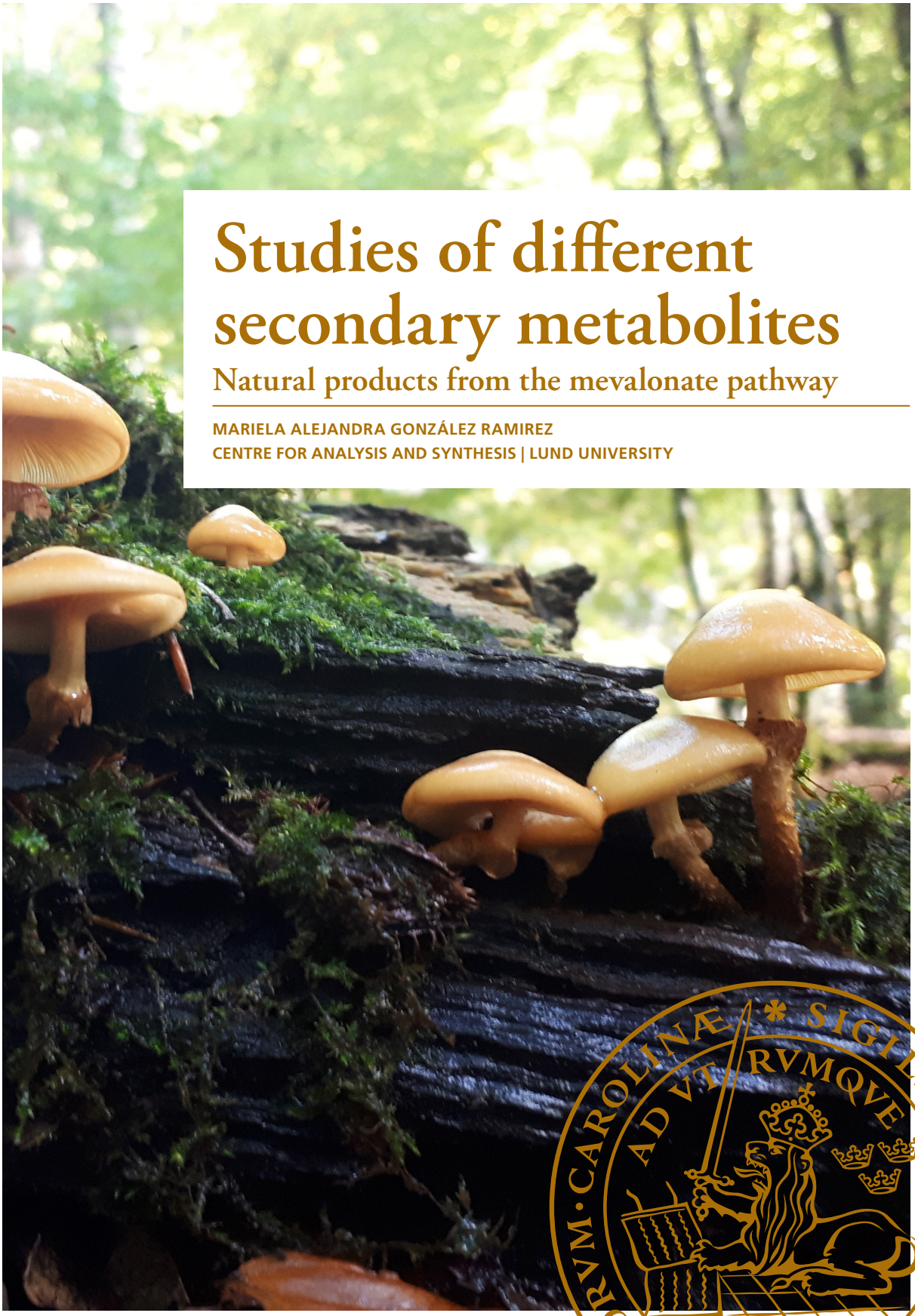
Studies of different secondary metabolites

Natural products from the mevalonate pathway

Gonzales Ramirez, Mariela Alejandra



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Natural products from the mevalonate pathway

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DOCTORAL DISSERTATION

by due permission of the Faculty of Science, Lund University, Sweden.
To be defended at Centre of chemistry on 26th August at 14.00, lecture hall K: C

Faculty opponent
Professor Luis Peña
Centro de Investigación Científica de Yucatán, México

Organization	Document name
LUND UNIVERSITY Centre for Analysis and Synthesis, CAS Department of Chemistry, Box 124 SE-221 00 Lund, Sweden	Doctoral Thesis
	Date of issue 26-8-2021
Author(s) Mariela González Ramírez	Sponsoring organization: CONICYT/ ANID

Title and subtitle
Studies of different secondary metabolites
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Abstract

Natural products, also called secondary metabolites, are small molecules that are not involved in main life processes. These can be produced by any living organism and play important roles as chemical weapons, protecting the organism from the competitive environment.

Due to their bioactivity, natural products present in medicinal plants, mushrooms (and other organisms such as bacteria and algae) have been the source of medicine and the inspiration of modern dug discovery since they are characterized by an enormous scaffold diversity and structural complexity.

In the search of new natural products, this thesis comprises the study of natural products from two fungal species, *Aleurodiscus sp.* and *Galiella Rufa*.

From *Aleurodiscus sp.,* 8 molecules in total were isolated, belonging to the benzofuran, chromane and chromene-type compounds.

From *G. rufa* no novel molecules were isolated since its chemistry is well known, however, this thesis comprises the use of its enzymatic potential to biotransform synthetic analogues of desoxygaliellalactone into galiellalactone and galiellalactam analogues.

Finally, from the medicinal plant *Trichilia adolfi*, nine novel limonoids-type natural products were isolated and evaluated as anti-parasitic agents.

Key words
Natural Products, secondary metabolites, fungi, medicinal plants, galiellalactone, limonoids.

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title		**ISBN** 978-91-7895-935-8 (print) 978-91-7895-936-5 (pdf)
Recipient's notes	**Number of pages**	Price
	Security classification	

Signature  Date 2021-06-4

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Coverphoto by: Mariela González Ramírez

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ISBN 978-91-7895-935-8 (print)
ISSN 978-91-7895-936-5 (pdf)

Printed in Sweden by Media-Tryck, Lund University
Lund 2021

To my teacher and friend
Sergio Triviño

Table of contents

Abstract

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Due to their bioactivity, natural products present in medicinal plants, mushrooms (and other organisms such as bacteria and algae) have been the source of medicine and the inspiration of modern dug discovery since they are characterized by an enormous scaffold diversity and structural complexity.

In the search of new natural products, this thesis comprises the study of natural products from two fungal species, *Aleurodiscus sp.* and *Galiella Rufa*.

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Finally, from the medicinal plant *Trichilia adolfi*, nine novel limonoids-type natural products were isolated and evaluated as anti-parasitic agents.

Popular summary

From the beginning of human history, human beings have used nature to satisfy all their needs. The search for new medicine is not an exception. Since ancestral times, many civilizations used medicinal plants, as well as other organisms such as fungi and algae, to treat pain or cure illness. With the development of the scientific method, chemists started to isolate the compounds with medicinal properties from plants/fungi.

At present, we know that secondary metabolites, well known as natural products, are responsible for these medicinal properties. Examples are several, from morphine to penicillin, and despite most of the drugs available on the market are inspired by natural products, the development of new conditions or diseases, makes the search of new drugs urgent. Despite many strategies are focused on the development of synthetic agents, the search of new natural products is still active, since they offer advantages based on their enormous scaffold diversity and structural complexity.

In the context of searching for new natural products, this thesis comprises the study of secondary metabolites from two fungal species and one medicinal plant.

List of papers

Paper 1
González-Ramírez, M., Cajas-Madriaga, D., Rajchenberg, M., Cabrera, J., Becerra, J., Sterner, O. Benzofuran derivatives from Andean-Patagonian *Aleurodiscus* fungi. *In manuscript.*

Contribution: Planning the project, performed *in vitro* cultivation of fungi, extraction of crude extract, isolation and elucidation of molecules, and to write the manuscript.

Paper 2
González-Ramírez, M., Escobar, Z., Ruano, A., Johansson, M., Sterner, O. Oxidation of desoxygaliellalactone derivatives by *Galiella rufa* P450-mediated biotransformation. *In manuscript.*

Contribution: Performed the isolation of enantiomers, fungal *in vitro* cultivation, feeding experiments, LC-MS analysis, isolation of products, interpretation of the data and to write the manuscript.

Paper 3
González Ramírez, M., Escobar, Z., Sterner, O. Galiellalactam: a synthetic/biosynthetic combinatory approach. *In manuscript.*

Contribution: Performed all the synthetic work, fungal *in vitro* cultivation, feeding experiments, isolation of galiellalactam, and to write the manuscript.

Paper 4
Limachi, I., **González-Ramírez, M**., Manner, S., Ticona, J.C., Salamanca, E., Gimenez, A., Sterner, O. 2021. Trichilianones A-D, novel cyclopropane-type limonoids from *Trichilia adolfi*. Molecules. 26:1019-1032

Contribution: Performed the isolation of molecules, helping with elucidation and to write the manuscript.

Paper 5

González-Ramírez, M., Limachi, I., Manner, S., Ticona, J.C., Salamanca, E., Gimenez, A., Sterner, O. 2021. Trichilones A-E: New Limonoids from *Trichilia adolfi.* Molecules. 26: 3070- 3085.

Contribution: Performed the isolation and elucidation of molecules, and to write the manuscript.

Abbreviations

TK: Traditional knowledge

NPs: Natural products

SMs: Secondary metabolites

HTS: High-throughput screening

EtOAc: Ethyl Acetate

CC: Column chromatography

HPLC: High performance liquid chromatography

HR-MS: High resolution mass spectrometry

LC-MS: Liquid chromatography mass spectrometry

NMR: Nuclear magnetic resonance

DCM: Dichloromethane

MPA: Methoxyphenylacetic acid

ADH: Alcohol dehydrogenase

DAAE: Ethyldiazoacetate

TMSBr: Trimethylsilyl bromide

TMSCl: Trimethylsilyl chloride

DIBAL: Diisobutylaluminium hydride

MsCl: Methanesulfonyl chloride

TEA: Triethylamine

THF: Tetrahydrofuran

DMF: Dimethylformamide

1. Introduction

1.1 The search for medicines since primitive times

Even before the agricultural revolution began, the human species, as well as other primates and animals, developed a sense for the use of plants as a source of medicine – a phenomenon called self-medication - understanding it as the use of natural materials or chemical substances to reduce or eliminate deleterious symptoms of parasites or pathogens[1](Figure 1A).

For hominids, the idea of self-medication in the paleolithic is supported by archeological records, where for instance, an analysis of dental calculus from a hominid found in the Neanderthal Sidrón cave, showed the presence of the medicinal plants *Achillea millefolium* (Figure 1B) and *Matricaria chamomilla.*[2] Despite reconstructing plant use before domestication is challenging, anthropologic research demonstrates that the deliberate selection and use of medicinal plants was a universal feature of the human past, and an integral part of broad survival strategies conducted by all paleolithic hominids and essential for them to survive, thrive, and successfully reproduce.[3]

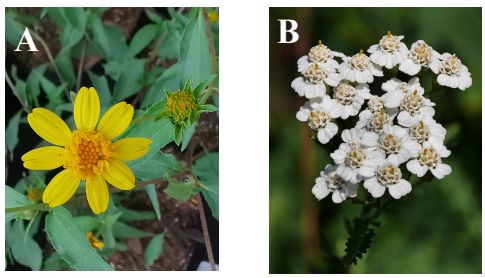


Figure 1. **A**. *Aspilia* mossambicensis, a plant used by African great apes for self-medication purposes[4] (Picture by Paul Venter, CC BY-SA 4.0 downloaded from https://en.wikipedia.org/). **B**. *Achillea millefolium*, plant consumed by the early Neanderthals[2] (Picture by Petar Milošević, CC BY-SA 4.0 downloaded from https://en.wikipedia.org/).

The coevolution of *Homo* with the natural environment conducted a *Homo sapiens* with a deep sense for the search of medicinal plants. This has been shown historically, as the ancient civilizations and many indigenous people experimented with plants and other organisms to determine the effect they might have, developing through trial and error a vast TK about medicinal plants.

