (K562) and human fibroblast (NFF) cell lines at 1/5000 dilutions.

Grifolin (6.1), a known antibiotic of the Albatrellaceae family was identified as the main component in Igura hivi. Figure 6.2 shows the chemical structure of grifolin. Thus, this chapter entails an investigation of Igura hivi, the isolation and elucidation of its major active compound responsible for its biological activity.

![Chemical structure of grifolin](image)

**Figure 6.2: Chemical structure of grifolin**

### 6.2 The genus *Albatrellus* Gray and its signature compound

The genus *Albatrellus* Gray (1821) has a widespread distribution especially in the temperate areas of the northern hemisphere.$^{139}$ The genus *Albatrellus* Gray was thought to be a member of the Polyporaceae family, due to its porous tissue layer bearing structure.$^{140}$ Although more recent phylogenetic analysis based on DNA sequences have suggested that its closest relatives are of the Russulaceae family, this type of fungi are commonly placed under the Albatrellaceae Nuss family.$^{141}$

A farnesylresorcinol derivative was originally isolated from the fruiting bodies of *Grifola confluens* (*Albatrellus confluens* (Alb. & Schwein.) Kotl. & & Pouzar) and named grifolin (6.1) by Hirata and Nakanishi$^{142}$ in 1949. According to the disc diffusion assay used by these authors, grifolin appeared inactive against Gram (-) bacteria such as
Bacillus dysenteriae and Salmonella typhi Le Minor & Popoff. However, when grifolin was tested against Gram (+) organisms such as S. aureus and Bacillus subtilis, this farnesylresorcinol derivative appeared active and hence it was classified as an antibiotic.\textsuperscript{142}

Recent studies have shown that grifolin (6.1) possesses different biological properties of interest in medicine, such as antioxidant, anti-inflammatory and antitumoral.\textsuperscript{143-145} Although grifolin (6.1) has been recognised as the signature compound amongst Albatrellus spp., other novel biological active compounds of interest have been isolated from this genus.

Ishii and co-workers,\textsuperscript{146} on their investigations on A. confluens, isolated grifolin (6.1), its monomethyl ester (6.2), neogrifolin (6.3) and grifolic acid (6.4), and their structures are shown in Figure 6.3.\textsuperscript{146} Although 6.1 and 6.2 showed very similar resonances in their respective \textsuperscript{1}H NMR spectra corresponding to the isoprene derived chain, grifolin monomethyl ester (6.2) possessed a desymmetrised ring and was thus readily distinguishable. The structure of grifolic acid (6.4) was determined based on comparisons with existing NMR data and experimentally upon treatment with diazomethane, which yielded its methylester, as expected.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.3.png}
\caption{Chemical structures of grifolin (6.1), grifolin monomethyl ester (6.2), neogrifolin (6.3) and grifolic acid (6.4)}
\end{figure}
Ishii and co-workers\textsuperscript{146} also isolated four new farnesylphenol derivatives (6.5 – 6.8) from Albatrellus dispansus (Lloyd) Canf. & Gilb. and their structures are depicted in Figure 6.4. Compounds 6.5 and 6.6 were isolated via HPLC and exhibited the same molecular formula C\textsubscript{22}H\textsubscript{32}O\textsubscript{3} and specific rotation value of [\(\alpha\)]\textsubscript{D} (\(\pm\) 0). In addition, NMR data comparisons with that for grifolin (6.1) showed several differences especially in the downfield region. These authors concluded that compounds 6.5 and 6.6 were racemic and based on the chemical shifts the orientation of the hydroxyl group on position 2’ was assigned as \textit{trans} to the methyl group on position 3’ for 6.5 and \textit{cis} for 6.6 respectively. Compound 6.7 was found to be a methyl ether of grifolic acid (6.4). In addition, an isopentenylphenol methylester (6.8) was also isolated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6_4.png}
\caption{Chemical structures of novel farnesylphenol derivatives (6.5 – 6.8)}
\end{figure}

Nukata and co-workers,\textsuperscript{143} during their investigations on the antioxidative potential of mushrooms also isolated grifolin (6.1), its isomer neogrigolin (6.3) and grifolic acid (6.4) from A. ovinus. In addition, these authors also isolated three novel neogrigolin derivatives (6.9 – 6.11) and their structures are shown on Figure 6.5.\textsuperscript{143} Regarding 3-hydroxyneogrigolin (6.9), its structure was elucidated based on NMR analyses. In addition, methylation with MeI/K\textsubscript{2}CO\textsubscript{3} under reflux yielded three methylethers, which suggested the presence of three phenolic hydroxyl groups in the molecule. The chemical structures of the remaining two compounds, 1-formylneogrigolin (6.10) and 1-formyl-3-
hydroxyneogrifolin (6.11), were also elucidated based on NMR analyses. The key similarity between 6.10 and 6.11 was the presence of an aromatic aldehyde, and the key difference was that 6.10 was lacking a phenolic hydroxyl group.

