Discovery of active secondary metabolites from Paenibacillus odorifer, a lichen-associated bacterium

Thi Bach Le Nguyen

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Thi Bach Le NGUYEN

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Rapporteurs avant soutenance :

Angèle LENGO-MAMBU	Professeur à l'Université de Limoges
Véronique EPARVIER	Ingénieur de Recherche 1 - CNRS - HDR

Composition du Jury :

Angèle LENGO-MAMBU	Professeur à l'Université de Limoges/rapporteur
Véronique EPARVIER	Ingénieur de Recherche 1 - CNRS - HDR /rapporteur
Nicolas PAPON	Professeur à l'Université d'Angers /examinateur
Nicolas RUIZ	Maître de Conférences à l'Université de Nantes/ examinateur
Sylvain TRANCHIMAND	Maître de Conférences à l' ENSCR/examinateur
Sophie TOMASI	Professeur à l'Université de Rennes 1/ Directeur de thèse

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ABREVIATIONS

ACN	Acetonitrile
$CHCl_3$	Chloroform
CoA	Coenzyme A
COSY	Correlation Spectroscopy
CFU	Colony- forming unit
Da	Dalton
DAD	Diode Array Detector
DCM	DiChloroMethane
DMSO	DiMethylSulfOxide
OD	Optical Density
DPPH	DiPhenylPicrylHydrazine
EDTA	EthyleDiamine Tetra Acetic acid
ESI	ElectroSpray Ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
FISH	Fluorescent In Situ Hybridization
FTIR	Fourier Transform Infra-Red
GC	Gas Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution ElectroSpray Ionization
HRMS	High Resolution Mass Spectrometry

HSQC	Heteronuclear Single Quantum Coherence spectroscopy
IC_{50}	Inhibitory Concentration of 50%
IR	Infra-Red
MeOH	Methanol
MS	Mass Spectrometry
NBT	NitroBlueTetrazolium
NI	Negative Ionization
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
ORF	Open Reading Frame
P	Para-phenylene diamine
PCB	Poly Chlorinated Biphenyl
PCR	Polymerase Chain Reaction
PDA	Photo Diode Array
PI	Positive Ionization
ppm	part per million
Rf	Retention front
rpm	rotation per minute
TLC	Thin Layer Chromatography
t_R	Retention time
UV/Vis	UltraViolet/Visible

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INTRODUCTION

INTRODUCTION

Since the discovery of penicillin at the beginning of the twentieth century, natural products have become candidates for the development of new pharmaceutical agents. Over 50% of anticancer drugs approved by United States Foods and Drugs Administration since 1960 derived from natural products (Lammer et al., 2017). A large percentage of natural products have been isolated from a variety of microorganisms. Over 7600 compounds have been isolated from bacteria and almost from *Streptomyces* genus (Keohn et al., 2005). Thus finding metabolites from other bacterial lineages represent new interests for chemists. Among that, lichens are admitted as a rich source of new bacterial lineages and novel bacterial compounds (Suzuki et al., 2015). Therefore, microorganism communities associated with lichens became interesting subjects with a great potential for the production of active natural compounds.

In this thesis, we focus our work on the isolation of bacterial lineages from the lichen *Rhizocarpon geogaphicum*, one of the most popular crustose lichen dwelling on the rock. From the strains isolated, a bacterial species was selected for further work to produce active compounds. Therefore, this thesis is divided into two parts.

In **part I**, a state of the art about lichens detailing the morphology of lichens, metabolites from lichens, bacteria associated with lichens, metabolites from lichen-associated bacteria is introduced. Besides, a general view about *Rhizocarpon geographicum* is described.

Part II reports the achievements of this work and is divided into 6 chapters.

Chapter 1 deals with the isolation of bacterial lineages from the lichen *Rhizocarpon geographicum*.

Chapter 2 gives details about various active metabolites which have been already isolated from strains closer to the isolates. The reasons for the selection of *Paenibacillus odorifer* for the production of metabolites of interest will be given.

Chapter 3 describes the optimization process to find the best conditions to produce active compounds from the culture of *P. odorifer*.

Chapter 4 reports all the metabolites isolated from *P. odorifer*. In this chapter, the results will be displayed either as an article or as a common part of the thesis.

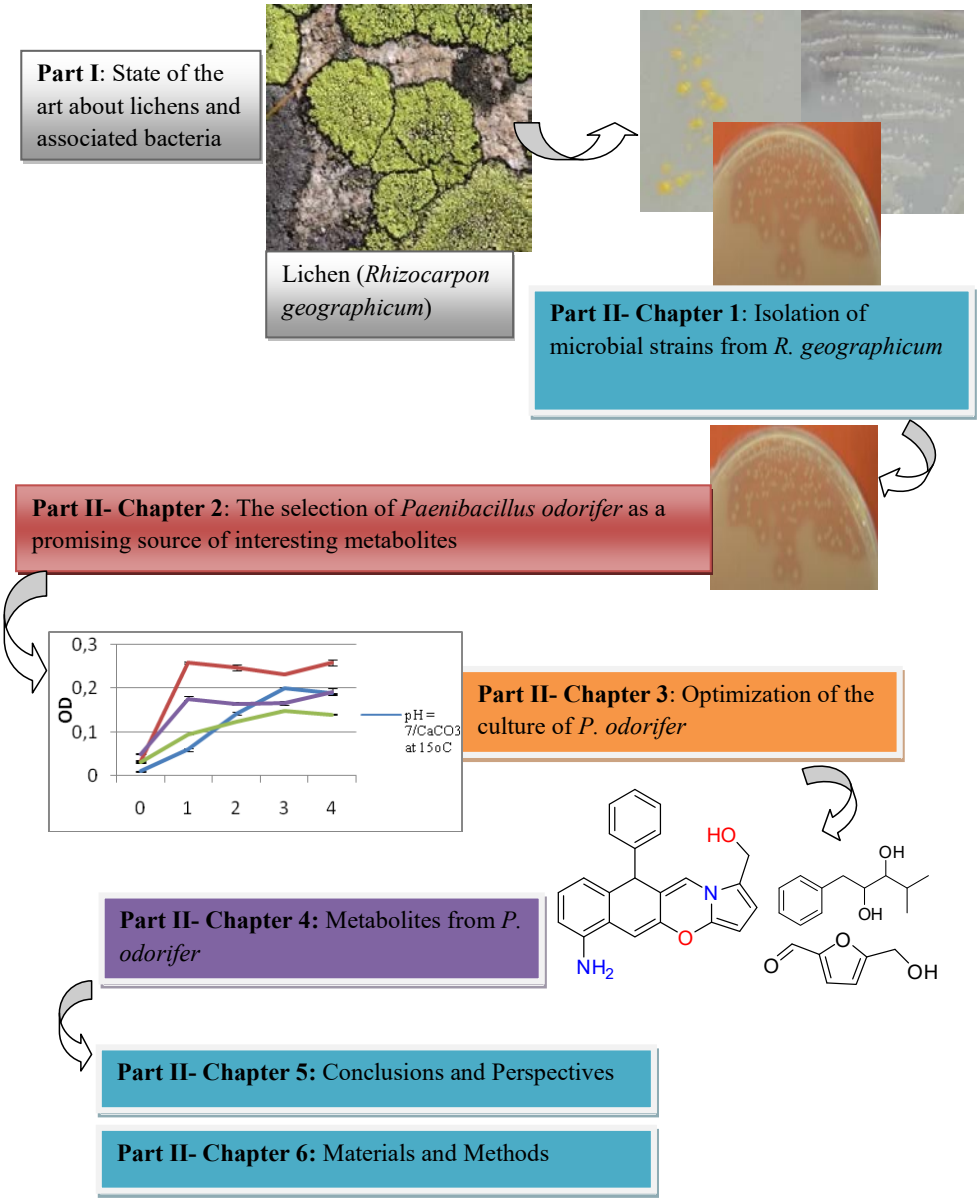
Chapter 5 corresponds to conclusions and perspectives.

Chapter 6 details materials and methods used for this work.

The annexes provide the spectroscopic data of the metabolites isolated.

All strategies were summarized in Scheme 1.

Scheme 1: General strategies used for the study described in the thesis



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PART 1: STATE OF THE ART ON LICHEN-ASSOCIATED BACTERIA

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This general part will be divided into 5 items describing general information on:

- Lichens

- Bacteria associated with lichens and their role in the symbiotic association. This part will report the recent studies focused on the bacterial communities found on lichens.

- Production of metabolites from lichen-associated bacteria.

- Natural cytotoxic compounds produced by bacteria.

- Reasons for the selection of *Rhizocarpon geographicum* as a subject of our research.

1.1. INTRODUCTION ON LICHENS

1.1.1. The lichens

1.1.1.1 Description

Lichen, one of the oldest life-forms, appears in several places on our planet (Grube and Berge, 2009). It can grow on most surfaces in the earth and even amazingly on some extreme environments (Boustie, Tomasi and Grube, 2010). It is estimated that 8% of Earth's land surface is covered by lichens (Grube et al., 2013). Around 18500 distinct lichen species have been characterized and they can adapt themselves to a drastic array of ecological conditions (Shukla et al., 2010). Lichen is a self-support system, a mini-ecosystem with a perfect combination between symbiotic parts including a fungal (mycobiont), green alga and/ or cyanobacterium (photobiont), forming a unique structure called the thallus, but also non-photobiont bacteria (Hodkinson and Lutzoni 2009; Cardinale et al., 2006, 2008; Grube and Berg, 2009). The majority of fungi adopting this lifestyle correspond to ascomycetes and more rarely to basidiomycetes. More recently Spribille and co-workers (2016) have discovered the presence of Cyphobasidiales yeasts in *Bryoria fremontii* and *B. tortuosa* which conducted Pr Grube Martin to define lichens as a "symbiotic network" (personal communication). The photobionts produce carbohydrate by photosynthesis, bacteria provide to lichens nutrients by fixing nitrogen in the atmosphere (Grube et al., 2015), while fungal counterparts supply water, mineral elements and protection for all system

(Parrot et al. 2016a, Benedict 2009) (Figure 1.1). Fungal endophytes have been also described from various thalli (Wang et al., 2016; Park et al., 2015). In this thesis we will not give details on this group of microorganisms existing in lichen thalli.

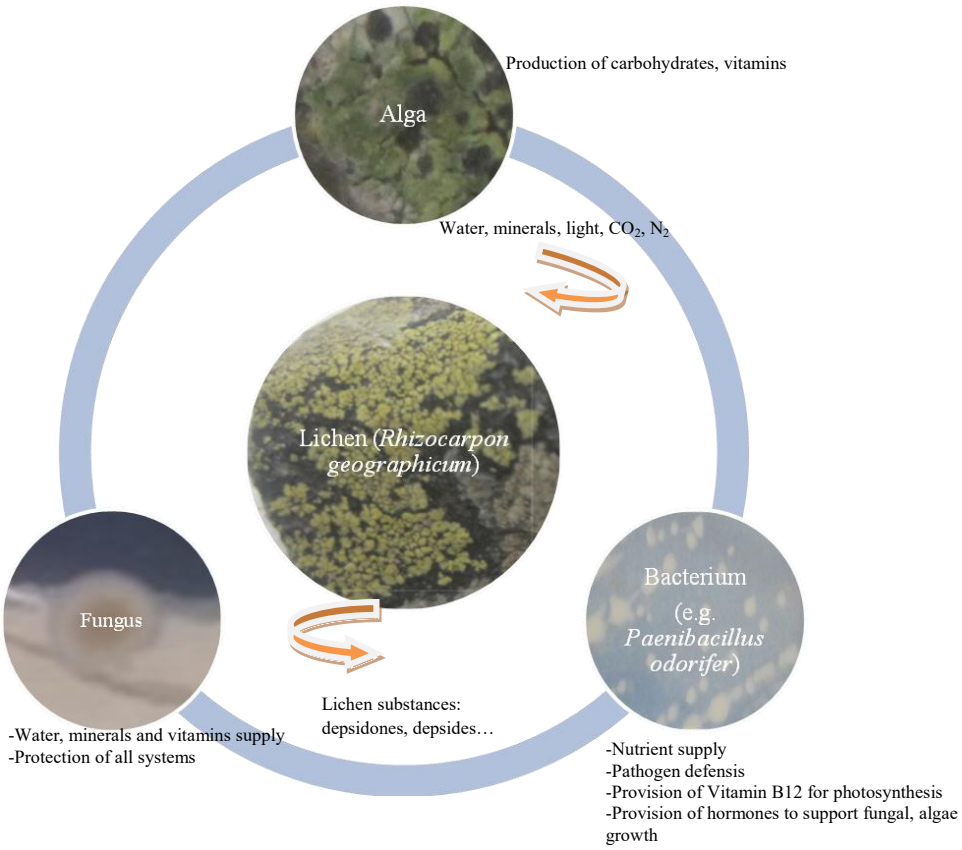


Figure 1.1 Exchange nutrients between lichen symbiotic partners (*R. geographicum* as the model) (adapted from Grube et al., 2015).

1.1.1.2 Morphology

The appearance of the thallus is predominantly determined by the mycobiont and could be divided into three main morphological groups: crustose, foliose and fruticose types (Figure 1.2). An additional type corresponds to the gelatinous thallus which possesses a particular aspect due to the presence of cyanobacteria as photobiont (Büdel and Scheidegger 2008). The identification of lichens is based on their morphological characteristics but also on thalline reactions which correspond to the chemical reaction after addition of various chemical reagents (Huneck and Yoshimura 1996).

There are several tests which are commonly used to identify lichen species; they include the C, Pd, K and KC test. The C test is executed using sodium hypochlorite (NaClO), readily accessible as it is contained in an appropriate dilution in most commercial sources of bleach. The Pd test is performed using the chemical reagent *para*-phenylenediamine. This chemical is known to be carcinogenic but generally accepted as a rather week carcinogen and relatively safe to use for chemical tests on lichens. The K test is carried out with the chemical compound potassium hydroxide. The final test, the KC test, is a combined test where the K test is performed followed by the application of the C test to achieve a stronger reaction (see Table 1.1). These tests are designed to create a chemical reaction when they come into contact with the lichen and are used as color spot tests to identify the various types of lichen. The tests are not always helpful in the identification of a specific lichen species. These thalline reactions correspond to a chemical reaction between one or some lichen compound(s) found on the thallus and the reagent deposited on it. Moreover, depending on the location where the drop of reagent was added (medulla, cortex, apothecia…) the reaction will be different due to the host localization of some lichen compounds (Parrot et al., 2014).

Parmotrema perlatum — Foliose lichen

Usnea subfloridana — Fruticose lichen

Rhizocarpon geographicum — Crustose lichen

Figure 1.2 Types of the lichens

(http://www.lichens.lastdragon.org/faq/lichenthallustypes.html)

Table 1.1 Some lichen subtances which give colour reactions with chemical reagents (Huneck and Yoshimura, 1996)

Chemical Test				**Substances in lichens**
K+	K+ purple			Parietine
	K+ brown-yellow			Fumarprotocetraric acid
	K+ yellow or orange		P+ yellow	Atranorine
			P+ brick red	Physodalic acid
			P+ orange	Stictic acid
	K+ yellow to red		P+ pale yellow	Hypostictic acid
			P+ red	Salazinic acid
K-	C+	C+ red		Gyrophoric acid
		C+ pink		Olivetoric acid
	C-	KC+	KC+ yellow	Usnic acid
			KC+ red	Lobaric acid
			KC+ orange yellow	Barbatic acid
			KC+ red	Norlobaridone
		KC-	P+ red	Alectoric acid
			P+ sulfur yellow	Psoromic acid

1.1.1.3 Metabolites produced by lichen thallus

Metabolites could be divided into two main classes. The first class concerns primary metabolites e.g. proteins, amino acids, carotenoids, polyols, polysaccharides, vitamins which are water-soluble and more often produced by the fungus than by the alga. The second class corresponds to secondary or specialized metabolites most often found on the surface of the hyphae than intracellular. In the Figure 1.3 are reported the biosynthetic pathways of the major groups of lichen metabolites such as moroaromatic phenolic compounds, depsides, depsidones, diphenyl ethers, dibenzofurane derivatives (Elix. and Stocker-Wörgötter 2008); and in Figure 1.4, 1.5 and 1.6 were described the structures of some secondary metabolites from lichens corresponding with the different pathways.

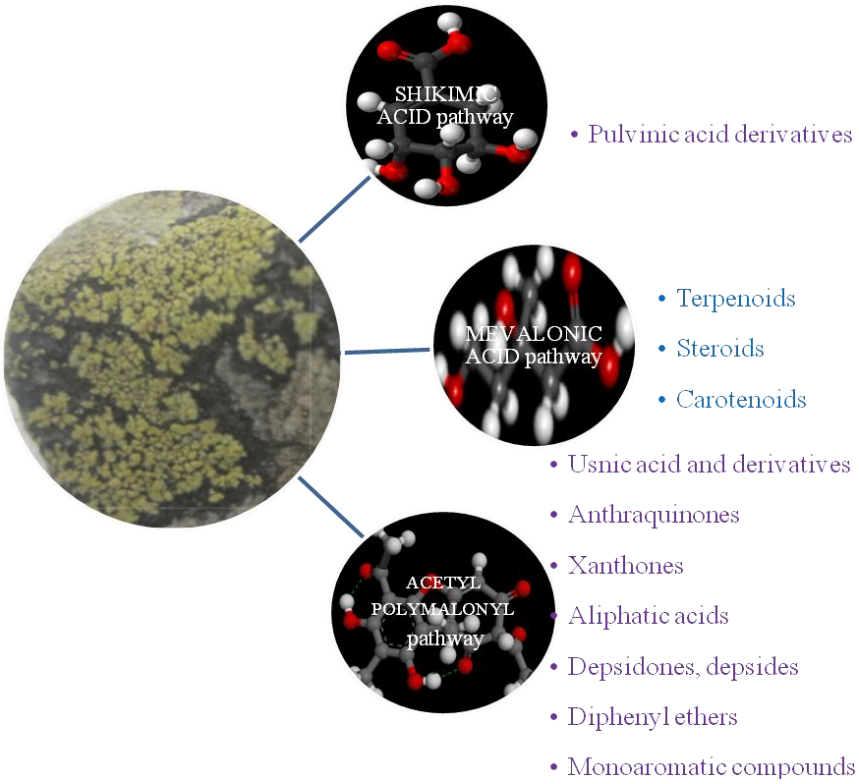


Figure 1.3 Putative pathways of the major groups of lichen metabolites (adapted from Elix and Stocker-Wörgötter 2008)

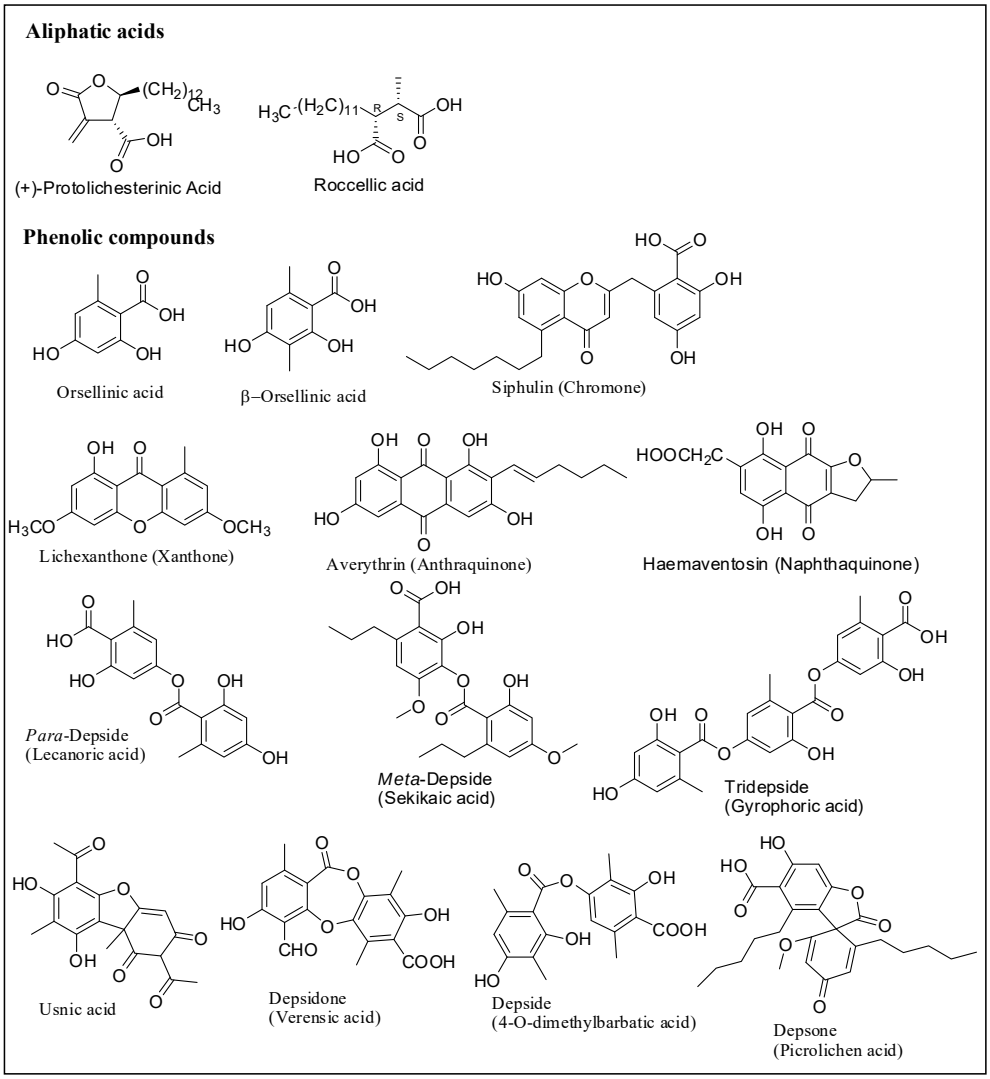


Figure 1.4 Structure of typical lichen products derived from the Acetyl-polymalonyl pathway

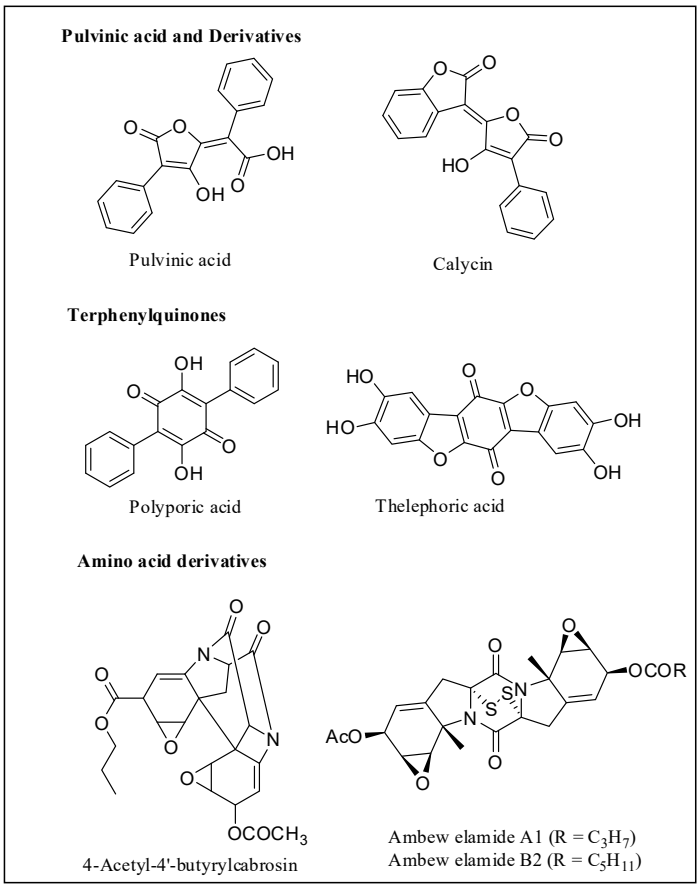


Figure 1.5 Structure of typical lichen products derived from the Shikimic acid pathway

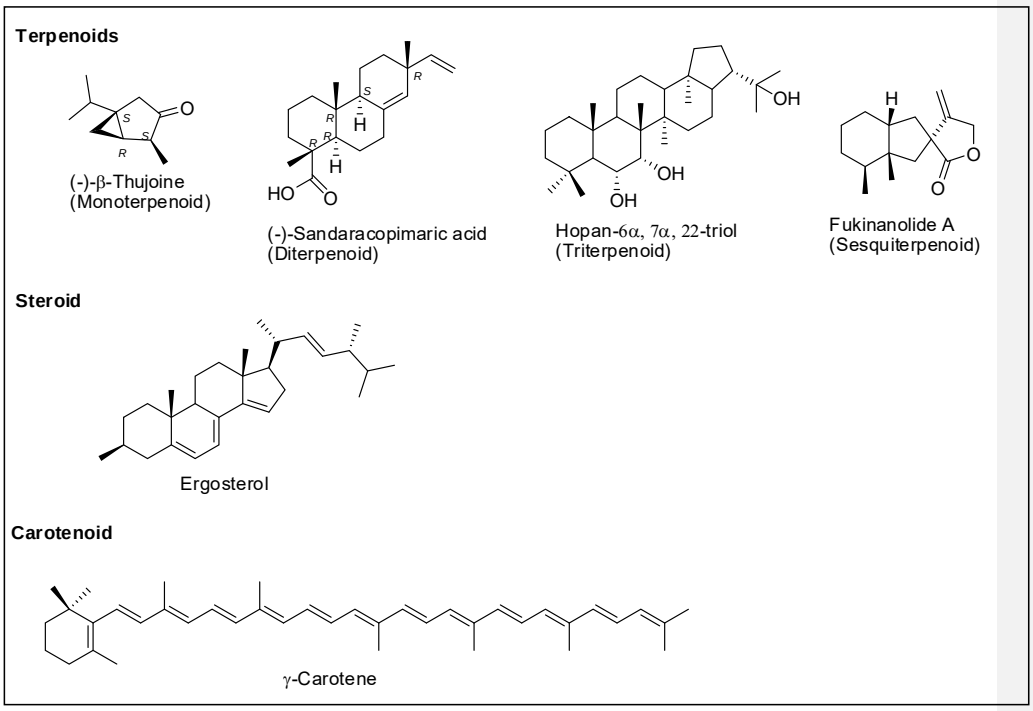


Figure 1.6 Structure of typical lichen products derived from the Mevalonic acid pathway

1.2. BACTERIA ASSOCIATED WITH LICHENS

1.2.1. State of art about bacterial communities on lichens

Although common knowledge dictated that the lichen thallus was formed by a fungus that constructed a symbiotic relationship with an alga and/or a cyanobacterium (photobiont), the non-photobiont bacteria were increasingly considered as an integral component of lichen thalli (Hodkinson et al., 2009). Therefore, the classical view of this symbiotic relationship should be expanded to include bacteria. This concept has taken its origin from researches in the beginning of this century using either cultivation method or cultivation independent method to study these communities. Cardinale and co-workers (2006) has used cultivation methods investigated on eleven different lichen samples collected on different sites and highlighted interesting results about the microorganism communities. More than 100 bacteria were identified belonging to *Firmicutes*, *Actinobacteria* and *Proteobacteria* phylum. Although the dominant community among these

bacterial strains was not presented in this report, *Paenibacilllus* and *Burkholderia* phylotypes were commonly found in almost lichens of this study. A distinct research also reported in the same year (Liba et al. 2006) used cultivation-dependent method with a nitrogen-free minimal medium to screen five species of cyanobacteria-deprive lichens *Canoparmelia caroliniana*, *crozalsiana*, *texana*; *Parmotrema sancti-angeli* and *tinctorum* harvested in San Paulo state (Brazil). The results demonstrated that seventeen strains were isolated and all isolates were belonged to Gamaproteobacteria (*Proteobacteria*) group.

Two years later, Cardinale and co-workers (2008) studied bacterial communities found on the shrub-like reindeer lichen *Cladonia arbuscula*. This research was based on cultivation independent method using general DNA staining and fluorescent *in situ* hydridization (FISH) coupled with confocal laser scanning microscopy (CLSM). This work exhibited that about 6.10^7 bacteria g^{-1} hosted on *C. arbuscula*. The report also showed that the dominance on bacterial communities corresponded to *Alphaproteobacteria* with more than 60% of all bacteria following by *Actinobacteria* and *Betaproteobacteria* phylum (lower than 10% for each). Whereas *Firmicutes* were rarely detected, no *Gammaproteobacteria* was found.

A non-cultivation method using FISH, CLSM and imaging analysis applied on three lichens (*Cladonia arbuscula*, *Lecanora polytropa* and *Umbilicaria cylindrica*) highlighted that the abundance of bacterial colonies was up to 10^8 cells per gram fresh weight (Grube and Berg 2009). This experiment also indicated the predominance of the bacterial communities on these three lichens being *Alphaproteobacteria* followed by *Firmicutes* with *Paenibacillus* cited as an example. Moreover, a different subsequent report from non-culture method carried out on *Cladonia arbuscula* lichens concluded that lichen-associated microbial communities consisted of diverse taxonomic groups and the majority of bacteria belonged to *Alphaproteobacteria* (Grube et al 2009). Another study used a set of PCR-based methods applied on different lichens from several sites in United States to examine the putative microorganism communities associated with lichen thalli. This work has revealed the identity of several bacterial associates consisting of the extremophilic *Acidobacteria*, *Brucellaceae*, and members of an undescribed lineage belonging to the *Rhizobiales* (Hodkinson and Lutzoni 2009). Nevertheless, a distinct research using culture dependent method from thirteen species of lichens collecting in different locations in the United

States highlighted the presence of thirty pure strains from main bacterial lineages as *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Deinococcus-Thermus* (Selbmann et al., 2009).

The next studies published in 2011 by research groups such as Bates et al., Schneider et al. and Bjelland et al. using unculture-based methods led to the results that *Proteobacteria* (*Alphaproteobacteria* or *Betaproteobacteria*) expressed again its dominance in bacterial communities from the studied lichens. It is then noted that a novel *Actinobacterium* belonging to the family *Microbacteriaceae* was first found in lichen *Cladonia arbuscula* via cultivation method and identified by 16S rRNA sequencing technology (Cardinale et al., 2011). This study led to the discovery of a novel member among the microorganism colonies associated with lichens. During the following years, the revolution of discovery on lichen-associated prokaryotic colonies bloomed throughout a series of surveys on many distinct lichens collected in the same site (Cardinale et al., 2012b) or on only lichen species collected at different locations (Cardinal et al., 2012a; Printzen el al., 2012). Most studies used non-culture based methods and they gave the same results about the dominant fraction of *Alphaproteobacteria* in bacteria communities. A different result, however, was provided by the team of Grube and his co-workers (2012b) where *Acidobacteria* was proposed as predominant colonies for all bacterial symbiotic partner of *Solorina crocea* found in the Alpine then followed by *Planctomycetes* and *Proteobacteria*.