For instance, the oldest written evidence of medicinal plants usage for preparation of drugs, was found on a Sumerian clay slab from Nagpur of approximately 5.000 years old, which comprises 12 recipes for drug preparation based on over 250 plants;[5] or the case of Tapputi-Belate-Kallim, who is considered to be the world's first recorded chemist, mentioned in a cuneiform tablet dated around 1200 BC in Babylonian Mesopotamia[6] (Figure 2A), who used natural materials such as flowers, cyperus, myrrh and calamus to prepare perfumes; and finally the case of the Egyptian medical Ebers Papyrus[9] (Figure 2B), dated about 1550 BC, which refers a large number of prescriptions and recipes including the use of opium, cannabis, myrrh, frankincense, fennel, cassia, senna, thyme, henna, juniper, aloe, linseed and castor oil.[7]

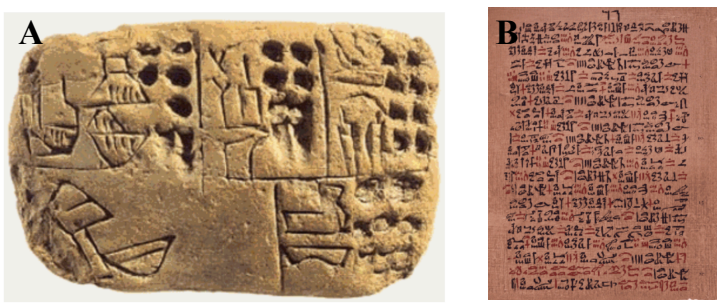


Figure 2. **A**. Cuneiform tablet dated 1200 BC referring to Tapputi[8] (Picture modified from https://www.wikidata.org/wiki/Q4355213). **B**. Ebers Papyrus[9] (Picture of public domain downloaded from https://en.wikipedia.org/).

1.2 Traditional knowledge of medicinal plants

As human societies became more aware of the use of plants and other organisms as a source of medicine, the constant experimentation with the environment to satisfy their needs, allowed them to empirically develop a deep knowledge of medicinal plants. This knowledge has been transmitted from generation to generation and is still used and practiced in many societies, being the primary healthcare system for about 80% of the world's population, especially in the developing countries.[10]

There are many examples of TK from ancestral times, in both, the old and new world. For instance, in Europe, *Digitalis purpurea* was traditionally used in folk medicine to treat dropsy.[11] In medieval times *Galega officinalis* was said to relieve the intense urination accompanying the disease that came to have the name of diabetes mellitus,[12] and even the use of *Salix alba*'s bark as an analgesic.[13] In South America, Quechua people used the bark of *Cinchona* trees as a muscle relaxant to cure shivering,[14] and Mapuche people used the stem of *Equisetum bogotense* as a detoxicant agent to clean kidneys,[15] knowledge that is practiced even nowadays by the Chilean contemporary society.

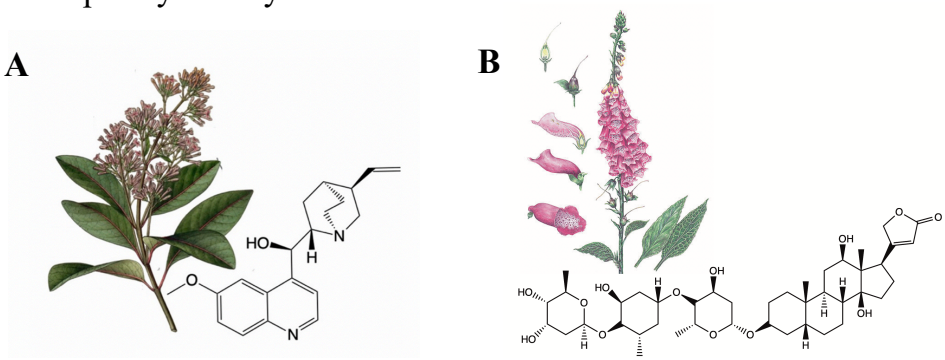


Figure 3. Structures of **A**. Quinine molecule isolated from *Cinchona* trees. (Modified from https://upload.wikimedia.org/wikipedia/commons/5/5d/Cinchona_calisaya_-_K%C3%B6hler%E2%80%93s_Medizinal-Pflanzen-179.jpg) **B**. Digoxin molecule isolated from *Digitalis purpurea.* (Modified from https://commons.wikimedia.org/wiki/File:Digitalis_purpurea_Elisabete_Ferreira.jpg)

However, it was not until the 19th century that man began to isolate the active principles of medicinal plants and one landmark was the discovery of quinine (Figure 3A), from *cinchona* bark by the French scientists Caventou and Pelletier,[16] a plant that as mentioned before, has been widely used in South America since ancestral times.

1.3 Natural Products

At present, it is well known that the active principles of medicinal plants -as well as other organisms-, are NPs. However, the term "natural product" is often ambiguous since it can be referred to:

(a) an entire organism,
(b) a part of an organism,
(c) an extract of an organism,
(d) pure compounds isolated from plants, animals or microorganisms.

However, in most cases, a NP refers to SMs produced by any living organism,[17] which are small molecules (< 2000 Da), that are not involved in main life processes for the growth, development, and reproduction (as primary metabolites), thus are not essential, however, they play a defensive role protecting the organism from the competitive environment. For example, from the herbivory, as allelopathic agents (Figure 4).

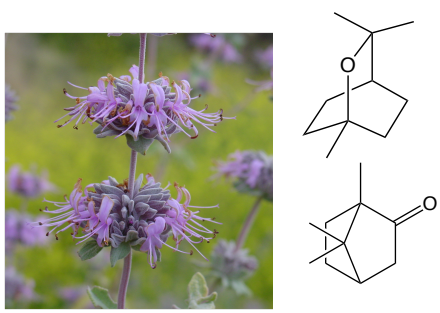


Figure 4. The sage bush, *Salvia leucophylla*, contain compounds Eucalyptol (top-right) and Camphor (bottom-right) that deter other plants from germinating[18]. (Picture modified, Author: Noah Elhardt, CC BY-SA 2.5 downloaded from https://en.wikipedia.org/).

Due the bioactivity of SMs against other organisms, these molecules are also able to act towards targets involved in human diseases. For instance, the main bioactive SMs isolated from the medicinal plants mentioned above are digoxin (Figure 3B), which nowadays is a medication used to treat various heart conditions, galegine (Figure 5A) whose structure inspired the development of the drug metformin used for the treatment of type 2 diabetes, and salicin (Figure 5B) which is an anti-inflammatory agent whose structure inspired the development of the drug aspirin.

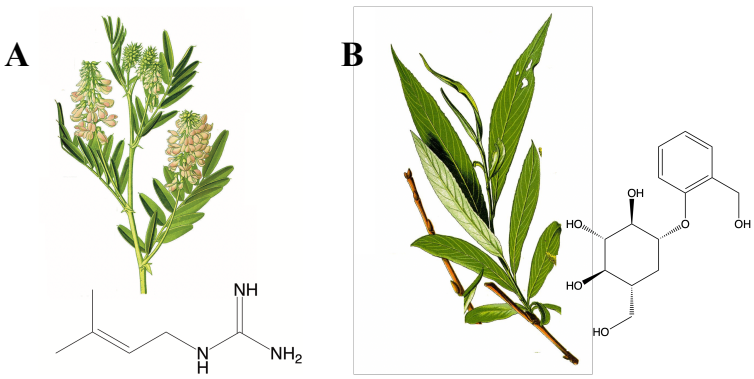


Figure 5. Structures of **A** Galegine molecule isolated from *Galega oficinalis*. (Picture modified from http://www.biolib.de/thome/band3/tafel_121.html) **B** Salicin molecule isolated from *Salix alba.* (Picture modified from https://sv.wikipedia.org/wiki/Vitpil)

1.4 Natural Products as source of drugs

Prior to the 19th century, the active principles of the medicines based on TK were unknown, however, the development of the scientific method and the curiosity about what substances medicinal plants are composed of, conducted to the isolation of NPs. For instance, investigations of opium, the dried latex from *Papaver somniferum*, resulted in the isolation of several alkaloids, including morphine (Figure 6), as first reported in 1803[19] by Friedrich Sertürner.

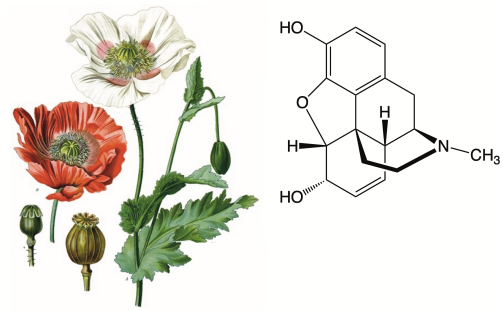


Figure 6. Structure of morphine, isolated from *P. somniferum* (Modified from https://en.wikipedia.org/wiki/Papaver_somniferum#/media/File:Papaver_somniferum_-_Köhler–s_Medizinal-Pflanzen-102.jpg)

Since then, with the development of isolation techniques such as chromatography, and strategies for elucidating structures, such as chemical and spectroscopic methods, a plethora of bioactive NPs have been isolated.[20] Over the last century, several top selling drugs have been developed from NPs; e.g. vincristine from *Vinca rosea* (Figure 7A), morphine from *P. somniferum*, and Taxol® from *Taxus brevifolia* (Figure 7B).[17]

A **B**

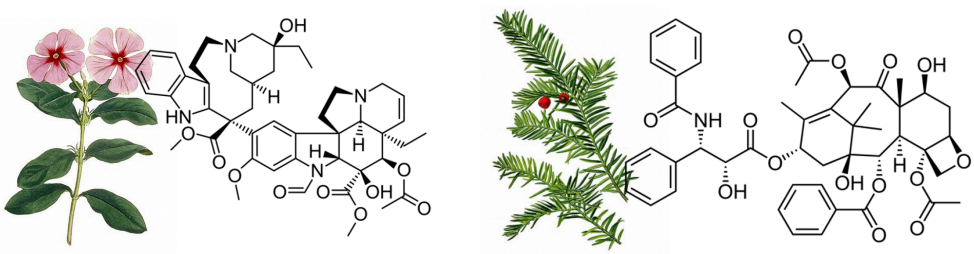


Figure 7. Structures of **A.** Vincristine isolated from *V. rosea*. **B.** Taxol® isolated from *T. brevifolia*. (Both pictures modified from https://commons.wikimedia.org/)

From the 1940s until 2010, 41% of anticancer and 65% of antibacterial small molecule drugs were either NPs or semi-synthetic derivatives of NPs.[21] Despite the fact that pharmaceutical companies traditionally have relied on nature as a source for drug discovery, the often frustrating, expensive and time-consuming process involved in the discovery of new

medicinal agents from NPs, triggered a movement away from NPs in the 1990. Instead, the screening of collections of synthetic compounds became more common.[22] Due the advent of genomic sciences, rapid DNA sequencing, combinatorial chemistry, cell-based assays, and automated HTS[23] that has led to a new concept of drug discovery based on synthetic chemistry, it is frequently assumed that NPs are no longer a source of drugs in the twenty-first century, however, this is not all true (Figure 8).[24]

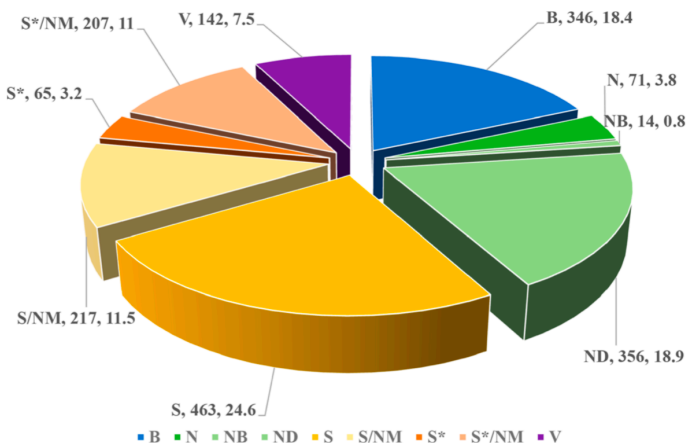


Figure 8. All new approved drugs from 01-Jan-81 to 30-Sep-19; *n* = 1881. B: biological macromolecule, N: unaltered natural product, NB: botanical drug (defined mixture), ND: natural product derivative, S: synthetic drug, S*: synthetic drug (NP pharmacophore), V: vaccine, /NM: mimic of natural product[24]