Figure 6.5: Neogrifolin derivatives isolated from *A. ovinus*.

Nukata and co-workers\textsuperscript{143} used the DPPH\textsuperscript{−} assay to compare the radical scavenging activity (RSA) of the three neogrifolin derivatives against the signature compounds of the genus *Albatrellus* Gray (grifolin (6.1)). Although grifolic acid (6.4) was practically inactive (RSA < 10%), grifolin (6.1) and neogrifolin (6.3) showed a RSA of 30\% - 40\%. However, when the number of substituents increased around the benzene ring, as for the neogrifolin derivatives, the RSA was almost double the RSA reported for neogrifolin (6.3) alone. 3-Hydroxyneogrifolin (6.9) and 1-formyl-3-hydroxyneogrifolin (6.11) (RSA 68\% - 75\%) were more active in the DPPH\textsuperscript{−} assay than the antioxidant used as a positive control (α-tocopherol, RSA = 45\%).
In 2006, Quang and co-workers,\textsuperscript{144} in the course of investigating new biologically active compounds, isolated two novel farnesyl phenol derivatives, namely grifolinone A (6.12) and grifolinone B (6.13), their chemical structures are depicted in Figure 6.6.\textsuperscript{144}

Both grifolinones (6.12 and 6.13) were able to inhibit nitric oxide production stimulated by lipopolysaccharides in murine leukemia virus cells (RAW 264.7), suggesting that both compounds might have the potential of being used in the treatment of inflammation.

![Figure 6.6: Grifolinones A and B (6.12 and 6.13) isolated from A. caeruleoporus.](image-url)

Recently in 2012, whilst screening mushroom extracts used by the Kiovi tribe from PNG, as reported in 0 section 2.6.6 (Table 2.5), the antibacterial effect of several mushroom extracts was tested against \textit{S. epidermidis} and \textit{E. coli β(-) ATCC 25922}.

Amongst the mushroom extracts tested was Igura hivi (\textit{Albatrellus sp.} Gray), which appeared inactive against the Gram (-) strain tested, but active against \textit{S. epidermidis} (IC\textsubscript{50} = 168 µg.mL\textsuperscript{-1}). These findings led to the identification and characterisation of the compound responsible for the biological activity exhibited by Igura hivi (\textit{Albatrellus sp.} Gray) – grifolin (6.1).
6.3 Characterisation of Igura hivi and isolation of grifolin

The macrofungus used in this study was collected from the forest area that surrounds the Kiovi village, Lufa District, Eastern Highlands Province, Papua New Guinea (PNG). See map on Figure 2.1 for location of study area.

Ethanolic extracts of this fungus possessed antibacterial activity against the Gram (+) *S. epidermidis* and thus its taxonomy and chemistry were further pursued. Morphological comparisons between Igura hivi with mushroom samples of known taxonomy kept, as vouchers, at the Centre for Natural Resources Research and Development (CNRRD) at the University of Goroka (UoG), provided indication that Igura hivi belongs to the Albatrellaceae family.

After initial extraction, as described in 0, section 2.6.1, ethanolic extracts of Igura hivi (*Albatrellus sp.* Gray) were partitioned under a modified Kupchan scheme to obtain five new extracts (Figure 6.7): hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and aqueous.

![Figure 6.7: Modified Kupchan solvent partition.](image)
Kupchan extracts were re-assayed using the turbidity-MTT assay described in Chapter 2, section 2.9.6. Results from the antimicrobial assay suggested activity in the DCM fraction and hence it was chosen to continue with the isolation of the active metabolites.

The DCM fraction was further partitioned via size exclusion chromatography, using sephadex LH-20 in MeOH as the mobile phase. A total of 40 fractions each containing about 10 mL of eluent were collected and pooled into four major fractions on the basis of their thin layer chromatography (TLC) profiles, \(^1\)H NMR spectra, GC-MS experiments and comparisons with crude extracts of Igura hivi (Albatrellus sp. Gray).

These fractions were re-assayed for their antibacterial potential with the turbidity-MTT method. Fraction two was active against the Gram (+) strain used and hence subjected to further purification. The active LH20 fraction was fractionated on silica gel 60 (230 – 400 mesh) column using 100% DCM as the mobile phase. A total of 25 fractions of approximately 5 mL of eluent were collected and pooled into three fractions with fraction one containing the major active compound, identified according to 1D and 2D NMR, MS experiments and comparisons with existing data, as grifolin.