Further study of Kim et al. (2012) performed on the Arctic lichen *Stereocaulon sp.* using culture method exhibited the presence of three bacterial members (*Pseudomonas graminis*, *Mucilaginibacter rugui*, *Bosea vestrisii*). The study also specified the antimicrobial properties of these bacteria against six tested bacteria (such as *Staphylococus aureus*, *Bacillus bacillus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*). A different research during this year using cultivation-independent method instead of cultivation method screened abundance of bacterial communities on lichens from Alpine soil crusts and led to the result that *Alphaproteobacteria* and *Acidobacteria* were predominant in these lichen-associated prokaryotic colonies (Muggia et al., 2013).

The most recent report using unculture method compared the lichen-associated bacterial community compositions on both thallus and isidioid soredia of the lichen *Lobaria pulmonaria*. The results showed that *Alphaproteobacteria* were the predominant phylum on both samples followed by *Sphingobacteria* (Aschenbrenner *et al*., 2014). Another study based on culture-based

method investigated on 4 lichen species found in northern Iceland (*Lecanora helicopis*, *Verrucaria ceuthocarpa*, *Hydropunctaria maura* and *Caloplaca verruculifera*) indicated that strains which were found belonged to 7 classes: *Alphaproteobacteria*, *Bacilli*, *Actinobacteria*, *Flavobacteria*, *Cytophagia*, *Sphingobacteria*, and *Gammaproteobacteria* (Sigurbjornsdottir et al. 2014). A report about 20 cultivable bacteria species isolated from the Antarctic lichen *Psoroma* sp. by Kim et al (2014) provided new knowledge about antimicrobial and antioxidant properties as a potential application of these symbiotic partners of lichens.

In summary

A series of researches have been undertaken to affiliate the third symbiotic partners of lichens using well-definite approaches, either unculturable or culturable methods, depending on the purpose of the studies. Each method displayed its advantages and disadvantages. While uncultivable methods led to discover a greater bacterial diversity on lichens, culturable approaches only supported information about cultivable bacterial strains and it can lead to wrong conclusions about the diversity of these microorganisms. A strong point for these cultivable methods, however, is to easily permit to study properties and production of metabolites from isolated bacteria which cannot be performed in non-culture based methods.

The detailed data indicated in Table 1.2 summarized information about bacterial communities (up to genus level) from lichen species with their names noted in alphabetical order. One lichen species can be studied by either a team or many teams. The methods used in these studies were noted clearly as cultivation-dependent or the independent one. From 52 lichen species mentioned in Table 1.2, *Proteobacteria* always exhibited as dominant phylum (with 39.5%) in a total of 119 genera reported. This value was over more twice than those of *Acidobacteria* (16.8%) and *Firmicutes* (15.1%). These data permit to conclude that three phyla were commonly presented in bacteria associated with lichens. In the dominant phylum *Proteobacteria*, the class present in the most cases was *Alphaproteobacteria*, following by *Gammaproteobacteria* and *Betaproteobacteria*, while *Deltaproteobacteria* rarely appeared from this phylum. The other phyla sometimes found in lichens were *Actinobacteria* (with 10.7%) and *Bacteroidetes* (about 5.8%). These two bacterial phyla were often present in foliose and crustose lichens. Indeed, *Actinobacteria* were found in the total of 11 lichen species consisting of 8 foliose lichens, 3 crustose lichens, 3 placodioid lichens and only one fructicose lichen; *Bacteroidetes* were found in 7 lichen species consisting of 3 foliose

lichens, 3 crustose lichens and 1 placodioid lichen. In addition, diverse bacterial phyla as *Deinococcus-thermus*, *Verrucomicrobia* … were presented in a little part from lichens. It is finally to note that, an ancient phylum also reported as a partner with lichens was *Chloroflexi* found from the following lichens *Ophioparma ventosa*, *Pertusaria corallina* and *Rhizocarpon geographicum*.

Table 1.2 Summary of bacterial communities associated with lichens

Lichen	Bacterial communities			Method	References
	Phylum	**Class**	**Family or genus**		
Arthrorhaphis citrinella	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria*		Cultivation independent method (FISH/CLSM)	Muggia et al., (2013)
Caloplaca verruculifera	*Proteobacteria* *Bacteroidetes* *Actinobacteria*	*Alphaproteobacteria* *Gammaproteobacteria* *Flavobacteria* *Sphingobacteria* *Actinobacteria*	*Jannaschia pohagensis/* *Sphingopysix marina* */S. desiccabilis/Aurantimonas coralicida/* *Aurantimonas kwanggyangensis/* *Sphingomonas suberifaciens* *Psychrobacter frigidicola.* *Micrococcus luteus/ Micrococcus flavus* *Aquimarina intermedia* *Lewinella antartica*	Cultivation dependent method	Sigurbjörnsdóttir et al., (2014)
Canoparmelia caroliniana	*Proteobacteria*	*Gammaproteobacteria*	*Stenotrophomonas maltophilia*	Cultivation dependent method	Liba et al. (2006)
Canoparmelia crozalsiana	*Proteobacteria*	*Gammaproteobacteria*	*Stenotrophomonas maltophilia/ Serratia marcescens*	Cultivation dependent method	
Canoparmelia texana	*Proteobacteria*	*Gammaproteobacteria*	*Serratia marcescens/ Pseudomonas stutzeri*	Cultivation dependent method	
Cetraria islandica	*Proteobacteria* *Actinobacteria* *Firmicutes*	*Alphaproteobacteria* *Betaproteobacteria* *Gammaproteobacteria* *Actinobacteria*		Cultivation independent method (FISH/CLSM)	Cardinale et al. (2012a) Grube et al. (2009)
Cetraria sp.	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonas faebi*	Cultivation dependent method	Kim et al. (2012)

Cetraria aculeata	*Proteobacteria*	*Alphaproteobacteria*		Cultivation independent (DNA/PCR)	Printzen et al. (2012)
Cladonia arbusculla	*Proteobacteria* *Firmicutes*	*Alphaproteobacteria* *Betaproteobacteria* *Bacilli*	*Burkholderia* sp./ *Paenibacillus* *Bacillus*	Cultivation independent (SSCP)	Grube et al.,(2009); Cardinale et al. (2012a)
Cladonia coccifera	*Proteobacteria* *Firmicutes*	*Alphaproteobacteria* *Betaproteobacteria* *Deltaproteobacteria*	*Burkolderia phenazinium* *Paenibacillus amylolyticus/* *Paenibacillus agarexedens*	Cultivation dependent ITS (Cultivation independent method)	Cardinale et al. (2006); Cardinale et al. (2012a)
Cladonia cristatella	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*	Cultivation independent method (16S rRNA, PCR)	Hodkinson et al., (2009)
Cladonia cryptochlorophaea	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*		
Cladonia peziziformis	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*		
Cladonia gracilis	*Actinobacteria*	*Actinobacteria*	*Streptomyces* sp.	Cultivation dependent method (16S rRNA)	Cheenpracha et al., (2010)
Cladonia sp.	*Proteobacteria*	*Betaproteobacteria*	*Burkholderia sordidicola*	Cultivation dependent method	Kim et al. (2012)
Cladonia pyxidata	*Proteobacteria*	*Betaproteobacteria*	*Burkholderia glathei/ Burkholderia sordidicola*	Cultivation dependent method	Cardinale et al., (2006)
Cladonia rangiferina	*Proteobacteria* *Firmicutes*	*Betaproteobacteria* *Bacilli*	*Burkholderia glathei/ Burkholderia sordidicola* *Paenibacillus pabuli*	Cultivation dependent method	
Cladonia subtenuis	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*	Cultivation dependent method (16S rRNA, PCR)	Hodkinson et al., (2009)
Cladonia uncialis	*Actinobacteria*	*Actinobacteria*	*Streptomyces uncialis*	Cultivation dependent method	Davies et al., (2005), Williams et al. (2008)
Flavoparmelia	*Proteobacteria*	*Alphaproteobacteria*	*Acetobacteriacae*	Cultivation	Hodkinson et al.

caperata	*Acidobacteria*	*Gammaproteobacteria*	*Brucellaceae* *Methylobacterium*	independent method (16S rRNA, PCR)	(2009)
Hydropunctaria maura	*Proteobacteria* *Bacterioidetes* *Actinobacteria* *Firmicutes* *Crenarchaeota* *Deinococcus-thermus*	*Alphaproteobacteria* *Gammaproteobacteria* *Sphingobacteria* *Bacilli* *Actinobacteria*	*Jannaschia helgolandensis/ J. pohangensis/J. donghaensis* *Altererythrobacter luteolus,* *Aurantimonas anganoxydans* *Psychrobacter namhaensis,* *Pseudoalteromonas paragorgicola* *Lewinella marina* *Bacillus aerius/ B. safensis/ B. idriensis* *Deinococcus Micrococcus flavus*	Cultivation independent method (DGGE-based DNA fingerprinting) Cultivation dependent method	Bjelland et al., (2011) Sigurbjörnsdóttir et al., (2014)
Hypogymnia physodes	*Firmicutes*	*Bacilli*	*Paenibacillus pabuli/ Paenibacillus amylolytocus*	Cultivation dependent method	Cardinale et al., (2006)
Icmadophila ericetorum	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria*		Cultivation independent method (FISH/CLSM)	Muggia et al., (2013)
Lasallia pensylvanica	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*	Cultivation independent method (16S rRNA, PCR)	Hodkinson et al. (2009)
Lecanora helicopis	*Actinobacteria* *Proteobacteria* *Firmicutes* *Bacterioidetes*	*Actinobacteria* *Gammaproteobacteria* *Bacilli* *Flavobacteria* *Cytophagia*	*Streptomyces microflavus,* *Salinibacterium amurskyense,* *Agrococcus baldri, A. antarcticus, A. jenensis,* *Micrococcus antarcticus* *Psychrobacter faesalis* *Staphycoccus cohnii, Salinicoccus roseus,* *Bacillus asahii.* *Eudoraea adriatica* *Hymenobacter actinosclerus*	Cultivation dependent method	Sigurbjörnsdóttir et al.,(2014)
Lecanora polytropa	*Proteobacteria*	*Alphaproteobacteria*	*Burkholderia sp. Paenibacillus*	Cultivation	Grube et al. (2009)

	Firmicutes	*Betaproteobacteria* *Gammaproteobacteria* *Deltaproteobacteria*	*Bacillus/ Acinebacter* sp.	independent method (SSCP)	Cardinale et al. (2012a)
Lecanora fuscobrunnea	*Actinobacteria* *Proteobacteria* *Firmicutes* *Deinoccocus-thermus*	*Actinobacteria*		Cultivation independent method	Selbmann et al., (2009)
Lobaria pulmonaria	*Proteobacteria* *Bacterioidetes* *Actinobacteria* *Cyanobacteria*	*Alphaproteobacteria* *Betaproteobacteria* *Gammaproteobacteria* *Deltaproteobacteria* *Sphingobacteria* *Actinobacteria* *Verrucomicrobia*	*Rhizobiles/* *Methylobacteriaceae/Bradyrhizobiaceae/* *Rhizobiaceae/ Xanthobacteraceae/* *Beijerinckiaceae/ Phyllobacteriaceae* *Sphingomonadaceae* *Caulobacterales* *Rhodospirillales* *Rhodobacterales* *Burkholderiales* *Myxococales, Polyangiaceae,* *Cystobacterineae* *Chitinophagaceae, Sphingobacteriaceae,* *Flexibacteraceae* *Pseudonocardiaceae, Frankiaceae,* *Microbacteriaceae,* *Micromonosporaceae* *Nostocaceae* *Chthoniabacteraceae*	Cultivation independent method (FISH/CLSM)	Cardinale et al. (2012a), Aschenbrenner et al. (2014), Grube et al., (2015)
Ophioparma ventosa	*Proteobacteria* *Chloroflexi* *Acidobacteria*	*Betaproteobacteria* *Gammaproteobacteria*		Cultivation independent method (DGGE, PCR)	Bjelland et al., (2011)
Parmelia sulcata	*Proteobacteria* *Firmicutes* *Actinobacteria* *Acidobacteria* *Verrucomicrobia*	*Alphaproteobacteria* *Actinobacteria* *Acidobacteria* *Planctomycetes*	*Rhodospirillales* *Rhizobiales* *Sphingomonas spp* *Actinomycetales bacterium* *Terriglobus roseus, Acidobacterium capsulatum*	Cultivation independent method (PCR ,16S rRNA genes and bar-coded pyrosequencing)	Bates et al. (2011)

			Nostocoida limicola *Rubritalea spp.*		
Parmotrema perforatum	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*	Cultivation independent method (16S rRNA, PCR)	Hodkinson et al. (2009) Liba et al. (2006)
Parmotrema sancti-angeli	*Proteobacteria*	*Gammaproteobacteria*	*Serratia marcesens/ Acinetobacter calcoaceticus*		
Parmotrema tinctirum	*Proteobacteria*	*Gammaproteobacteria*	*Serratia marcesens/ Pseodomonas sp.* *Stenotrophomonas maltophilia/ Pentoea sp.*		
Pertusaria corallina	*Proteobacteria* *Acidobacteria* *Chloroflexi*	*Alphaproteobacteria* *Betaproteobacteria*		Cultivation independent method(DGGE, PCR)	Bjelland et al. (2011)
Peltigera membranacea	*Proteobacteria* *Actinobacteria* *Bacteriodetes*	*Alphaproteobacteria* *Betaproteobacteria*		Cultivation independent (Pyrosequencing)	Grube et al. (2014)
Peltigera phyllidosia	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteriacae* *Brucellaceae* *Methylobacterium*	Cultivation independent (16S rRNA, PCR)	Hodkinson et al. (2009)
Pseudevenia furfuracea	*Firmicutes*	*Bacilli*	*Paenibacillus mendilii/ Paenibacillus phyllosphaerae*	Cultivation dependent	Cardinale et al. (2006)
Psora decipiens	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria*		Cultivation independent method(FISH/CLSM)	Muggia et al. (2013)
Rhizocarpon geographicum	*Proteobacteria* *Acidobacteria* *Chloroflexi*	*Alphaproteobacteria* *Betaproteobacteria*		Cultivation independent method (DGGE, PCR)	Bjelland et al (2011)
Rhizocarpon chrysoleuca	*Proteobacteria* *Acidobacteria* *Firmicutes* *Verrucomicrobia*	*Alphaproteobacteria* *Acidobacteria* *Firmicutes* *Verrucimicrobia* *Planctomycetes*	*Rhodospirillales/ Acetobacteria/Acidiphilum sp* *Rhizobiales* *Sphingomonas spp.* *Acidobacteriales/Terriglobus roseus*	Cultivation independent method (Pyrosequencing)	Bates et al. (2011)

			Rubritalea spp. *Nostocoida limicola* *Desulfotomaculum sp.*		
Solorina crocea	*Acidobacteria* *Planctomycetes* *Proteobacteria*	*Acidobacteria* *Planctomycetes* *Alphaproteobacteria*	*Acidobacterium sp./Edaphobacter* *Isosphaera sp./Gemmata* *Novosphingobium* *Sphingomonas*	Cultivation independent method	Grube et al. (2012a)
Stereocaulon sp.	*Proteobacteria*	*Betaprotebacteria*	*Burkholderia sordidicola*	Cultivation dependent method	Kim et al. (2012)
Trapeliopsis granulosa	*Proteobacteria* *Acidobacteria*	*Alphaproteobacteria*		Cultivation independent method (FISH/CLSM)	Muggia et al. (2013)
Umbilicaria americana	*Proteobacteria* *Acidobacteria* *Actinobacteria* *Firmicutes* *Bacteroidetes*	*Alphaproteobacteria* *Acidobacteria* *Actinobacteria* *Firmicutes* *Bacteroidetes*	*Rhodospirillales/ Acidiphilum sp.* *Phenylobacterium spp.* *Sphingomonas spp.* *Acidobacteriales/Terriglobus roseus* *Actinomycetsles bacterium* *Desulfotomaculum sp.* *Sphingobacteria/Pedobacter solani*	Cultivation independent method (pyrosequencing)	Bates et al. (2011)
Umbilicaria cylindrica	*Proteobacteria* *Firmicutes*	*Alphaproteobacteria* *Betaproteobacteria* *Gammaproteobacteria* *Bacilli*	*Acetobacteraceae* *Burkholderia sp.* *Acinebacter* *Bacillus sp.*	Cultivation independent method (SSCP)	Grube et al. (2009), Cardinale et al. (2012a)
Umbilicaria decussata	*Actinobacteria* *Proteobacteria* *Firmicutes* *Deinococcusthermus*	*Actinobacteria*	*Actinomycetales/ Intrasporangiaceae* *Knoellia*	Cultivation dependent method	Selbmann et al. (2009)
Umbilicarria phaea	*Proteobacteria* *Acidobacteria* *Firmicutes* *Planctomycetes*	*Alphaproteobacteria* *Acidobacteria* *Firmicutes* *Planctomycetes*	*Rhodospirillales/Acidiphilum sp.* *Acidobacterium apsulatum* *Desulfotomaculum sp.* *Nostocoida limicola*	Cultivation independent method (Pyrosequencing)	Bates et al., (2011)
Umbilicaria mammulata	*Acidobacteria* *Proteobacteria*	*Acidobacteria* *Alphaproteobacteria* *Gammaproteobacteria*	*Acetobacteraceae* *Brucellaceae* *Methylobacterium*	Cultivation independent method (16s rRNA, PCR)	Hodkinson et al. (2009)

Umbilicaria sp.	*Proteobacteria*	*Betaproteobacteria*	*Burkholderia sordidicola*	Cultivation dependent method	Kim et al. (2012)
Usnea antarctica	*Actinobacteria* *Proteobacteria* *Firmicutes* *Deinococcusthermus*	*Actinobacteria* *Gammaproteobacteria*	*Micrococcaceae/Arthrobacter* *Pseudomonadaceae/Pseudomonas*	Cultivation dependent method	Selbmann et al. (2009)
Xanthoria elegans	*Actinobacteria* *Proteobacteria* *Firmicutes* *Deinococcusthermus*	*Gammaproteobacteria* *Actinobacteria* *Firmicutes*	*Pseudomonadaceae/Pseudomonas* *Mycobacterriun* *Paenibacillus* *Bacillus*		
Verrucaria ceuthocarpa	*Proteobacteria* *Actinobacteria* *Firmicutes* *Bacteroidetes*	*Alphaproteobacteria* *Gammaproteobacteria* *Actinobacteria* *Bacilli* *Bacteroidetes*	*Sphingopysix baekryungensis* *Loktanella salsilacus* *Altererythrobacter luteolus* *Psychrobacter maritimus* *Micrococcus antarcticus* *Micrococcus luteus* *Bacillus murimartini* *Bacillus safensis* *Rhodothermus marinus*	Cultivation dependent method	Sigurbjörnsdóttir et al., (2014)

1.2.2. The roles of bacteria

Bacterial communities were identified as stable, specific and structurally integrated partners of the lichen symbiosis, but their role has remained largely elusive in comparison to the well-known functions of the fungal and algal partners. One of the first studies to question about the roles of the bacterial communities in lichens was those of Grube (2015) which found that more than 800 bacterial species have the ability to contribute multiple aspects to the symbiotic system, including essential functions such as (i) nutrient supply, especially nitrogen, phosphorous and sulfur, (ii) resistance against biotic stress factors (pathogen defenses as an example), (iii) resistance against abiotic factors, (iv) support of photosynthesis by provision of vitamin B12, (v) fungal and algal growth support by provision of hormones, (vi) detoxification of metabolites, and (vii) degradation of older parts of the lichen thallus (Grube et al., 2015).

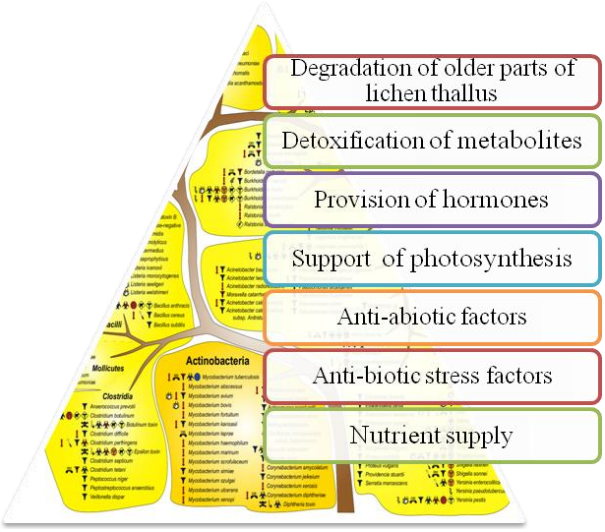


Figure 1.7 Roles of bacteria in lichens (adapted from Grube et al., 2015)

1.3. METABOLITES FROM THE LICHEN-ASSOCIATED BACTERIA

As lichens are a promising source of bioactive metabolites (Boustie and Grube, 2005), bacteria from lichens possess an unexploited potential of effective new metabolites (Grube et al., 2012b). Recent investigations focused on a strain of *Streptomyces* isolated from the lichen *Cladonia uncialis*. The extracts from the broth of the cultivation of this strain produced a series of novel active compounds. Among them, uncialamycin, an enediyne compound (Davies et al., 2005) exhibited a strong antibacterial activity against the human pathogens *Burkholderia cepacia* and *Staphylococcus aureus* but also as potent cytotoxic against MCF-7 cells. This strain also produces seven new bis-indole alkaloids, named cladoniamides, which possess an unprecedented skeleton in natural products (Williams et al., 2008). In these alkaloids, cladoniamide G presented a potential as the anticancer compound by its cytotoxicity against human breast cancer MCF-7 cells in vitro at 10 µg/mL. Further investigations on this strain in 2015 (Williams et al., 2015) yielded a new compound, unciaphenol, resulted from the expected Bergman cycloaromatization of uncialamycin. It is interesting that the unciaphenol presented in vitro anti-HIV activity against drug-resistant isolates of the virus. Another lichen-derived *Streptomyces* strain (*Streptomyces* sp.) from unidentified lichen in Japan produced a new angucycline which also displayed inhibitory effects on certain cell lines and on bacterial strains and a new butenolide (Motohashi et al., 2010). Another research on *Streptomyces caeruleus* obtained from the lichen *Cladonia gracilis* afforded six compounds, coumabiocins A-F, which exhibited significant inhibition activity against *Streptomyces*-85 using agar diffusion assay (Cheenpracha et al., 2010).

Moreover, a culture of bacterium *Streptomyces cyaneofuscatus* isolated from a marine lichen *Lichina confinis* yielded a new bioactive compound cyaneodimycin (Parrot et al., 2016b). This compound showed activity against human keratinocyte HaCaT and murine melanoma B16 cell lines [Inhibitory concentration (IC_{50}) values of 47 ± 11 and 27 ± 4 µM, respectively]. A potent cytotoxic compound *N*-methyldactinomycine derived from the known anticancer drug actinomycine D was also isolated from this same species. Although diketopiperazines, also found from this species, did not display noticeable cytotoxic properties, they seem becoming as chemical signals for lichen-associated bacteria. They were not only found from the strain cited above (Parrot

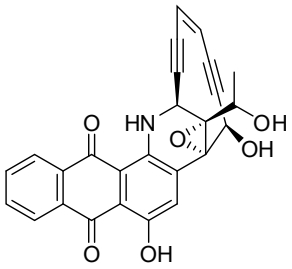
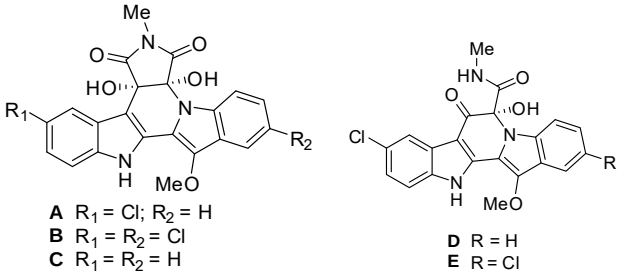
et al. 2016b), but they also are produced by another lichen-associated actinobacterium as *Nocardia sp* associated to *Collema auriforme* (Noël el al., 2017).

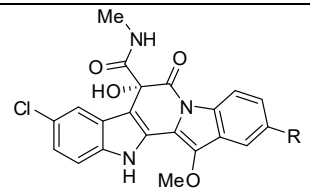
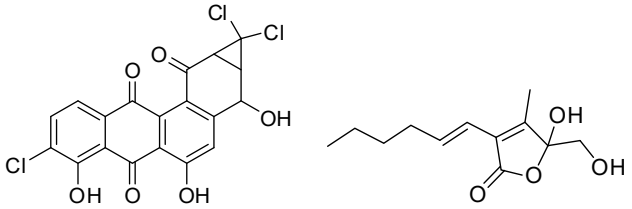
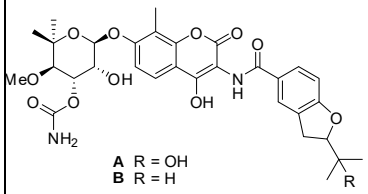
IN SUMMARY

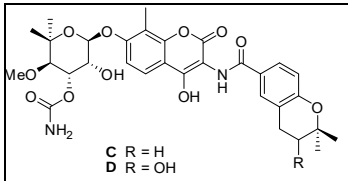
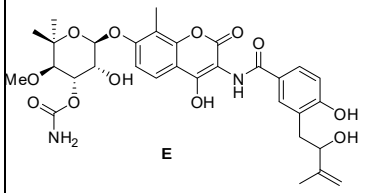
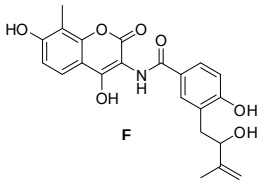
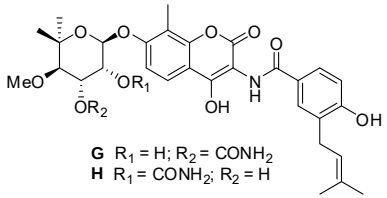
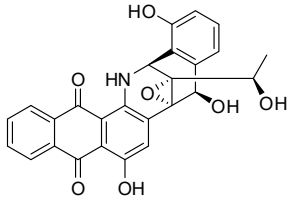
Up to now, the rare studies on bioactive metabolites from lichen-associated bacteria have almost focused on *Streptomyces* strains isolated from *Cladonia* genus or *Lichina* sp. That is one of the reasons that encouraged us to investigate another type of lichen to study its bacterial symbiotic communities and to study the interesting metabolites produced by one of these strains. The reasons for the selection of the bacterial species will be displayed in part 1.5. The bacteria belonging to *Streptomyces* genus, isolated from different natural origins, are an important source to produce bioactive metabolites such as antibiotic, antitumor and immunosuppressant drugs (Lucas et al., 2013). Around two-thirds of all known natural antibiotics are produced from these kinds of bacteria which belong to *Actinobacteria* phylum. *Streptomyces* genus from lichens confirmed again their crucial roles via antibacterial properties, cytotoxicity, or even, anti-HIV virus isolates.

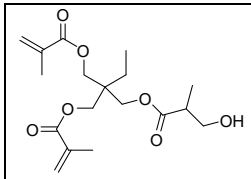
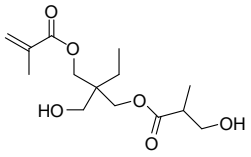
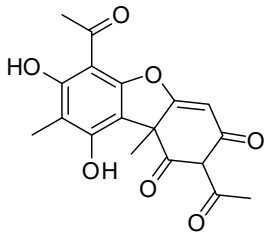
The Table 1.3 cited below showed the summary of metabolites isolated from lichen-associated bacteria. The information including origin, structure and biological activities were displayed in Table 1.3 organized following the year of publication.