Even today in the search for novel agents against manifold diseases, NPs structures are often the foundation for compounds that are either recently approved (last 5-10 years), or that are now in clinical trials.[24] The trend nowadays is a shift away from large combinatorial libraries, with the emphasis on small collections that contain much of the structural aspects of NPs, so the utilization of NPs and/or synthetic variations using their novel structures, in order to discover and develop a final drug entity, is still alive and well.[24]

1.5 Advantages of Natural Products

Despite the advances in synthetic chemistry, that have been conducted into HTS as a dominant method to identify lead compounds in drug discovery, most compound screening collections consist principally of planar molecules with little structural or stereochemical complexity, which do not offer the arrangement of chemical functionality necessary for modulation of many drug targets.[22] In front of that challenging scenario, NPs are still a source of inspiration for drug design as they are characterized by an enormous scaffold diversity and structural complexity.[25] In comparison to synthetic compound libraries, the structural advantages of NPs are:

- Much larger fraction of sp^3-hybridized bridgehead atoms.[26]
- Fewer aromatic rings and rotatable bonds.[27]
- Larger number of chiral centers.[28]
- Higher number of H-bond acceptors and donors.[26,29]
- Lower calculated octanol-water partition coefficients.[29]
- Greater molecular rigidity.[27]
- More oxygen but fewer nitrogen atoms.[27, 30]

All these features imply NPs as non-flat three-dimensional structures, displaying a constellation of properties and multiple functions integrated into a compact, highly functionalized molecule,[31] offering offer many advantages in terms of efficiency and selectivity of molecular targets,[32] which may positively influence the probability of clinical success of drug candidates.[33]

Additionally, it's important to consider that the production of NPs is not just framed for plants, but for all living organisms with SM including the kingdoms of Bacteria and Fungi, as well as species from aquatic ecosystems such as algae and deep-sea bacteria, thus, expanding the diversity of molecules given the class of compounds can be differentiated by their source organism, biosphere of origin and biological role.[2]

2. Scope of this thesis

The aim of this thesis is to investigate natural products from the fungi *Aleurodiscus sp.* and *Galiella rufa*, along with the plant *Trichilia adolfi.*

This thesis comprises five articles regarding the study of natural products from fungi (*Aleurodiscus sp.* and *Galiella rufa*) and the plant *Trichilia adolfi.*

Paper 1 describes the isolation of fungal benzofuran molecules from *Aleurodiscus sp. in vitro* cultures, whose fruiting bodies were collected in Patagonia.

Paper 2 describes the synthesis of desoxygaliellalactone analogues and their biotransformation to galiellalactone derivatives performed by *Galiella rufa in vitro* cultures.

Paper 3 describes the synthesis of desoxygaliellalactam, and its conversion to galiellalactam carried out by biotransformation from *Galiella rufa in vitro* cultures.

Paper 4 and **5** describes the isolation of novel limonoids from the plant *Trichilia adolfi* along with their biological activity.

3. Fungal natural products

Nature has not only provided plants as a source of NPs, but also other organisms, such as the fungi. Probably, one of the most important molecules that changed the history of medicine is the group of penicillin antibiotics (Figure 3.1A), discovered from *Penicillium sp.* by Alexander Fleming,[34] whose β-lactam structure was established in 1945 by Dorothy Hodgkin.[35]

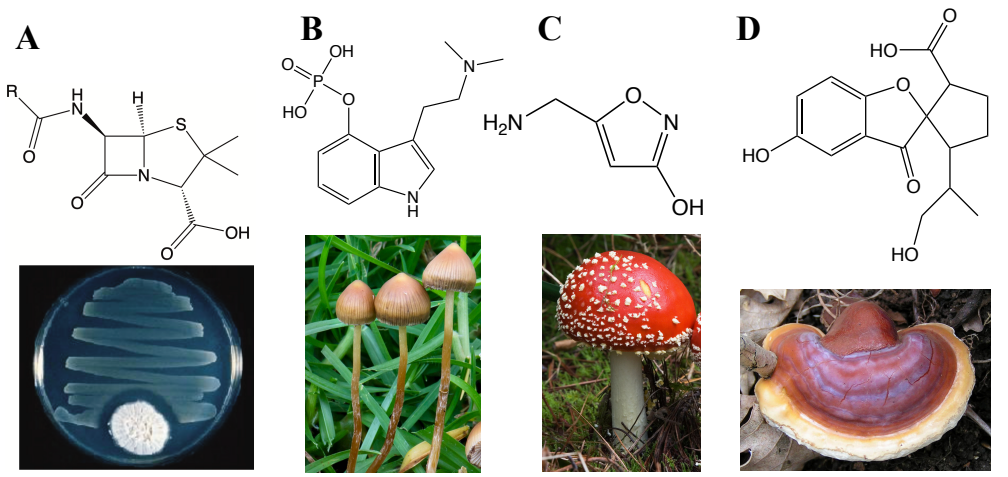


Figure 3.1 Structures of **A**. Penicillin group of antibiotics, isolated from *Penicillium sp.* molds. (Picture modified from http://www.accessexcellence.org/) **B**. Psilocybin, isolated from *Psilocybe sp.* Mushrooms[36] (Picture modified, author: Alan Rockefeller, CC BY-SA 4.0) **C**. Muscimol, isolated from *A. muscaria*[37] (Picture modified, author: JJ Harrison, CC BY-SA 3.0) **D**. Spirolingzhine A isolated from *G. lucidum*[38] (Picture modified, author: Eric Steinert, CC BY-SA 3.0). Last three pictures download from https://en.wikipedia.org/

Other examples of important fungal NPs, are:

- Psilocybin (Figure 3.1B): a psychoactive alkaloid present in *Psilocybe sp*., mushrooms consumed since ancestral times in Mesoamerica for spiritual and divinatory purposes, whose main active principle it's nowadays being evaluated as an antidepressant agent.[39-41]

- Muscimol (Figure 3.1C): a psychoactive constituent from *Amanita muscaria*, which acts as a selective agonist for the $GABA_A$ receptors, being the lead compound in the development of a range of GABAergic agents.[42]
- Spirolingzhine A (Figure 3.1D): a meroterpene with a spiro[benzofuran-2.1'-cyclopentane] motif, isolated from the medicinal mushroom *Ganoderma lingzhi*, which is a neuroprotective agent acting as a stimulator of neural stem cell proliferation.[38]

Fungi are microorganisms with unusual biochemical pathways,[43] allowing the production of a variety of NPs, including all important categories (i.e. terpenes, alkaloids, polyketides, lactones, polysaccharides), and representing an important source of highly selective candidate molecules. Some reasons of the production of complex NPs are:

- Fungi are widespread, non-photosynthetic microorganisms that gain their energy from the degradation of matter present in the environment, fulfilling a recycling role.[44]
- Fungi secrete enzymes to externally break down their surrounding food and absorb the products of this catabolic activity from the medium.[45]
- Their variety of enzymes allows to carry out chemically complex transformations.
- Fungi have evolved to release a diversity of SMs as "chemical weapons" to protect their food from competitors.[46]
- Many fungi are pathogens to plants and animals, and SMs have the key role as signals and toxins in these interactions.
- Fungal primary and secondary metabolic pathways are often encoded by metabolic gene clusters (MGCs) that facilitates the adaptation to changing environments,[46] so their chemistry can vary with the conditions under which it is grown.[44]

4. Benzofuran derivatives from Andean-Patagonian *Aleurodiscus sp.* Fungi (paper 1)

The oxygen-containing heterocycles are an important class of compounds in organic chemistry mainly due their natural abundance and diverse biological functions.[47] Some examples of these structures are benzofurans, chromane, chromene, coumarins and xanthene derivatives (Figure 4.1 A-E, respectively), whose main scaffolds have been an inspiration in drug discovery, e.g., bergapten, nebivolol, troglitazone, warfarin, theophylline.

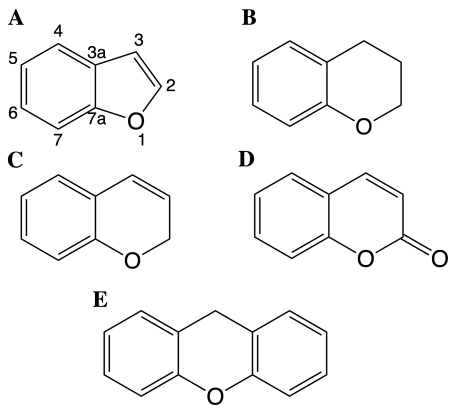


Figure 4.1. Examples of oxygen-containing heterocycles structures. A: benzofuran, B: chromane, C: chromene, D: coumarin, E: xanthene.

Among these, several derivatives of benzofuran (Figure 4.1A) have been recognized as biologically and pharmacologically relevant molecules,[48] whose bioactivity vary as antioxidants,[49] antimicrobials,[50] enzyme inhibitors,[51-53]enzyme activators,[54,55] receptor agonists and antagonists,[56-58] anti-inflammatory compounds,[59-61]anticancer agents[62,63] and antiviral agents.[64-66]

As the major group of biologically active heterocycles,[67] benzofurans also have attracted the attention as anti-Alzheimer agents, where diverse synthetic derivatives have shown to inhibit the main targets involved, such as the enzymes acetylcholinesterase (AChE),[68-70,75] butyrylcholinesterase (BuChE), [69,77] monoamine oxidase (MAO),[71,72] and the aggregation of amyloid- β peptide[68,69,73,74,75] and TAU protein.[76,78]

The majority of the anti-Alzheimer benzofurans has been found through synthetic approaches. However, considering that approximately ~84% of approved drugs for CNS diseases are NPs or NP-inspired, where 20 NPs have provided more than 400 clinically approved CNS drugs,[79] it seems reasonable to search for natural benzofuran structures.

In this sense, it has been shown that fungi belonging to *Russulales* order (Basidiomycota) produces benzofuran derivatives[80-86] with a huge diversity of structures and functional groups. Following this approach, from a previous work the benzofuran Fomannoxin was isolated from the Andean-Patagonian fungus *Aleurodiscus vitellinus* (Figure 4.2) and showed potent neuroprotective properties in a cellular model of amyloid- β peptide toxicity,[87] opening up the possibility to find new benzofuran structures in other *Aleurodiscus* species.

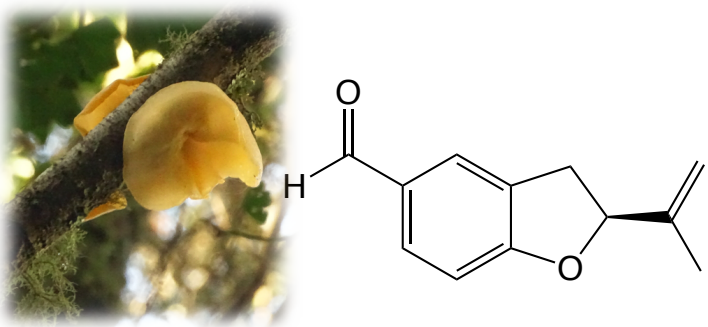


Figure 4.2 Structure of Fomannoxin, isolated from the fungus *Aleurodiscus vitellinus*[87]

In this work, three fruiting bodies of fungi belonging to *Aleurodiscus* genus were collected in different spots from Andean-Patagonian forests, in the Chilean and Argentinean Southern cone (Figure 4.3). Considering

this region as a rich source of fungi (whose chemistry is just starting to be studied), as well as the extreme environmental conditions of this area (such as low temperatures, high UV-irradiation and high endemism of their forests), this territory is a promising source of novel NPs.

Figure 4.3. Location of Patagonia in the Southern cone (framed in red). (Modified from https://legacy.lib.utexas.edu/maps/world_maps/world_pol_2011_nov.pdf)

Fungal tissue from the fruiting bodies was isolated and cultivated *in vitro*. Subsequent liquid fermentation for 21 days allowed the production of SMs. Culture broth was separated from the mycelia and extracted with EtOAc by liquid-liquid extraction. Isolation of the NPs was carried out by silica gel CC and further purification by HPLC. Structures were confirmed by 1D and 2D NMR experiments and HR-MS.

4.1 *Aleurodiscus antarcticus*

From *A. antarcticus*, the known compounds **1** and **3**, reported before from *Stereum subpileatum*[84] were identified and confirmed by their ^{1}H- and ^{13}C-NMR spectral data. An additional diastereomer of the diol **1** was identified (**2**).