### 6.4 Elucidation of grifolin

![Figure 6.8: Grifolin (6.1)](image)

HRESIMS suggested a molecular formula of \(\text{C}_{22}\text{H}_{32}\text{O}_2\) through the appearance of a deprotonated molecule \([M – H]^–\) of \(m/z\) 237.2324 (calculated for \(\text{C}_{22}\text{H}_{31}\text{O}_2\), 237.2324). This molecular formula implied seven degrees of unsaturation, as per Equation 3, shown in Chapter 3.
Moreover, the $^1\text{H}$ NMR spectrum (Figure 6.9) displayed ten resonances with five distinctive methyl groups. One methyl resonance was relatively more deshielded (E, $\delta$ 2.21 ppm) than the rest (F – I) suggesting the presence of an aromatic ring. The appearance of a singlet (A) at $\delta$ 6.24 ppm in the $^1\text{H}$ NMR spectrum integrating for two protons was characteristic of a benzene ring with symmetry.

Two distinctive resonances in the $^1\text{H}$ NMR spectrum comprised an AM$_2$ spin system (B) and a singlet (C), both integrating for a total of three olefinic protons between $\delta$ 5.0 - $\delta$ 5.3 ppm. In addition, a clear “methylene envelope” between $\delta$ 1.9 - $\delta$ 2.2 ppm and an A$_2$M spin system (D, $\delta$ 3.39 ppm) deshielded due to its proximity to the paramagnetic region of the benzene ring.

The $^{13}\text{C}$ NMR spectrum displayed 20 discrete resonances and with HSQC analysis, allowed the confirmation of a number of proton-carbon assignments. Carbons on positions 1 and 3 on the benzene ring (Figure 6.8) had the same chemical shift ($\delta$154.9).
In addition, carbons on positions 4 and 6 also on the benzene ring appeared with the same chemical shift (δ109.2), which suggested symmetry in the aromatic ring effectively bringing a total of 22 carbons assigned matching the molecular formula (C_{22}H_{31}O_{2}) calculated based on HRESIMS experiments. Finally, HMBC correlations allowed the elucidation of grifolin. Because grifolin is not a new compound, data was also compared against that of Ishii and co-workers.\textsuperscript{146} A summary of $^1$H and $^{13}$C NMR correlations and a comparison with that of the literature can be seen in Table 6.1.

| Position | $^1$H-NMR δ (ppm), $|J|$ (Hz) | $^{13}$C-NMR δ (ppm) | HMBC | $^1$H-NMR $^1$H - $^{13}$C (Literature)$^{146}$ | $^{13}$C-NMR $^{13}$C (Literature)$^{146}$ |
|----------|-------------------------------|----------------------|-------|------------------------------------------|------------------------------------------|
| 1        |                               | 154.9                |       |                                          | 154.6                                    |
| 2        |                               | 110.6                |       |                                          | 111.0                                    |
| 3        |                               | 154.5                |       |                                          | 154.6                                    |
| 4        | 6.24 (s)                      | 109.2                | C3, C5, C7 | 6.20 (s) | 109.3                                    |
| 5        |                               | 137.6                |       |                                          | 137.2                                    |
| 6        | 6.24 (s)                      | 109.2                | C1, C5, C7 | 6.20 (s) | 109.3                                    |
| 7        | 2.21 (s)                      | 21.2                 | C4, C5, C6 | 2.18 (s) | 21.0                                     |
| 1’       | 3.39 (d, 7.1)                | 22.3                 | C1, C2, C2’ | 3.37 (d, 7.0) | 22.3                                    |
| 2’       | 5.27 (t, 7.1)                | 121.8               | C1’, C3’ | 5.26 (t, 7.0) | 122.0                                    |
| 3’       |                               | 139.0                |       |                                          | 138.4                                    |
| 4’       | 1.87 – 2.10 (m)              | 39.8                 |       | 1.90 – 2.10 (m) | 39.7                                    |
| 5’       | 1.87 – 2.10 (m)              | 26.5                 |       | 1.90 – 2.10 (m) | 26.7                                    |
| 6’       | 5.09 (s)                     | 123.7                |       | 5.06 (m) | 123.7                                    |
| 7’       |                               | 135.7                |       |                                          | 135.4                                    |
| 8’       | 1.87 – 2.10 (m)              | 39.8                 |       | 1.90 – 2.10 (m) | 39.7                                    |
| 9’       | 1.87 – 2.10 (m)              | 26.8                 |       | 1.90 – 2.10 (m) | 26.7                                    |
| 10’      | 5.09 (s)                     | 124.5                |       | 5.06 (m) | 124.5                                    |
| 11’      |                               | 131.4                |       |                                          | 131.0                                    |
| 12’      | 1.68 (s)                     | 25.8                 |       | 1.66 (s) | 25.7                                     |
| 13’      | 1.60 (s)                     | 17.8                 |       | 1.57 (s) | 17.7                                     |
| 14’      | 1.59 (s)                     | 16.4                 |       | 1.57 (s) | 16.1                                     |
| 15’      | 1.82 (s)                     | 16.2                 |       | 1.80 (s) | 16.0                                     |
6.5 Conclusion

Investigation of the major isolated compound of the mushroom Igura hivi (*Albatrellus sp.* Gray), collected in the Eastern Highlands Province of Papua New Guinea and used traditionally by the Kiovi tribe as medicine, led to the discovery of a known antibiotic named Grifolin.