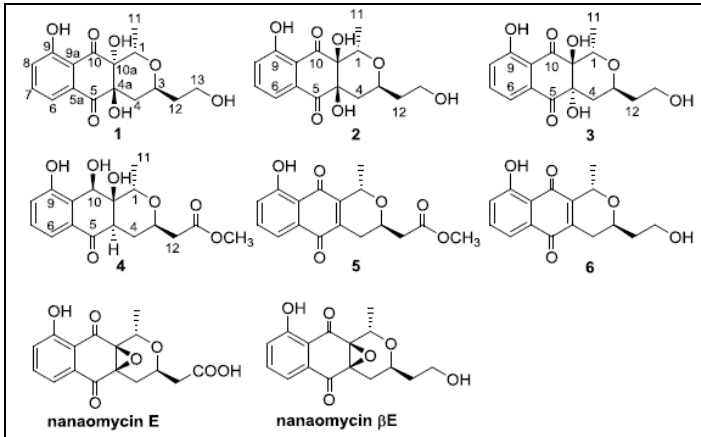
Table 1.3 Summary of metabolites from lichen-associated bacteria

Compounds	Biological activities	Origin		References
		Bacterial species	**Lichen species**	
➢ Uncialamycin 	Antibacterial activity against *Burkholderia cepacia* (MIC 0.001 µg/mL). *Staphylococcus aureus* ((MIC 0.0000064 µg/mL), *Escherichia coli* (MIC 0.002 µg/mL)	*Streptomyces uncialis*	*Cladonia uncialis*	Davies et al., 2005
➢ Cladoniamide A-G  **A** R_1 = Cl; R_2 = H **B** R_1 = R_2 = Cl **C** R_1 = R_2 = H **D** R = H **E** R = Cl	Cytotoxicity against human breast cancer MCF-7 cells (for cladoniamide G) in vitro at 10 µg/mL	*Streptomyces uncialis*	*Cladonia uncialis*	Williams et al., 2008

 **F** R = H **G** R = Cl				
➢ Angucyline derivative Butenolide derivative 	Angucyline: - cytotoxic activity against human cervical carcinoma Hela (IC_{50} values of 36 µM) and human malignant pleural mesothelioma ACC-MESO-1 cell lines (IC_{50} of 52 µM) - Antibacterial against *Micrococcus luteus*, at 25 µg.	*Streptomyces sp.*	Unidentified lichen	Motohashi et al., 2010
➢ Coumabiocins A – F  **A** R = OH **B** R = H	Coumabiocins A-E : significant inhibition activity against *Streptomyces* 85E at concentration of 10 µg Coumabiocin F : inactivity	*Streptomyces caeruleus*	*Cladonia gracilis*	Cheenpracha et al., 2010

 **C** R = H **D** R = OH  **E**  **F** ➢ Novobiocin (G) and isonovobiocin (H)  **G** R_1 = H; R_2 = $CONH_2$ **H** R_1 = $CONH_2$; R_2 = H	Novobiocin : potent inhibition activity Isonovobiocin : weaker activity			
➢ Unciaphenol 	in vitro anti-HIV activity against drug-resistant isolates of the virus.	*Streptomyces uncialis*	*Cladonia uncialis*	Williams et al., 2015
➢ Cyaneodimycin Cyaneomycin	Cyaneodimycin : cytotoxic	*Streptomyces*	*Lichina*	Parrot et al.,

  ➢ Diketopiperazines : Cyclo-(L-Phe-L-Pro); Cyclo-(L-Leu-L-Pro) ➢ (3-hydroxyacetyl)indole ➢ *N*-methyldactinomycin ➢ Usnic acid 	against human keratinocyte HaCaT (IC_{50} of 47±11 µM), murine melanoma B16 (IC_{50} of 27±4 µM) and leukemic (Jurkat) cell lines (IC_{50} = 18.5±0.5 µM). ➢ *N*-methyldactinomycin cytotoxic activities (IC_{50} ≈ 0.05 µM) against various normal or cancer cell lines after 48 h of incubation	*cyaneofuscatus*	*confinis*	2016b
➢ Diketopiperazines : cyclo (L-Pro-L-OMet), cyclo (L-Pro-L-Tyr), cyclo (D-Pro-L-Tyr), cyclo (L-Pro-L-Val), cyclo (L-Pro-L-Leu), cyclo (D-Pro-L-Br-Tyr), cyclo (L-Pro-L-Br-Tyr) ➢ Indole-carboxaldehyde	No cytotoxic activities	*Nocardia ignorata*	*Collema auriforme*	Noël et al., 2017
Nanaomycin	Compounds **1**- **4**: inactive	*Streptomyces*	*Lepidostroma*	Liu et al.,

	antibacterial at concentration of 100 µg/mL. Compounds **5-6** : antibacterial activities against *Staphylococcus aureus, Candida albicans* and *Bacillus subtilis* with MIC values ranging from 3.13 to 100 µg/mL.	*hebeiensis* *Lepidostroma* sp.,	sp.,	2017

1.4. FOCUS ON *RHIZOCARPON GEOGRAPHICUM*

1.4.1. Description of *Rhizocarpon* genus

Rhizocarpon is a large lichen genus containing approximately 200 species worldwide. They dwelled mainly on siliceous rocks; a few are the parasite of other lichens. *Rhizocarpon* is green to yellow-green, white, grey, brown or rust-red, rarely sorediate or blastidiate. Its prothallus usually appears with black color or sometimes white to brown-grey (Benedict 1998). This cosmopolitan kind of lichen is generally found in temperate, Arctic and Antarctic areas (Ihlen et al., 2004).

The *Rhizocarpon* genus is divided into two large sub-genera *Phaeothallus* and *Rhizocarpon* based on the presence or absence of rhizocarpic acid (Innes, 1985; Benedict, 2009), but this classification has recently been questioned based on genetic studies (Ihlen & Ekman, 2002). The subgenus *Rhizocarpon* can be further subdivided into four sections: *Alpicola, Rhizocarpon, Superficiale* and *Viridiatrum,* based on thallus morphology, spore characteristics, and chemistry of the secondary metabolites found in the medulla (Innes, 1985) (see Table 1.4).

These sections can be broadly subdivided based on the number of cells in a spore (Benedict, 1988). *Superficiale* and *Alpicola* have uniseptate spores (two cells) whereas *Rhizocarpon* and *Viridiatrum* contain pluriseptate (usually muriform of submuriform) spores (several cells) (Innes, 1985). Section *Alpicola* and section *Superficiale* can then be further subdivided by the size of the spores, the hymenial dimensions and colour of the apihymenium. In the sections with pluriseptate spores, *Rhizocarpon* and *Viridiatrum* apihymenium colour and the reaction of the medulla to iodine (turning blue) are common distinguishing characteristics (Benedict, 1988). A cross-section of a *Rhizocarpon* thallus can be found in Figure 1.8 and demonstrates the characteristic of a stratified thallus concluding three parts: upper cortex, alga layer and medulla part.

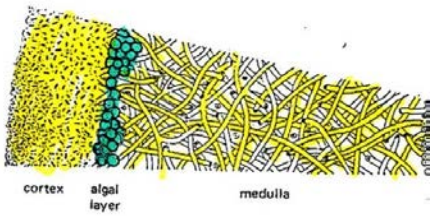


Figure 1.8: Scheme of the cross section of *R. geographicum*

http://www.imlichenit.com/imlichenit/about.html)

Table 1.4 Classification of Rhizocarpon subgenus **(Innes, 1985)**

Genus	Section	Species
Rhizocarpon	*Superficiale*	*R. dispersum* Runem. *R. ejjiguratum* (Anzi) Th.Fr. *R. norvegicum* Ras. *R. parvum* Runem. *R. pusillum* Runem. *R. superjiciale* (Schaer.) Vain
	Alpicola	*R. alpicola* (Hepp.) Rahb. *R. eupetraeoides* (Nyl.) 810mb. *R. inarense* (Vain.) Vain.
	Viridiatrum	*R. atrovirellum* (Nyl.) Zahlbr. *R. kakurgon* Poelt *R. lusitanicum* (Nyl.) Arnold *R. oportense* (Vain.) Ras. *R. subtile* Runem. *R. tetras porum* Runem. *R. viridiatrum* (Wulf.) Koerb
	Rhizocarpon	*R. atroglavescens* Lynge *R. carpaticum* Runem. *R. ferax* H. Magn. *R.furax* Poelt et V. ***R. geographicum* (L.) DC.** *R. intermediellum* Ras. *R. lecanorinum* Anders *R. macros porum* Ras. *R. pulverulentum* (Schaer.) Ras. *R. rapax* V. Wirth et Poelt *R. riparium* Ras. *R. saanense* Ras. *R. sphaerosporum* Ras. *R. sublucidum*

1.4.2. Morphology of *R. geographicum*

The most well-known representative of this genus is perhaps *Rhizocarpon geographicum* (L.) DC (Figure 1.10), well-known as the crustose lichen, grow on the coast areas as indicated in France into the Figure 1.9. It belongs to the *Rhizocarpon* section. The photobiont belongs to Chlorococcoid and is *Trebouxia* sp. The morphology of this lichen is reported in Figure 1.10 and can be described as follows (Frank S. Dobson, 2005)

- Thallus up to 15 cm across, areolate and mosaic-shaped ; presence of angular areolates, 0.2-2 mm diameter, flat to slightly convex, smooth, yellowish, yellow-greenish ; presence of prothallus black distinctly visible at the margin or between the areoles.

- Apothecia irregular to 1-1.5 mm, immersed in the thallus, circular of slightly angular, disc flat to slightly convex, not pruinose, black, exciple variable, rather thick at first but not very distinct at maturity.

- Ascospores brown-black, broardly ellipsoid, muriform with 5-22 cells in section, 20-50 x 10-20 µm.

- Thalline reactions: Medulla: C+/- : red ; Pd+/- : yellow-orange ; K+ (C: Sodium hypochorite ; K: KOH ; Pd : *para*-phenylenediamine)

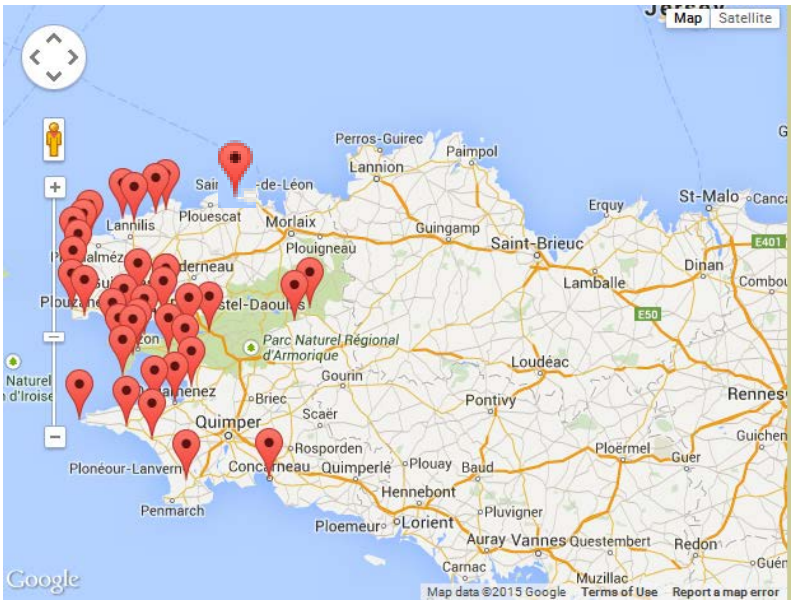


Figure 1.9: Map of the sites of Rhizocarpon *geographicum* in France

(http://www.lichensmaritimes.org)

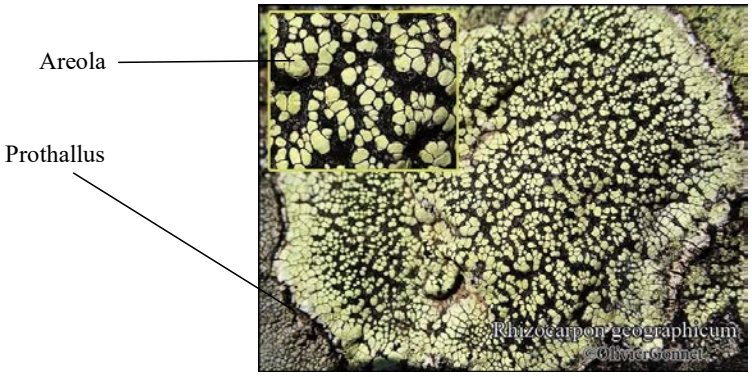


Figure 1.10 Morphology of *Rhizocarpon geographicum*

(by Olivier Gonner at http://www.afl-lichenologie.fr/Photos_AFL/Photos_AFL_R/Rhizocarpon_geographicum.htm)

1.4.3. Studies on *R. geographicum*

In the report of McCathay and Elix (2014), rhizocarpic acid and psoromic acid and barbatic acid were considered major organic compounds from *R. geographicum* originated from Australia. Besides, alectoronic acid, α-collatolic acid, bourgeanic acid, confluentic acid, 2-*O*'-methylperlatolic acid, 2-*O*'-methylanziaic acid and gyrophoric acid were also found in this lichen. The structure of these compounds were shown in Figure 1.11.

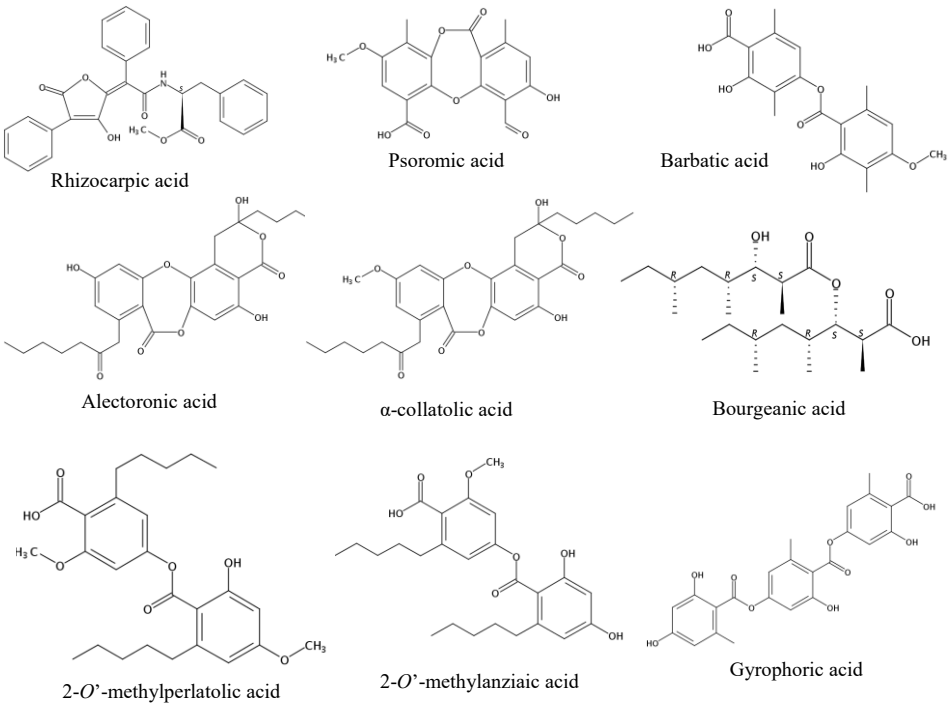


Figure 1.11 Structure of some compounds from *R. geographicum*

This yellow-green species grows very slowly. It is estimated that its annual radial growth rate is from 0.02 to 2.0 mm per year (Armstrong et al., 1996). The exceptionally slow growth rate and longevity of these species have made them especially valuable in lichenometry studies (Innes et al., 1985) and were the focus of many geographical researches (Table 1.5). The lichenometry used the lichen growth to determine the age of exposed rock based on a presumed specific rate of increase in radial size time by time. The Radial Growth Rate (RGR) of *R. geographicum* depended on many environmental factors as temperature, wind, humidity, sunny and geographical regions (Armstrong et al., 2006, Bench et al., 2001, Bradwell et al., 2007, O'Neal et al., 2003). Studies suggested that although a number of parameters affected RGR, the most important influence appeared to be with temperature. The RGR of *R. geographicum* was increased by high temperature and inhibited by low temperature. The maximum growth of *R. geographicum* was period of July-September (Armstrong et al., 2006). The Table 1.5 summarized studies of lichenometry on *R. geographicum* displayed by the location studied, period time, and growth size and the technique used as well.

Author	Location	Study period	Size range (mm)	Technique
Armstrong (1983)	Ordovician slate rock field, Gwynedd, North Wales	1.5 years (6 month intervals)	2-65	Beck Kassal lens (fitted with a 1 cm micrometer with x8 magnification
Proctor (1983)	Valsorey moraine, Switzerland	4 years (2 year intervals)	2-18	Photographs with a 0.05 mm scale micrometer placed next to the lichen
Haworth et al. (1986)	Brooks Range, Alaska	4-6 years	3-107	Tracing thallus area onto acetate and converting to equivalent diameter
McCarthy (2012)	Illecillewaet glacier, GNP, British Columbia	Annual intervals over 4 years	5-50	Digital caliper (+/-0.02mm)
Armstrong (2005)	Cascade mountains, Washington State	6 years	3-102	Lens with x 8 magnification with a 1 cm micrometer scale
Bradwell and Armstrong (2007)	Gigjokuk glacier, Iceland	4.33 years	<78	Field-ruler and 8xhand lens (precision is probably 0.5mm)

Bradwell (2010)	Lochiniver, Scotland	Churchyard-5 years Mill-7 years	2-28 2-43	Digital camera and Adobe Photoshop 8.0
McCarthy (2012)	Selkirk Mountains of Canada's glacier, British Columbia	3-7 years	<5	Adobe, photoshop CS3 extended software

Table 1.5 Summary of studies on *Rhizocarpon geographicum*

In summary, most studies on *R. geographicum* were focus on its lichenometry and some metabolites were presented from it. This species is promising as interesting objective to discover.

1.4.4. Bacteria communities of *R. geographicum*

As mentioned above, almost all studies on this lichen species mainly focused on its characteristic for lichenometry. However, a few rare investigations about bacterial communities on *R. geographicum* were conducted and they only applied cultivation independent method to discover these microorganisms.

Bacterial communities involved in this yellow-green colonization of rocks were mainly composed of *Acidobacteria*, *Proteobacteria* with distinct variations among sites (Bjelland et al., 2011 and Bates et al., 2011). The study of Esposito and co-workers (2013) also showed that bacterial communities were mainly composed of *Acidobacteria, Proteobacteria*, and *Cyanobacteria* with distinct variations among sites. In another study (reviewed by Bjelland et al., 2011) the bacterial community of *R. geographicum* consisted mainly of *Acidobacteria*, *Proteobacteria* (*Alpha-* and *Betaproteobacteria*) and *Chloroflexi* classes (Table 1.1). Therefore, regarding previous researches about the third symbiotic on lichens, *Proteobacteria* (specify *as Alphaproteobacteria*) was not found as dominant in bacterial communities of *R. geographicum*. Instead of, it seems that the dominant community on this yellow-green lichen was *Acidobacteria* using cultivation-independent method.

Because of no reports using culture-based method to isolate bacterial colonies associated with *R. geographicum,* so far, no metabolites produced from isolates have been described. This is one of the reasons of the focus of our study on this lichen and its bacterial communities.

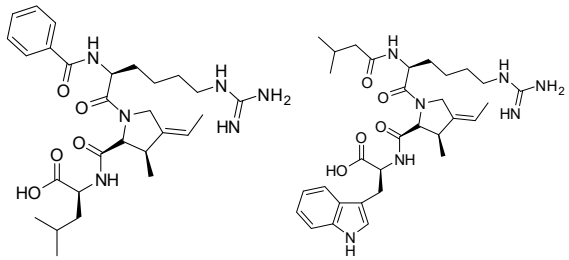
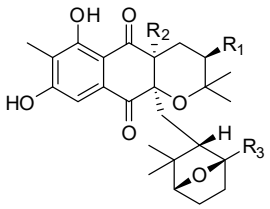
IN SUMMARY

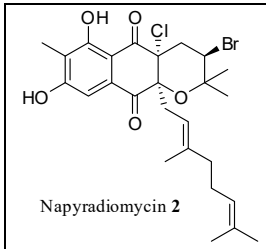
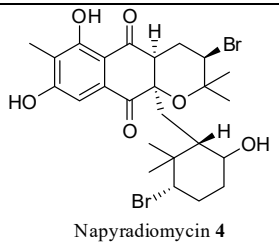
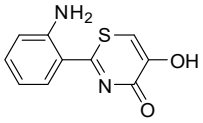
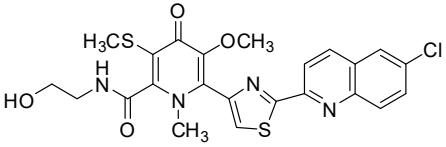
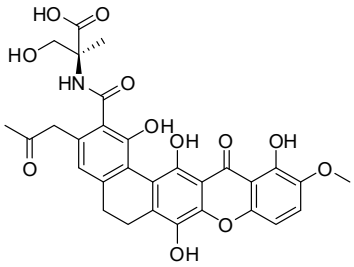
A number of studies of *Rhizocarpon geographicum* have almost focused on the lichenometry. Moreover, the research about the bacterial communities as well as the secondary metabolites of this lichen has been still limited. As a result, for a deeper understanding of *Rhizocarpon geographicum* and particularly to learn more about its culturable bacterial communities and their ability to produce metabolites of interest, we have focused on *Rhizocarpon geographicum* in our work.

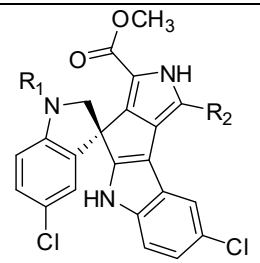
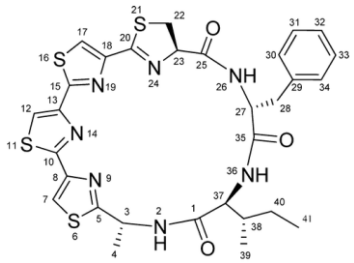
1.5. NATURAL CYTOTOXIC COMPOUNDS FROM BACTERIAL CULTURE

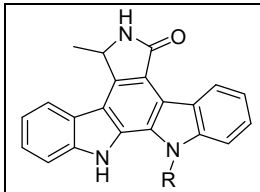
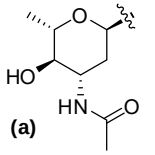
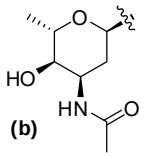
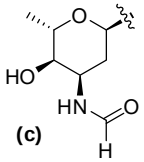
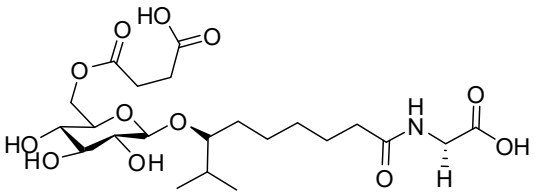
Natural products are an important source in the development of drugs due to their significant activities as antioxidant, antibiotic, cytotoxic … Among them, the natural cytotoxic compounds become potential candidates for the development of novel anticancer agents. Over 50% of anticancer drugs were derived from natural products (Kim and Park, 2002, Ruiz-Torres et al., 2017). Most of these compounds were isolated from plants and some were produced from other sources as fungi (Debbab et al., 2010) or marine sponges (Mioso et al., 2017)…. To date, bacteria have become a potential source in the production of active compounds. Obviously, the bacterial cytotoxic compounds have also attracted many chemists. Therefore, many new cytotoxic compounds derived from bacteria were continuously reported. Some examples are reported in Table 1.6 as lucentamycins isolated from *Actinomycete* (Cho et al., 2007), napyradiomycin derivatives from a marine *Actinomycete* (Farnaes et al., 2014), thiasporine A from *Actinomycetospora chlora* (Fu and MacMillan, 2015) e.g. and cytotoxic compounds from lichen-associated bacteria mentioned in part 1.3 (Table 1.3). Therefore, the efforts to isolate cytotoxic compounds from selected bacteria associated with *R. geographicum* are one of our aims in this thesis.

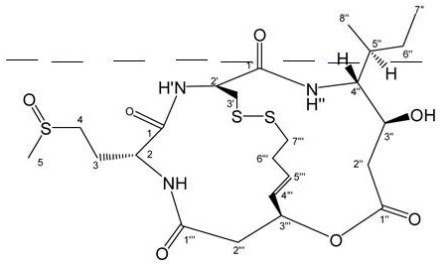
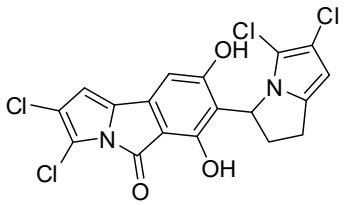
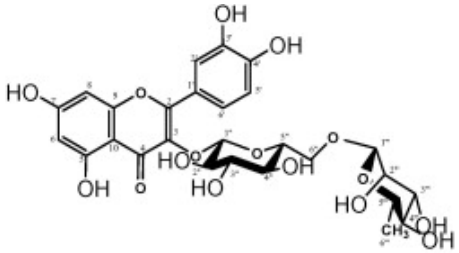
Table 1.6 Summary of some cytotoxic compounds produced by bacteria

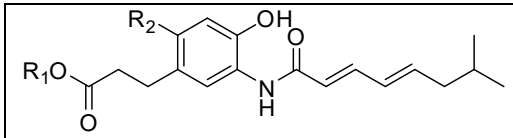
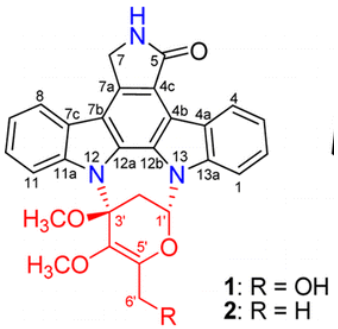
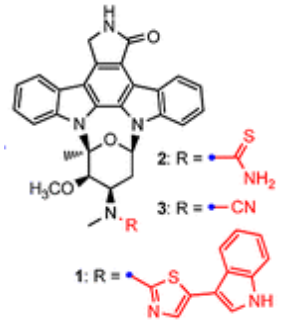
Compounds	Cytotoxicity, IC_{50}	Bacterial origin	References
Lucentamycin A **Lucentamycin B** 	*in Vitro* against HCT-116 human colon carcinoma cell line IC_{50} values of 0.20 µM and 11 μM (for Lucentamycin A and B, respectively)	*Nocardiopsis lucentensis*	Cho et al., 2007
Napyradiomycin  Napyradiomycin **1** (R_1 = Cl, R_2 = Cl, R_3=Me) Napyradiomycin **3** (R_1 = Br, R_2 = Cl, R_3=Me)	Against HCT-116 colon carcinoma at concentrations of 17, 6, 49 µM for Napyradiomycin 1, 2 and 4, respectively	Actinomycete strain CNQ525	Farnaes et al., 2014

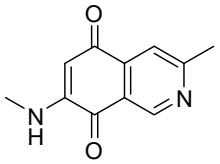
 Napyradiomycin **2**  Napyradiomycin **4**			
Thiasporine A 	Against the non-small-cell lung cancer cell line H2122 , IC_{50} of 5.4 µM	*Actinomycetospora chlora*	Fu and MacMillan, 2015
Lodopyridone 	Against HCT-116 human colon cancer cells, IC_{50} of 3.6 µM.	*Saccharomonospora* sp	Maloney et al., 2009
Buanmycin 	Against human carcinoma cell lines: A594 (lung cancer – IC_{50} of 1.7 µM), HCT116 (colon cancer – IC_{50} of 0.9 µM), SNU638 (gastric cancer – IC_{50} of 0.8 µM), SKHEP1 (liver cancer – IC_{50} of 1.9 µM), MDA-MB231 (breast cancer – IC_{50} of 1.2 µM)	*Streptomyces cyaneus*	Moon et al., 2015
Spiroindimicins	**B**: against cell lines: CCRF-CEM (human leukemia	*Streptomyces* sp.	Zhang et al., 2012

 Spiroindimicin **B** ($R_1 = CH_3$, $R_2 = H$) Spiroindimicin **D** ($R_1 = CH_3$, $R_2 = COOCH3$)	– IC_{50} of 4 µg/mL), B16 (mouse melanoma – IC_{50} of 5 µg/mL), H460 (human lung cancer – IC_{50} of 12 µg/mL). **D**: against cell lines: B16 (mouse melanoma – IC_{50} of 20 µg/mL), H460 (human lung cancer – IC_{50} of 18 µg/mL), HepG2 (human hepatocellular liver carcinoma – IC_{50} 22 µg/mL).		
Marthiapeptid 	Against human carcinoma cell lines: SF-268 (glioblastoma cancer - IC_{50} of 0.38 ± 0.02 µM), MCF-7 (breast cancer –IC_{50} of 0.43 ± 0.005 µM), NCI-H460 (lung cancer – IC_{50} of 0.47 ± 0.003 µM) and HepG2 (hepatocarcinoma cancer – IC_{50} of 0.52 ± 0.01 µM)	*Marinactinospora thermotolerans*	Zhou et al., 2012
Indolocarbazole	Compound **1, 2** and **3:** against human prostate PC-3 cancer cell line, IC_{50} of 0.8 ; 16.2 and 1.5 µM, respectively	*Streptomyces* sp.	Qin et al., 2018

 Indolocarbazole **1** (R = a) Indolocarbazole **2** (R = b) Indolocarbazole **3** (R = ac  (a)  (b)  (c)			
Ieodoglucomide B 	Against lung cancer and stomach cancer cell lines, GI_{50} of 25.18 and 17.78 µg/mL, respectively.	*Bacillus licheniformis*	Tareq et al., 2012
Spiruchostatin C		*Burkholderia thailandensis*	Klausmeyer et al., 2011

			
Chlorizidin 	Against HCT-116 human colon cancer cells, IC_{50} = 3.2 - 4.9 µM	*Streptomyces* sp	Alvarez-Mico et al., 2013
Flavonoid 	Against A549 lung cancer cell line	*Streptomyces* sp	Balachandran et al., 2014
Carpatamides	**A**: against human carcinoma cell line HCC-366 (IC_{50} of 2.8 µM), A549 (IC_{50} of 4.1 µM), HCC44 (IC_{50} of 8.4 µM)	*Streptomyces* sp	Fu et al., 2014

 Carpatamide **A** ($R_1 = CH_3$, $R_2 = OH$) Carpatamide **C** ($R_1 = R_2 = H$)	**B**: against human carcinoma cell line HCC-366 (IC_{50} of 2.2 µM), A549 (IC_{50} of 3.7 µM)		
Streptocarbazoles 	**Compound 1:** against cell lines HL-60 (IC_{50} of 1.4 µM), A459 (IC_{50} of 5.0 µM), P388 (IC_{50} of 18.9 µM) and Hela (IC_{50} of 34.5 µM). **Compound 2**: against P388 (IC_{50} of 12.8 – 22.5 µM) cell line	*Streptomyces* sp	Fu et al., 2012a
Indolocarbazoles 	**1**: against human carcinoma cell lines as HL-60 (IC_{50} of 1.3 µM), K-562 (IC_{50} of 4.58 µM), A-549 (IC_{50} of 1.41 µM) and BEL-7402 (IC_{50} of 3.26 µM) **2**: against human carcinoma cell lines as HL-60 (IC_{50} of 1.60 µM), K-562 (IC_{50} of 1.47 µM), A-549 (IC_{50} of 0.001 µM) and BEL-7402 (IC_{50} of 1.74 µM). **3**: against human carcinoma cell lines as HL-60	*Streptomyces fradiae*	Fu et al., 2012b

	(IC_{50} of 0.13 µM), K-562 (IC_{50} of 0.43 µM), A-549 (IC_{50} of 0.02 µM) and BEL-7402 (IC_{50} of 0.68 µM)		
Mansouramycin B 	Against lung cancer (LXFA 629 - IC_{50} 1.63 µM) and melanoma cells (MEXF 276 and MEXF 514, IC_{50} 0.33 and 0.05 µM)	*Streptomyces* sp	Hawas et al., 2009

Summary

Throughout a screening of the data of metabolites isolated from bacterial cultures, bacteria showed a great potential source of cytotoxic metabolites that might serve as useful leads in the development of new pharmaceutical agents. Moreover, the information from Table 1.6 highlighted that most of these cytotoxic compounds are alkaloids and they were frequently produced from *Streptomyces* genus, while different bacterial genera are still unexploited. Thus, these data can give us helps in the selection of the genus producing cytotoxic metabolites.