Due to the lack of data regarding the absolute configuration of **1** and **2**, a ^{1}H-NMR experiment was carried out, in which the assignment of the secondary diol was deduced by comparing the NMR spectra of the

compounds after derivatization with the (R) - and (S) - enantiomers of an auxiliary reagent (MPA in this case). In this way, the absolute configuration of the asymmetric centers were determined based on predictions that depends upon the ^{1}H-NMR $\Delta\delta^{RS}$ values, given the anisotropy produced by MPA (shielding/deshielding effects).

Based on the $\Delta\delta^{RS}$ values of the resulting (R)- and (S)- bis esters, the absolute configuration of the diol **1** was assigned as *anti* (1S, 2R). Unfortunately, due the low yield of the diastereomer **2,** it was not possible to carry out the same experiment.

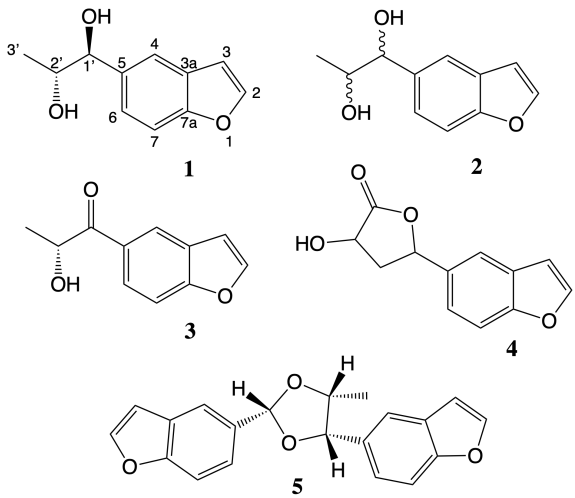


Figure 4.4 Benzofuran derivatives isolated from the fungus *Aleurodiscus antarcticus.*

The benzofuran containing a hydroxy γ-lactone **4** was identified and confirmed by ^{1}H- and ^{13}C-NMR along with HRMS analysis.

Finally, the dioxolane derivative **5** was identified and confirmed by 1D, 2D NMR experiments and HRMS analysis. This structure has never been reported before.

4.2 *Aleurodiscus parmuliformis*

Compounds **1** and **3** were also isolated from this species, suggesting that both molecules are common precursors in the biosynthetic pathway of *Aleurodiscus* species.

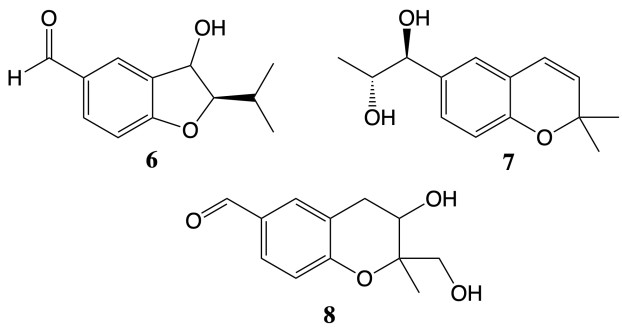


Figure 4.5 Structures identified from the fungus *Aleurodiscus parmuliformis*

Despite the isolation of **1** and **3**, no more benzofurans were identified from this species, but the isolation of the dihydrobenzofuran **6** which shares structural similarities with Fomannoxin (reported before from *A. vitellinus*[87]).

The chromene **7,** which has been reported before from culture broth of the fungus *Hericium erinaceus* (named as Erinachromane B[88]) was isolated. Additionally, the chromane **8** was identified, which has never been reported before as a fungal NP.

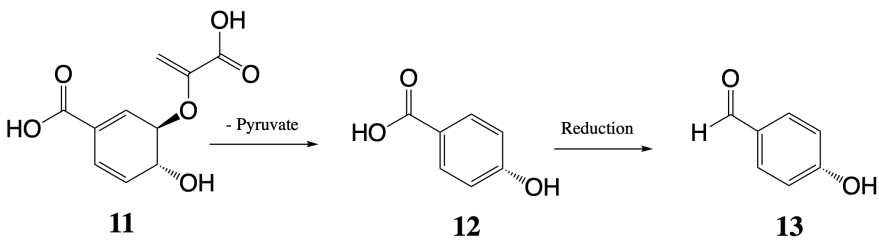
4.3 *Aleurodiscus triviale*

Compounds **1**, **3** and **5** were also identified for this fungus, demonstrating again the existence of common precursors in the biosynthesis of SMs from *Aleurodiscus* species.

4.4 Benzofuran biosynthesis

From previous studies of a related fungal species, *Heterobasidion annosum*,[89] a biosynthetic route for the production of benzofuran derivatives has been suggested. Based on the structural similarities of the molecules present in *H. annosum* compared with those of *Aleurodiscus* genus isolated in this work, the following biosynthesis is proposed:

Chorismic acid **11** (Scheme 4.1, produced from the shikimic acid pathway), loses a pyruvate unit by activity of chorismate pyruvate-lyase, giving *p*-hydroxybenzoic acid **12**,[90] which undergoes a reduction to give *p*-hydroxybenzaldehyde **13**.



11 **12** **13**

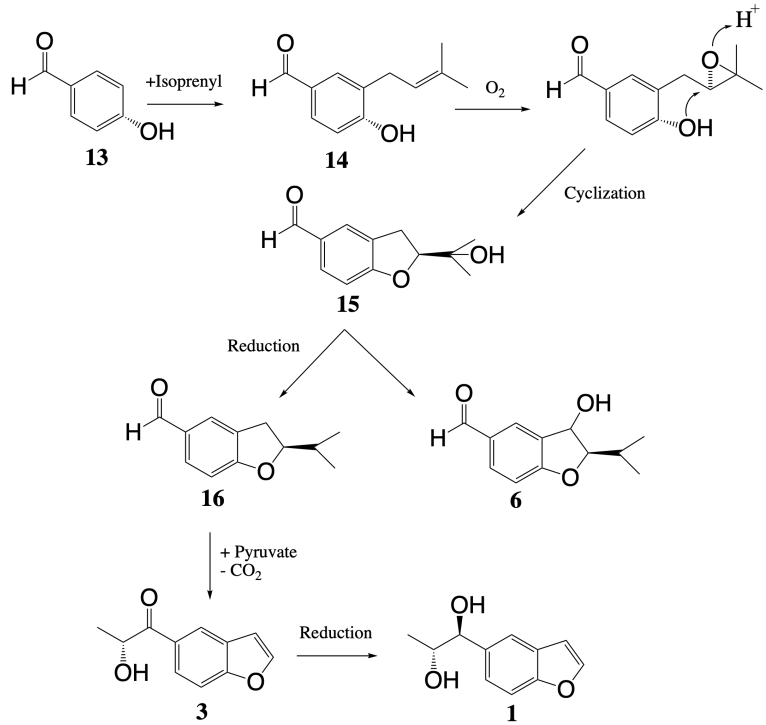
Scheme 4.1 Production of *p*-hydroxybenzaldehyde intermediate in the biosynthesis of benzofurans

A prenyl group, from the mevalonic pathway, is added to **13** by alkylation of the aromatic ring[91,92] (Scheme 4.2). The intermediate **14** goes through cyclization via epoxidation,[93] leading to formation of the dihydrobenzofuran system **15**.

From **15** two different mechanisms are possible: a) Enzymatic migration of the -OH group to C-3 forming **6**, or b) Reduction forming the intermediate **16**.

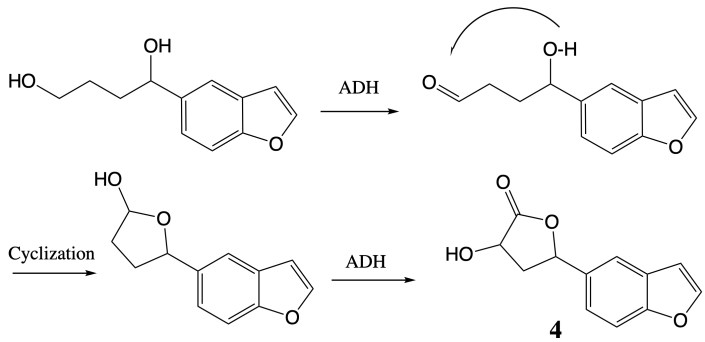
Cleavage of the C-2 isopropyl group in **16** and further addition of a pyruvate unit in the C-5 formyl group, followed by loss of CO_2 via

pyruvate decarboxylase,[94] gives compound **3**. Finally, reduction of the C-1' carbonyl group forms the diol **1** present in all the fungi studied.



Scheme 4.2 Proposed biosynthesis of benzofurans in *Aleurodiscus sp.*

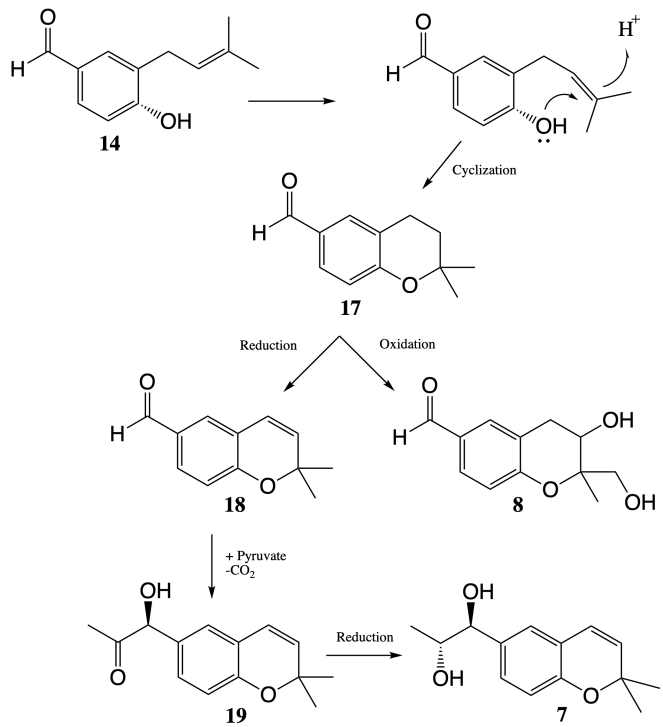
From the diol **1**, additional modifications in the C-5 side chain forming a 1,4-diol intermediate, can yield the derivative **4** *via* oxidative diol lactonization (Scheme 4.3)[95,96] by activity of alcohol dehydrogenase (ADH).



Scheme 4.3. Proposed mechanism of oxidative diol lactonization forming **4**

The heterocyclic ring formation from **14** can take place in two different ways giving rise to 2-isopropylenebenzofurans as showed before, or to 2,2-dimethylchromenes[97] as in structure **7**.

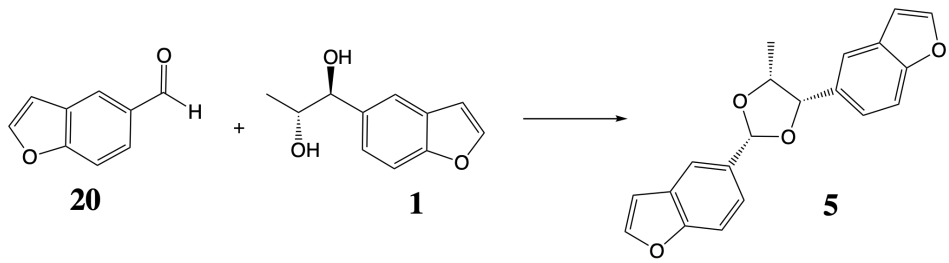
In the case of **7** (Scheme 4.3), a cyclization of **14** to a 6-membered ring via protonation of the double bond results in the formation of chromane **17**. Two different paths may happen afterwards. a) Oxidation conducting to **8**, and b) Reduction of the tetrahydropyran moiety conducting to intermediate **18**, which subsequent pyruvate addition and reduction will produce chromene **7**.



Scheme 4.3. Proposed biosynthesis of chromene derivative **7** in *Aleurodiscus parmuliformis.*

Finally, due the structural features of the dioxolane **5,** it seems reasonable the possibility that **5** was formed as an artifact from a side

reaction between compound **1** and an aldehyde **20** *via* standard acid-catalysed acetalization[98,99] (Scheme 4.4).



Scheme **4.4**. Plausible side reaction between **1** and **20**, yielding **5**.