6.6 Experimental

For general information on instrumental methods used for the characterisation of grifolin, see experimental section in Chapter 3, section 3.8.1 to 3.8.6.

6.6.1 Metabolite data

Grifolin (6.1): $^1$H NMR (CDCl$_3$, 400 MHZ); and $^{13}$C NMR (CDCl$_3$, 100 MHZ) see Table 6.1. HRESIMS $m/z$ 237.2324 [M – H]$^-$ (calculated for C$_{22}$H$_{31}$O$_2$, 237.2324).
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    National University, **2014**.
132. Fang, C.; Oruganti, B.; Durbeej, B., Computational study of the working 
    mechanism and rate acceleration of overcrowded alkene-based light-driven rotary 
133. Dhakshinamoorthy, A.; Alvaro, M.; Garcia, H., Aerobic Oxidation of Styrenes 
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Appendices
Appendix 1: Mushrooms used by the Kiovi

<table>
<thead>
<tr>
<th>Specimens and collection code</th>
<th>Species (Family)</th>
<th>Local name (English name)</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kiovi-MSp1</td>
<td><em>Entoloma sp.</em> (Bull.) P. Kumm. (Entolomataceae)</td>
<td>Jokoni</td>
<td>• Inedible mushroom&lt;br&gt;• Regarded as potentially poisonous</td>
</tr>
<tr>
<td>Kiovi-MSp2</td>
<td><em>Cantharellus sp.</em> Adans. ex Fr. (Cantharellaceae)</td>
<td>Lamana hivi</td>
<td>• Edible mushroom&lt;br&gt;• Used as a food source</td>
</tr>
<tr>
<td>Kiovi-MSp3</td>
<td><em>Xylaria sp.</em> Hill ex Schrank (Xylaraceae)</td>
<td>Kula avara</td>
<td>• Situated at soil level&lt;br&gt;• Inedible mushroom&lt;br&gt;• Regarded as potentially toxic</td>
</tr>
<tr>
<td>Kiovi-MSp4</td>
<td>Missing data</td>
<td>Cofy candy</td>
<td>• Edible mushroom&lt;br&gt;• Grows on grounds next to coffee plants</td>
</tr>
<tr>
<td>Kiovi-MSp5</td>
<td>Missing data</td>
<td>Mamu hivi</td>
<td>• Edible mushroom&lt;br&gt;• Grows on soil&lt;br&gt;• Used as a food source</td>
</tr>
<tr>
<td>Kiovi-MSp6</td>
<td><em>Lactarius sp.</em> Pers. (Russulaceae)</td>
<td>Dagaidy</td>
<td>• Large fleshy mushroom&lt;br&gt;• Could be edible if marinated before cooking</td>
</tr>
<tr>
<td>Kiovi-MSp7</td>
<td><em>Russula sp.</em> Pers. (Russulaceae)</td>
<td>Kokai</td>
<td>• Strong red coloured cap and white gills&lt;br&gt;• Hot in taste&lt;br&gt;• Regarded as potentially poisonous</td>
</tr>
<tr>
<td>Kiovi-MSp8</td>
<td><em>Ramaria sp.</em> Fr. Ex Bonord. (Gomphaceae)</td>
<td>Dekeja-hava 2</td>
<td>• Edible mushroom&lt;br&gt;• Boiled and then cooked in oil before eating</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Image</th>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><em>Ramaria sp.</em> Fr. Ex Bonord. (Gomphaceae)</td>
<td>Dekeja-hava 3</td>
<td>Edible mushroom, boiled and then cooked in oil before eating</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>Missing data</td>
<td>Kaudi klodi</td>
<td>Edible mushroom, cooked in oil before eating</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><em>Ramaria sp.</em> Fr. Ex Bonord. (Gomphaceae)</td>
<td>Dekeja-hava 1</td>
<td>Edible mushroom, club shape, grows on grounds</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td><em>Albatrellus sp.</em> Gray (Albatrellaceae)</td>
<td>Fagotave hivi</td>
<td>Edible mushroom, used medicinally to treat stomach complaints</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td><em>Albatrellus sp.</em> Gray (Albatrellaceae)</td>
<td>Nupa duguripa</td>
<td>Edible mushroom, used medicinally to treat stomach complaints</td>
</tr>
<tr>
<td><img src="image6.png" alt="Image" /></td>
<td><em>Amanita sp.</em> Pers. (Amanitaceae)</td>
<td>Fulaga dive</td>
<td>Edible mushroom, grows on soil, used as a food source, Kiovi people particularly reported that eating this mushroom makes them feel well</td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td><em>Amanita sp.</em> Pers. (Amanitaceae)</td>