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Webography

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1.6. THE OBJECTIVES OF THE WORK

According to the state of the art detailed in the previous part, we have decided to focus our research work on the isolation of bioactive novel metabolites from interesting and original sources such as lichen-associated bacteria which are poorly studied. The first step was the selection of the lichen which could possess specific and novel bacterial partners. Due to the ubiquitary presence of the lichen *Rhizocarpon geographicum* in coastal areas which could afford particular environments to their hosted organisms (Delmail et al., 2013) and due to the lack of knowledge of culturable bacterial communities associated with this organism, we decided to select this lichen to study its associated bacteria via a culture-based approach. These data will be presented in chapter 1 of part II of this manuscript.

After isolation of various pure strains from *R. geographicum*, a species *Paenibacillus odorifer* with special characteristics known through several references would be preferred for studying its ability to produce secondary metabolites of interest (reported in chapter 2). We have focused our research on the discovery of potential cytotoxic compounds (tested on two HaCaT and B16 cell lines) as we have seen previously the particular ability of some bacteria to produce novel and potent cytotoxic metabolites possessing original skeletons. The optimization of the culture in order to increase the production of bioactive compounds has then been applied for this selected strain and described in chapter 3. Two optimization steps using various vessels (bioreactor, Erlenmeyer flask) have been undertaken. The parameters selected for this process included pH of medium, temperature, stirring, inoculum ratio, and the quantity and bioactivity of extracts from the broth of the culture as well. The best cultured conditions were applied for a large volume culture as explained in chapter 3.

Finally, the isolation, identification as well as the evaluation of bioactivities of a number of pure bacterial compounds were performed and has detailed in chapter 4. The chapter 5 was displayed conclusions and perspectives. The chapter 6 finally reported the materials and methods used during this work.

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PART II: RESULTS

PART II. RESULTS

This part displayed the results of this PhD work divided into distinct 5 sections which are reported either as articles (in progress) or as classical parts.

In the first part, the isolation and identification of thirteen pure strains from *Rhizocarpon geographicum* will be presented. This result would be proposed as an article in progress. (Chapter 1)

The second section detailed the selection of bacterial species with the objective to produce metabolites. It is classically reported in this thesis as Chapter 2. After the strain selected, the optimization to find the best conditions for the culture will be displayed in Chapter 3 in a classical form.

The metabolites isolated will be introduced in Chapter 4 including a fraction of polysaccharide, two *tert*-butylphenol compounds and a cytotoxic alkaloid that are presented as articles (in progress), while other metabolites with no cytotoxicity are displayed as a part in the thesis.

After conclusions and perspectives (Chapter 5), the final section will be the description of materials and methods used in this work (Chapter 6).

Chapter 1: ISOLATION OF BACTERIAL STRAINS FROM *R. GEOGRAPHICUM*

CHAPTER 1: ISOLATION OF BACTERIAL STRAINS FROM *R. GEOGRAPHICUM*

The results of chapter 1 were reported as an article (in preparation).

Culturable bacterial communities from the lichen *Rhizocarpon greographicum*

An article in progress

Nguyen Thi Bach Le, Delmail David, Tomasi Sophie

Univ Rennes, CNRS, ISCR – UMR 6226, F-35000 Rennes, France

Abstract

Eleven microorganisms associated with the lichen *Rhizocarpon geographicum* were isolated using a culture-based method. They were identified based on their 16s rRNA gene sequence analysis and led to their affiliation as 11 microbial strains along with 8 ones belonged to the Bacteria kingdom. The results first demonstrated that the cultured bacterial communities in *Rhizocarpon geographicum* were dominated by Bacilli class (*Firmicutes* phylum) followed by *Proteobacteria*.

INTRODUCTION

Many studies in previous decades had not admitted bacterial communities as an important part of lichens which were only considered as a result of the symbiotic association between a mycobiont (known as fungus) and a photobiont (alga or cyanobacterium). These previous studies, therefore, cannot address the question why lichens can grow on extremely nutrient-poor life conditions (Brodo and Irwin 1973) such as rocky surface, frosted polar or barren desert where fungi or algae cannot survive alone. Several recent studies have demonstrated that microorganisms mostly supplied food for lichens by fixing nitrogen, carbon dioxide in the atmosphere to produce a substantial source of crucial nutrients (González et al. 2005; Liba et al. 2006; Hodkinson et al.

2012; Grube et al. 2015). As a result, lichens, forming the symbiotic association between fungi, algae/cyanobacteria and with bacterial cohabitation, are one of the most successful life-form on the earth which can grow in any harshly environmental conditions (Boustie and Grube 2005; Boustie, Tomasi, and Grube 2010). Moreover, bacterial communities played significant roles in the ecology of lichens (Cardinale, Puglia, and Grube 2006; Grube and Berg, 2009; Liba et al. 2006 , Hodkinson and Lutzoni 2009) and recent years, these communities became an interesting subject for researches in the field of Lichenology.

Several studies demonstrated that the majority of lichen-associated bacteria live on or near the fungal surfaces of lichen thalli and they formed biofilm-like coatings on lichen surfaces (Cardinale et al. 2008; Grube and Berg 2009). As a result, bacteria provide to lichens nutrients by fixing nitrogen in the atmosphere whereas green algae cannot (Grube et al., 2015). Therefore, this hypothesis could address the question why lichen can grow in any habitat and geographic area on the Earth.

These microorganisms were often described by culture or unculture-based methods. While the latter method highlighted a great diversity of bacteria in each lichen thallus (Cardinale, Puglia, and Grube 2006), the culture-based methods cannot collect uncultured bacteria and cannot afford the identification of the vast majority of microorganisms in the laboratory (Amann, Ludwig, and Schleifer 1995). It may lead to false conclusions about the abundance and importance of certain bacteria in nature. However, the significant advantages of culture-based methods are the abilities to isolate bacterial strains as well as to produce metabolites from these populations and to check bioactivities of the substances produced.

Rhizocarpon geographicum, one of the most popular species of the crustose lichen family (Ihlen et al., 2004) grows mainly on nutrient-poor substrates as rock surface (especially rocky promontory) or under harsh coastal conditions (Armstrong and Bradwell 2010, Armstrong and Smith 2009). However, the information on bacterial communities from this lichen remains still poor. One unusual study on microorganism communities of *Rhizocarpon geographicum* was performed by Bjelland and co-workers (2011). The results provided by this literature using uncultured-based method indicated that *Alphaproteobacteria* was predominant in bacterial population associated with *Rhizocarpon geographicum*.

In this study, we first report the bacterial communities associated with *R. geographicum* by a culture-based method and identify selected strains at the genus level by partial 16S rRNA sequencing. Interestingly, the results from our study illustrated a dominance of *Bacilli* class instead of *Alphaproteobacteria* class which is previously presented as dominant in previous studies performed on microorganisms of crustose lichens (Bjelland et al., 2011; Bates et al., 2011).

MATERIALS AND METHODS

Sample collection

The lichen samples of *Rhizocarpon geographicum* were collected at a small rocky promontory in Bretagne, France near Saint-Malo in February 2015 as reported in Figures 1 and 2. Three points of the collection in the same site have been labeled using specific colors following the distance to the sea (blue for the close site, pink one for the medium distance then green one for the farthest site). At each location, fragments of lichen species were collected together with rock surface below them using a sterile hammer and a chisel. The samples were transferred into sterile Eppendorf® tubes before being brought to the laboratory.

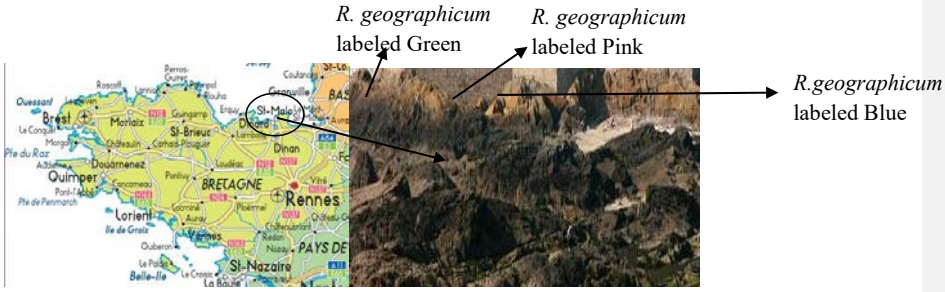


Figure 1: **The sites of *R. geographicum* harvest and their associated color**

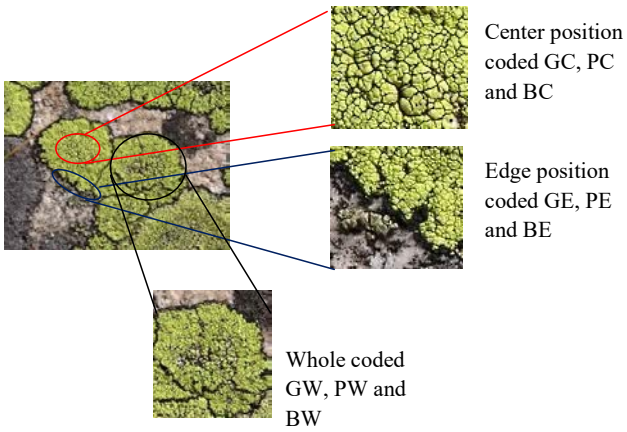


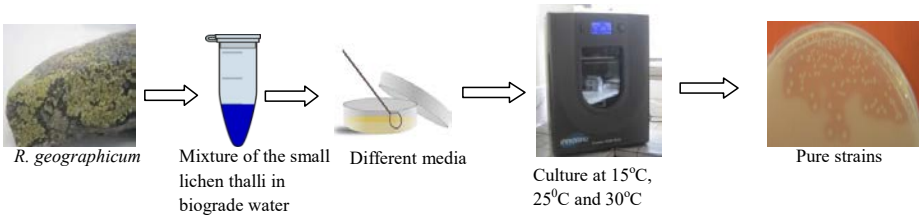
Figure 2: The description of cuttings on the surface of *R. geographicum*
(G: green, P: pink, B: blue)

Microbial isolation

Samples were studied without surface treatment. The isolation of the external bacteria portion was performed as follows : the lichen upper cortex was cut into small fragments with very thin layers which were characterized as ~~-e~~enter", ~~—d~~ge" or ~~-w~~hole" (see Figure 2) and vortexed with 100 µL bio-grade water for 5 minutes. Then, 100µL of the resulting suspension was streaked on various agar media following German collection of microorganisms and cell culture (DSMZ) (Bacillus acidocaldarius medium, Gym Streptomyces medium, Gym Streptomyces with 10% NaCl medium, Thiobacillus acidiphillus medium and Marine broth agar). Before preparing agar plate the pH was adjusted to an appropriate value between 3.5 and 7.5 with 0.1N NaOH or 0.1N H_2SO_4.

Plates were incubated in an orbital incubator at 15°C, 25°C and 30°C during 15-30 days until no new colonies appeared. The colony morphology wwas analyzed based on color, texture, opaqueness, concavity, size and other visible features. All the colonies with distinctive phenotypes were purified by seeding single colony on a new plate filled with the same medium. Colonies representatives of a typical morphology were cultured on fresh media to obtain pure cultures.

Finally, thirteen pure strains were collected and identified by 16S rRNA gene sequencing analysis. The process of isolation was demonstrated in Scheme.1.



Scheme 1: Process of isolation of microbial strains from *R. geographicum*

The cryopreservation was prepared by suspending a loop full of colonies in 1.0 mL of the 47.5% (v/v) glycerol and 5% (v/v) of dimethyl sulfoxide (DMSO) in 50% (v/v) of stetile water and stored at -80^{0}C in the laboratory of the University of Rennes 1 (CORINT team).

The isolated strains were coded following the sites of the lichen harvest, the position of cutting on the surface of *R. geographicum*, the growth medium and their morphological characteristics. For instance, the strain coded GC-MA-OP means that it was isolated from lichen labeled Green, cut at the center position on the surface of the lichen, cultured on Marine agar medium, and with characteristics as orange-pink color (Table 1).

Phylogenetic analysis

Isolated strains were selected for identification based on colony morphology and phylogenetic analysis. For DNA extraction from pure cultures, a Kit Promega® was used according to the manufacturer's instruction and performed by L. Intertaglia (Plateform, Banyuls/Mer, France). Extracted DNA was PCR amplified in an ABI- Applied Biosystems thermocycler (Observatoire Océanologique de Banyuls sur Mer, France) using the universal primer pair 27F mod (5'-AGRGTTTGATCMTGGCTCAG-3') and 1492R mod (5'-TACGGYTACCTTGTTAYGACTT-3') at a final concentration of 1.0µmol/L in total volume of 10µL of PCR mixture. The PCR reaction was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30s, 51°C for 30s, 72°C for 1.30 min, and final extension was performed at 72°C for 10

min. PCR products were then separated by electrophoresis on 1% agarose gel. The separated bands were visualized using a UV transilluminator. Partial sequencing of the purified PCR products was performed with a Big Dye terminator kit run on DNA analyzer. Characterization by 16S rRNA sequencing showed one strain to be related with percentage after comparison using the EZ Taxon server.

RESULTS AND DISCUSSION

Bacterial isolation

Cultivable bacterial communities found on the surface of *Rhizocarpon geographicum* were isolated at 15°C, 25°C and 30°C from distinguishable media named as *Bacillus acido*, *Gym Streptomyces*, *Gym Streptomyces* with 10% NaCl, *Thiobacillus* and Marine agar. These media were selected from the marine media (following references of DSMZ catalogue) in order to easily harvest corresponding maritime bacteria. The results reported in Table 1 highlighted that thirteen pure strains were isolated using our culture-based method from *R. geographicum* samples collected near the coasts. The most number of strains were obtained at 15°C (eight strains) in comparison to any other temperatures, such as at 25°C (four isolates) and at 30°C (only one strain). Moreover, the results also demonstrated that the strains were more collected from Gym Streptomyces and Marine agar media (with 38.5 % for each) than from the three other media (7.7% for each). On the other hand, when we consider the position of the harvest on lichen thalli, the data showed that the most of the strains were isolated from all the surface on lichen thalli (61 % of the total of the isolated strains), the rest of the strains were harvested from center position of the lichen thalli, while no strains were found at the edge location. Interestingly while the main groups of the isolates (69%) were found on samples harvested on the site at the medium distance to the sea (labeled P) no strains were isolated from samples located closer to the sea in the culture conditions used in this study.

Table 1: Factors for microbial isolation from *R. geographicum*.

Bacterial strains (code)	Part collected on lichen samples	Media	Temperature °C		
			15	25	30
GC-GYM-YT	Center	Gym Streptomyces	+	-	-
PW-GYM-WS	Whole	Gym Streptomyces	-	+	-
PC-GYM-TO	Center	Gym Streptomyces	+	+	-
PW-GYM-LY	Whole	Gym Streptomyces	+	-	-
PW-GYM-CY	Whole	Gym Streptomyces	+	-	-
PW-GYM+10%NaCl-PY	Whole	Gym Streptomyces plus 10% NaCl	-	-	+
PW-MA-YF	Whole	Marine agar	-	+	-
PW-MA-LG	Whole	Marine agar	+	-	-
PW-MA-LB	Whole	Marine agar	+	-	-
PW-MA-OF	Whole	Marine agar	-	+	-
GC-MA-OP	Center	Marine agar	+	-	-
GC-Thio-DG	Center	Thiobacillus acidphillus	+	-	-
GC-Bac-LW	Center	Bacillus acidocaldarius	+	-	-

A total of thirteen strains were isolated from *Rhizocarpon geographicum* based on their morphology characteristics such as color, texture, opaqueness, concavity, size and other visible features (Table 2 and Figure 3). Following all these features these isolates could be classified into 11 types (Figure 3). These strains were finally stored with a solution of 47,5% (v/v) glycerol and

5%(v/v) dimethylsulfoxide (DMSO) at -80^{0}C. Reculturability from frozen stocks has been confirmed for all isolates.

Table 2: Morphology of isolated strains from *Rhizocarpon geographicum*

Type	Color	Sheen	Convexity	Other features	Representative strain
A	Bright yellow	+	+		GY-GYM-YT
B	Pale grey	+	+	Whole, thick colonies, lumpy form	PW-GYM-WS
C	Colorless	-	-	Change yellow medium into colorless one	PC-GYM-TO
D	Pastel yellow	+	-		PW-GYM-LC; PW-GYM-CY
E	Pure yellow	-	-	Change yellow medium into colorless one	PW-GYM+10%NaCl-PY
F	Pastel yellow	-	-	Whole, thick trace	PW-MA-YF ; PW-MA-LG
G	Light brown	+	-		PW-MA-LB
H	Orange - yellow	-	+		PW-MA-OF
I	Orange-pink	-	-	Incrusted in the surface Nucleus with filamentous around	GC-MA-OP
J	Dark green	+	+	Lumpy form	GC-Thio-DG
K	Light white	-	+	Turbidity	GC-Bac-LW

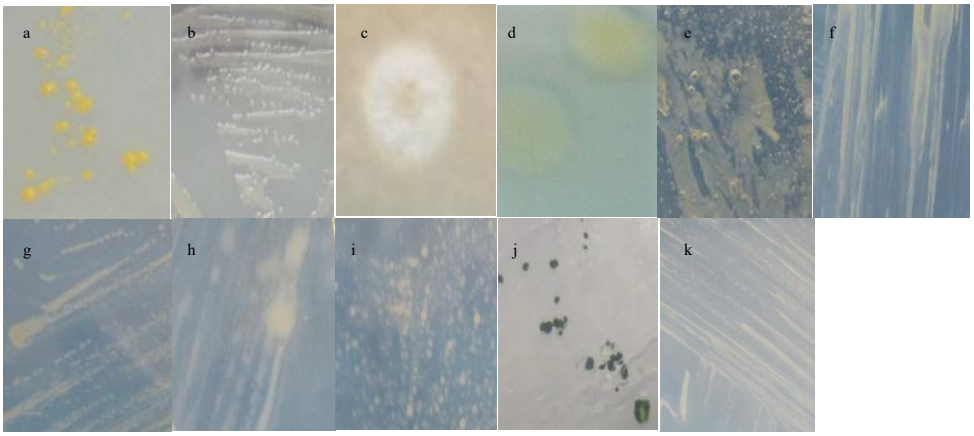


Figure 3: The pure strains isolated from *Rhizocarpon geographicum*. (a:GC-GYM-YT; b: PW-GYM-WS; c: PC-GYM-TO; d: PW-GYM-LY and PW-GYM-CY; e: PY-GYM +10%NaCl-PY; f: PW-MA-YF and PW-MA-LG; g: PW-MA-LB; h: PW-MA-OF; i: GC-MA-OP; j: GC-Thio-DG; k: GC-Bac-LW)

Table 3 : The most common colony types in samples after 14 days of incubation

Samples	Dominant colonies	Other colony types
GC	A	I,J,K
PC	C	
PW	D,F	B,E,G,H

GC: thin layer at the center position on the surface of *R. geographicum* collected at the farthest distance from the sea
PC: thin layer at the center position on the surface of *R. geographicum* collected at the medium distance
PW: whole of *Rhizocarpon geographicum* collected at the medium distance

A clear difference in the dominance of the colony morphology was observed in agar plates obtained from *Rhizocarpon geographicum* samples collected at the different locations on the thallus. Most colony types were scarce, with one or few representatives per plate, the samples being strongly dominated by one or few colony types (See Table 3). The crustose lichen-associated strains appear to be characterized by a strong presence of bacteria forming colony type D, F, A, C. However, the individual samples appear to harbor populations distinct from another type with GC

samples harboring populations strongly dominated by colony type A, whereas colony type C dominated on PC samples and the PW samples are co-dominated by bacteria forming colony types D and F.

Microbial identification

The isolates were identified by partial 16S rRNA gene sequence analysis (see Table 4). All sequences matched with entries in GenBank with similarity ranging from 85% to 100%. The collection was found to contain members of three classes such as *Alphaproteobacteria*, *Betaproteobacteria* and *Bacilli*. The taxonomic diversity of selected strains is shown in Figure 4.

Table 4: Identify of cultured strains as revealed by partial 16S rRNA gene sequencing

Strain	Colony type	Sequence length (pb)	GenBank accession number	Most similar strains	% Identity	Family	Order	Classe
GC-GYM-YT	A	1445	AF131295	*Sphingomonas aquatilis*	98.39	*Sphingomonadaceae*	*Sphingomonadales*	*Alphaproteobacteria*
PW-GYM-WS	B	1489	AF512826	*Burkholderia sordidicola*	98.98	*Burkholderiaceae*	*Burkholderiales*	*Betaproteobacteria*
PC-GYM-TO	C	1512	AJ223990	*Paenibacillus odorifer*	98.46	*Paenibacillaceae*	*Bacillales*	*Bacilli*
PW-GYM-LY	D	1503	EU099594	*Paenibacillus castaneae*	98.34	*Paenibacillaceae*	*Bacillales*	*Bacilli*
PW-GYM-CY	D	1503	EU099594	*Paenibacillus castaneae*	98.34	*Paenibacillaceae*	*Bacillales*	*Bacilli*
PW-GYM+10%NaCl- PY	E	1508	AJ316316	*Bacillus murimartini*	99.93	*Bacillaceae*	*Bacillales*	*Bacilli*
PW-MA-YF	F	1291	AJ277984	*Psychrobacillus psychrodurans*	99.84	*Bacillaceae*	*Bacillales*	*Bacilli*
PW-MA-LG	F	1291	AJ277984	*Psychrobacillus psychrodurans*	99.84	*Bacillaceae*	*Bacillales*	*Bacilli*
PW-MA-LB	G	1494	HM054474	*Bacillus xiaoxiensis*	99.11	*Bacillaceae*	*Bacillales*	*Bacilli*
PW-MA-OF	H	1487	AB300598	*Lysinibacillus parviboronicapiens*	99.12	*Planococcaceae*	*Bacillales*	*Bacilli*
GC-MA-OP	I	556	EF600972	*Elsinoe verbenae*	84.19	*Elsinoaceae*	*Myriangiales*	*Dothideomycetes*
GC-Thio-DG	J	1572		*Coccomyxa sp.*	100.00	*Coccomyxaceae*	*Trebouxiophyceae ordo incertae sedis*	*trebouxiophyceae*
GC-Bac-LW	K	1493	AXCJ01000001	*Candidatus "Xenolissoclinum pacificiencis"*	68.78			

Among the strains harvested from *R. geographicum*, ten isolates were bacteria; one was a fungus (*Sphaceloma* sp.), one was a cyanobacterium and one seems to be not pure regarding its identity (coded GC-Bac-LW) (see Table 4 and Figure 3).Two isolates with the codes PW-GYM-LY and PW-GYM-CY were affiliated to the same species named *Paenibacillus castaneae* with a percentage of identity as 98.34 %. This result was obvious because of their similarity of the growth medium and their morphological characteristics as well. Additionally, two other isolates named PW-MA-YF and PW-MA-LC belonged to *Psychrobacillus psychrodurans* with a percentage of similarity up to 99.84%. Two species belonging to *Proteobacteria* phylum were identified as *Sphingomonas aqualitis* and *Burkholderia sordidicola*. The yellow pigmented *Sphingomonas aqualitis* species was firstly reported from the several mineral water sources in Korea by Lee and co-workers (2001). Herein, this strain was the first example found from *R. geographicum*. However, the *Sphingomonas* genus was already described commonly from lichens in some previous studies. It was isolated from *Caloplaca verruculifera* (Sigurbjörnsdóttir et al., 2014)

Cetraria sp. (Kim et al., 2012), *Lobaria pulmonaria* (Cardinale et al., 2011 ; Aschenbrenner et al., 2014; Grube et al., 2015), *Solorina crocea* (Grube et al., 2012), *Umbilicaria americana* (Bates et al., 2011). Therefore, it is the first report of *Sphingomonas aqualitis* found from *R. geographicum*. Another species of Proteobacteria, *Burkholderia sordidicola* which was firstly identified from white-rot fungus Phanerochaete (Lim et al., 2003) was commonly found in the third symbiotic communities of lichens such as from *Cladonia pyxidata* and *Cladonia rangiferina* (Cardinale et al., 2006) and from *Cladonia* sp., *Steroacaulon* sp. and *Umbilicaria* sp. in the Arctic (Kim et al., 2012). This study is thus considered as the first description of this species from *R. geographicum* samples.

The other six bacterial species all belonged to *Bacilli* class, *Firmicute*s phylum consisting of two *Paenibacillus*, two *Bacillus*, one *Psychrobacillus* and one *Lysinibacillus*. Among these, two species *Psychrobacillus psychrodurans* and *Lysinibacillus parviboronicapiens* (or their genera) were for the first time discovered from lichens. Although it is the first report of the other species *Paenibacillus odorifer*, *P. castaneae*, *Bacillus murimartini* and *B. xiaoxiensis* from *R. geographicum*, the genera from which they belong were often found in lichens. For instance, *Paenibacillus* genus was recorded from *Cladonia* sp. lichens (Grube et al., 2009; Cardinale et al., 2011; Cardinale et al., 2006; Hodkinson et al., 2009), *Hypogymnia physodes* (Cardinale et al., 2006), *Lecanora polytropa* (Grube et al., 2009; Cardinale et al., 2011) ; *Bacillus* species were found from *Cladonia arbuscula* (Grube et al., 2009; Cardinale et al., 2011), *Hydropunctaria maura* (Sigurbjörnsdóttir et al., 2014), *Lecanora* species (Sigurbjörnsdóttir et al., 2014 ; Grube et al., 2009; Cardinale et al., 2011), *Umbilicaria cylindrica* (Grube et al., 2009; Cardinale et al., 2011) and *Xanthoria elegans* (Selbmann et al., 2009). As a result, most isolates from *R. geographicum* were for the first time reported from lichens, excepted *B. sordidicola* species which was already reported from *Umbilicaria* sp. (Kim et al., 2012) and *Cladonia pyxidata* (Cardinale et al., 2006). Further, we need to investigate biological interests of these species and detailed investigations about their activities are essential.

Besides, two non-bacterial isolates were *Coccomyxa* sp., a genus of green alga, and *Sphaceloma araliae*, a genus of *Ascomycete* fungi. The *Coccomyxa* genus was already found from *Rhizocarpon*

lecanorinum (Clayden 1998). The presence of this alga can be explained by the fact that it came from the process without surface treatment of the lichen before cutting it.

Figure 4: Taxonomic diversity of selected strains

CONCLUSION

Herein, our culture-based strategy led to the isolation of thirteen solates and among them 11 were bacteria. These data highlighted that the method used was not enough selective to the bacteria kingdom. *Alphaprotebacteria* was not the major part of cultivable lichen-associated microbial communities on lichen *R. geographicum*. *Bacilli* belonging to Firmicutes phylum became a dominant class for these communities. This result was similar to those reported from Cardinale's team (2006) where Firmicutes dominated all bacterial communities associated with lichens studied.

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Lim, Y. W., Baik K. S., Han S. K., Kim S. B., and Bae K. S.. 2003. ―Burkholderia Sordidicola Sp. Nov., Isolated from the White-Rot Fungus Phanerochaete Sordida.‖ *International Journal of Systematic and Evolutionary Microbiology* 53 (5): 1631–36. https://doi.org/10.1099/ijs.0.02456-0.

Selbmann, L., Zucconi L., Ruisi S., Grube M., Cardinale M., and Onofri S.. 2009. ―Culturable Bacteria Associated with Antarctic Lichens: Affiliation and Psychrotolerance.‖ *Polar Biology* 33 (1): 71–83. https://doi.org/10.1007/s00300-009-0686-2.

Sigurbjörnsdóttir, M. A., Heiðmarsson S., Jónsdóttir A. R. and Vilhelmsson O.. 2014. ―Novel Bacteria Associated with Arctic Seashore Lichens Have Potential Roles in Nutrient Scavenging.‖ *Canadian Journal of Microbiology* 60 (5): 307–17. https://doi.org/10.1139/cjm-2013-0888.

CHAPTER 2: THE SELECTION OF *PAENIBACILLUS ODORIFER* AS A PROMISING SOURCE OF INTERESTING METABOLITES

CHAPTER 2: THE SELECTION OF *PAENIBACILLUS ODORIFER* AS A PROMISING SOURCE OF INTERESTING METABOLITES

Because we have focused our work on the isolation of bioactive bacterial metabolites, the bibliographic data concerning the production of interesting compounds from the isolated strains from *R. geographicum* had to be studied. In this chapter, the chemical production of these strains will be introduced and we will give details on studies from which we will be based for our selection of the strain(s) for further researches.

2.1. State of art on the chemical production of the isolated strains

Firstly, it seems that no report had described metabolites from *Sphingomonas aquatilis* belonging to *Sphingomonas* genus. However this genus produced some primary metabolites like enzymes (Kmunícek et al., 2005) or glycosphingolipids (Kawahara et al., 1991; Kubota et al., 2009) which demonstrated an activity on invariant natural killer T (iNKT) cells. The Table 2.2.1 provides data on the metabolites already isolated from this genus.

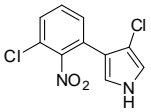
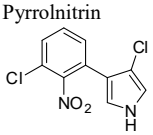
Table 2.2.1 Summary about chemical studies on *Sphingomonas* genus

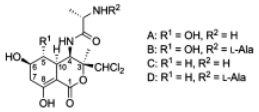
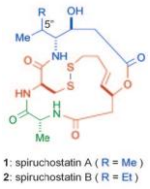
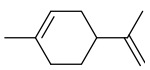
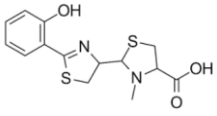
Producing strains	Compounds	Nature of bioactivities	References
S. paucimobilis	Haloalkane dehalogenase LiB	Dehalogenation	Kmunícek et al., 2005
	Two novel glycosphingolipids		Kawahara et al., 1991
S. yanoikyae	Glycosphingolipid (GSL-7)	Activity on invariant natural killer T (iNKT) cells	Kubota et al., 2009
S. terrae	Glycosphingolipid (GSL-13)		
S. adhaesiva	Glycosphingolipid (GSL-4B)		Kawahara et al., 1991

Studies on *Burkholderia* genus described many active compounds (primary or secondary metabolites) isolated from this genus such as antifungal pyrrolnitrin (El-Banna and Winkelmann, 1998; Hwang et al., 2002; Keum et al., 2009); diketopiperazines (Wang et al., 2010) or antitumor spiruchostatin (Klausmeyer et al., 2011). The summary of some compounds isolated from *Burkholderia* genus was shown in Table 2.2.2. We can conclude the

existence of several studies focused on this genus and many significant bioactive metabolites were produced from this one.