4.5 Conclusions

Structures **1**, **3** and **7** have been reported before in related fungal species, while molecules **4**, **5**, **6** and **8** are reported for the first time.

All the molecules in this work are substituted at C-5 and/or C-2, which it seems to be a typical feature of benzofurans isolated from the Russulales fungal order.[81,84,85,87]

In addition to the biosynthesis of benzofurans, the production of chromene-type molecules is also reported, which can also be a particular feature for this taxon, where the enzyme responsible for the cyclization of the intermediate **14** can give rise to benzofuran or chromene molecules from the same common precursor.

5. Galiellalactone

Galiellalactone (**3**) (Figure 5.1) is a SM produced by fungus *Galiella rufa* (Pezizales, Ascomycota) which was isolated for first time as a phytotoxin in a screening of NPs from Basidiomycetes and Ascomycetes for plant growth regulating substances.[100] Compound **3** was shown to be an inhibitor of gibberellic acid-induced *de novo* synthesis of α-amylases in wheat seeds germination. Later, compound **3** was reported as an inhibitor of the interleukin-6 (IL-6)-mediated signal transduction in HepG2 cells,[101] and suggested as a lead compound for the development of new therapeutic agents for diseases originating from the unappropriated expression of IL-6.

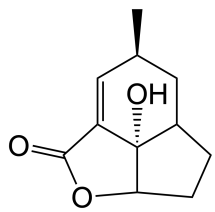
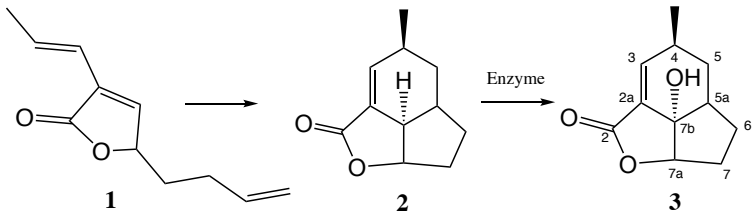


Figure 5.1 (+)-Galiellalactone

Further studies into the bioactivity of **3** as a selective inhibitor of IL-6 pathway,[101,102] demonstrated that it interferes with the STAT3 signaling. As STAT3 is a transcription factor involved in the proliferation of several cancer types,[103-106] **3** was proposed as an anti-cancer agent. In this sense, **3** has shown growth-inhibitory effects on p-STAT3 expressing prostatic cancer cells,[107] and thus have a potential as an interesting compound for the development of future prostate cancer drugs.[108,109]

Prompted by the activity of **3** toward IL-6 signaling, attempts for its total synthesis were undertaken.[110] To develop a synthetic strategy for **3**, a biosynthetic route was proposed based on other SMs isolated from *G. rufa* (Scheme 4.1). It was suggested that (-)-pregaliellalactone (**1**) through an intramolecular Diels-Alder cyclisation form

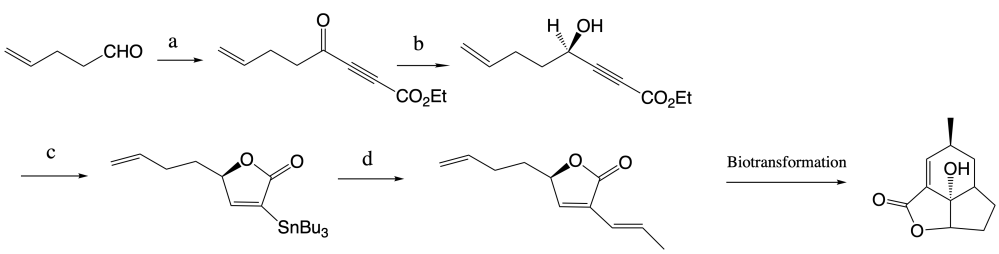
desoxygaliellalactone (**2**). Finally, the last step involves oxidation of the C-H bond at C-7b, to form **3**.



Scheme 4.1. Proposed Galiellalactone biosynthesis in *Galiella rufa.*

Despite the attempts to develop a total synthesis for **3**, the last step involving an aliphatic oxidation on the tertiary central bridgehead C-7b in **2** was not successful. Selective bond functionalization of aliphatic C-H bonds of low acidity is very complicated.[111] In contrast heme and non-heme iron enzymes in *G. rufa* installs oxygen functionalities independently and remotely from existing functionality.[112]

In this context, the first effort to obtain **3** was based on a synthetic/biosynthetic approach[110], in which an enantioselective synthesis of **1** was achieved (Scheme 4.2), followed by a biotransformation experiment, taking advantage of the enzymatic potential of *G. rufa*. Thus **1** was converted to **3** through a feeding experiment, in which **1** was added to an aqueous suspension of *G. rufa* mycelium under constant stirring, finally yielding the desired molecule.



Scheme 4.2. (a) (i) *n*-Buli, HMDS, ethylpropiolate, THF (ii) Jones reagent, acetone, 58%. (b) (R)-Alpine-borane, neat, 70, 83% ee. (c) Bu_3SnH, $Pd(Ph_3)_2Cl_2$, THF, 60% (d) 1-Bromopropene, $Pd(PhCN)_2Cl_2$, CuI, $AsPh_3$, NMP, 50%.

The successful hydroxylation at C-7b was proposed to be performed by the enzyme Cytochrome P450 (P450), i.e. as a large superfamily of heme thiolate proteins that catalyzes the conversion of a variety of chemically diverse compounds by insertion of an activated oxygen atom into an inert C-H bond.[111] This system is common in the fungal kingdom[114] and one of Nature's most potent mono-oxygenating enzymes in aerobic organisms,[113] well known for carry out this kind of highly selective oxidations at inactivated C-H bonds by the known oxygen rebound mechanism[115] (Figure 5.2).

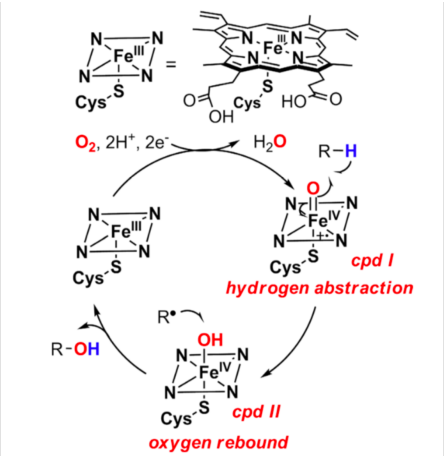


Figure 5.2. Mechanism of aliphatic C-H hydroxylation catalyzed by Cytochrome P450 (the oxygen rebound mechanism)[115]

The biotransformation experiment opened the door to a new approach that combine organic synthesis with biotechnology, using the enzymatic potential of a fungus to yield a molecule that it is impossible to reach by synthetic procedures.

6. Biotransformation of desoxygaliellalactone derivatives (Paper 2)

Further studies on Galiellalactone **3**, have demonstrated that the presence of the hydroxyl group located at the central carbon of the molecule (C7b) is essential for its biological activity.[101,102] Thus, the (+) desoxygaliellalactone (**2**)[116] which lacks the hydroxyl group at C7b, shows considerably lower bioactivity than 3.[101,117]

After the development of a synthetic route for (-)-pregaliellalactone (**1**), the efforts were focused on the synthesis of **2**, where an efficient method was developed, and opened the possibility to synthesize many galiellalactone analogues in an efficient way. Unfortunately, the last step involving the C7b hydroxylation was still unfeasible by all the potential alternatives known for tertiary hydroxylations such as dioxiranes, iminium salt, ruthenium catalysts and the well-known iron-based White-Chen catalyst (A-G). All the options caused oxidations but not at the desired position, resulting in for example epoxidation on the double bond or hydroxylation on C4 (Figure 6.1). Another attractive choice was the use of pure P450 enzymes that are commercially available, however, even if the choice of those where carefully selected based on similar functional groups as in **3**, none of those gave the desired result.

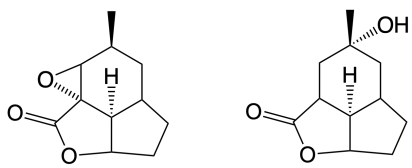
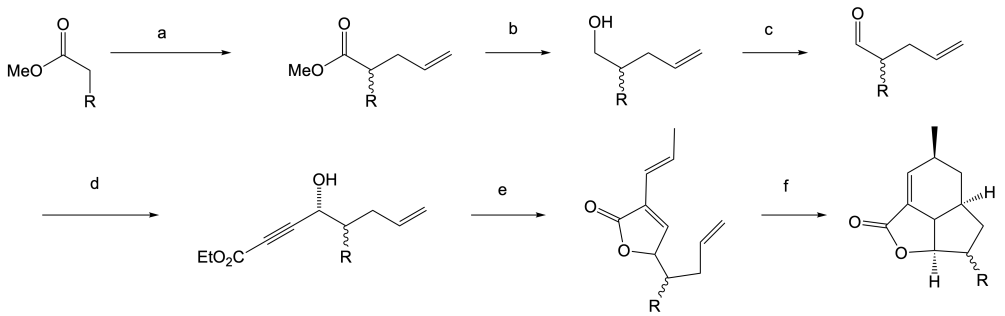


Figure 6.1. Oxidation of desoxygaliellalactone obtained by synthetic methods

In front of this scenario and based on the previous approach that synthetic **1** could be converted into **3** by feeding a suspension of *G. rufa*

mycelium with the molecule, it was proposed to follow the same synthetic/biosynthetic strategy. In this sense, various desoxygaliellalactone analogues were synthesized, applying a synthetic route developed previously (Scheme 6.1).



Scheme 6.1. Synthetic route of desoxygaliellactone derivatives. a) Allyl bromide, LDA, THF, -78°C 3 h. b) DIBAL, DCM (dry), -78°C, 2h. c) Dess-Martin periodinane, DCM (dry), 0°C, 30 min. d) ethyl propiolate, LDA, THF, -78°C, 5 h. e) trans-1-propen-1-ylboronic acid, $Pd(OAc)_2$,(t-Bu)3P, AcOH, THF, 60°C, 6 h. f) toluene reflux, overnight.

As it was proposed that the enzymatic oxidation might be exerted by P450, and with the aim to enhance the biotransformation, a new feeding experiment protocol was stablished, in which instead of giving the synthetic molecule to an aqueous suspension of the mycelium, the compounds were added to the *in vitro* culture broth of *G. rufa*, under parameters that would ensure: proper mycelium growth (the right nutrients); optimal production of P450, such as the addition of iron(II)sulphate (considering that iron is essential for the coordination complex of heme as the cofactor of P450); and the proper feeding timing when the fungus is able to receive the molecule.

In order to analyze the selectivity of the enzymatic oxidation (in terms of enantio-, stereo-, regio- and chemoselectivity), the following desoxygaliellalactone synthetic analogues (Figure 6.2) were tested in *G. rufa* liquid *in vitro* cultures:

- The unnatural (-)-desoxygaliellalactone enantiomer (**4**).
- An analogue with the unsubstituted ring increased (**5**).

- (-) and (+) enantiomers of an analogue with a methyl group at C-7a (**6**).
- (-) and (+) enantiomers of analogues with phenyl group at C-7 (**7**).
- (-) and (+) enantiomers of the diastereomers **8, 9** and **10**.

The biotransformations were monitored everyday by LC-MS analysis of aliquots from the culture broth. Compound isolation was carried out by CC, purification (if needed) by HPLC. Products were confirmed by 1D and 2D NMR.