<td>Opoiya</td>
<td>White cap with fleecy spots, inedible, regarded as potentially poisonous</td>
</tr>
<tr>
<td><img src="image8.png" alt="Image" /></td>
<td><em>Polyporus sp.</em> P. Micheli ex Adans. (Polyporaceae)</td>
<td>Dugulipa2</td>
<td>Edible mushroom, given specially to pigs for fattening</td>
</tr>
<tr>
<td>Image</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp18</td>
<td>Missing data • Edible mushroom • Kiovi use this mushroom as fertiliser</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp19</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp20</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp21</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp22</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp23</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp24</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp25</td>
<td>Missing data • Edible mushroom • Used as a food source</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Image</th>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kiovi-MSp26</td>
<td><em>Polyporus sp.</em> P. Micheli ex Adans. (Polyporaceae)</td>
<td>Yausivi1</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Kiovi-MSp27</td>
<td><em>Polyporus sp.</em> P. Micheli ex Adans. (Polyporaceae)</td>
<td>Unale kaula</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Kiovi-MSp29</td>
<td><em>Boletus sp.</em> L. (Boletaceae)</td>
<td>Nimi-hove</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp30</td>
<td>Missing data</td>
<td>Kanaravo</td>
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<tr>
<td>Kiovi-MSp31</td>
<td><em>Boletus sp.</em> L. (Boletaceae)</td>
<td>Nama-hove</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp32</td>
<td><em>Boletus sp.</em> L. (Boletaceae)</td>
<td>Demose</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Kiovi-MSp33</td>
<td>Missing data</td>
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<tr>
<td>Kiovi-MSp34</td>
<td><em>Polyporus sp.</em> P. Micheli ex Adans. (Polyporaceae)</td>
<td>Yausivi3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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| Kiovi-MSp35 | *Albatrellus sp.* Gray (Albatrellaceae) | Dugulipal | Edible mushroom  
|             |                                       |           | Used medicinally to treat stomach complaints |
| Kiovi-MSp36 | *Trametes sp.* L. (Polyporaceae)       | Yausivi2  | Edible mushroom  
|             |                                       |           | Boiled in water before eating |
| Kiovi-MSp37 | *Leccinum* sp. Gray (Boletaceae)       | Agajowa1  | Edible mushroom  
|             |                                       |           | Used as a food source |
| Kiovi-MSp38 | Missing data                          | Agajowa2  | Edible mushroom  
|             |                                       |           | Used as a food source |
| Kiovi-MSp40 | Missing data                          | Fu-aulga dive | Edible mushroom  
|             |                                       |           | Used as a food source |
| Kiovi-MSp41 | Missing data                          | Fanu      | Edible mushroom  
|             |                                       |           | Used as a food source |
| Kiovi-MSp42 | *Albatrellus sp.* Gray (Albatrellaceae) | Igura hivi | Edible mushroom  
|             |                                       |           | Grows on dead trunks  
|             |                                       |           | Kiovi people use this mushroom medicinally to treat stomach upset |

Mushroom photographs by Anson Barish (UoG). Reproduced with permission
Appendix 2: Mushrooms used by the Waefo