Table 2.2.2 Summary about chemical studies from *Burkholderia* genus (*Betaproteobacteria*)

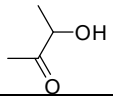
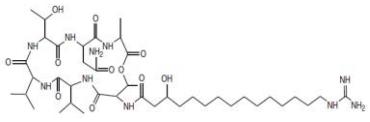
Producing strains	**Compounds**	**Nature of bioactivities**	**References**
Burkholderia plantarii	6-deoxy-α-D-talan polymer (O-acetylated homopolysaccharide)		Zähringer et al., 1997
Burkholderia cepacia	Pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chloro phenyl)pyrrol] 	Antibiotic activity against fungi and against *Streptomyces antibioticus* using agar diffusion test at 0.2 µg/mL	El-Banna and Winkelmann, 1998
	Pyrrolnitrin 	Biocontrol of Rhizoctonia stem rot of poinsettia	Hwang et al., 2002
	Lipopolysaccharide (LPS)	Decreasing the binding capability of Polymycin B	Shimomura et al., 2003
	Polyhydroxyalkanoates (PHAs)		Pan et al., 2011
	Diketopiperazines : cyclo(Pro–Tyr), cyclo(Ala–Val), cyclo(Pro–Leu), and cyclo(Pro–Val),	Inactive against *Candidas*	Wang et al., 2010
Burkholderia sp.	Pyrrolnitrin, *N*-Acylhomoserine lactones		Keum et al., 2009
	Lipase	Biocatalyst	Tran et al., 2012
	N-Acylhomoserine lactones: *N*-hexanoylhomoserine lactone (C6-NSL) *N*-octanoylhomoserine lactone (C8-NSL)		Chen et al., 2013
	N-Acylhomoserine lactones (AHLs): C6-NSL, C8-NSL, C10-NSL, C12-NSL		Goh et al., 2014

Burkholderia vietnamiensis	Endotoxin	Immunostimulatory activity on human myelomonocytic U937 cells.	Ieranò et al., 2009
Burkholderia thailandensis	Bactobolin 	Bactobolins A, B: antibiotics against MRSA and *V. parahemolyticus* with MICs < 1 µg/mL	Seyedsayamdost et al., 2010
	Spiruchostatins 	Antitumor towards LOX IMVI melanoma cells using murine hollow fiber assay	Klausmeyer et al., 2011
B. dolosa	Endotoxin		Lorenzo et al., 2013
Burkholderia gladioli pv. *agaricicola*	Volatile organic compounds (major compound as 1-methyl-4-(1-methylethenyl)-cyclohexene) 	Antifungal activity against *Botrytis cinerea*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium digitatum*, *Penicillium expansum*, *Sclerotinia sclerotiorum and Phytophthora cactorum* using agar diffusion test	Elshafie et al., 2012
	Exopolysaccharide		Andolfi et al., 2008
	O-specific polysaccharide (containing : D-mannose, D-rhamnose, D-galactose)		Karapetyan et al., 2006
Burkholderia arboris	Pyochelin 	Phytotoxicity against pine callus at EC_{50} of 171.98 µg ml^{-1}	Dang et al., 2011

The *Paenibacillus* genus possess the ability to produce some metabolites with industrial applications such as xylanase in paper industry (Zheng et al., 2012; Yeasmin et al., 2010),

acetoin in cosmetics and foods (Zhang et al., 2012). Especially, the genus produced fusaracidin - a compound having remarkable antibiotic activity against *Micrococcus luteus* (Kim et al., 2014) or abling to control *Phytophthora* blight infection (a disease blighted leaves) caused by *Phytophthora capsic* (Lee et al., 2012). This genus thus is promising as a source of interesting compounds. The data of some species production belonging to this genus was reported in Table 2.2.3.

Table 2.2.3 Summary about chemical studies on *Paenibacillus* genus (*Bacilli*)

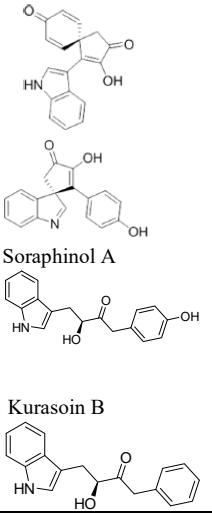
Producing strains	**Compounds**	**Nature of bioactivities**	**References**
P. campinasensis	Xylanase	Industrial application: pulp bleaching pretreatment.	Zheng et al., 2012
P. polymyxa	Acetoin 	Cosmetic, food	Zhang et al., 2012
	Xylanase enzyme	Paper industry	Yeasmin et al., 2010
	Lipopeptide	Anti-phytopathogen *Xanthomonas campestris* using agar diffusion test	Mageshwaran et al., 2011
	Fusaracidin 	Antibiotic : control Phytophthora blight infection caused by *Phytophthora capsic* at concentration 0.1 ppm	Lee et al., 2012
		Antimicrobial activity against *Micrococcus luteus* using agar diffusion test	Kim et al., 2014
	β-1,4-mannanase	Production of mannooligosaccharide	Hori et al., 2014
Paenibacillus sp.	Fibrinolytic enzyme		Vijayaraghavan et

			al, 2014
	Mutanase (α-1,3-glucan)	An oral hygiene product	Shimotsuura et al., 2008
	Chitinase		Meena et al., 2013
	KB 425796-A (nikkomycin) KB 425796-B	Antifungal activity against *Aspergillus fumigatus* (MIC of 0.5 and 2.5 µg.mL^{-1}, respectively.)	Kai et al., 2013b
	β-1,3-glucanase PgIA	Fungal disease biocontrol	Cheng et al., 2013
	KB 425796-C macrocyclic lipopeptidolactone	Anifungal activity activities against *T. asahii* (MEC of 1.56 mg.ml^{-1}, MIC of 3.13 mg.ml^{-1}) and *Aspergillus fumigatus* (MEC of 3.13 mg.ml^{-1}, MIC > 50 mg.ml^{-1}).	Kai et al., 2013a
P. terrae	Xylanase (endo-β-1,4-xylanase KRICT PX-3)		Song et al., 2014
P. woosongensis	Alkaline keratinolytic protease	In the laundry industry	Paul et al., 2014
P.alvei	Cyclic lipopeptide	Antimicrobial activity against *E. coli*, *Salmonella*, and *Staphylococcus aureus* using agar diffusion test	Knolhoff et al., 2015
P. elgii	Lipopeptide	Antibiotic	Ding et al., 2011

To date, to our knowledge, the genus *Lysinibacillus* was not already found as symbiotic partner of lichens. Herein, it was firstly reported from *R. geographicum*. The studies describing the production of its metabolites were also limited. Some researches (summarized in Table 2.2.4) highlighted that some bioactive compounds were isolated from this genus as spirobacillenes, soraphinol A and kurasoin B (Park et al., 2012), biosulfactants which possess

potent activity as anticancer agent with cytotoxic effect on human embryonic kidney cancerous cell (HEK-293) with LC_{50} 75 µg.ml^{-1} (Pradhan et al., 2014).

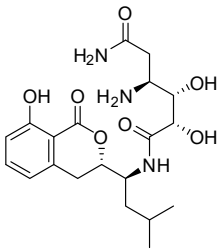
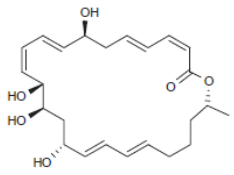
Table 2.2.4 Summary about chemical studies on *Lysinibacillus* genus (*Bacilli*)

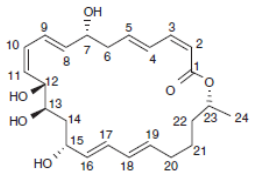
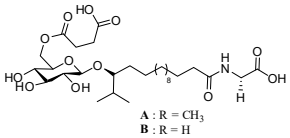
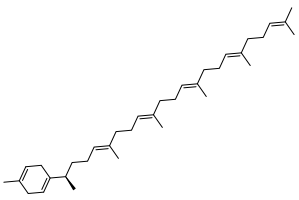
Producing strains	**Compounds**	**Nature of bioactivities**	**References**
Lysinibacillus fusiformis	Spirobacillenes A and B:  Soraphinol A Kurasoin B	Activity against the production of nitric oxide (NO) and reactive oxygen species	Park et al., 2012
	Protease and esterase		Prabha et al., 2014
	Biosurfactants	Cytotoxicity effect on human embryonic kidney cancerous cells (HEK-293) with LC_{50} 75 µg ml^{-1}	Pradhan et al., 2014

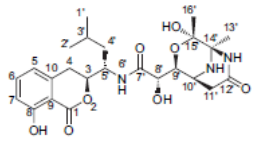
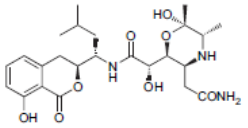
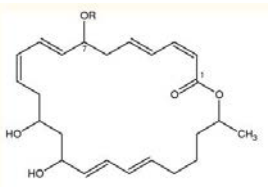
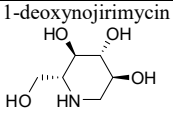
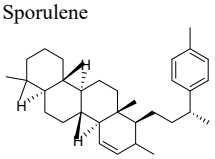
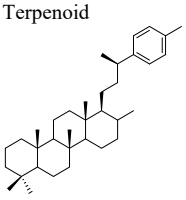
To date, the *Bacillus* genus has already attracted many interests for its metabolites. It produced many significant bioactive compounds (see Table 2.2.5). Diverse metabolites extracted from its culture were isolated e.g. enzymes as β-mannanase (Zang et al., 2015), lipoamide (Berrue et al., 2009), a series of bioactive compounds as surfactin, macrolactin (Nastro et al., 2013; Mondol et al., 2011a, 2011b) and diketopiperazines (Kumar et al., 2013).

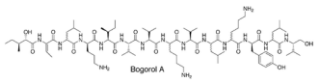
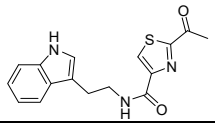
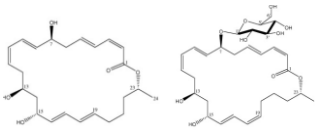
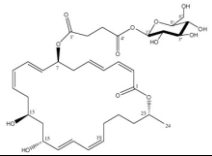
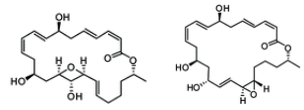
Table 2.2.5 Chemical studies on *Bacillus* genus (*Bacilli*)

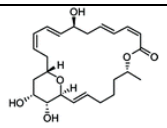
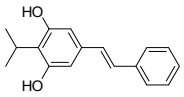
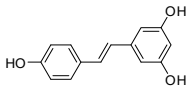
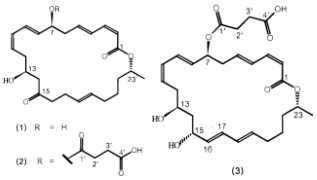
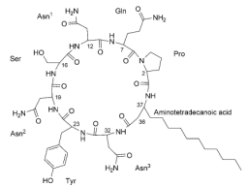
Producing strains	**Compounds**	**Nature of bioactivities**	**References**

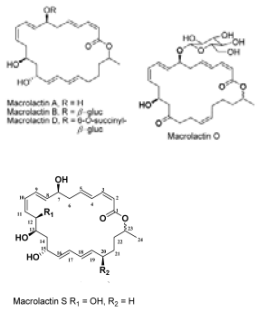
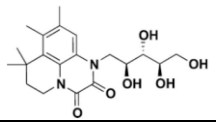
Bacillus pumilus	β-mannanase	Monno-oligosaccharides production	Zang et al., 2015
	Lipoamide A Lipopeptides 1-4 Amicoumacins A, B 	No active Antibacterial activity against *S. aureus, P. vulgaris, E. faecalis* (MIC from 6.5-25 µg/mL) Antibacterial activity against *S. aureus, P. vulgaris, E. faecalis, S. aureus* (MIC from 6.5-50 µg/mL)	Berrue et al., 2009
Bacillus amyloliquefaciens	Surfactin Fengycin Iturin A Macrolactin Difficidin Bacillaene		Nastro et al., 2013
	Difficidin Oxidifficidin Bacillaene		Chen et al., 2006
	Macrolactin S  Macrolactin V	Macrolactin S : antibacterial activity against *E.coli* and *S.aureus* (MIC of 0.3 and 0.1µg.mL^{-1}, respectively) Macrolactin V : antibacterial activity against *Bacillus subtilis*, *E.coli*, *S.aureus* (MIC of 0.1µg.mL^{-1})	Gao et al., 2010

			
	Bacillopeptin B1 and B	Antifungal activity	Ma et al., 2014
Bacillus lichenformis	Glycolipids : ieodoglucomide C and ieodoglycolipid	Antibiotic properties against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa* with MICs ranging from 0.01 to 0.05 µM	Tareq et al., 2015
	Ieodoglucomides A and B  **A** : R = CH_3 **B** : R = H	Antimicrobial activity **B**: Anti-lung cancer (GI_{50} 25.18 µg.mL^{1}), stomach cancer (GI_{50} 17.78 µg.mL^{1})	Tareq et al., 2012
	Bacteriocin BL8	Antimicrobial activity	Smitha and Bhat, 2013
Bacillus subtilis	Tetraprenyl-β-curcumene  Tetraprenyl-α-curcumene C35-terpenol		Takigawa et al., 2010
	Chitinase emzyme	Antifungal activity Anti-insecticid pests	Senol et al., 2014

	Bacilosarcins A  Bacilosarcins B 	Bacilosarcin A showed 82% inhibition at 50 mM against growth of barnyard millet	Azumi et al., 2008
	7-*O*-malonyl macrolactins A  R = CO-CH_2-COOH	Antibacterial activity	Romero-Tabarez et al., 2006
	1-deoxynojirimycin 	Increasing of GLUT4 and glucose uptake into adipocytes at 0.5 µM	Lee et al., 2013
	Sporulene  Terpenoid 		Kontnik et al., 2008
Bacillus claucii	β-geranylfarnesene β-hexaprene		Sato et al., 2013

Bacillus circulans	Cycloisomalto-oligosaccharide glucanotransferase (CITase)		Oguma et al., 2014
Bacillus sp.	Cyclo-(L-Pro-L-Leu) Cyclo-(D-Pro-L-Leu) Cyclo-(D-Pro-L-Tyr) Cyclo-(D-Pro-L-Phe) Cyclo-(L-Pro-L-Met)	Antimycobacterial activity : cyclo-(L-Pro-L-Met): MIC values of 4 µg/ml against *M. tuberculosis* $H_{37}Rv$	Kumar and Mohandas, 2014
	Bogorol A 	Antibacterial activity: against MRSA (MIC 2 μg/mL) and VRE (10 μg/mL), *E. coli* (35 μg/mL)	Barsby et al., 2001
	Loloatin B	Antibacterial activity: against *Staphylococcus aureus, Enterococcus sp, Streptococcus pneumoniae* with MICs of 1-2 µg/mL	Gerard et al., 1996
	Bacillamide 	Antialgal activity against *Cochlodinium polykrikoides* with LC_{50} of 3.2 µg/ml.	Jeong et al., 2003
	Macrolactin A Macrolactin Q  Macrolactin W 	Macrolactin W: antibacterial activity: against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* at MIC of 64 µg/mL	Mondol et al., 2011a
	Macrolactins **1** Macrolactins **2**  Macrolactins **3**	Antibacterial activity: MIC of 0.16 µM against *Bacillus subtilis* and *Escherichia coli* ; MICs against *Saccharomyces cerevisiae* 0.16, 0.02, and 0.16 µM, respectively.	Mondol et al., 2011b

			
	Lichenase		Maktouf et al., 2015
	Semiquinone glucoside	Antioxidant	Mishra et al., 2014
	3,5-dihydroxy-4-isopropyl stilbene 	Antioxidant at concentration 100 µg/ml Anticancer against cervical cancer cell line (HeLa), growth inhibition at IC_{50} of 25 µg/ml	Kumar et al., 2013
	3,4',5-trihydroxystilbene  3,5-dihydroxy-4-isopropyl stilbene	Antiphytopathogen: *P. expansum* (MIC of 4 µg/mL) *Fusarium oxysporum* (MIC of 2 µg/mL) Antifungal *: P. expansum* (MIC of 8 µg/mL), *Rhizotocnia solani* (MIC of 8 µg/mL)	Kumar et al., 2012
	Macrolactin F (1) and 7-*O*-succinyl macrolactin F (2), and A (3) 	Antibacterial activity against *B. subtilis* and *S. aureus* (inhibition zones of 8-28 mm at 50-100 µg/disk	Jaruchoktaweechai et al., 2000
Bacillus marinus	Marihysin A 	Antifungal activity against *Alternaria solani, Fusarium oxysporum, Verticillium alboatrum, F.graminearum, Sclerotium sp., Penicillium sp., Rhizoctonia solani,* and *Colletotrichum sp.* with MIC values of 100 – 200 mg/ml	Liu et al., 2010
	Macrolactins T and U	Macrolactin B: antifungal activity against *Alternaria solani* with MIC values of	Xue et al., 2008

	Macrolactins A,B,D,O and S 	7.5 and 20.1 µg/mL *Pyricularia oryzae* and antibacterial *Staphylococcus aureus* (MIC of 4.5 µg/mL)	
Bacillus hunanensis	Hunanamycin A 	Antibacterial activity against *Salmonella enterica* (MIC of 12.4 µM)	Hu et al., 2013

In summary, via a screening of the references dealing with bacterial compounds of some genera isolated from *R. geographicum*, *Paenibacillus* genus exhibited a great promising potential in production of bioactive metabolites. So far, this genus has almost produced macromolecules as enzyme, polysaccharide, macrolactins… (See Table 2.2.3) that presented significant antifungal and antibacterial activities. Therefore, this genus was selected for further studies to isolate other interesting metabolites.

2.2. Chemical studies on *Paenibacillus odorifer*

To our knowledge, there are no reports about the production of metabolites from *P. odorifer* belonging to *Paenibacillus* genus to date. We have decided to work on the unexpoited *P. odorifer* isolated from *R. geographicum* for further bioactive metabolites study. We have focused our research on the discovery of new and potent cytotoxic compounds as bacteria have often been highlighted as a promising source of active metabolites (Newman and Cragg, 2016).

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CHAPTER 3: OPTIMIZATION OF THE CULTURE OF *PAENIBACILLUS ODORIFER*

CHAPTER 3: OPTIMIZATION OF THE CULTURE OF *PAENIBACILLUS ODORIFER*

When *P. odorifer* is cultivated in liquid media, the selection of culture parameters in this process is crucial because they could dramatically affect the production of bioactive compounds. We have decided to perform the optimization of the culture in order to obtain sufficient amount of active extracts for further purification steps.

In this chapter, we will describe two optimal processes used for the culture of this strain. The first optimization was based on the selection of culture parameters as pH, temperature, and $CaCO_3$ supplementation in medium to obtain the best bacterial growth. The second process was then applied using the results of the first one and was set up using new parameters corresponding to stirring rate, inoculum ratio, quantity and biological activities of the crude extracts obtained.

3.1. THE FIRST OPTIMIZATION OF PROCESS

3.1.1. The selected parameters

P. odorifer was isolated from Gym *Streptomyces* agar medium at 15° and 25°C. The DSMZ (German collection of microorganisms and cell culture) suggested that $CaCO_3$ can be removed if Gym *Streptomyces* medium was liquid. The supplementation with or without $CaCO_3$ in liquid medium was selected as factors for optimal process. Beside, the pH values of medium were also chosen to measure its eventual impact on bacterial growth. Indeed, the parameters selected for the first optimization were pH of medium (4, 7, 8, 9 and 10), temperature (15°C and 25°C) and medium supplementation with or without $CaCO_3$.

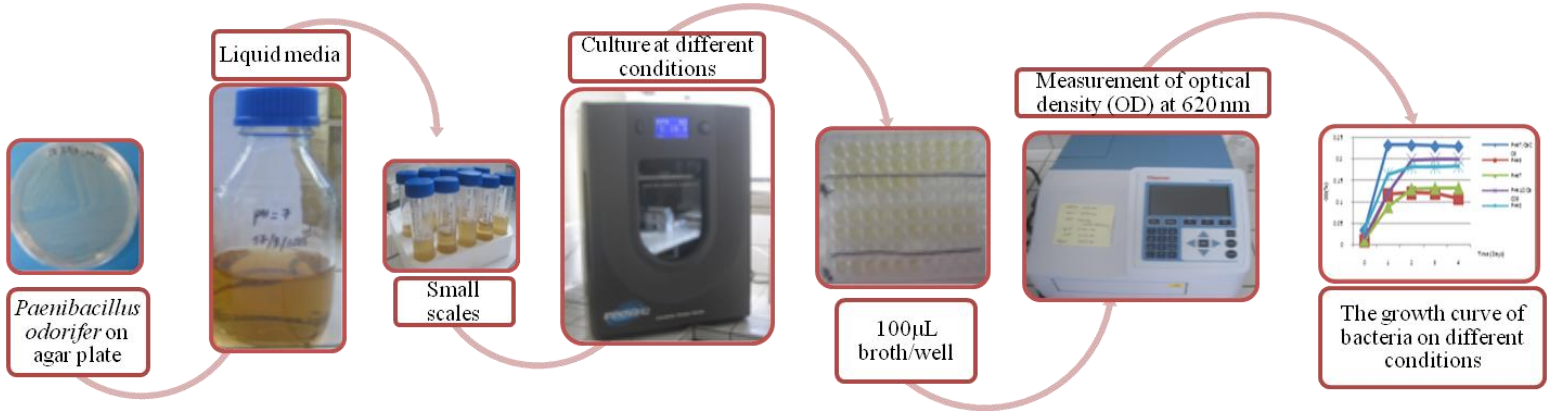
The experimental plans were carried out at small scale (25 mL of medium). The cultures were performed in Gym *Streptomyces* medium with parameters shown in Table 2.3.1. However, the stirring rate was fixed at 150 rpm.

Table 2.3.1 Parameters for the first optimization

pH	4		4		7		7		8		8		9		9		10		10	
Supplementation in medium			$CaCO_3$				$CaCO_3$				$CaCO_3$				$CaCO_3$				$CaCO_3$	
Temperature (°C)	15	25	15	25	15	25	15	25	15	25	15	25	15	25	15	25	15	25	15	25

The growth of bacterium was checked each day by measurement of optical density (OD) at 620 nm. The curves of bacterial growth provide information to select the best conditions for culture. The process is reported in Scheme 2.3.1.

Scheme 2.3.1: Diagram of the first optimal process



3.1.2. Results

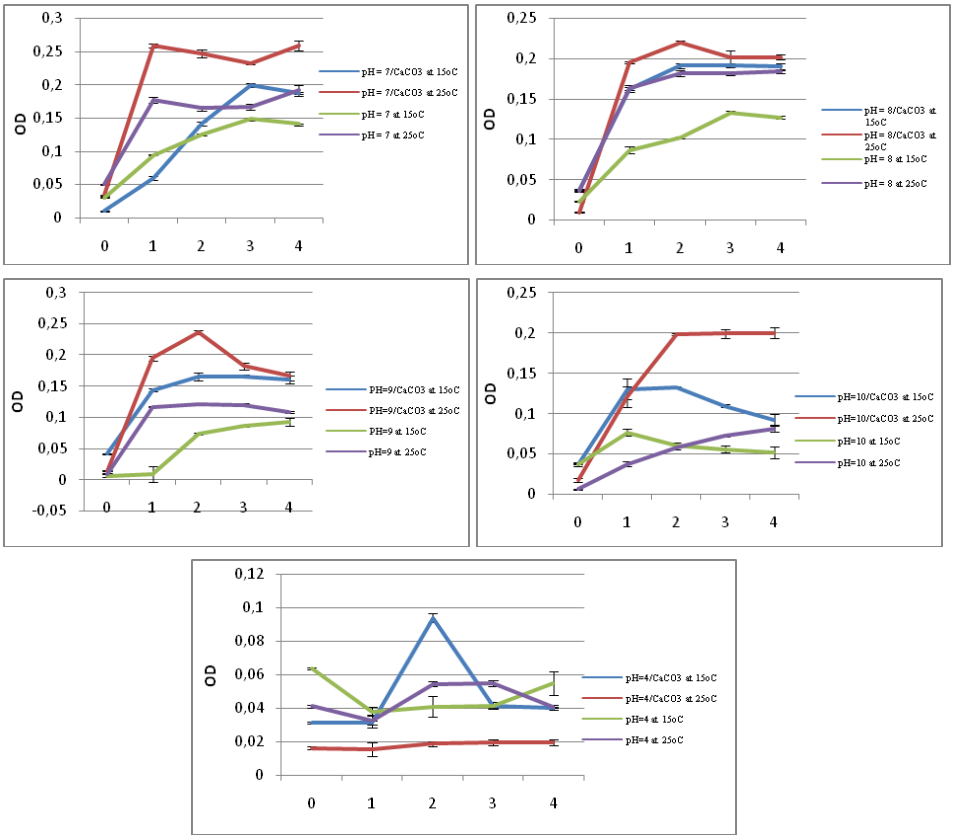


Figure 2.3.1 The curves of bacterial growth at 15°C and at 25°C with different culture conditions

The results in Figure 2.3.1 indicated that *P. odorifer* did not grow at pH 4 at any temperature in medium with or without $CaCO_3$. This strain grew well at other pH values. Based on OD measurement, the growth was higher at 25°C than at 15°C. The comparison between OD values led to a conclusion that the best conditions for *P. odorifer* growth was in Gym *Streptomyces* medium supplemented with $CaCO_3$ at pH 7 and 25°C.

3.1.3. The application to bioreactor fermentation

The first optimization provided the best parameters of pH and kind of medium for the culture of *P.odorifer* in liquid medium with small scale. These conditions were applied to the fermentation step using a bioreactor (Figure 2.3.2).

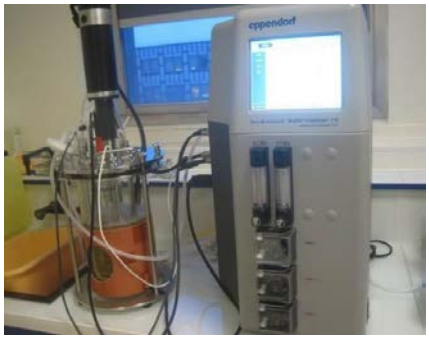


Figure 2.3.2 Bioreactor *(BioFlo® 115)*

The process was set up in 4.5 litres of Gym *Streptomyces* medium supplemented with $CaCO_3$, at pH 7, 25°C, with a 1/4.5 ratio of VVM during 4 days. The bacterial growth in bioreactor was also checked by measurement of OD at 620 nm (Figure 2.3.3).

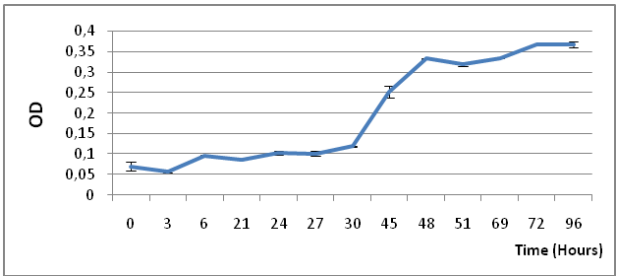


Figure 2.3.3 The curves of bacterial growth cultured using a bioreactor

After a 4-day culture, the crude extracts from the fermentation were collected following the Scheme 2.4.1 (Chapter 4, part 4.1). LC-UV and LC-MS analyses were undertaken on the obtained extracts (Chapter 6, part 6.2.2) to study the production of metabolites (reported in Chapter 4, part 4.4).

The metabolites isolated from the fermentation using bioreactor corresponded to a fraction of polysaccharide, to two diol derivatives together with some different metabolites. The polysaccharide structure will be detailed in an article (Chapter 4, part 4.2, 4.2.2), the diol derivatives along with other metabolites will be presented in Chapter 4, part 4.5 as a report of thesis. Unfortunately other compounds, due to their presence in a too low amount (< 0.8 mg), could not be submitted to structural elucidation and biological evaluation steps. Therefore, to increase the amount of secondary metabolites produced, a second optimization process was established using Erlenmeyer flasks.

3.2. THE SECOND OPTIMIZATION PROCESS

3.2.1. The selected parameters

In the first optimization step, the factors consisting of stirring, inoculum ratio injected in the culture, the quantities and the activities of crude extracts from the broth were not chosen. Therefore, these factors became the parameters of the second optimization. Moreover, the best culture conditions determined from the first optimization were also applied in this stage.

Firstly, experimental plans were performed with parameters as inoculum ratio and stirring (See Table 2.3.2). Each experiment was carried out at 25°C using 4 liters of each medium supplemented with $CaCO_3$ at pH=7 in Erlenmeyer flasks (300 mL volume). The culture process was checked by OD measurement and it was stopped until decreasing of the OD values which corresponded to the reach of the stationary phase. It is well known that the production of secondary metabolites is made at this step of growth. Besides, the colony forming unit (CFU) was counted at the same time with OD measurement. The method to determine CFU was shown in Chapter 6, part 6.2.