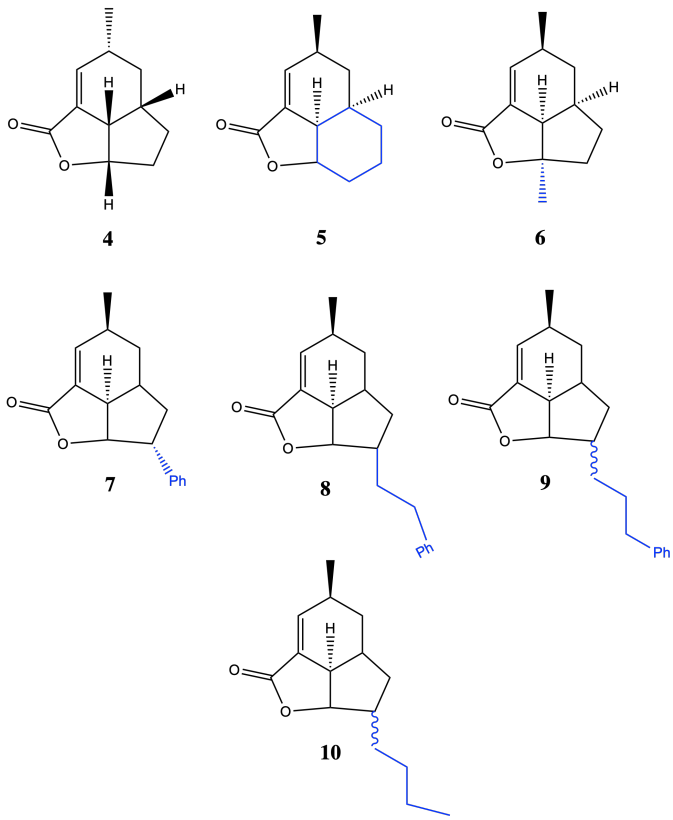


Figure 6.2 Desoxygaliellalactone derivatives tested in feeding experiments. In blue differences with respect to **3**.

Starting with **4**, no oxidation happened (Figure 6.3), suggesting that **4** is not a substrate for the fungal oxidative enzyme, therefore, showing a highly specific enantioselective mechanism.

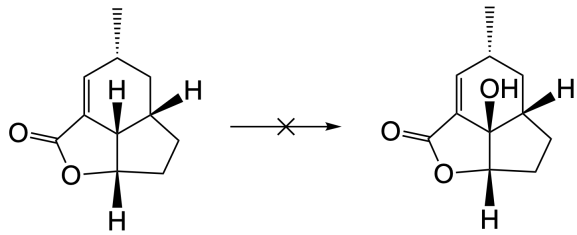


Figure 6.3 No oxidation when *G. rufa* was fed with (**-**)-desoxygaliellalactone (**4**)

With the aim to analyze how the size of the molecule interferes with the enzymatic oxidation, compound **5** was tested (which is very similar to the natural **2** but just possessing an extra carbon on the unsubstituted ring). NMR analysis revealed that an allylic oxidation took place at C-4 (Figure 6.4), indicating that increasing the ring size impedes the desired hydroxylation at C-7b.

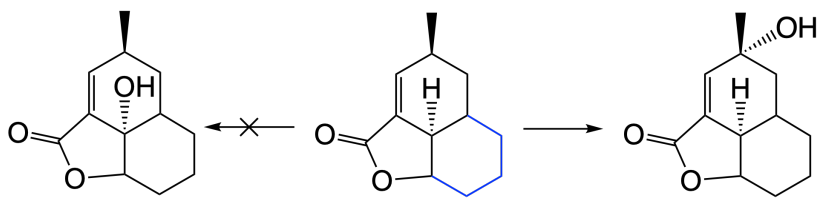


Figure 6.4. Enzymatic hydroxylation of **5** by *Galiella rufa*

In case of derivatives carrying a methyl group at C-7a *syn* to H-7b (**6**), the hydroxylation succeeded. However, the regioselectivity depended on the optical activity of the enantiomer (Figure 6.5). As in the natural process, where **2** is a dextrorotary molecule, the desired product **6A'** was obtained from the (+) enantiomer **6A**, giving a product with the hydroxyl group attached at C-7b as the natural product **3**. On the other side, the feeding experiment with the enantiomer (**-**)**6B** gave a hydroxylated

product whose oxidation occurred at C-4 (same as **5'**). This result confirmed the enantioselectivity of the mechanism.

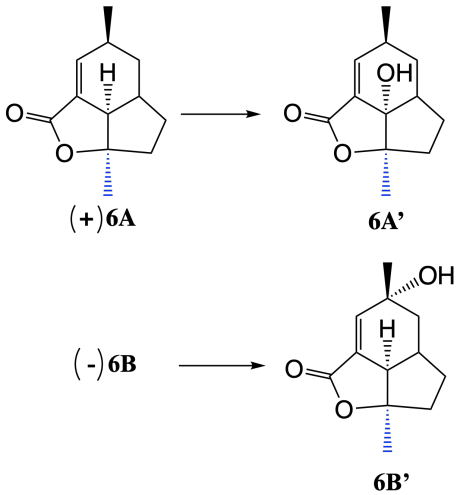


Figure 6.5 Hydroxylated products obtained from 7-Methyl-desoxygaliellalactone enantiomers by *G. rufa*-mediated biotransformation. **6A** (+)7-Methyl-desoxygaliellalactone, **6B** (-) 7-Methyl-desoxygaliellalactone.

Continuing with the enzymatic mechanism elucidation, analogues carrying substitutions at C-7 were tested. Analogue **7**, which is carrying a phenyl group at C-7 *syn* to H-7b, both enantiomers (+) and (-) were tested. Despite both enantiomers were hydroxylated (Figure 6.6), none gave the desired product. In this case steric hindrance (from phenyl group) can be considered as an additional factor impeding the optimal enzymatic activity. This idea was confirmed when derivatives with longer substitutions were tested, such as the case of structures **8**, **9**, and **10**, where no oxidation was detected for any diastereomer.

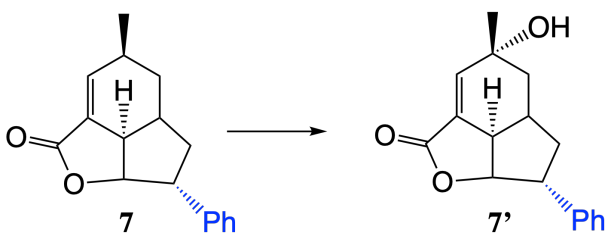


Figure 6.6 Biotransformation of 7-Phenyl desoxygaliellalactone (both enantiomers)

From all the biotransformations achieved, the hydroxylation was carried out with retention of stereochemistry with to the respect natural product **3**. Both, **2** and **3**, have an unusual highly congested tricyclic ring system, that generates tension. The structure of **2**, akin an umbrella, comprises a convex plane where the bridgehead hydrogen atoms (C-7b, C-5a and C-7a) are situated, and a concave side where the methyl group at C-4 is positioned. The isolated hydroxylated products, all presented the -OH group on the convex plane of the molecule, however, differing the regioselectivity depending on the analogue. The only case of success in regioselectivity with respect to **3** was in compound **6A** (oxidizing C-H bond at C-7b), whose optical rotation and relative configuration is same as **2**, but just differing by the presence of a methyl group in C-7a. It is possible that substrates with the same relative configuration and ring sizes than **2** are suitable substrates for the fungal enzymes, as was shown by other analogues.

Among all the oxidative fungal enzymes, it is reported that monooxygenase enzymes belonging to the Cytochrome P450 superfamily (P450s) are the responsible for these complex hydroxylations by introducing an oxygen atom into non-activated C-H bond under normal pressure and room temperature,[118] a pathway known as oxygen rebound mechanism.[115] P450s are heme-thiolate proteins found in all forms of life.[119] Their role in the synthesis of NPs has been investigated intensively, particularly to understand and predict chemo-, regio- and stereoselectivity of hydroxylation[118] over a variety of substrates,[121-130] aiming to use their catalytic potential in molecules with bonds difficult to oxidize by organic synthesis, as in this case.

From this work it was possible to contribute about the mechanism exerted by P450 from *Galiella rufa*, as the expected enzyme responsible for the tertiary hydroxylation of **2** to **3**, as the last step of the biosynthetic route. It is important to note that this fungus can be used to carry out complex C-H oxidations, as in this case, where all the hydroxylations were accomplished on inactivated tertiary carbons, reactions that are still challenging to achieve by synthetic approaches.

7. Galiellalactam (Paper 3)

As have been described earlier, galiellalactone (**3**) is a potent inhibitor of the transcription factor STAT3 mediated IL-6 signaling pathway,[99] whose overexpression is contributed to cancer cell proliferation.[102] As STAT3 is observed in the majority of human cancers,[131] it is currently a promising therapeutic target. In this sense, **3** has been shown to inhibit the growth, both *in vitro* and *in vivo*, of prostate cancer cells expressing active STAT3, and inducing apoptosis of prostate cancer cells expressing phosphorylated STAT3.[132-134]

Studies of the molecular mechanism of **3** suggests that it inhibits STAT3 by forming a covalent bond with the cysteines residues Cys-367 and Cys-468, leading to DNA-binding inhibition and therefore, repressing STAT3 signaling.[135] The efficiency and selectivity of **3** depends on its reactivity as a Michael acceptor, where the C-2a double bond is the requisite to react with nucleophiles[136] (in this case the cysteine residues). Additionally, it has been demonstrated that the presence of the hydroxyl group at C-7b is pivotal for the biological activity, where semi-empirical calculations have shown that -OH has a significant effect on lowering the LUMO energy of the conjugated double bond.[106,134]

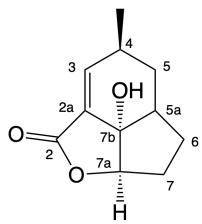


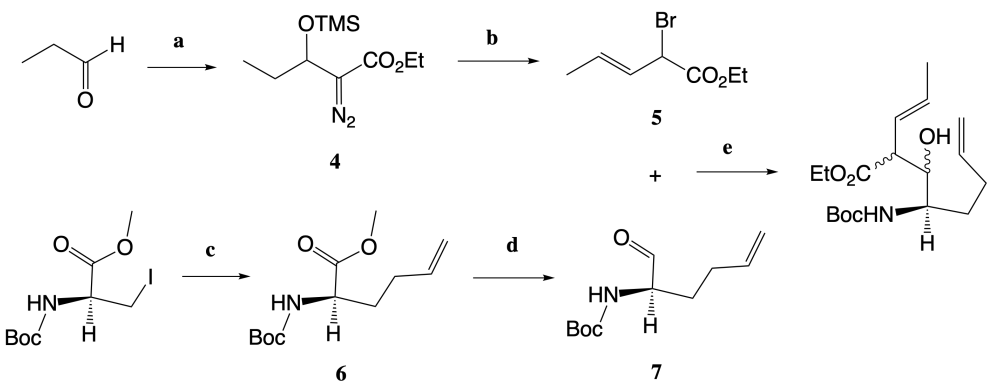
Figure 7.1 Galiellalactone

As shown before, many attempts to produce galiellalactone analogues have been accomplished with the aim to elucidate the bioactivity of **3** versus STAT3. Due to the complexity to achieve the oxidation of H-7b

by organic synthesis, the synthetic/biosynthetic approach ensures a straightforward path to achieve galiellalactone analogues.

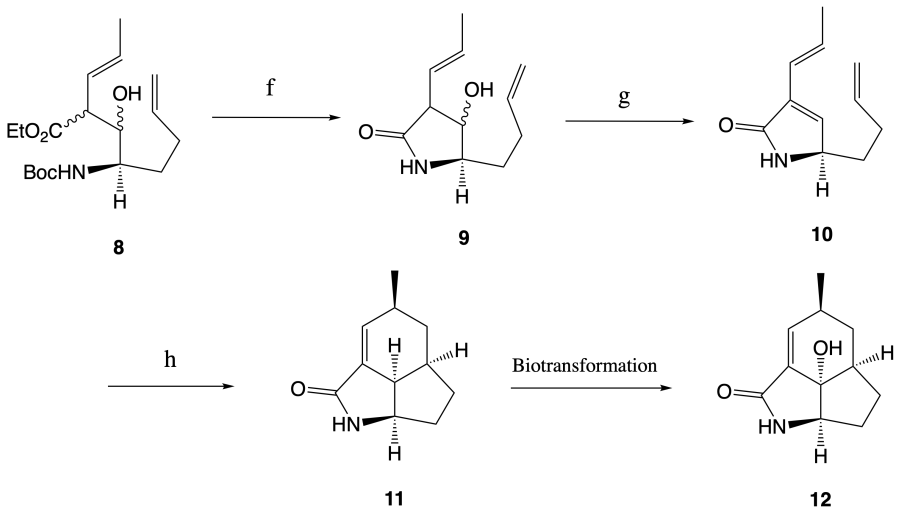
From the previous results, mentioned in chapter six, it was concluded that the regioselectivity of hydroxylation can be predicted. Desoxygaliellalactone analogues with the same optical activity as **2** or carrying substitutions at C-7a, can be hydroxylated successfully at C-7b.