<table>
<thead>
<tr>
<th>Specimens and collection code</th>
<th>Species (Family)</th>
<th>Local name (English name)</th>
<th>Uses</th>
</tr>
</thead>
</table>
| Waefo-MSp1                    | *Polyporus sp.* P. Micheli ex Adans. (Polyporaceae)  | Holipa lua hefola         | • Edible mushroom  
• Grows on dead trunks  
• Associated with mosses under moist conditions  
• Cooked in wooden drums before eating |
| Waefo-MSp2                    | *Polyporus sp.* P. Micheli ex Adans. (Polyporaceae)  | Yomba lua mulita          | • Inedible mushroom  
• Grows on dead trunks  
• Associated with mosses and lichens  
• Short-medium stipe  
• Grey, brown and white colours in circular intervals on cap |
| Waefo-MSp3                    | (Sclerodermataceae)                                    | Nokondi agamula lua       | • Inedible mushroom  
• Waefo believes this mushroom protects the forest  
• Grows on dead logs and branches |
| Waefo-MSp4                    | *Polyporus sp.* P. Micheli ex Adans. (Polyporaceae)  | Heleme lua                | • Edible mushroom  
• Grows on soil  
• Waefo made emphasis on the good taste of this mushroom |
| Waefo-MSp5                    | Missing data                                           | He lua                    | • Edible mushroom  
• Grows on the bark of specific living trees  
• Culturally of great value as it is specially given to pigs to fatten them |
| Waefo-MSp6                    | *Polyporus sp.* P. Micheli ex Adans. (Polyporaceae)  | Mamona lua homulege       | • Edible mushroom  
• Grows on dead trunks  
• Associated with high moisture  
• Raw mushroom induces vomiting  
• Cooked before eating |
| Waefo-MSp7                    | *Amanita sp.* Pers. (Amanitaceae)                      | Fiona fululu lua (slippery/waxy) | • Edible mushroom  
• Grows on soil and on dead trunks  
• Mushroom is cooked before eating |
|                               | *Polyporus sp.* P. Micheli ex Adans. (Polyporaceae)  | Nege lua fana             | • Inedible mushroom  
• Grows on dead logs and fallen branches |
| Waefo-MSp8  | (Ganodermataceae) Holipa lua namba | Inedible mushroom  
Grows on dead logs |
|-------------|-----------------------------------|------------------|
| Waefo-MSp9  | (Auriculareaceae) Hoinomba lua lafa (red roster’s comb) | Edible mushroom  
Grows on decomposing dead logs  
Waxy surface and brown in colour |
| Waefo-MSp11 | (Polyporaceae) Nege lua lina | Inedible mushroom  
Grows on fallen logs  
Porous cap on underside |
| Waefo-MSp12 | (Polyporaceae) Ya lua | Inedible mushroom  
Grows on dead trunks |
| Waefo-MSp13 | | Inedible mushroom  
Grows on dead trunks |
| Waefo-MSp14 | | Inedible mushroom  
Grows on dead trunks |
| Waefo-MSp15 | *Auricularia sp.* (Dicks.) Pers. (Auriculareaceae) Hoinomba lua feke (white rooster’s comb) | Edible mushroom  
Grows on dead trunks  
Associated with mosses and lichens |
| Waefo-MSp16 | (Fistulinaceae) Yafo welahuluma lua (pig’s tongue) | Edible mushroom  
Cooked twice before eating  
Regarded as poisonous  
Grows on the bark of living trees |
| Waefo-MSp17 | *Polyporus sp.* P. Micheli ex Adans. (Polyporaceae) Lambi lua | Inedible  
Grows on dead logs |
| Waefo-MSp18 | (Ganodermataceae) Holipa lua muliti | Inedible  
Grows on dead logs |
| | *Boletus sp.* L. (Boletaceae) Lua horu | Edible mushroom  
Cooked or smoked on fire before eating  
Sprouts from where the soil is rich in decomposed matter |
Appendix 3: Mushrooms used by the Kopanka