Table 2.3.2 The parameters for second optimization

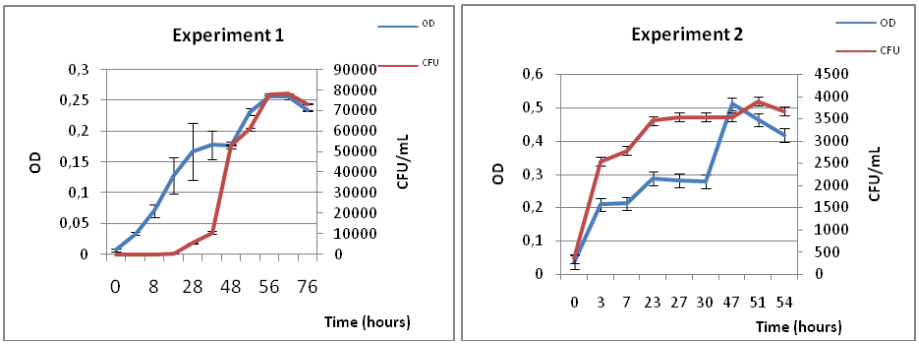
Experimental number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Factor	Inoculum (%) (v/v)	1%	5%	10%	1%	5%	10%	1%	5%	10%
	Stirring (rpm)	150	150	150	180	180	180	120	120	120

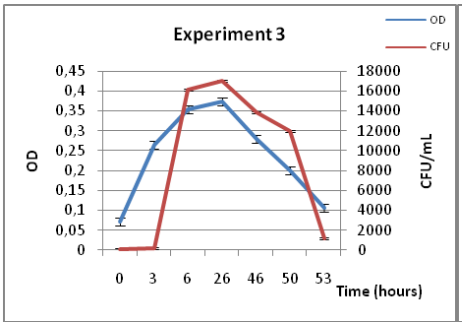
The crude extracts from these broths were obtained following the process reported in Scheme 2.4.1- Chapter 4 and were analyzed by HPLC-UV and submitted to biological evaluation (cytotoxicity using a MTT assay). Finally, the combined data derived from mass of crude extract, HPLC data and bioactive properties afforded the best conditions for the culture of *P. odorifer.*

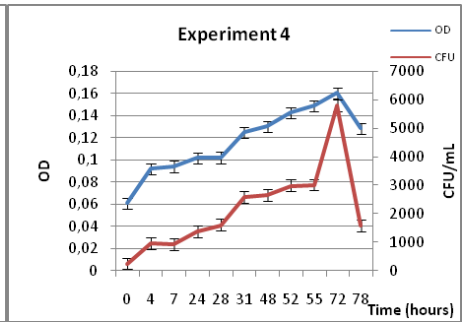
3.2.2. Results

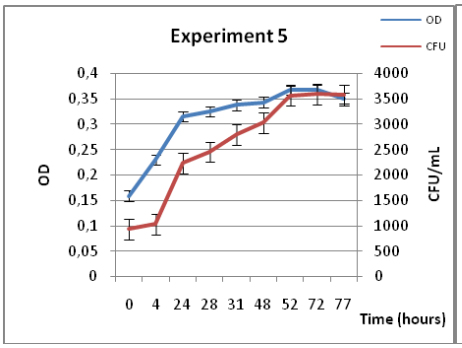
a) OD values and CFU data

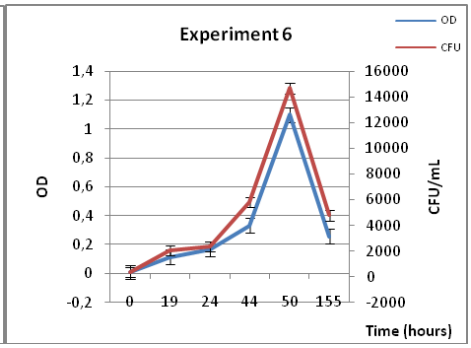
The OD values and the number of CFU/mL calculated for each experiment were reported in the same line chart (see Figure 2.3.4).

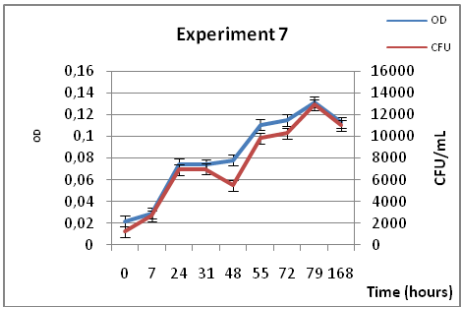


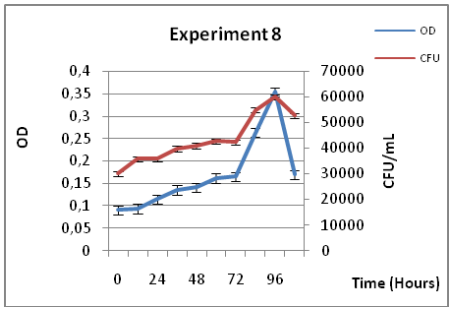

Experiment 3
OD
CFU
0,45 0,4 0,35 0,3 0,25 0,2 0,15 0,1 0,05 0
18000 16000 14000 12000 10000 8000 6000 4000 2000 0
0 3 6 26 46 50 53
Time (hours)
CFU/mL


Experiment 4
OD
CFU
0,18 0,16 0,14 0,12 0,1 0,08 0,06 0,04 0,02 0
7000 6000 5000 4000 3000 2000 1000 0
0 4 7 24 28 31 48 52 55 72 78
Time (hours)
CFU/mL


Experiment 5
OD
CFU
0,4 0,35 0,3 0,25 0,2 0,15 0,1 0,05 0
4000 3500 3000 2500 2000 1500 1000 500 0
0 4 24 28 31 48 52 72 77
Time (hours)
CFU/mL


Experiment 6
OD
CFU
1,4 1,2 1 0,8 0,6 0,4 0,2 0 -0,2
16000 14000 12000 10000 8000 6000 4000 2000 0 -2000
0 19 24 44 50 155
Time (hours)
CFU/mL


Experiment 7
OD
CFU
0,16 0,14 0,12 0,1 0,08 0,06 0,04 0,02 0
16000 14000 12000 10000 8000 6000 4000 2000 0
0 7 24 31 48 55 72 79 168
Time (hours)
CFU/mL


Experiment 8
OD
CFU
0,4 0,35 0,3 0,25 0,2 0,15 0,1 0,05 0
70000 60000 50000 40000 30000 20000 10000 0
0 24 48 72 96
Time (Hours)
CFU/mL

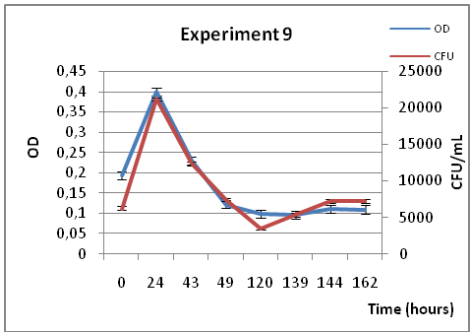


Figure 2.3.4 The OD and number of CFU/mL for each experiment at different culture conditions

As seen in Figure 2.3.4, the OD and CFU/mL data has evolved for each experiment in the same manner indicating that there is a linear relationship between these two parameters.

b) The mass of the crude extracts

The mass of crude extracts were shown in Table 2.3.3. Following the process described in Scheme 2.4.1 in Chapter 4 – part 4.1 two different crudes were obtained for each experiment labeled resin or supernatant extracts. In most experiments, the mass of resin extracts was higher than those of the supernatant extracts expected for experiments **5** and **9**.

Table 2.3.3 The mass of crude extracts (mg) from experiments obtained during the second optimization step

Experiment No		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
mass of crude extracts (mg)	Resin extract	176.2	164.2	200.4	229.8	237.6	176	439.5	307.9	136.0
	Supernatant extract	59.0	39.6	85.0	103.9	264.8	128.0	61.9	111.6	196.3

c) The analysis of chemical profile of each extract by HPLC

The analysis by HPLC-DAD was performed using the same concentration of 1 mg/mL for each sample, with a volume injection of 20 µL, using a reverse phase (Prevail C_{18} column), and a gradient of CH_3CN and H_2O as mobile phase (See Chapter 6, part 6.2; 6.2.2.4).

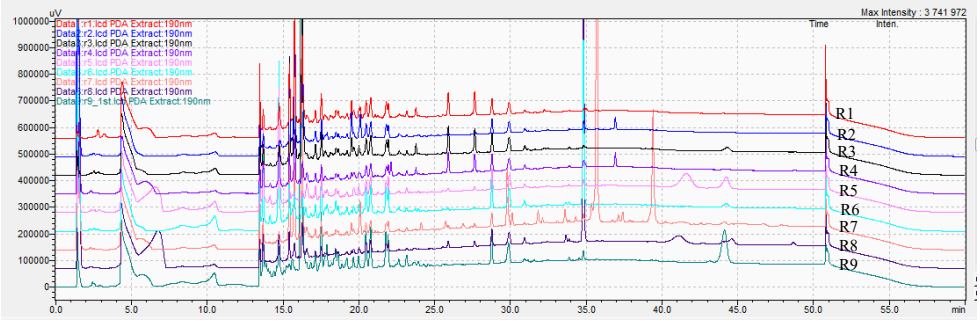


Figure 2.3.5 Chemical profiles obtained by HPLC-DAD of crude extracts from resin (R1: extract from resin of experiment number 1, similar for R2, R3, R4, R5, R7, R8, and R9)

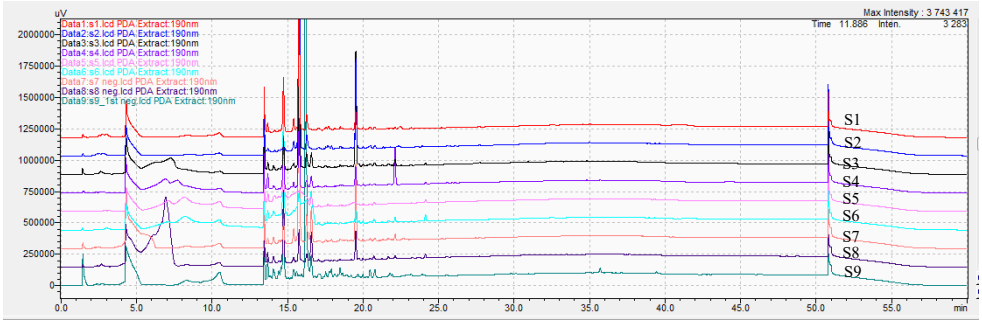


Figure 2.3.6 Chemical profiles obtained by HPLC--DAD of crude extracts from supernatant (S1: extract from supernatant of experiment number 1)

The data reported in Figures 2.3.5 and 2.3.6 highlighted a similar chemical profiling of extracts of all the experiments depending on the nature of these extracts (resin or supernatant). Moreover, the resin extracts seem richer in term of metabolites than the supernatants which contained more polar metabolites (compounds with T_r between around 13 and 24 min).

d) The evaluation of biological activities

The cytotoxic effect on all the crude extracts were evaluated using a MTT assay against HaCaT human keratinocyte and B16 murine melanoma cell lines with doxorubicine as positive control (See Chapter 6 - part 6.2 - 6.2.7). The results showed that the extracts possessing the highest cytotoxicity (IC_{50} of 26 ± 3 and 17 ± 7) against the two cell lines were the resin extract 7, which was harvested from culture at 25°C, 120 rpm of stirring with 1% inoculum in medium supplemented with $CaCO_3$ at pH 7, followed by resin extract 5. However the supernatant extract of experiment 7 (in the same conditions of culture) exhibited low values of cytotoxicity (IC_{50} of 93±11 and 150±10 for HaCaT and B16, respectively). The difference can be explained that the vast majority of cytotoxic metabolites in the broth number 7 were present on extract resin in comparison to the supernatant extract.

Table 2.3.4 The results of the second optimization (in Gym *Streptomyces* medium supplemented with $CaCO_3$ at 25°C, pH = 7)

Experimental conditions			**Results**					
			Crude extract from resin			**Crude extract from supernatant**		
Experiment	**Stirring (rpm)**	**Inoculum (%)**	**Amount (mg) (in 4.0 L medium) (mg/L yield)**	**IC_{50} (µg/mL)**		**Amount (mg) (in 4.0 L medium) mg/mL yield)**	**IC_{50} (µg/mL)**	
				HaCaT	B16		HaCaT	B16
1	150	1	**176.2, (43.25)**	193±23	190±40	**59.0, (14.75)**	>200	>200
2	150	5	**164.2, (41.05)**	>200	>200	**29.6, (7.40)**	158±31	190±42
3	150	10	**200.4, (50.1)**	>200	>200	**85.0, (21.25)**	123±17	133±15
4	180	1	**229.8, (57.45)**	152±24	190±34	**103.9, (25.96)**	118±13	110±21
5	180	5	**237.6, (59.4)**	63±5	50±18	**246.8, (61.7)**	47±11	100±16
6	180	10	**176.0, (44.0)**	130±10	46±20	**128.0, (32.0)**	56±5	96±8
7	120	1	**439.5, (108.86)**	26±3	17±7	**61.9, (15.48)**	93±11	150±10
8	120	5	**307.9, (76.96)**	105±15	45±18	**111.6, (27.9)**	39±6	115±6
9	120	10	**136.0, (34.0)**	167±17	18±8	**196.0, (49.0)**	187±34	>200

e) Conclusions

The combination of the data from the secondary optimization (see Table 2.3.4) indicated that the best conditions of culture to produce bioactive compounds were those of experiment noted **5** and **7**. Although the OD values of experiment **7** was lower than that of experiment **5**, its yield (mg/L) and cytotoxic properties exhibited values better than those of experiment **5**. Therefore, the parameters of experiment **7**, corresponding to culture in Gym Streptomyces medium supplemented with $CaCO_3$, pH 7 at 25°C with stirring at 120 rpm, inoculation of 1% (v/v) during 7 days, were selected as the best conditions for culture of *P. odorifer*.

Besides that, the summary of results in Table 2.3.4 emphasized that the parameters including stirring and inoculum ratio were important factors dramatically affecting the production of cytotoxic metabolites. For instance, using the same inoculum ratio, the stirring of experiment **1** was stronger than that of experiment **7**, but the mass and biological effects of extracts from experiment **1** were less important than those of experiment **7**. Similarly, the cultures with the same stirring rate gave noticeable differences on the mass and cytotoxicity of crude extracts as displayed for experiments **6**, **7**.

3.2.3. The application of these results for cytotoxic compounds discovery

The crude extracts from experiments number **7** (**R7** and **S7**) which exhibited higher activities were directly used to isolate metabolites. The extract **R7** (439.5 mg) was submitted to various chromatography approaches as flash chromatography, analytical and semi-preparative HPLC (See Chapter 6, part 6.2) to afford 9 compounds as two *tert*-butyl compounds, furfural derivatives … Among them, one of the two *tert*-butyl compounds possessed a significant cytotoxic effect against HaCaT human keratinocyte and B16 murine melanoma cell lines. Moreover, these compounds are considered to be rare in nature. The isolation, structural elucidation and evaluation of bioactive properties of these compounds were detailed as an article (Chapter 4 - part 4.3). The other compounds were classically reported in this manuscript in Chapter 4 - part 4.5.

Further, the culture with a large volume (40 L in Erlenmeyer) using the best conditions selected following the results of the second optimization was carried out to find bioactive

metabolites with higher amounts. Bioassay-guided fractionation of the resin extract led to the identification of a new alkaloid formed by a dihydronaphthalen part fused to a pyrrolooxazine unit. The details of the isolation, structural elucidation and cytotoxic activity of this alkaloid were reported as an article (see Chapter 4 - part 4.4).

CHAPTER 4: ISOLATION OF METABOLITES FROM *PAENIBACILLUS ODORIFER*

molecules

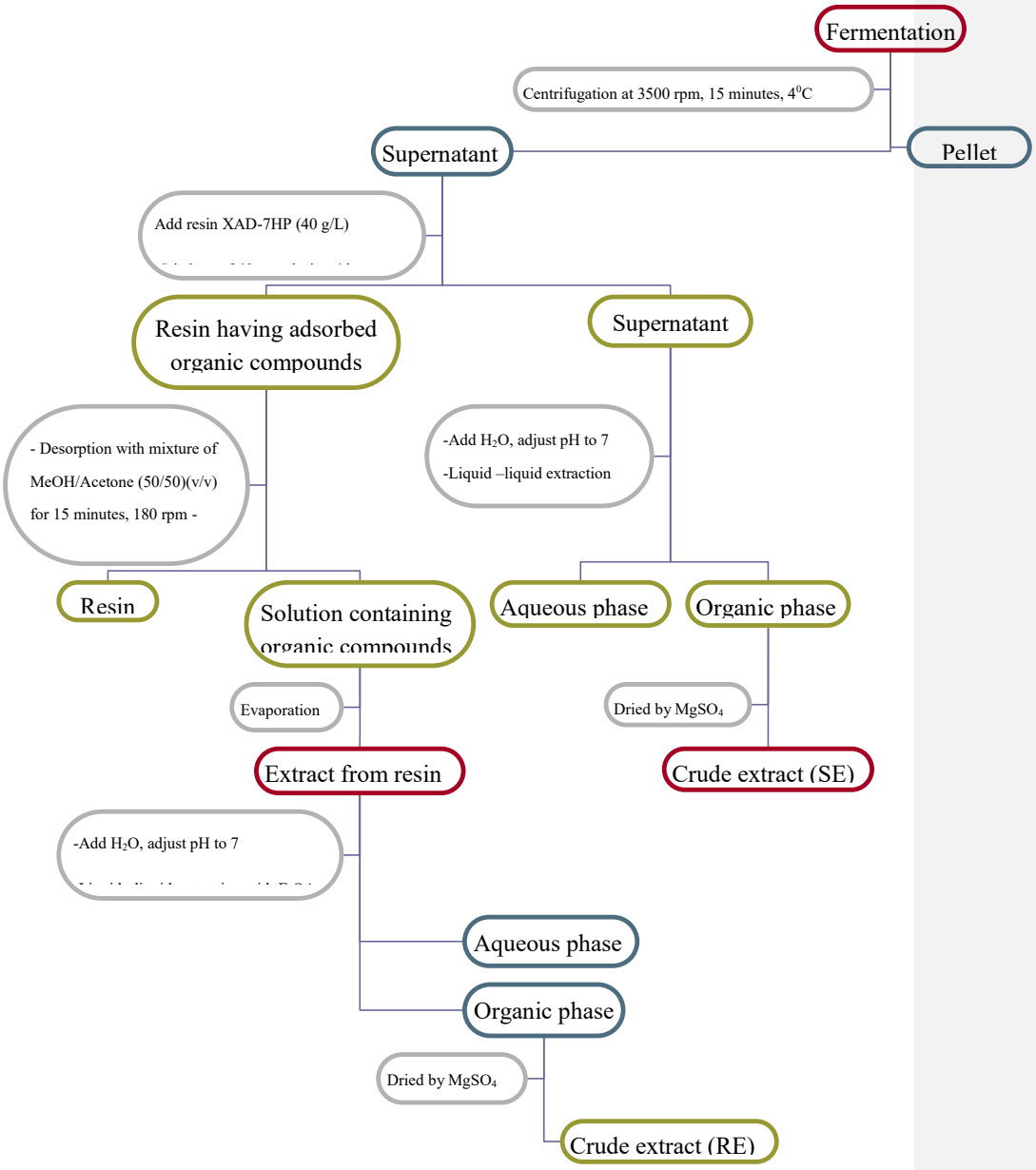
CHAPTER 4: ISOLATION OF METABOLITES FROM *PAENIBACILLUS ODORIFER*

Once the parameters of culture of *P. odorifer* have been selected we have carried out the cultures either by Erlenmeyer Flasks or by Bioreactor following the general process described below (chapter 4.1). We have focused our work on the resin or supernatant extracts and a bio-guided approach has been attempted in order to highlight the metabolites of interest. This chapter will be divided into 5 parts including part 4.1 being introduction of the process for the production of crude extracts from many fermentations, part 4.2 dealing with the presentation of an oligosaccharide obtained in high amount during a culture in bioreactor, the isolation of *tert*-butyl compounds will be discussed into part 4.3, the selection of an active novel alkaloid will be described into part 4.4 and finally a report of the other metabolites produced by this species following various kind of cultures will be done on part 4.5.

4.1 THE PROCESS FOR THE PRODUCTION OF CRUDE EXTRACTS FROM FERMENTATION

After separation of supernatant and bacterial cells by centrifugation, a XAD-7HP resin was added to the supernatant to absorb organic compounds with stirring for 4 hours. After filtration, the resulting supernatant was extracted by EtOAc to afford supernatant extract. While the resin was desorbed using MeOH : acetone 50:50 (v/v) three times, the resulting phase was concentrated then extracted by EtOAc to afford a crude extract labeled resin extract. For each culture, two different crude extracts were each time obtained and called supernatant extract (SE) and resin extract (RE). The general process was reported in Scheme 2.4.1.

Scheme 2.4.1: Process of the obtention of the crude extracts from fermentation



4.2. ISOLATION OF A POLYSACCHARIDE UNIT

During our work, a polysaccharide fraction was isolated and its antioxidant and cytotoxic activities were exhibited. This part will be described in chapter 4 – part 4.2.2 as an article. We will first describe a general presentation on bacterial polysaccharides and the polysaccharide fraction isolated from P. *odorifer* culture from bioreactor will be then reported.

4.2.1. General presentation of bacterial polysaccharides: structure and properties

Polysaccharides are polymers of high molecular weights formed by chains of sugar units connected by many ether glycosidic linkages. They are ubiquitary present in all living organisms and are important for the life due to their properties such as responsible of membrane rigidity or gel formation, energy storage, protection against dehydration etc. Bacterial polysaccharides have recently attracted much attention from chemists due to their easier biotechnological production and their significant properties (see in Ruas-Madiedo et al., 2002). Thus, many active bacterial polysaccharides were reported continuously such as an exopolysaccharide isolated from *Micrococcus luteus* possessing antioxidant property using DPPH assay (EC_{50} of 180 µg/mL) (Asker et al., 2014); an extracellular polysaccharide from *Streptomyces* sp. exhibiting antioxidant DPPH activity and cytotoxicity using MTT assay against human breast cancer (MDA-MB-231) and mouse breast cancer (4T1) cell lines (Elnahas et al., 2017); an exopolysaccharide from *Bacillus coagulans* with an effect of lipid peroxidation inhibition (TBA assay) (Kodali et al., 2011); an extracellular polysaccharide from *Paenibacillus polymyxa* having antioxidant activity using NBT assay (Raza et al., 2011), an exopolysaccharide from *Bacillus amyloliquefaciens* sp. owning antitumor ability (MTT assay) against gastric carcinoma cell lines MC-4 (IC_{50} of 19.7 µg/µL) and SGC-7901 (IC_{50} of 26.8 µg/µL) (Chen et al., 2013); a sulfate polysaccharide from *Pseudomonas* sp. displaying its cytotoxicity against many human cancer cell lines (Matsuda et al., 2011).

Bacterial polysaccharides represent a diverse range of macromolecules that include peptidoglycan (Manna et al., 2017) consisting of sugars and aminoacids; lipopolysaccharides (Kokoulin et al., 2016; Ravenscroft et al., 2015) containing lipid and polysaccharide, capsular polysaccharides (Kadirvelraj et al., 2006; Petersen et al., 2014) and exopolysaccharides (Asker et al., 2014; Hung et al., 2005) containing polysaccharide and protein. These compounds demonstrate functions like structural cell-wall components (e.g. peptidoglycan (Schleifer et al., 1972)), immunostimulating effect (Manna et al., 2017), as important virulence factors (e.g. Poly-*N*-acetylglucosamine in *Staphylococcus aureus* (Kropec et al., 2005)) or permitting the bacterium to survive in harsh environments (e.g. *Pseudomonas aeruginosa* in the human lung (Sadikot et al., 2005)).

4.2.2. Production of one polysaccharide fraction from *P. odorifer*

The polysaccharide fraction was extracted from the broth of the culture carried out on bioreactor (4.5 L) at 25°C, with Gym Streptomyces medium supplemented with $CaCO_3$ at pH =7 (Chapter 3 - 3.1). The crude extract (353.7 mg) collected from the culture (following Scheme 2.4.1) was separated by flash chromatography using a reverse phase Reveleris (Grace) C_{18} column with parameters described in 5.2.2 and Figure 2.5.2 (Chapter 5) to afford 21 fractions. The twentieth fraction was purified several times by methanol to give a polysaccharide fraction (116.4 mg) (Table 2.5.1) yielding a 0.0073 g/L.

The polysaccharide fraction was first identified by IR spectrum; the elucidation of its structure performed via various steps and approaches was reported in an article (in progress).

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Article

Isolation and structural characterization of an antioxidant and cytotoxic fraction of polysaccharide purified from culture of *Paenibacillus odorifer* – a bacterium associated with the lichen *Rhizocarpon geographicum*

An article in progress

Le Thi Bach Nguyen[1,2], Isabelle Rouard[1], Aurélie Sauvager[1], and Sophie Tomasi [1,*]

[1] Univ Rennes, CNRS, ISCR – UMR 6226, F-35000 Rennes, France

[2] Faculty of Chemical and Food technology, HCMC University of Technology and Education, Vietnam
* Correspondence: sophie.tomasi@univ-rennes1.fr ; Tel.: +33(0)2-23-23-48-17

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Abstract: Extracellular polysaccharide (EPS), coded as PO-QL1, was isolated from *Paenibacillus odorifer* – a bacterium associated with the lichen *Rhizocarpon geographicum*. The EPS was obtained from methanol extraction by successive purification via flash chromatography using C_{18} reserve phase column, with elution gradient as H_2O and acetonitrile mixture. Its structural feature analyzed by IR, HPLC, NMR, LC-MS indicated that it was mainly composed of *β*-D-glucuronic acid, *β*-D-fructose and *β*-L-fucose as monomeric constituent units with an approximate molar ratio as 4:2:1. Further, the process sequencing comprising methylation, hydrolysis, reduction and acetylation was applied to determine the structure of PO-QL1. The results based on GC-MS data indicated that the carbohydrate contained a fraction of linked monosaccharides as →2)- β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→4)-β-D-Fru*f*-(2→4)-β-D-Fru*f*-(2→4)-β-L-Fuc*p*-(1→. This extracellular polymeric substance also displayed a moderate antioxidant activity using NBT assay (IC_{50} of 22.5 ± 1.5 μg/mL), while it possessed a significant cytotoxicity measured by MTT assay with IC_{50} value of 19 μg/mL and 27 μg/mL on HaCaT human keratinocyte and B16 murine melanoma cell lines, respectively. This may provide a potential source of antitumor agents as microbial polysaccharide fraction from *P. odorifer*.

Keywords: Extracellular polysaccharide fraction; *Paenibacillus odorifer*; *Rhizocarpon geographicum*.

1. Introduction

Natural polysaccharides, including those secreted by bacteria, have drawn the attention of many researchers during the last decades due to their significant biological activities. Indeed, the microbial polysaccharides, or extracellular polysaccharides (EPSs), extracted from the broth of culture process from bacteria were recently postulated as a potential source of antioxidants [1] – for

important and useful applications in pharmaceutical and cosmetic industries [2] and for antitumor agents discovery [3,4]. Recently, EPSs derived from *Paenibacillus* genus have attracted much attention because of their biotechnological potential in many distinct fields such as the production of antioxidant active EPSs [5];; of EPS for application in cosmetic field [6]; of polysaccharides useful in wastewater treatment [7]; of bioflocculant polymeric substance [8]; of curdlan with potential in pharmaceutical industry [9]; of removal heavy metal EPS [10], of a multi-functional EPS [11]; or of β-glucan with enhancing immunity ability for animals [12]. However, no EPSs has been reported from *Paenibacillus odorifer* species. Herein, we first report structural characterization of a EPS fraction isolated from *P. odorifer* that exhibited remarkable antioxidant and cytotoxic activities.

2. Results and Discussion

2.1. Characterization of a polysaccharide fraction PO-QL1 isolated from Paenibacillus odorifer

2.1.1. Analysis by Fourier transform Infrared (FT-IR) spectroscopy

The main structural groups of polysaccharide PO-QL1 were first identified by characteristic absorbance band maxima highlighted by FT-IR spectrum analysis (Figure 1). It revealed a typical major broad stretching vibration at 3342 cm^{-1} for hydroxyl groups that were assigned to functional groups of polysaccharides. The sharp bands at 2917 and 2849 cm^{-1} introduced the C-H bond that was always present in carbohydrates. The strong absorption at 1656 cm^{-1} is characteristic to stretching band of carbonyl groups (C=O). Moreover, the prominent band at 1062 cm^{-1} was attributed to stretching vibration of C-O-C bond of glycosidic linkages. Interestingly, the band at the frequency of 950 cm^{-1} suggested the presence of *β*-anomer linkage in PO-QL1 [13].

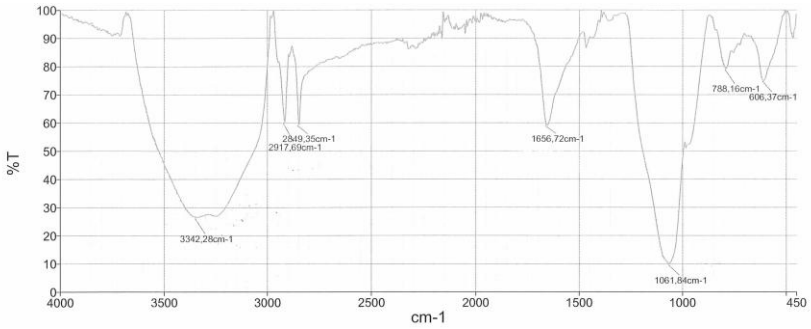


Figure 1. Fourier transform infrared (FT-IR) spectrum of polysaccharide QL1 in the range of 400 – 4000 cm^{-1}

2.1.2. Analysis of ^{1}H-NMR data of PO-QL1

The structure of the polysaccharide fraction PO-QL1 was further identified by one-dimensional (1D) ^{1}H-NMR spectrum (Figure 2). The ^{1}H-NMR data showed signals at δ_H 2.8-2.9 ppm corresponding to acetyl groups [14], and it also presented an overlap signal region at δ_H 3.0-4.0 ppm assigned to protons belonging to ring monosaccharide of many sugar units – a typical structure of polysaccharide. One interesting point was highlighted in the ^{1}H-NMR with the presence of a signal at δ_H 4.5-5.5 ppm characteristic of β-anomer linkage between monosaccharide units. These results are similar to those shown by FTIR spectrum of PO-QL1 (Figure 1).

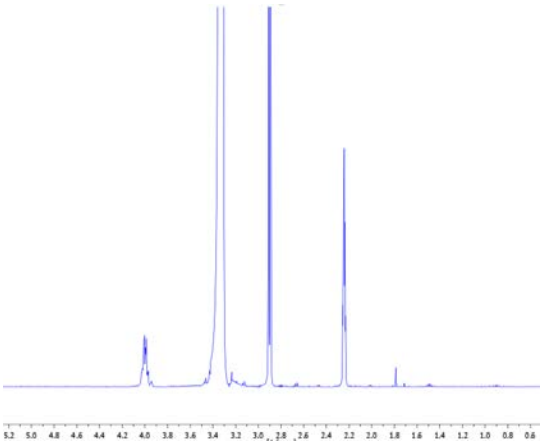
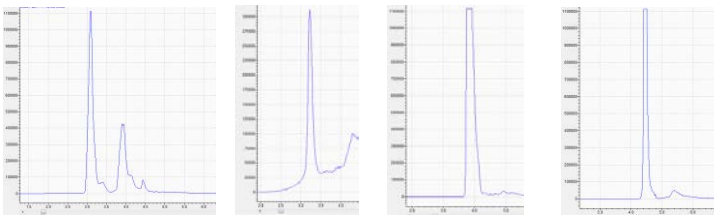


Figure 2. The ^{1}H-NMR spectrum of polysaccharide PO-QL1 in DMSO-d_6 recorded at 300 MHz.