Following the same approach, we decided to produce a lactam analog of 3. The lactam moiety would be less electron withdrawing, thus changing the electron density of the double bond.[117] Therefore, the lactam would be expected to have a different biological activity compared to **3**. In this sense, an enantioselective synthesis of desoxygaliellalactam (**11**) was performed as described before[137] (Scheme 7.1), in which a particular strategy to synthesize **11** was carried out by producing their intermediates in parallel blocks. Based on previous reports,[138,139] compound **5** and the aldehyde **7** were synthesized, whose further coupling by a Reformatsky-type reaction using SmI_2 gave the intermediate **8** as a mixture of diastereomers.



Scheme 7.1 Synthetic/biosynthetic approach to produce Galiellalactam **12**. a) 1. DAAE, *T*-BuOK, 0°C, 2. TMSCl, TEA, DCM, 0°C. b) TMSBr, DCM, 0°C. c) 1. Zn, DMF. 2. Allyl chloride, CuBr*Sme_2. d) DIBAL, DCM, -78°C. e) SmI_2, THF, -78°C.

Further deprotection, hydrolysis and lactonization gave the intermediate **9** (Scheme 7.2), which through a mesylated intermediate and followed by an α-elimination under basic conditions gave pregaliellalactam **10**. The last step in the synthesis was a Diels-Alder cyclization, giving **11**.



Scheme 7.2 Synthetic/biosynthetic approach to produce Galiellalactam **12**.f) 1. HCl 4M in dioxane, rt. 2. $NaHCO_3$ aq., THF, 40°C. g) MsCl, TEA, DCM, 0°C. h) Toluene, 120°C.

Finally, *G. rufa* culture broth was fed with **11**, whose biotransformation to **12** was analysed every day by LC-MS. Production of **12** was indicated by detection of 194 $[M+H]^+$ and 216 $[M+Na]^+$. Finally, **12** was isolated from ethyl acetate culture broth extract using SEPHADEX LH-20 and a chiral HPLC method. Despite the isolation was challenging due the polarity of **12**, and the similarity with the other NPs produced by *G. rufa,* it was possible to yield an amount to confirm the structure by NMR analysis.

The production of **12** by biotransformation, gives an additional insight of the mechanism of the oxidative enzyme, expected to be P450. In case of this analogue, the modification from lactone to lactam suggest that changes in electron density is not a limitation, but only the size and optical rotation of the analogues. Again, this result confirms the advantage of using fungal oxidative enzymes in modifications that are impossible to achieve by organic synthesis.

8. Limonoids from *Trichilia adolfi* (Paper 4 and 5)

The *Trichilia* genus is a flowering plant taxon belonging to the Meliaceae family, comprising about 90 species[140] represented as trees or groves,[141] mainly distributed in tropical America and Africa.

Several species are known to be used in TM, where for instance, in Mali and South Africa the leaf decoction of *Trichilia emetica* is employed for the treatment of malaria;[142] In Cameroon, the bark decoction of *Trichilia rubescens* is used to treat diarrhea, bronchitis and sexual impotence;[141] and in Bolivia, the bark infusion of many *Trichilia* species such as *T. inaeqilatera*, *T. pleeanea* and *T. adolfi* are used by Tacana people to treat lung, kidney, and liver pains.[144]

In the search for the active principles of *Trichilia* species, several studies indicates that limonoids are typical constituents,[141]which are also the most characteristic SMs present in the Meliaceae and Rutaceae family,[143] therefore being a chemotaxonomic indicator.

Structurally, limonoids are highly oxygenated triterpenoids, also called tetranortriterpenoids as the loss of four carbon atoms of the side chain occurs during the oxidative process, eventually forming a 17 β-furan ring.[146] Limonoids possess various carbon skeletons, normally furan and lactone rings, being structurally classified by their basic four rings[147](A-D, Figure 8.1), as well as modifications in these.

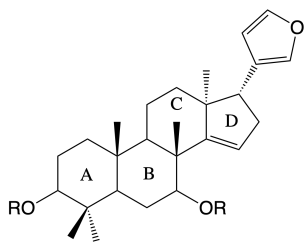


Figure 8.1. Basic skeleton of the limonoids from *Trichilia sp.*

Limonoids can be classified into three major groups based on the variety of oxidations and rearrangements on their rings:[148]

1. Intact ring type: which conserves the four-ring system of the triterpene precursor intact.
2. Ring-*seco* type: based on which of the four rings is oxidized and clivated.
3. Rearranged type: limonoids produced by rearrangements after ring openings.

In case of the genus *Trichilia*, some examples of the most representative types are the Azadirone and Trichilin-type limonoids (belonging to the intact ring class), the Prieurianin-type (belonging to the A, B- *seco* group) and the Mexicanolide type (belonging to the rearranged group) (basic skeleton in Figure 8.2).

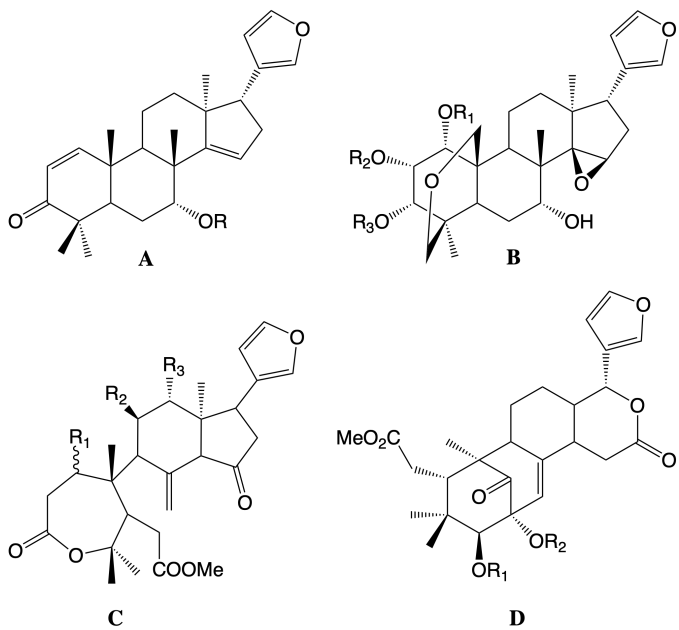


Figure 8.2. Representative limonoids from *Trichilia sp.* **A**. Azadirone-type. **B** Trichillin type. **C**. Prieurianin-type. **D** Mexicanolide-type.

Regarding this work, a previous study focused on a screening for SMs from Bolivian plants with antiparasitic activities, showed that an ethanol extract of *Trichilia adolfi* bark possessed antileishmanial properties,[149] and as the SMs of this species are poorly investigated, the aim of this study was focused on the characterization of the SMs present in a bark ethanol extract of *Trichilia adolfi* along with their biological activity in terms of cytotoxicity (evaluated in *in vitro* cell cultures of murine macrophages), and anti-leishmanial activity in two leishmania-promastigote strains; *Leishmania amazoniensis* and *Leishmania braziliensis.*

According to the chemotaxonomically features of *Trichilia* species, suggesting the production of limonoids, *T. adolfi* was not the exception and 9 novel limonoids were isolated. The structures of the metabolites were elucidated by an extensive spectroscopic analysis based on 1D and 2D NMR experiments as well as HRMS.

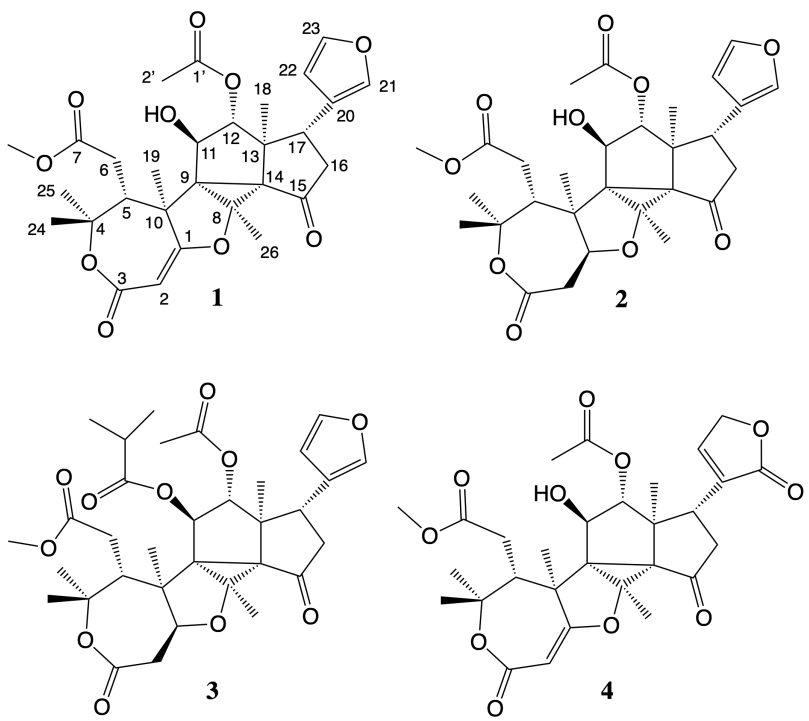


Figure 8.3 Trichilianones A (**1**), B (**2**), C (**3**), D (**4**) isolated from *Trichilia adolfi.*

The first four limonoids isolated, named as Trichilianones A-D (**1-4**, Figure 8.3), have structures that are related to the hortiolide-type limonoids.[150] From the structural elucidation we confirmed the presence of an α, β- unsaturated ε-lactone ring, attached to a tetrahydrofuran ring and connected to an unusual bicyclo [5.1.0] hexane system that is joined with a cyclopentanone carrying a 3-furanyl substituent (except in **4** that possess a (2-oxo)-furan-(5H)-3-yl group).

In terms of bioactivity, despite the initial study on the crude ethanol extract that showed antileishmanial properties,[149] all four compounds tested independently lacks leishmanicidal activity for both strains (*L. amazoniensis* and *L. braziliensis* promastigotes) (Table 8.1). In the case of cytotoxicity on murine macrophage cells, moderate bioactivity was noted (IC_{50} values around 70 μg/ml), but still lower compared with the control (Table 8.1).

Table 8.1. Leishmanicidal activity against *leishmanial* promastigotes (*L.a: Leishmania amazonensis; L.b: L. braziliensis*) and cytotoxicity in Raw murine macrophage cell cultures (Raw), of compounds **1-9** and the positive control Miltefosine. Data given as IC_{50} values in μg/ml.

Compound	*L.a*	*L.b*	Raw
1	>100	>100	64 ± 23
2	>100	>100	75 ± 18
3	>100	>100	70 ± 27
4	>100	>100	76 ± 23
5	73.6 ± 5.1	58.0 ± 18.0	42.0 ± 0.9
6	>100	70.1±3.0	30.0 ±10.0
7	>100	87.2 ± 13.0	60.0 ± 17.0
8	>100	98.5 ± 1.5	94.1 ± 4.2
9	92.0 ± 7.0	68.1 ± 6.0	41.5 ± 24.0
Miltefosine	5.0±0.2	4.07±0.5	21.0±2.0

In addition to the isolation of Trichilianones A—D, five new limonoids were identified (Trichilones A-E, Figure 8.4), whose structures differs from **1-4** by having 4 fused rings instead of 5. Trichilones A-E (compounds **5-9**, respectively) are examples of prieurianin-type

limonoids, having an ε-lactone ring (which in **8** and **9** is α, β-unsaturated) fused with a tetra-hydrofuran ring which is connected to an oxidized hexane ring joined with a cyclopentanone having a 3-furanyl substituent.