<table>
<thead>
<tr>
<th>Specimens and collection code</th>
<th>Species (Family)</th>
<th>Local name (English name)</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tapiaku-MSp1</td>
<td>Ramaria sp. Fr. Ex Bonord. (Ramariaceae)</td>
<td>Ombnantskim (Sugar cane skin)</td>
<td>Edible mushroom • Used as an analgesic</td>
</tr>
<tr>
<td>Tapiaku-MSp2</td>
<td>Ramaria sp. Fr. Ex Bonord. (Ramariaceae)</td>
<td>Ombnantskim (Sugar cane skin)</td>
<td>Edible mushroom • Used as an analgesic</td>
</tr>
<tr>
<td>Tapiaku-MSp3</td>
<td>Clavaria sp. Vaill. ex L. (Clavariaceae)</td>
<td>Ombnantskim (Sugar cane skin)</td>
<td>Edible mushroom • Used as an analgesic</td>
</tr>
<tr>
<td>Tapiaku-MSp4</td>
<td>Ramaria sp. Fr. Ex Bonord. (Ramariaceae)</td>
<td>Ombnantskim (Sugar cane skin)</td>
<td>Edible mushroom • Used as an analgesic</td>
</tr>
<tr>
<td>Tapiaku-MSp5</td>
<td>Ramaria sp. Fr. Ex Bonord. (Ramariaceae)</td>
<td>Ombnantskim (Sugar cane skin)</td>
<td>Edible mushroom • Used as an analgesic</td>
</tr>
<tr>
<td>Tapiaku-MSp6</td>
<td>Thelephora sp. Ehrh. ex Willd. (Thelephoraceae)</td>
<td>Ombnantskim (Sugar cane skin)</td>
<td>Edible mushroom • Used as an analgesic</td>
</tr>
<tr>
<td>Tapiaku-MSp7</td>
<td>Tricholoma sp. Fries (Tricholomataceae)</td>
<td>Makbunde (Ground mushroom)</td>
<td>Edible mushroom • Eaten cooked, boiled or fried • Source of food and also, used as a fertilizer</td>
</tr>
</tbody>
</table>
| Tapiaku-MSp8 | Hygrocybe sp. (Fr.) P. Kumm. (Hygrophoraceae) | Amto (Breast milk) | • Edible mushroom
• Eaten cooked or raw
• Given to lactating mothers for breast milk stimulation |
| Tapiaku-MSp9 | Auricularia sp. (Dicks.) Pers. (Auriculariaceae) | Koikamuk (Rat ears) | • Edible mushroom
• Eaten cooked or boiled
• Given to mothers post-partum for womb healing |
| Tapiaku-MSp10 | Rugosomyces sp. L. (Tricholomataceae) | Kombsar (Lightning) | • Edible mushroom
• Mushroom only appears after lightning strikes
• Kopanka believes that this mushroom provides them with protection from lightning strikes during the wet season |
| Tapiaku-MSp11 | Macrocystidia sp. Joss. (Marasmiaceae) | Kopi nonda (coffee mushroom) | • Edible mushroom
• Grows on soil next to coffee plantations
• Eaten cooked |
| Tapiaku-MSp12 | Echinoderma sp. (Pers.) Bon (Agaricaceae) | Nakents (baby faeces) | • Edible mushroom
• Grows on soil
• Given to babies as anti-diarrhoeal |
| Tapiaku-MSp13 | Missing data | Komugtai (Mushroom madness) | • Edible mushroom
• Eaten raw
• Makes consumers go crazy
• Men want to show their strengths/fighting capabilities
• Kopanka reported taking this mushroom in time of war with other tribes |
| Tapiaku-MSp14 | Missing data | Kalap nonda | • Edible mushroom
• Cooked in oil before eating |
| Tapiaku-MSp15 | Missing data | Kalap | • Inedible mushroom
• Used as medicine topically to treat skin growths |
<table>
<thead>
<tr>
<th>Image</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tapiaku-MSp16</td>
<td><em>Amanita sp.</em> Pers. (Amanitaceae) Tokangi (Swollen body)  • Edible mushroom  • Eaten cooked  • Used medicinally as anti-inflammatory remedy</td>
</tr>
<tr>
<td>Tapiaku-MSp17</td>
<td>Missing data Gesu  • Edible mushroom  • Kopanka reported that eating this mushroom makes them strong and gives them confidence</td>
</tr>
<tr>
<td>Tapiaku-MSp18</td>
<td><em>Microporus sp.</em> P. Beauv. (Polyporaceae) Willikimik  • Edible mushroom  • Grows on dead trunks  • Used for the treatment of pimples and scar removal</td>
</tr>
<tr>
<td>Tapiaku-MSp19</td>
<td><em>Microporus sp.</em> P. Beauv. (Polyporaceae) Willi-toli (Strong)  • Edible mushroom  • Kopanka does not eat it because it is very hard and dry, hence its name “strong”</td>
</tr>
<tr>
<td>Tapiaku-MSp20</td>
<td>Missing data Dange  • Edible mushroom  • Eaten cooked  • Given to women post-partum to aid with the removal of scars associated with delivery</td>
</tr>
<tr>
<td>Tapiaku-MSp21</td>
<td><em>Morchella sp.</em> (L.) Pers (Morchellaceae) Kipenembik (devil’s penis)  • Edible mushroom  • Grows on garden soil  • Eaten cooked and used as a food source</td>
</tr>
<tr>
<td>Tapiaku-MSp22</td>
<td>Missing data Dukdedek (Eye water)  • Inedible mushroom  • Sap is extracted and rub on eyes for conjunctivitis (used topically)</td>
</tr>
<tr>
<td>Tapiaku-MSp23</td>
<td>Missing data Tuarbik (Dog’s tongue)  • Edible mushroom  • Sour taste</td>
</tr>
<tr>
<td>Tapiaku-MSp24</td>
<td><em>Russula sp.</em> Pers. (Russulaceae) Dekmindemp (Earth worm)  • Edible mushroom  • Used to boost up their immune system</td>
</tr>
<tr>
<td>Image</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
</tbody>
</table>
| Tapiaku-MSp25 | Missing data | Binge bang (Red) | • Inedible mushroom  
• Boiled and water is drank,  
• Used as emetic and body cleanser (potentially toxic) |
| Tapiaku-MSp26 | Russula sp. Pers. (Russulaceae) | Binge | • Edible mushroom  
• Used as a food source |
| Tapiaku-MSp27 | Missing data | Bongnengs | • Edible mushroom  
• Grows on soil  
• Eaten cooked  
• Given to babies to prevent diseases and to grow healthy |
| Tapiaku-MSp28 | Missing data | Angen-muk | • Edible mushroom  
• Highly valuable for Kopanka  
• Given to pigs as a fattening source |
| Tapiaku-MSp29 | Missing data | Kupsik-kupsik | • Edible mushroom  
• Sour in taste  
• Induce individuals to sweat when eaten. |
| Tapiaku-MSp30 | Missing data | Kupsik nonda | • Edible mushroom  
• Does not make you sweat  
• Nonda added to differ from Kupsik-kupsik |
| Tapiaku-MSp31 | Boletus sp. L. (Boletaceae) | Kirmapik (Bat mushroom) | • Edible mushroom  
• Eaten cooked or raw  
• Sour in taste  
• It is believe that bats bring this mushroom to the village |
| Tapiaku-MSp32 | Russula sp. Pers. (Russulaceae) | Kaimuk (Bird’s egg) | • Edible mushroom  
• Used as a food source |
| Tapiaku-MSp33 | Missing data | Mos-1 | • Inedible mushroom  
• Regarded as potentially toxic |
### Appendices