2.1.3. Determination of sugar units of the polysaccharide PO-QL1

The sugar units of PO-QL1 was investigated using the method of Sulkowska-Ziaja and co-authors [15] with some modifications. PO-QL1 (10 mg) was hydrolyzed with 3 ml of trifluroroacetic acid (TFA) 2 M at 120^{o}C, 550 rpm during 4 h to cut glycosidic linkages between monosaccharide units. After hydrolysis, the monosaccharide compositions of the obtained solution were determined by HPLC method and the identification of these units was carried out by comparison of their retention time with those of standard monosaccharides including glucose, galactose, mannose, fructose, xylose, rhamnose, fucose, arabinose and glucuronic acid in the same conditions of HPLC analysis. The HPLC profiles showed three distinguishable peaks (Figure 3) at retention time corresponding with those of D-glucuronic acid, D-fructose and L-fucose (Table 1). Moreover, the analysis of intensities of the peaks in HPLC data indicated that PO-QL1 contained glucuronic acid, fructose and fucose in relative molar ratio of 4:2:1, respectively.



(a) (b) (c) (d)

Figure 3. HPLC chromatograms (using Prevail C18 column (250 mm x 4.6 mm, 5μm) mobile phase with gradient elution as various concentration of H_2O : Acetonitrile, flow rate of 0.8 mL/min, injection volume of 20 μL, concentration of sample of 1 mg/mL) of PO-QL1 (a) and standard glucuronic acid (b), fructose (c) and fucose (d)

Table 1. Comparison of retention time between polysaccharide after treatment by TFA with standard monosaccharides in HPLC analysis using reserve-phase Prevail column and elution with a gradient of mixture H_2O/Acetonitrile.

Polysaccharide PO-QL1	Retention time (min)	Standard monosaccharide	Retention time (min)
Peak 1	3.12	Glucuronic acid	3.16
Peak 2	3.92	Fructose	3.92
Peak 3	4.4	Fucose	4.4

2.1.4. Determination of the order of linked sugar units by LC-MS

NMR spectroscopy and HPLC analysis were valuable tools for the structural elucidation of polysaccharides, affording saccharide compositions, ring glycosidic linkage informations... Nevertheless these techniques were limited by the requirement of relatively large amounts of polysaccharides. Mass spectrometry (MS) is another powerful technique for structural elucidation of carbohydrates, including those based on electrospray ionization (ESI-MS) and matrix-assisted laser desorption/ionization time–of-flight (MALDI-TOF-MS). Both ESI-MS and MALDI-TOF-MS possess particularly advantages in the analysis of macromolecules [16]. In MALDI-TOF-MS, the parent ion can be easily formed, but this ion cannot accurately give data on major ion in order to determine molecule weight of polysaccharide because of the easily damaged structure occurring during the analysis. While ESI-MS, combined with on-line liquid phase chromatography (LC), provided very gentle ionization that lead to more informations about mono-, di-, tri-… oligosaccharides from fragments collected in the analysis process [17,18,19] without having ability to give true molecular weight of polysaccharides. In this report, a LC-MS method was applied to analysis structural characterization of polysaccharide PO-QL1 (Figure 4). The results from the most abundant ion series were characteristic of the small saccharides formed in ionization source. The major components of PO-QL1 were mainly glucuronic acid, fructose, fucose according to HPLC analysis. Indeed the analysis of LC-MS data focused on these three kinds of monosaccharides and the results were reported in Table 2. After analysis of all these data a small structure of polysaccharide PO-QL1 corresponding to an oligosaccharide fraction was described in Figure 5.

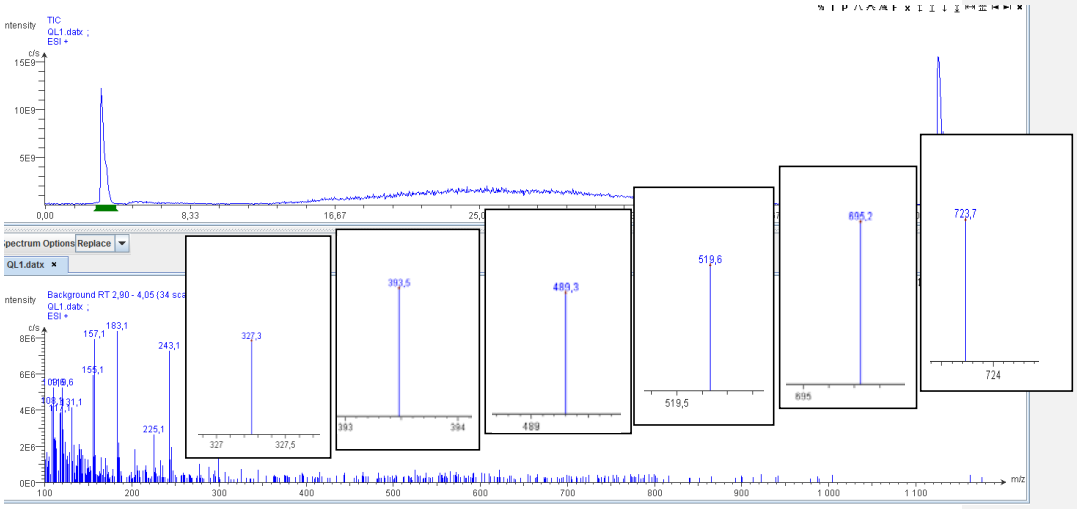


Figure 4. TIC (Total ion current) chromatogram and full mass rang scan ESI-MS spectrum in positive mode of polysaccharide PO-QL1

Table 2. The observed fragments of PO-QL1 from LC-MS data.

Oligosaccharide size	*m/z*	Ion observed	Oligosaccharide calculated mass	Theoretical mass	Unit/ fragments
Monosaccharide	165.0	$[M+H]^+$	164.0	164.1565	Fuc
	195.3	$[M+H]^+$	194.3	194.1394	GlcA
	243.1	$[M+DMSO+H]^+$	164.1	164.1565	Fuc
Disaccharide	183.1	$[M+Na+H]^{2+}$	342.1	342.2965	Fru-Fru
	203.1	$[M+DMSO+2H]^{2+}$	326.2	326.2971	Fru-Fuc
	225.1	$[M+DMSO+2H]^{2+}$	370.2	370.2635	GlcA-GlcA
	327.3	$[M+H]^+$	326.3	326.2971	Fru-Fuc
	393.5	$[M+Na]^+$	370.5	370.2635	GlcA-GlcA
Trisaccharide	157.1	$[M+DMSO+4H]^{4+}$	546.4	546.3876	GlcA-GlcA-GlcA
	489.3	$[M+H]^+$	488.3	488.44352	Fru-Fru-Fuc
	503.5	$[M+H]^+$	502.5	502.4212	GlcA-Fru- Fuc
	517.5	$[M+H]^+$	516.5	516.4047	GlcA-GlcA-Fuc
	519.6	$[M+H]^+$	518.6	518.4206	GlcA-Fru-Fru
Tetrasaccharide	155.1	$[M+DMSO+5H]^{5+}$	692.5	692.5288	GlcA-GlcA-GlcA-Fuc
	693.5	$[M+H]^+$	692.5	692.5288	GlcA-GlcA-GlcA-Fuc
	695.2	$[M+H]^+$	694.2	694.5447	GlcA-GlcA-Fru-Fru
	723.7	$[M+H]^+$	722.7	722.5118	GlcA-GlcA-GlcA-GlcA
Hexasaccharide	1017.8	$[M+H]^+$	1016.8	1016.8100	GlcA-GlcA-GlcA-Fru-Fru-Fuc

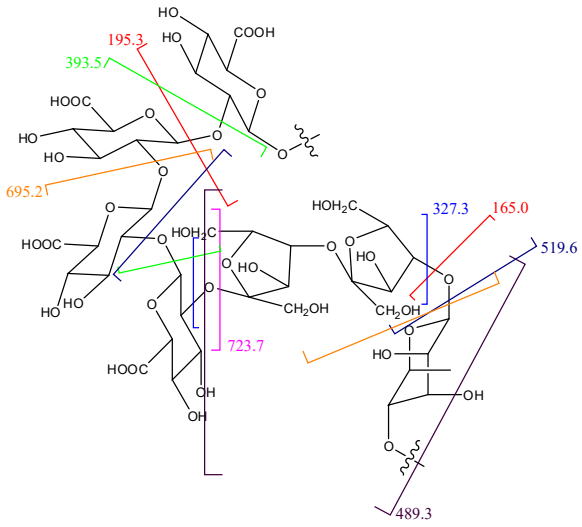
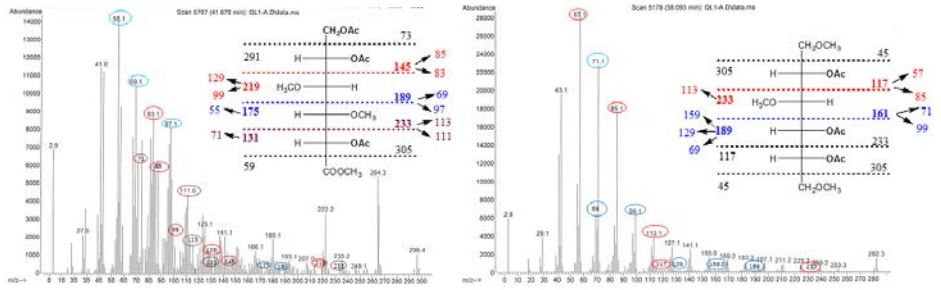


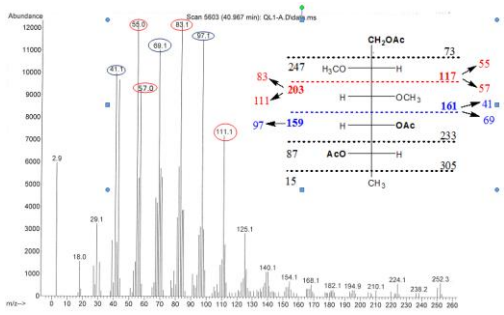
Figure 5. Structure of the oligosaccharide and observed fragments of polysaccharide fraction PO-QL1 formed during LC-MS process.

2.1.5. Determination of linkage position of sugar units in polysaccharide PO-QL1 by GC-MS

The alditol acetate and partially methylated alditol acetate method applied on carbohydrates and analyzed by gas chromatography mass spectrometry (GC-MS) is mostly employed [20]. This procedure involves the conversion of all the free hydroxyl groups on polysaccharide into methoxyl group followed by cleavage of glycosidic linkage, reduction reaction to open monosaccharide ring and acetylated process. The substitution patterns of *O*-methyl groups in monomers revealed the carbon atoms in polysaccharide that are not involved in glycosidic linkage. After a chain reaction, these monosaccharides were analyzed by GC-MS providing information of position linkage between sugar units in polysaccharides. In GC-MS, the collected fragments derived from the cleavage between two adjacent carbon atoms containing simultaneously two methoxyl groups or one bearing methoxyl group and one linked to acetyl group. The cleavage of two adjacent carbon atoms bearing two acetyl groups rarely happened [20]. Therefore, the linked positions of monosaccharide ring can be easily confirmed via the mechanism of the cleavage. The results of GC-MS analysis of polysaccharide PO-QL1 after treatment was shown in Figure 6 and exhibited that glycosidic linkage between units of glucuronic acid, fructose and fucose were at carbon atoms with position 1,2 ; 2,4 and 1,4 respectively (Table 3).



(a) (b)



(c)

Figure 6. The observed fragments of glucuronic acid (a), fructose (b) and fucose (c) from GC-MS.

Table 3. GC-MS data for alditol acetate derivatives from polysaccharide PO-QL1

Methylated sugar	Retention time (min)	Mass fragments (m/z)	Type of linkage
3,4,6-tri-*O*-Me-glcA[1]	41.65	55, 69, 71, 83, 85, 97, 99, 111, 113, 129, 131, 145, 175, 189, 219, 233	→2)-glcA-(1→
1,3-di-*O*-Me-fru	38.09	57, 69, 71, 85, 99, 113, 117, 129, 159, 161, 189, 233	→4)-fru-(2→
2,3-di-*O*-Me-fuc	40.98	41, 55, 57, 69, 83, 97, 111, 117, 159, 161, 203	→4)-fuc-(1→

[1] 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl glucuronic acid.

2.1.6. Conclusion of structural characterization of the polysaccharide fraction PO-QL1

No single method can be completely determined the structure of polysaccharide due to its complex structure. Therefore, the simultaneous combination of many evident data from IR, NMR, HPLC, LC-MS, GC-MS with distinguished chemical modifications led to the structural characterization of a fraction from PO- QL1 as a polysaccharide containing a chain of oligosaccharide as follows → 2)- β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(2→2)-β-D-Fru*f*-(2→4)-β-D-Fru*f*-(2→4)- β-L-Fuc*p*-(1→ (Figure 5).

2.2. Biological activities of PO-QL1

2.2.1. Antioxidant activity

The polysaccharides were recently reported as effective free radical scavengers and antioxidants, playing a critical role in protection against oxidation damages in living organisms [21,22,23]. Although they lacked phenolic-type structures which were essential for scavenging free radicals, the natural polysaccharides emerged as an important kind of antioxidants. Their antioxidant potentials were not determined by a single factor but a combination of several related factors [24] including molecular weight [25], monosaccharide components such as galacturonic acid, glucuronic acid...[26,27], the presence of electrophilic groups like keto, aldehyde…which facilitated the liberation of hydrogen from O-H bond [21,28].

2.2.1.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging

The DPPH assay is popular in natural product antioxidant studies. This assay is based on the theory that a hydrogen donor is an antioxidant. The antioxidant effect can be evaluated by following the decrease of UV absorption at 540 nm corresponding to the transformation of DPPH radical (purple) into DPPH (yellow). Antioxidant activity of polysaccharide PO-QL1 was investigated using DPPH assay and gallic acid, quecertine were used as positive controls. PO-QL1 did not display antioxidant activity by DPPH method whereas it was described in references for other natural polysaccharides.

2.2.1.2. Nitroblue tetrazolium (NBT)

The superoxide radical scavenging activity assay was performed using the method of nitroblue tetrazolium (NBT) reduction with Vitamin C as a control sample. Polysaccharide was tested at various concentrations from 0.25 - 50 mg/mL. The results exhibited antioxidant activity of PO-QL1 with IC_{50} value 22.5 ± 1.5 μg/mL, in comparison with a 6.1 ± 0.2 μg/mL IC_{50} value of Vitamin C.

2.2.2. MTT Assay

Cytotoxic activity of PO-QL1 was investigated by MTT assay on two cell lines as human keratinocyte cells (HaCaT) and murine melanoma cell (B16) with doxorubicine as positive control [29]. PO-QL1 demonstrated a significant cytotoxic activity with an IC_{50} value of 19 μg/mL and 27 μg/mL on HaCaT and B16, respectively.

3. Materials and Methods

3.1. Materials and Regents

2,2-Diphenyl-1-picrylhydazyl (DPPH), nitroblue tetrazolium (NBT), 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (NBT), trifluoroacetic acid (TFA), sodium borohydride ($NaBH_4$), glucose, malt extract, yeast extract, agar, XAD-7HP-resin were purchased from Sigma-Aldrich, Germany. RMPI 1640 medium was purchased from Eurobio-Abcys, France.

3.2. *PO-QL1 Isolation and Purification*

PO-QL1 was isolated from the Gym Streptomyces medium (containing 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$ in 1 L) for the culture broth of *Paenibacillus odorifer* – a bacterium identified from the lichen *Rhizocarpon geographicum*. After 7 days culture, the fermentation (4.2 L) was separated by centrifugation (Allegra Série X-12- Thermo Ficher Scientific, USA) at 3500 x g, 4°C for 15 min to eliminate the pellet. The XAD-7HP resin was then added into the broth (40 g/L, 180 rpm during 4 hours) and it was filtered and de-adsorbed by solvent mixture of Methanol : Acetone (1:1) (shaken at 180 rpm for 15 min). The mixture of solvent was removed under reduced pressure; the resulting aqueous layer was extracted with ethyl acetate (3 x 300 mL). The crude extract (439.5 mg) was collected after drying of the EtOAc – solute extract under vacuum.

This crude extract was subjected to flash chromatography (Puriflash INTERCHIM) with a reverse phase C_{18} Reveleris (Grace) column, using a sequential mixture of solvent of ACN and H_2O to furnish 21 fractions. The fraction 21 was washed several times by MeOH to give PO-QL1 (116.4 mg) yielding a 0.0073 g/L.

3.3. PO-QL1 Spectra Analysis

The infrared spectrum of PO-QL1 was recorded on Fourier transform infrared (FTIR) spectroscopy (Thermo Fisher, Germany) to identify structural groups of polysaccharide. A polysaccharide sample of two milligrams was pressed and scanned in the frequency range of 4000 – 400 cm^{-1}.

The NMR analysis was performed in DMSO-d_6 solution on a Bruker DMX 300 spectrometry [300 MHz for ^1H and 75 MHz for ^{13}C).

The LC-MS experiment was first carried out on HPLC system (Shimadzu- DGU-20AsA, Shimadzu Corporation, Kyoto, Japan) using Prevail C18 column (250 mm x 4.6 mm, 5 μm); mobile phase with gradient elution as various ratio of H_2O : Acetonitrile (beginning at 0% of ACN in 5 min, then increasing up to 100% of ACN at 35 min, and maintaining this concentration in next 10 min, finally decreasing to 0% at 55 min) ; flow rate of 0.8 mL/min; injection volume of 20 μL; concentration sample 1 mg/mL, UV wavelength of 220 nm. Advion Expression CMS plus ion-trap mass spectrometer was used for HPLC-MS analysis (Advion, Inc, NY, USA). The MS spectrum was recorded in scanning range of *m*/*z* 100 – 1200.

3.4. Compositional Analysis of PO-QL1

3.4.1. PO-QL1 acid hydrolysis.

The sugar units of polysaccharide QL1 was investigated using the method of Sulkowska-Ziaja [15] with some modifications. QL1 (10 mg) was hydrolyzed with 3 ml of 2 M triflouroacetic (TFA) in

the heat vial at 120°C, shaking of 550 rpm during 4 h to cut glycosidic linkages between monosaccharide units. After reaction, the excess acid was removed by evaporation on the water bath with a temperature of 40°C and under vacuum with water (3 x 5 mL). The product obtained after hydrolysis was investigated by HPLC with chromatographic conditions described as above.

3.4.2. Partially methylated alditol acetates

Methylation reaction. Polysaccharide was submitted to methylation according to the method of Osborn [30]. A sample of PO-QL1 (20 mg) and dimethylsulfoxide (DMSO) (2 mL) were placed into a 20 ml of glass vial and was left on overnight at room temperature with shaking of 610 rpm. 15 g powder NaOH was added the next day and the mixture was shaken for 10 min. Methyl iodide (2.5 mL) and more than 5 mg of powder NaOH were then added. The reaction was set at 20°C, shaking of 610 rpm during 4 hours. The mixture was then put for 5 min at ambient temperature and the milky white liquid was pulled off into a centrifuge tube. Dichloromethane (DCM) (2 mL) was added to remaining sample left in the tube and 2 mL of distilled water was then supplemented to centrifugation tube. The centrifugation was performed at 4000 rpm for 15 min and the water layer was removed. The process was repeated three times to wash the sample of any impurities. After the last centrifugation, the DCM layer was removed into the flask and was evaporated under reduced pressure.

Hydrolysis of product from methylation. The product from methylation was dissolved in mixture consisting of 10 drops of methanol and 1 mL of chloroform, and hydrolyzed by 2 mL of TFA (4M) at 120°C, 550 rpm during 4 hours. The mixture after reaction was treated as above.

Sodium Borohydride Reduction. The sample after hydrolysis was dissolved in 5 mL of deionized water and added by a 3 milligram of anhydrous sodium borohydride ($NaBH_4$). The vial was sealed with the Teflon cap and left for 22 hours at room temperature with periodic vortexing. Glacial acetic was then added to the reaction vial the next day until no effervescence was observed. The solution in glass vial was evaporated to dryness under reduced pressure.

Acetylation by Acetic anhydride. After reduction, the dried sample was dissolved in an 5mL of acidic methanol (95% methanol and 5% acetic acid) and evaporation to dryness to convert boric acid formed during the reduction stage to volatile methyl borate. This was repeated three times to completely remove boric acid which can interfere with the acetylation process. The product was dried and the formation of a white powder was observed. This powder was then extracted by DCM (3 x 3 mL) and dried by sodium sulfate.

GC-MS Analysis of Alditol Acetates. The final product obtained after derivatization was dissolved in 1 mL of DCM. This solution was subjected in GC-MS-system (Agilent Technologies GC-MS 7820A-GC, Agilent, Waldbronn, Germany) with Nitrogen as carrier gas, using DB-5 column (30 M x 0.25 mm x 0.25 µm) and injection volume of 1 µL. The initial 37°C oven temperature was set for 6s following injection, raised to 140°C for 30 min at a rate of 20°C/min, then to 180°C for 40 min at a rate of 40°C/min, and finally maintained at 230°C for 30 min. The detector temperature was held at 270°C.

3.5. Biological evaluation

3.5.1. DDPH assay

The free radical scavenging activity of PO-QL1 was measured by DPPH method with Quercetin and Galic acid as positive controls. 100 µL of DPPH methanol solution (freshly prepared at a concentration of 1 mg/mL) were mixed with 10 µL of PO-QL1 at different concentrations (4.00; 11.1; 33.3; 100 µg/mL) in DMSO. After 15 min culture in the dark, the absorbance was measured at 540 nm using a UV-visible spectrophotometer.

The DPPH scavenging percentage activity was calculated as follows [14]:

$$\text{Scavenging ability \%} = [1- (A_s - A_b)/A_c] \times 100$$

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Where A_s is the absorbance of polysaccharide sample with DPPH, A_b is the absorbance of polysaccharide sample without DPPH (blank), A_c is the absorbance of negative control.

3.5.2. NBT assay

Phenazine methosulphate in the presence of NADH and under aerobic conditions (+ O_2) produces superoxide anions whose formation is appreciated by a dye nitro blue tetrazolium (NBT). In the solution containing 40 µL of tris hydrochloride buffer (16 mM, pH = 8), 50 µL nicotinamid adenine dinucleotide (NADH) (78 µM), 50 µL NBT (50 µM) and 50 µL phenazin methosulphate 910 µM) were added to test with different concentrations of polysaccharide (0.25; 1.85; 2.5; 5.5; 16.0; 25.0; 50 mg/mL in DMSO). The color reaction of superoxide radical and NBT was monitored by measurement of the absorbance at 560 nm. Vitamin C was used as positive control. The experiment was repeated three times. The inhibition percentage was calculated [31] as follows :

$$\text{Scavenging effect (\%)} = (1 - A_{\text{Sample at 560 nm}}/A_{\text{Control at 560 nm}}) \times 100$$

3.5.3. MTT assay

PO-QL1 (40 mg/mL) was submitted to MTT assay against HaCaT human keratinocyte and B16 murine melanoma cell lines on 96-well plate as described in literature [29]. Firstly, the HaCaT (2000 cells/well) and B16 (1800 cells/well) were cultivated in RMPI 1640 medium (Thermo Fisher Scientific, USA) supplemented with 5% of foetal calf serum (FCS) and antibiotics (penicillin, streptomycin) in atmosphere of 5% CO_2 at 37°C. After 24 hours culture, the samples were added at different concentrations of PO-QL1 (1, 10, 50, 100 and 200 µg/mL) and each 96-well plate was continuously incubated at the same temperature and atmosphere as above. The cell growth after 48h culture were then measured at 540 nm by Microplate photometer (Thermo Scientific™ Multiskan™ FC, USA) using a MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay. The doxorubicine was used as a positive control. Each experiment was repeated three times.

4. Conclusion

A polysaccharide fraction was isolated from the culture of *Paenibacillus odorifer* – a bacterium associated with the lichen *Rhizocarpon geographicum*. Its chemical components were analyzed by IR, NMR, HPLC and LC-MS and indicated that it contains an oligosaccharide fraction including mainly D-glucuronic acid, D- fructose, L-fucose in a molar ratio of 4:2:1, respectively. The fraction structure of this saccharide determined via GC-MS spectrometry was →2)- β-D-GlcA*p*-(1→ 2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(2→2)-β-D-Fru*f*-(2→4)-β-D-Fru*f*-(2→4)- β-L-Fuc*p*-(1→. The polysaccharide possessed an antioxidant activity using NBT assay (IC_{50} of 22.5 ± 1.6 µg/mL), and it also presented significant cytotoxic activity measured by MTT with an IC_{50} value of 19 µg/mL and 27 µg/mL on HaCaT and B16 cell lines, respectively.

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Author Contributions: N.T.B.L designed and performed most of the experiments and wrote manuscript; I.R. evaluated biological tests; A.S. and S.F. were technical assistance in HPLC, LC-MS and NMR. S.T. designed, supervised the experiments and edited the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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4.3. ISOLATION OF *TERT*-BUTYL PHENOLIC COMPOUNDS

4.3.1. State of art about *tert*-butyl compounds

The *tert*-butylphenol compounds have become novel compounds to focus for chemists due to their interesting properties. Almost of *tert*-butylphenol compounds were volatile organic compounds and they were frequently discovered from plants (see in review of Dembitsky et al., 2006) and some characteristic structures were shown in Figure 2.4.1.

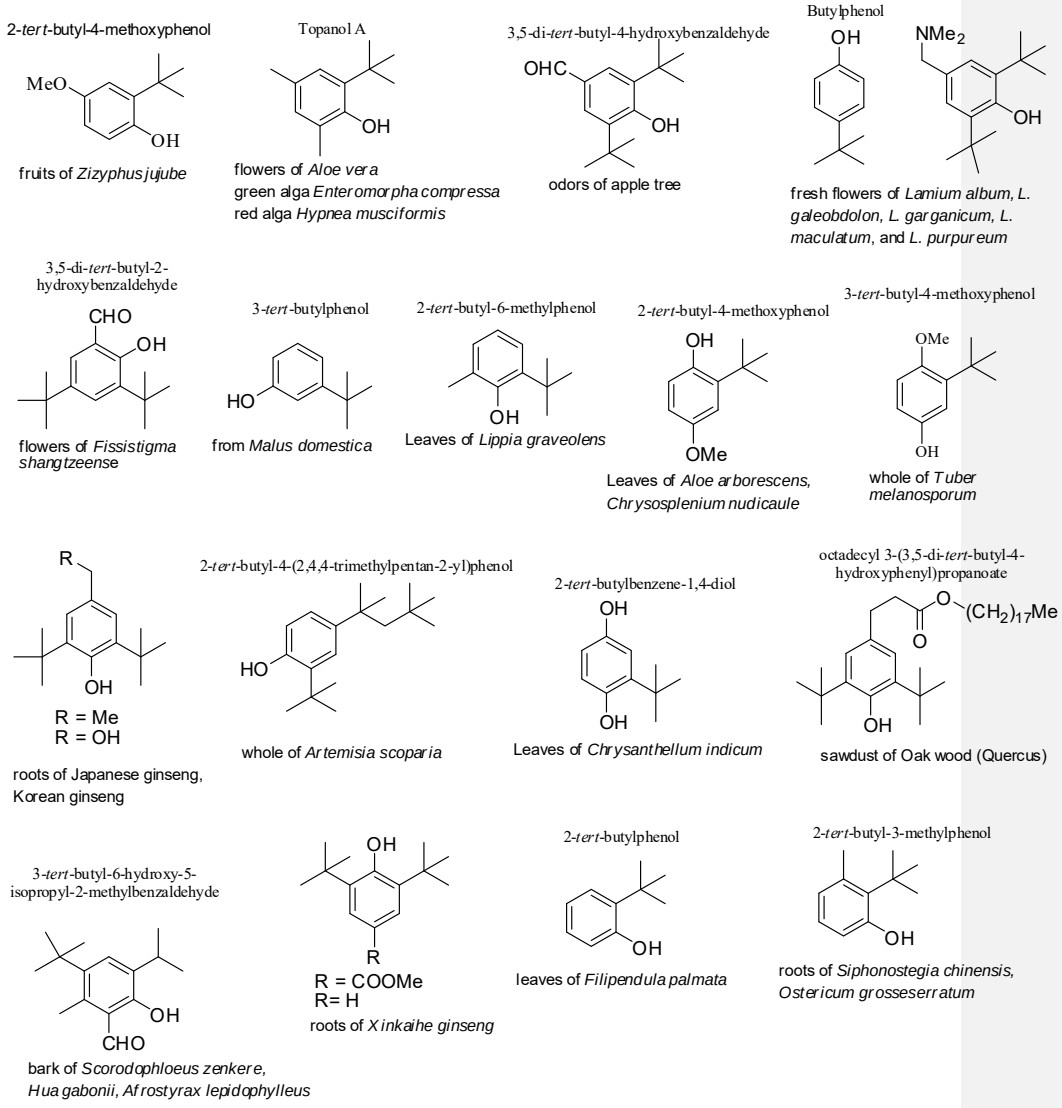
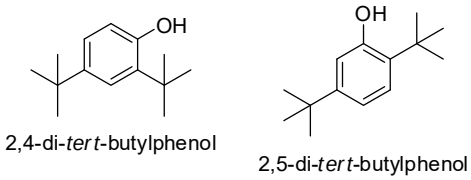


Figure 2.4.1 *Tert*-butylphenols isolated from nature (from Dembitsky et al., 2006)

Beside, some *tert*-butylphenol compounds produced from bacteria were recently reported. They included (Figure 2.4.2) 2,4-di-*tert*-butylphenol from *Streptomyces mutabilis* (Belghit et al., 2016), from *Pseudomonas monteilii* (Dharni et al., 2014) and from *Lactococcus* sp. (Varsha et al., 2015); 2,5-di-*tert*-butylphenol from *Streptomyces* sp. (Jannu et al., 2015)…etc. These *tert*-butylphenols exhibited an interesting antioxidant activity reported in many studies (Choi et al., 2013; Chuah et al., 2015; Varsha et al., 2015; Yoon et

al., 2006), antifungal properties (Varsha et al., 2015; Dharni et al., 2014) and cytotoxicity (Varsha et al., 2015) as well.



2,4-di-*tert*-butylphenol 2,5-di-*tert*-butylphenol

Figure 2.4.2 *Tert*-Butylphenols produced by various bacteria

In our study, two *tert*-butylphenol derivatives were isolated. One of the compounds possessed a strong cytotoxic effect against B16 murine melanoma and HaCaT human keratinocyte cell lines. This compound possess a symmetrical structure composed of two *tert*-butylphenol linked together by a sulfur-bond. The second compound was well-known but its NMR spectroscopic data was for the first time reported in this work.