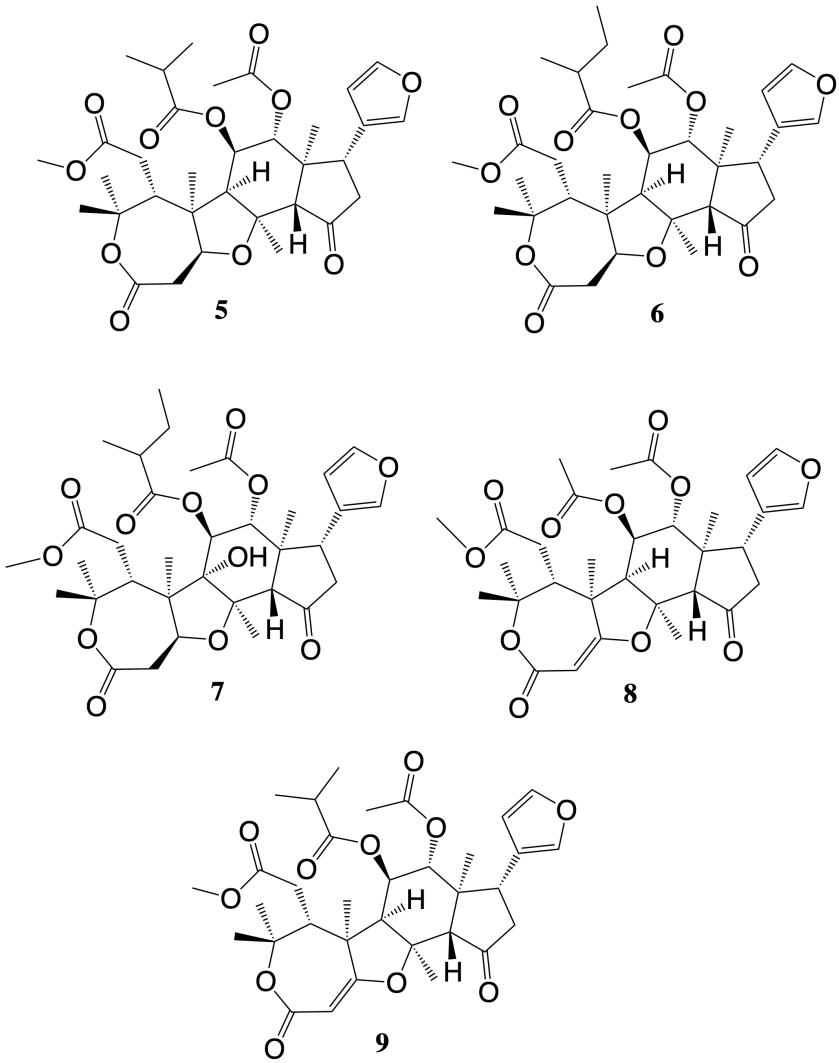


Figure 8.4 Trichilianones E (**5**), F (**6**), G (**7**), H (**8**) and I (**9**) isolated from *Trichilia adolfi.*

The compounds were also tested as cytotoxic and antiparasitic agents in an *in vitro* murine macro-phages cell model, and both strains of *L. amazoniensis* and *L. braziliensis*. Compounds **5**- **7** and **9** showed moderate cytotoxicity (between 30.0 and 60.0 µg/ml) (Table 8.1). However, it seems that they are not responsible for the antileishmanial effect of the ethanolic extract. In this sense, several studies have shown that the overall activity of botanical extracts can result from mixtures of compounds with synergistic, additive, or antagonistic activity.[151]

Trichilones E-I biosynthesis

Not much is known about the biogenesis of the Prieurianin-type limonoids, as well as the reaction mechanisms involved in the biosynthesis of these highly complex molecules. Nevertheless, a possibility for the biogenesis of **5** - **9** based on previous reports[148] is shown (Figure 8.5).

The limonoids have a triterpene origin, where the intermediate **10** (an azadirone-type metabolite) can be proposed as a precursor. Oxidation of the C-7/C-8 bond to a lactone, followed by hydrolysis and a 180° bond rotation around C-9/C-10 would facilitate a cyclization to form a tetrahydrofuran ring possible. A second oxidation of the C-3/C-4 could produce the ε-lactone ring and thereby make the four-fused ring system possible. Modifications on the carbon-carbon bonds (C-1/C-2 and C-9/C-11) could then yield metabolites **5**- **9** isolated in this investigation.

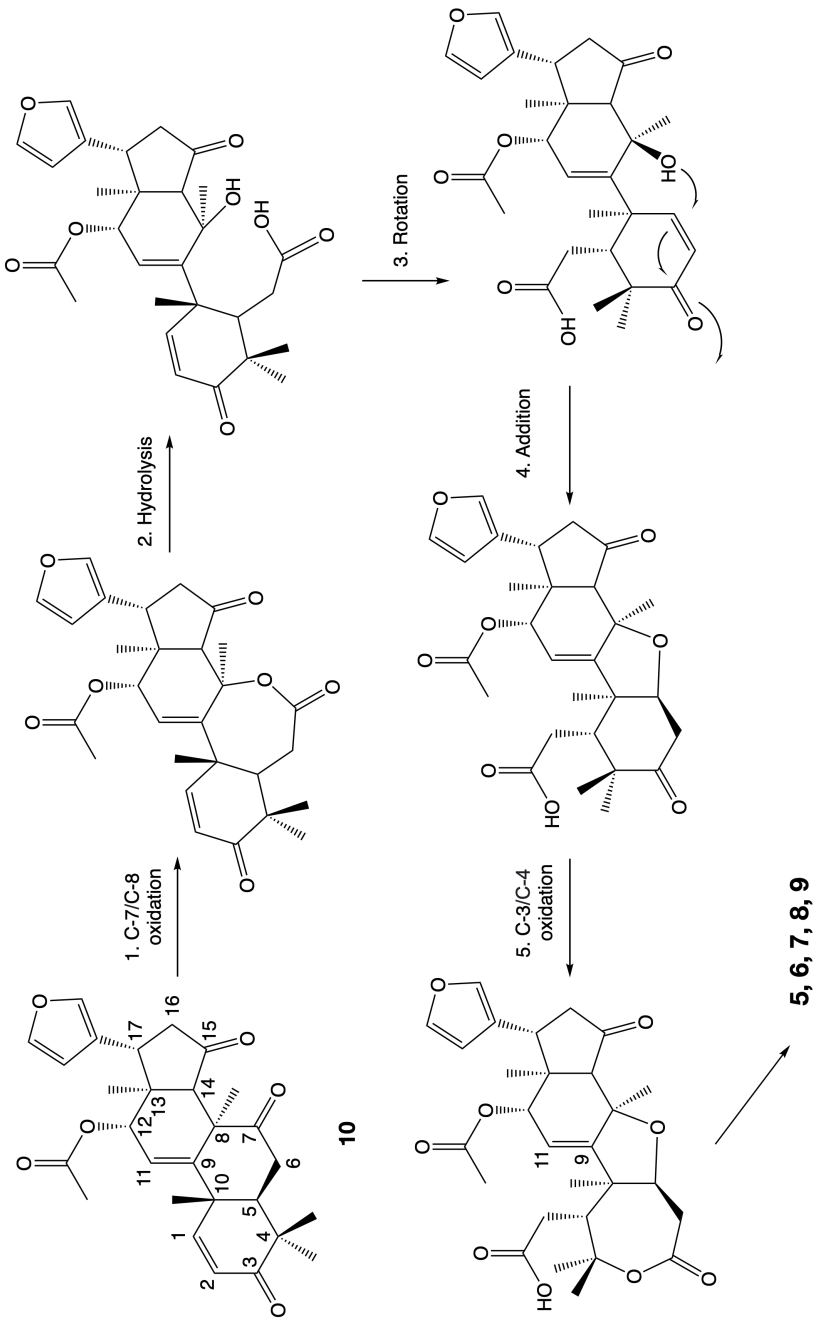


Figure 8.5. Suggested biosynthesis for compounds 5-9

Final conclusions

From this thesis, whose aim was the study of natural products from the fungi *Aleurodicus sp.* and *Galiella rufa*, along with the plant *Trichilia adolfi*, new secondary metabolites were reported.

From *Aleurodiscus sp.* fungi was confirmed the production of benzofuran and chromene-type molecules, contributing to the chemotaxonomy of fungi belonging to Russulales order.

From *Galiella rufa*, despite that their secondary metabolites are well studied, the results from this thesis confirmed the use of its enzymatic potential for an approach to new galiellalactone analogues combining organic synthesis and biotechnology.

From *Trichilia adolfi,* nine novel metabolites were reported. None of these compounds showed the expected anti-parasitic activity.

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Acknowledgments

Since I was a child, my dream was to study abroad and to start my "hero's journey" into the unknown. For some reason, when I left Chile and arrived at Sweden just with my baggage, some books of Carlos Castaneda and my guitar, I knew I had all the enough tools to survive, and that everything would be fine. Now, at the end of this cycle I realised my intuition was right, and this present is possible thanks to many wonderful people I met during this journey (that I hope is just starting).

First, thanks to my supervisor Olov Sterner, because from the beginning you were so accessible in accept me as your last PhD student. Thanks for gave me one of the most important opportunities in my life, and thanks for your good energy of always! Every time we had a meeting you transmitted me a lot of confidence that was essential for reaching my results! (For me, you are the evidence that chameleon effect exists)

Thanks to my colleague and dear friend Zilma Escobar. Thanks for being so humble and like my big sister. Thanks for introduced me in the incredible world of organic synthesis, I will be always grateful of your legacy on me. Thanks for trust on me.

Thanks to all the past members of OS group, Rodrigo, Maribel, Paola, Juliao and Ivan for being so kind people and helped me during my first year at CAS.

Thanks to Bodil, Maria and Katarina for your help in administrative matters, making the work at CAS so efficient.

Thanks to Sofia Essén for helping and teaching me very nice tips with mass spectrometry.

Thanks to Charlotta Turner for your help with my academic drawbacks during the complex moment of the pandemic, I will always remember your human quality.

Thanks to my co-supervisors Sophie Manner and Ulf Ellervik for your incredible support during the thesis development.

Thanks to CONICYT/ANID from the Chilean government for the economic support to study in Sweden.

In a personal context, as Carl Jung said, "The meeting of two personalities is like the contact of two chemical substances: if there is any reaction, both are transformed", I want to express my acknowledgments to all the beautiful friends I met in Sweden, who really shaped to transform me into a better version of myself these last 4 years.

First, thanks to Silva-Nilsson family, for helping me from the beginning with support and advice every time I asked for. Thanks for your generosity and your sense of embracing new students in Lund.

Thanks to my friend Paulina, my little sister, for all the good and wise times we shared. Thanks for made me laugh so much with your jokes, for joined me in the blues nights at Mässingshornet and for the walks into the woods looking for mushrooms.

Thanks to Sebastian, for all the scientific talks we had that really helped me to prepare myself in front of the real world of tricky questions. Thanks for all the funny moments as well, from the beer times to the sauna sessions with Urska.

Thanks to my dear friend Rafael, for all the bossa nova we played and for inspired me about what to be an artist means. Thanks also for bring me back the cumbia sound and the absurd Chilean humor.

Thanks to Ellen, chora querida, for have been my first Swedish friend who helped me to feel integrated in the Malmö-Swedish life. Thanks for all the music and laughs we shared! (And for teaching me funny Swedish vocabulary).

Thanks to my friend Armando, for always been there when I asked for some help (especially when you drove me to the hospital one day after I broke my ankle), and of course, thanks for all the music!

Thanks to all the new wonderful friends I met during this last year, Maria, Anna and Per, for your good vibes and good humor sense!

Thanks to my friends Charlie and Therese (and Grodis these last months). Thanks for somehow introduced me into the Swedish culture and always influence me in a positive way. Thanks for all the moments we shared, the beer times, the games, walks, climbing and swimming sessions (and of course, the childish humor… did you know?). Every day after having seen you, I always felt so happy, that sharing with you was essential for my well-being as a PhD student. You both have been my serotonin in these last 3 years.

Tack till alla mina fina vänner, ni är de finaste jag vet 

Finally, thanks to my mom and dad for your unconditional support and for taught me to be an optimistic and persistent person. Thanks for influenced me to love music, without that I could never be the holistic person I am, that helped me to have a mentally healthy and balanced life in the lovely Sweden.

And thanks to you, Universe, for giving me a life full of privileges, joy and understanding (particularly that I'm a group of organized molecules that wants to understand other molecules).

"While still a child, I experienced several more of these deeply euphoric moments on my rambles through forest and meadow. It was these experiences that shaped the main outlines of my world view and convinced me of the existence of a miraculous, powerful, unfathomable reality that was hidden from everyday sight" ... "Because I wanted to gain insight into the structure and essence of matter, I became a research chemist. Intrigued by the plant world since early childhood, I choose to specialize in research on the constituents of medicinal plants"

Albert Hofmann

Printed by Media-Tryck, Lund 2021 // NORDIC SWAN ECOLABEL 3041 0903

ISBN 978-91-7895-935-8

Centre for Analysis and Synthesis
Department of Chemistry
Faculty of Science
Lund University