<table>
<thead>
<tr>
<th>Photograph</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
</table>
| Tapiaku-MSp34 | Missing data Mos-2 | - Edible mushroom  
- Used as a food source  
- Gills must be removed before cooking  
- Gill regarded as potentially toxic |
| Schizophyllum sp. (Schizophyllaceae) Kombas (Skin dust) | - Edible mushroom  
- Used medicinally as internal cleansing |

Mushroom photographs by Anson Barish (UoG). Reproduced with permission
Appendix 4: Supporting information: $^1$H and $^{13}$C NMR spectra for synthesis of F-acids and T-acids

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 5-pentylfuran (4.26) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 5-pentylthiophene (5.4) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 2-iodo-5-pentylfuran (4.28) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 2-iodo-5-pentylthiophene (5.5) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 2-(prop-2-yn-1-yloxy)-tetrahydro-2H-pyran (4.30) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of hex-2-yn-1-ol (5.6) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of non-2-yn-1-ol (4.32) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of dodec-2-yn-1-ol (5.7) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of hex-5-yn-1-ol (5.8) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of non-8-yn-1-ol (4.33) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of dodec-11-yn-1-ol (5.9) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 3-(5-pentylfuran-2-yl)-prop-2-yn-1-ol (5.10) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 3-(5-pentylthiophene-2-y1)-prop-2-yn-1-ol (5.13) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 6-(5-pentylfuran-2-y1)-hex-5-yn-1-ol (5.11) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 6-(5-pentylthiophene-2-yl)-hex-5-yn-1-ol (5.14) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 9-(5-pentylfuran-2-yl)-non-8-yn-1-ol (4.34) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 9-(5-pentylthiophene-2-yl)-non-8-yn-1-ol (5.15) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 12-(5-pentylfuran-2-yl)-dodec-11-yn-1-ol (5.12) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 12-(5-pentylthiophene-2-yl)-dodec-11-yn-1-ol ($5.16$) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 3-(5-pentylfuran-2-yl)-prop-2-ynoic acid ($5.24$) in CD$_3$OD
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 3-(5-pentylthiophene-2-yl)-prop-2-ynoic acid (5.27) in CD$_3$OD

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 6-(5-pentylfuran-2-yl)-hex-5-ynoic acid (5.25) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 6-(5-pentylthiophene-2-yl)-hex-5-ynoic acid (5.28) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 9-(5-pentylfuran-2-yl)-non-8-ynoic acid (4.36) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 9-(5-pentylthiophene-2-yl)-non-8-ynoic acid (5.29) in CDCl$_3$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 12-(5-pentylfuran-2-yl)-dodec-11-ynoic acid (5.26) in CDCl$_3$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of 12-(5-pentylthiophene-2-yl)-dodec-11-ynoic acid (5.30) in CDCl$_3$.

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of (Z)-6-(5-pentylfuran-2-yl)-hex-5-enoic acid (5.31) in C$_6$D$_6$. 

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$^1$H and $^{13}$C NMR (800/200 MHz) spectra of (Z)-6-(5-pentylthiophene-2-yl)-hex-5-enolic acid (5.33) in C$_6$D$_6$

$^1$H and $^{13}$C NMR (800/200 MHz) spectra of (Z)-9-(5-pentylfuran-2-yl)-non-8-enolic acid (3.1) in C$_6$D$_6$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of (Z)-9-(5-pentylthiophene-2-yl)-non-8-enoic acid (5.34) in C$_6$D$_6$

$^1$H and $^{13}$C NMR (400/100 MHz) spectra of (Z)-12-(5-pentylfuran-2-yl)-dodec-11-enoic acid (5.32) in C$_6$D$_6$
$^1$H and $^{13}$C NMR (400/100 MHz) spectra of (Z)-12-(5-pentylthiophene-2-yl)-dodec-11-enoic acid (5.35) in C$_6$D$_6$