4.3.2. Production of the *tert*-butyl phenol compounds

The *tert*-butyl phenol compounds were isolated from the supernatant extract of the culture broth of *P. odorifer* obtained during the second optimization (Experiment 7 – Chapter 3 – 3.2 – 3.2.3). The supernatant extract (439.5 mg) was injected into flash chromatography using a silica gel Chromabond column with parameters described in Chapter 6 – 6.2.2.3 and Figure 2.6.1 to furnish 14 fractions.

The detailed process of isolation and the structural elucidation of these two *tert*-butyl phenol derivatives and their evaluation of biological properties were described as an article (Published by Molecules).

The NMR data for these compounds were introduced in ANNEXE 1.

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Article

tert-Butylphenolic Derivatives from *Paenibacillus odorifer*—A Case of Bioconversion

Thi-Bach-Le Nguyen [1], Olivier Delalande [2], Isabelle Rouaud [1], Solenn Ferron [1], Laura Chaillot [3], Rémy Pedeux [3] and Sophie Tomasi [1,*]

[1] University of Rennes 1, CNRS, ISCR—UMR 6226, F-35000 Rennes, France; nguyen.bachle@yahoo.com (T.-B.-L.N.); isabelle.rouaud@univ-rennes1.fr (I.R.); solenn.ferron@univ-rennes1.fr (S.F.)
[2] University of Rennes 1, CNRS, IGDR—UMR 6290, F-35000 Rennes, France; olivier.delalande@univ-rennes1.fr
[3] Chemistry, Oncogenesis, Stress, Signaling, Centre Eugène Marquis, Université de Rennes 1, INSERM U1242, 35000 Rennes, France; laura.chaillot@univ-rennes1.fr (L.C.); remy.pedeux@univ-rennes1.fr (R.P.)
* Correspondence: sophie.tomasi@univ-rennes1.fr; Tel.: +33-223-234-817

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Abstract: Two compounds (**1**) and (**2**) containing *tert*-butylphenol groups were, for the first time, produced during the culture of *Paenibacillus odorifer*, a bacterial strain associated with the crustose lichen, *Rhizocarpon geographicum*. Their entire structures were identified by one-dimensional (1D) and two-dimensional (2D) NMR and high-resolution electrospray ionisation mass spectrometry (HRESIMS) spectroscopic analyses. Among them, Compound **1** exhibited significant cytotoxicity against B16 murine melanoma and HaCaT human keratinocyte cell lines with micromolar half maximal inhibitory concentration (IC_{50}) values. Furthermore, after supplementation studies, a putative biosynthesis pathway was proposed for Compound **1** throughout a bioconversion by this bacterial strain of butylated hydroxyanisole (BHA), an antioxidant polymer additive.

Keywords: *tert*-butylphenols; *Paenibacillus odorifer*; *Rhizocarpon geographicum*; bioconversion

1. Introduction

Organic products with branched *tert*-butyl groups represent a relatively important number of active compounds [1,2]. In the past, this group was already exploited in many organic syntheses due to its chemical reactivity. There are more than 200 compounds containing *tert*-butyl groups, described as natural products with interesting bioactivities [3]. Indeed, the *tert*-butyl moiety can be found in a variety of compounds produced by various sources, such as plants, fungi, algae, cyanobacteria [4–9], and especially from bacteria which were admitted as a source of novel and interesting bioactive products [10]. The *tert*-butylated compounds from natural sources reported in these papers often belong to classes of terpenes or polyketides. Although metabolites containing *tert*-butyl functionality on the aromatic ring are considered to be rare, several *tert*-butylphenyl derivatives were already isolated from organisms and they also exhibited remarkable antitumor, antibacterial, and antioxidant activities [11–13]. Chemical studies on *Paenibacillus odorifer*, a bacterium associated with the crustose lichen, *Rhizocarpon geographicum,* led to the identification of two *tert*-butylphenol derivatives (**1**, **2**) described in Figure 1. Interestingly, aromatic compounds simultaneously bearing a *tert*-butyl group and a sulfur atom are really uncommon in nature. The aims of this work were to characterize these compounds, to give some hypothesis about their origin, and to evaluate their cytotoxic activities. Herein, to our knowledge, is the first report of the bacterial production of Compound **1** carrying a *tert*-butyl phenol moiety and a sulfur atom.

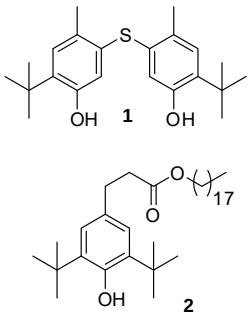


Figure 1. The structure of *tert*-butylphenolic derivatives found in the culture of *Paenibacillus odorifer*.

2. Results

2.1. Process of Purification

Paenibacillus odorifer (*P. odorifer*) was cultivated via a scale-up (4.0 L) shaken fermentation at 25 °C, pH 7, 120 rpm with 1% (*v*/*v*) inoculum in GYM *Streptomyces* medium supplemented with $CaCO_3$. From the broth, the excreted metabolites were collected using Amberlite® XAD-7-HP resin, and the resulting extract was separated via a combination of liquid/liquid extraction and normal-phase flash chromatography to give several fractions. Final purification combining silica gel chromatography, semi-preparative HPLC, and preparative silica thin-layer chromatography (TLC) afforded 5,5′-thiobis(2-*tert*-butyl-4-methylphenol) (**1**; 5.0 mg) and octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate (**2**; 5.9 mg).

2.2. Structural Elucidation

Compound **1** was obtained as a white amorphous powder. Its molecular formula was assigned as $C_{22}H_{30}O_2S$ from the $[M - H]^-$ ion at *m*/*z* 357.1983 according to high-resolution electrospray ionisation mass spectrometry (HRESIMS) data, and required eight degrees of unsaturation. Its ^{13}C-NMR data highlighted nine carbon signals, which were classified by Jmod and HSQC analyses as two aromatic methine carbons, four aromatic quaternary carbons, one aliphatic quaternary carbon, and four methyl carbons (Table 1). The lack of coupling between two aromatic protons at δ_H 6.56 ppm (1H, s, H-6) and 7.00 (1H, s, H-3) indicated a *para*-relationship between them, and the existence of a 1,2,4,5-tetrasubstituted phenyl nucleus. The presence of three methyl groups at δ_H 1.28 (9H, s, H-9/H-10/H-11) along with their HMBC correlations to a quaternary carbon at δ_C 34.4 (C-8) revealed the presence of a *tert*-butyl group which was one of the substituted groups on the aromatic ring. On the other hand, the ^{13}C shift of C-2 at δ_C 134.6 together with its HMBC correlations with H-9/H-10/H-11 confirmed the position of the *tert*-butyl group at C-2. In addition, analysis of the HMBC spectrum exhibiting the correlations from H-3 to C-8 (δ_C 34.4), C-4 (δ_C 125.5), C-5 (δ_C 137.8), and C-1 (δ_C 153.4) provided the presence of a methyl group at C-4, a hydroxyl group at C-1, and a sulfur-bond at C-5. The latter could also be indicated through the HMBC correlations from the aromatic methyl protons at δ_H 2.28 (3H, s, H-7) to C-4, C-5, and C-6 (δ_C 118.8). Moreover, HMBC correlations from H-6 to C-1, C-2, C-4, and C-7 were also observed. Interestingly, NOE correlations between two kinds of protons at δ_H 7.00 (H-3) and δ_H 1.28 (H-9/H-10/H-11) indicated that the *tert*-butyl group possessed a position close to C-3. The remaining structural assignment of Compound **1** (Figure 2) required $C_{11}H_{15}O$, which corresponds to the already assigned part of Compound **1**. On the basis of molecular formula and HMBC correlations, Compound **1** was suggested to possess a symmetrical structure containing two 1,2,4,5-tetrasubstituted phenyl nuclei which were linked via a sulfur bond This structure is close to that of a known synthetic compound Santonox (CAS registry number 96-69-5) 78 (Figure 3). This is the first natural isolation of Compound **1**.

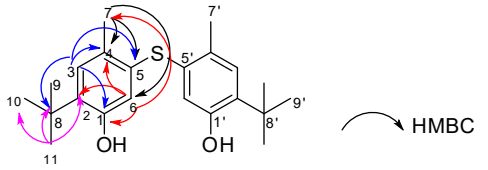
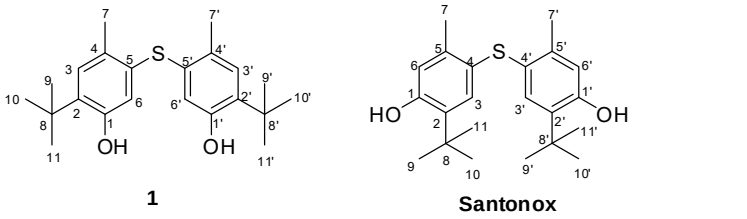


Figure 2. Key correlations for the structural assignment of **1**.



1

5,5'-thiobis(2-*tert*-butyl-4-methylphenol)

Santonox

4,4'-thiobis(2-*tert*-butyl-5-methylphenol)

Figure 3. Structures of Compound **1** and Santonox.

From the comparison of the NMR spectroscopic data, minor differences in the chemical shifts of protons and carbons were highlighted for Compound **1** and standard Santonox. Thus, the identification of the exact structures could not be based on these data, with the exception of NOE correlations. For Santonox, the NOE correlations were clearly displayed between two series of protons at δ_H 7.00 (aromatic proton, H-3) and δ_H 1.28 (*tert*-butyl protons), and at δ_H 6.54 (aromatic proton, H-6) and δ_H 2.27 (methyl protons). For Compound **1**, the NOE experiments only highlighted the correlations between δ_H 7.00 (aromatic proton, H-3) and δ_H 1.28 (*tert*-butyl protons). The absence of the NOE correlation between H-6 (δ_H 6.56) and protons of the *tert*-butyl group (δ_H 1.28) indicated that H-6 in Compound **1** was not close to the *tert*-butyl group. NOE predictions obtained through molecular dynamics simulations confirmed the NOE experimental data (see Supplementary Materials, Figure S20 and Table S1). As a result, the data from NOE correlations finally highlighted that Compound **1** was an isomer of Santonox. Moreover, to our best knowledge, it is the first report of the isolation of Compound **1** from a bacterial culture.

Table 1. One-dimensional (1D) and two-dimensional (2D) NMR data for Compound **1** and standard Santonox in $CDCl_3$ (300 MHz for ^{1}H-NMR, and 75 MHz for ^{13}C-NMR).

Compound 1						Standard Santonox					
Position	δ_C, ppm	δ_H, (ppm) mult. (*J* in Hz)	HMBC (H → C)	COSY	NOESY	Position	δ_C, ppm	δ_H, (ppm) mult. (*J* in Hz)	HMBC (H → C)	COSY	NOESY
1/1′	**153.4**	**-**	**-**	**-**		**1/1′**	**153.2**	**-**	**-**	**-**	**-**
2/2′	**134.6**	**-**	**-**	**-**		**2/2′**	**134.5**	**-**	**-**	**-**	**-**
3/3′	**130.8**	**7.00, s**	**1, 4, 5, 8**	**7, 9/10/11**	**9/10/11**	**3/3′**	**130.6**	**7.00, s**	**1, 4, 5, 6, 7, 8**	**7, 9/10/11**	**9/10/11**
4/4′	**125.5**	**-**	**-**	**-**		**4/4′**	**137.7**	**-**	**-**	**-**	**-**
5/5′	**137.8**	**-**	**-**	**-**		**5/5′**	**125.4**	**-**	**-**	**-**	**-**
6/6′	**118.8**	**6.56, s**	**1, 2, 4, 7**	**7**		**6/6′**	**118.7**	**6.54, s**	**1, 4, 5, 7, 8**	**7**	**7**
7/7′	**19.8**	**2.28, s**	**4, 5, 6**	**3, 6**		**7/7′**	**19.7**	**2.27, s**	**4, 5, 6**	**3, 6**	**6**
8/8′	**34.4**	**-**		**-**		**8/8′**	**34.3**	**-**	**-**	**-**	
9, 10, 11 /9′,10, 11′	**29.7**	**1.28, s**	**9/10/11, 2, 8**	**3**	**3**	**9, 10, 11/9′,10, 11′**	**29.5**	**1.28, s**	**9/10/11, 2, 8**	**3**	**3**
OH		**4.72, br**	**-**			**OH**					

Compound **2** was isolated as a white solid and had a $C_{35}H_{62}O_3$ molecular formula determined by the $[M + Na]^+$ peak at *m*/*z* 553.4592 from its (+)HRESIMS data. The analysis of ^{1}H-NMR and Jmod, along with HSQC data (Table 2), indicated the presence of two aromatic protons, 19 methylene groups (one of them oxygenated), seven methyl groups (six of them as singlets), four aromatic quaternary carbons, and one carbonyl carbon. The presence of two aromatic protons at δ_H 6.99 (2H, s, H-3′/H-5′) pointed to the existence of a 1,2,4,6-tetrasubstituted phenyl moiety (Figure 4). Four spin systems could be revealed via analysis of COSY correlations, corresponding to the C-1 to C-2, C-1″ to C-2″, C-3″ to C-17″, and C-17″ to C-18″ fragments. The HMBC correlations from an exchangeable proton (δ_H 5.07, 1H, bs) to C-1′ demonstrated that C-1′ might be substituted by a hydroxyl group, and it was confirmed by the ^{13}C shift of C-1′ at δ_C 152.1. Compound **2** also presented two *tert*-butyl groups containing six methyl groups at $\delta_{C/H}$ 30.3/1.43 (18H, s, H-8′, H-9′, H-10′, H-12′, H-13′, H-14′) connected with two quaternary carbons at δ_C 34.3 (C-7′/C-11′) as two of the substituted groups on the aromatic ring. In addition, the HMBC data provided correlations from the protons of *tert*-butyl groups to C-7′/C-11′, C-2′/C-6′ (δ_C 135.8), C-3′/C-5′ (δ_C 124.8), and C-4′ (δ_C 131.1); and from H-3′/H-5′ to C-1′ (δ_C 152.1), C-3′/C-5′ (δ_C 124.8), and C-1 (δ_C 31.0). Moreover, the presence of two coupled methylene groups at δ_H 2.85 (2H, dd, *J* = 9.1, 6.9 Hz, H-1) and δ_H 2.60 (2H, dd, *J* = 9.1, 6.9 Hz, H-2) was also observed. The HMBC correlations from H-1 to C-4′, C-3′/C-5′, C-2 (δ_C 36.5), and C-3 (δ_C 173.4); and from H-2 to C-4′, C-1, and C-3 provided more evidence that this group made a linkage between a phenyl nucleus and a carbonyl carbon (Figure 4). Furthermore, the oxygenated methylene at δ_H 4.07 (2H, t, *J* = 6.8Hz, H-1″), δ_C 64.6 (C-1″) was linked to carbonyl carbon C-3 and to other several methylene groups as indicated by the HMBC data. Thus, the structure of Compound **2** was established as octadecyl 1-(2′,6′-di-*tert*-butyl-1′-hydroxyphenyl)propanoate, introduced in Figure 4. This structure was already reported in the literature [3]; however, its full NMR data are not yet published, and this is the first report of its isolation from a culture of *P. odorifer*.

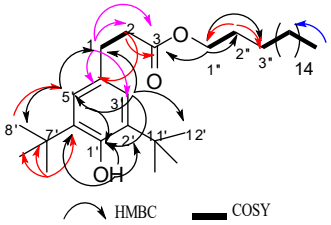


Figure 4. Key correlations for the structural assignment of **2**.

Table 2. 1D and 2D NMR data for Compound **2** in $CDCl_3$ (300 MHz for ^{1}H-NMR, and 75 MHz for ^{13}C-NMR).

Compound 2					
Position	δ_C	Type	δ_H, mult. (*J* in Hz)	COSY	HMBC (H → C)
1′	**152.1**	**C**	**-**	**-**	
2′	**135.8**	**C**	**-**	**-**	
3′	**124.8**	**CH**	**6.99, s**		**1′, 5′, 12′**
4′	**131.1**	**C**	**-**		
5′	**124.8**	**CH**	**6.99, s**		**1′, 3′, 1, 8′**
6′	**135.8**	**C**	**-**		
1	**31.0**	**CH_2**	**2.85, dd (9.1, 6.9)**	**2**	**3′/5′, 4′, 2, 3**
2	**36.5**	**CH_2**	**2.60, dd (9.1, 6.9)**	**1**	**5′, 1, 3**
3	**173.4**	**C**	**-**		
1″	**64.6**	**CH_2**	**4.07, t (6.8)**	**2″**	**3, 2″, 3″**
2″	**29.7**	**CH_2**	**1.56–1.61, m**	**1″, 3″**	**1″, 3″**
3″	**29.7**	**CH_2**	**1.56–1.61,m**	**2″**	**2″**
4″–17″	**22.7–32.0**	**CH_2**	**1.24, m**	**18″**	**5″–17″, 2″, 3″, 18″**
18″	**14.1**	**CH_3**	**0.88, t (6.7)**	**17″**	**17″**

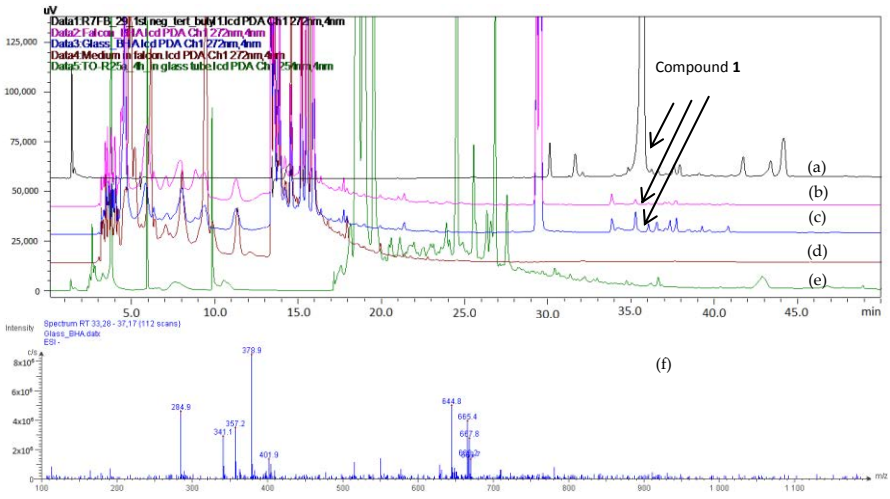
7′/11′ [c]	**34.3**	**C**	**-**	
8′,9′,10′/12′,13′,14′ [a]	**30.3**	**CH_3**	**1.43, s**	**8′,9′,10′/12′,13′,14′, 7′/11′, 2′, 6′, 3′/5′**
OH			**5.07, bs**	**1′, 2′, 6′**

[a] Carbons 7′/11′ and 8′,9′,10′/12′,13′,14′ form a single peak each.

2.3. Supplementation Assays

The structure of Compound **1** was determined to be closely related to that of butylated hydroxyanisole (BHA) which is approved as an antioxidant ingredient added to polymers, foods, and food-related products [14–16]. To respond to this issue, a supplementation of the culture of *P. odorifer* was carried out with standard BHA, put either in a culture flask (a kind of plastic vessel) or in an Erlenmeyer (a kind of glass vessel). Furthermore, the controls were based on the medium incubated in a culture flask and the culture of *P. odorifer* in an Erlenmeyer flask. The results from the LC–MS data are shown in Figure 5, and they highlighted that both extracts from the cultures supplemented with BHA in the culture flask and Erlenmeyer flask provided $[M - H]^-$ ions at *m*/*z* 357 with a retention time of 35.7 min, which is characteristic of Compound **1**. However, Compound **1** could neither be found in the extract from the medium incubated in the culture flask, nor from the culture of *P. odorifer* in the Erlenmeyer flask.

Additionally, the analysis of Fraction **1′,** which was a mixture of non-separable BHA and Compound **1**, partially purified from the culture supplemented with BHA in the Erlenmeyer flask, showed similar NOE correlations to Compound **1** between δ_H 6.99 and δ_H 1.28 (*tert*-butyl protons; see Supplementary Materials, Figure S13). Accordingly, we concluded that Compound **1** was converted by *P. odorifer* from BHA, which was detected in the medium incubated in the culture flask. Therefore, the biosynthetic pathway of Compound **1** is proposed in Figure 6, following the mechanism suggested by Fontecave [17,18] with some modifications. After an oxidative step of BHA, the formed phenoxy radical could react with cysteine as a sulfur donor to produce Compound **1** after further reactions. This reaction could be supported by an iron–sulfur cluster protein that was already reported in the genome of *P. odorifer* (gene symbol PODO_RS22860), described in the NCBI bank.



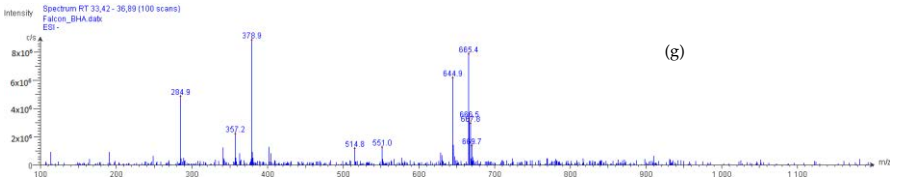


Figure 5. HPLC chromatograms of Compound **1** (**a**), of the extracts from *P. odorifer* culture supplemented with butylated hydroxyanisole (BHA) in the culture flask (**b**), or in the glass Erlenmeyer flask (**c**), of medium in the culture flask (**d**), and of the *Paenibacillus odorifer* (*P. odorifer*) culture in the Erlenmeyer flask (**e**). Electrospray ionisation (ESI)-MS (−) spectra of extracts from the *P odorifer* culture supplemented with BHA in the culture flask (**f**), or in the glass Erlenmeyer flask (**g**).

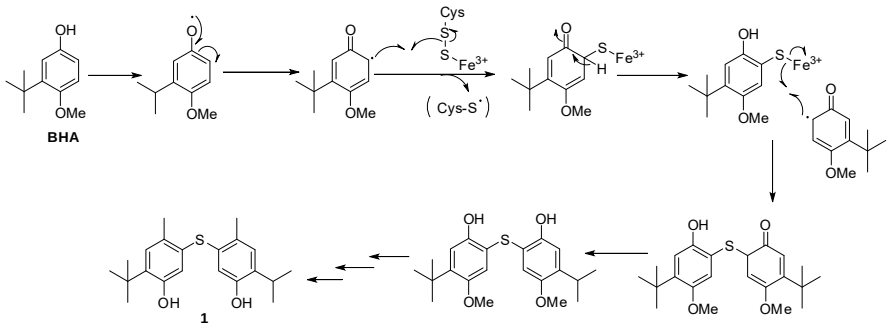
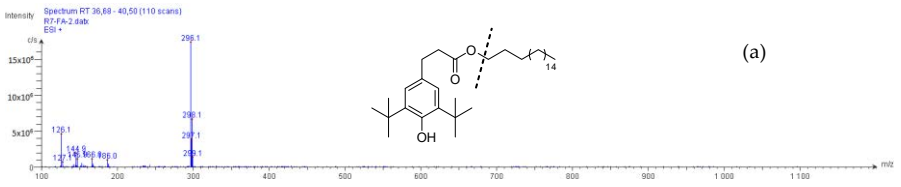


Figure 6. Putative biosynthetic pathway for Compound **1** from BHA supported by an iron–sulfur cluster protein with cysteine as a sulfur donor.

Compound **2,** as with Compound **1,** was isolated from the culture process using a culture flask in the pre-culture stage. In order to discover the origin of Compound **2**, butylated hydroxytoluene (BHT), with its close structure to that of Compound **2**, was used as a supplemented material during the culture of *P. odorifer*, put either in a culture flask or in an Erlenmeyer flask. The blank controls were the medium (without bacteria) in the culture flask and the culture of *P. odorifer* in the Erlenmeyer flask. The HPLC-MS data introduced in Figure 7 exhibited that Compound **2**, with a retention time at 38 min, was associated with an ion at *m*/*z* 296, which occurred in extracts from media supplemented with standard BHT in both the culture flask and the Erlenmeyer flask. This ion was detected in the MS spectrum of Compound **2** due to the hydrolysis of its ester group in LC–MS process. However, Compound **2** was not found in the medium put in the culture flask, but was found in the culture broth of *P. odorifer* in the Erlenmeyer flask. On the other hand, this compound was already reported in the literature [3] from Oakwood. Therefore, we propose that Compound **2** came from the bioconversion of BHT, or as a natural metabolite from the culture of *P. odorifer*. Furthermore, our *P. odorifer* strain could be considered as a new example of *tert*-butylphenol-utilizing bacterium.



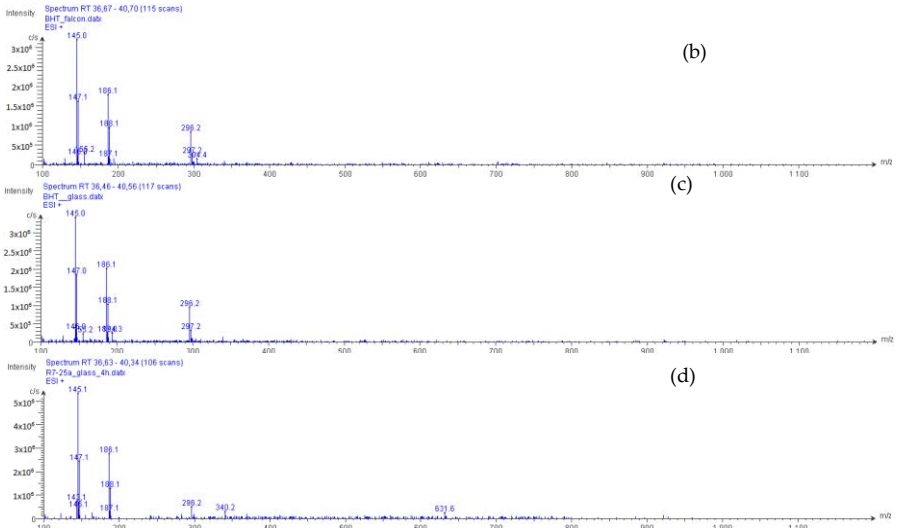


Figure 7. ESI-MS (+) spectra of Compound **2** (**a**), of the extracts from the culture supplemented with BHT in either the culture flask (**b**) or the Erlenmeyer flask (**c**), and of the culture of *P. odorifer* in the Erlenmeyer flask (**d**).

2.4. Cytotoxic Activity

The biological activities of Compounds **1** and **2** were tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on HaCaT (human keratinocytes) and B16 (murine melanoma) cell lines (Table 3) [19]. Although neither compound showed activity significantly greater than the positive control (doxorubicin) against the two cell lines, Compound **1** exhibited a significant half maximal inhibitory concentration (IC_{50}) on B16 (4.75 μM) and HaCaT (8.38 μM), while Compound **2** was less active. Additionally, DNA damage assays, using γH2AX as a biomarker, were performed with Compound **1** on U2OS cells (Table 4). These cells are frequently used since they are sensitive to DNA damage. Although the compound was highly cytotoxic at 1 μM (cell death > 90%), no significant induction in γH2AX foci was observed at 1 μM or 0.1 μM within the nuclei, suggesting that no significant DNA damage was triggered compared to untreated cells. These results suggest that the cytotoxicity of Compound **1** was not driven by DNA damage.

Table 3. Cytotoxic assay of Compounds **1** and **2**.

Compound	IC_{50} (μM)	
	HaCaT	B16
1	**8.38**	**4.75**
2	**>377.4**	**169.8 ± 1**
Doxorubicin	**0.096 ± 0.009**	**0.034 ± 0.001**

Table 4. DNA damage assay of Compound **1**.

Concentration (μM)	γH2AX Foci/Nuclei
0	**12.9 ± 0.4**
0.1	**12.8 ± 0.2**
1	**3.6 ± 0.3**

3. Materials and Methods

3.1. General Experimental Procedures

One-dimensional (1D) and two-dimensional (2D) NMR spectroscopic data were recorded in MeOH-d_4 and $CDCl_3$ on a Bruker DMX 300 spectrometer (300 MHz (^1H) and 75 MHz (^{13}C), Bruker BioSpin, Billerica, MA, USA). NMR spectroscopic data were processed using the MestRenoVa version 10.0 software (Mestrelab Research, S.L., Santiago de Compostela, Spain). HRMS measurements for exact mass determination were performed with a Q-Extractive Focus at the Centre Regional de Mesure Physique de l'Ouest (CRMPO), Rennes, France. Analytical HPLC and semi-preparative HPLC were performed on a 5-μm Prevail C_{18} column (250 mm × 4.6 mm for the former, and 250 mm × 10 mm for the later), GRACE, Columbia, MD, USA.

3.2. Collection and Phylogenetic Analysis of PC-GYM-TO Strain

The PC-GYM-TO strain was isolated from the crustose lichen, *Rhizocarpon geographicum*, collected in Brittany, France in February 2015. The strain was identified at Banyuls/mer Platform (L. Intertaglia) as *Paenibacillus odorifer* based on 16S ribosomal RNA (rRNA) gene sequence analysis (GenBank accession number AJ223990). A comparative BLAST similarities search of the 16S rRNA gene sequence gave a 98.46% similarity to that of *P. odorifer* (Gene bank entry PODO_RS03805). After culture in GYM *Streptomyces* medium (containing 4 g of glucose (Sigma-Aldrich, St Louis, MO, USA), 4 g of yeast extract (Sigma-Aldrich, St Louis, MO, USA), 10 g of malt extract (Sigma-Aldrich, St Louis, MO, USA), 2 g of $CaCO_3$ (Merck KGaA, St Frankfurter, Darmstadt, Germany), and 12 g of agar (Sigma-Aldrich, St Louis, MO, USA) in 1 L), the bacterium was stored in a mixture of 47.5% (*v*/*v*) glycerol, 47.5% (*v*/*v*) H_2O, and 5% (*v*/*v*) DMSO at −80 °C with a reference of PC-GYM-TO (CORINT collection).

3.3. Cultivation and Extraction

P. odorifer (strain PC-GYM-TO) was cultured on GYM *Streptomyces* medium agar (2 g of glucose, 4 g of yeast extract, 4 g of malt extract, 2 g of $CaCO_3$, and 12 g of agar in 1 L at pH 7). The inoculum was prepared by transferring one loop full of culture (PC-GYM-TO) from agar medium to a 250-mL culture flask, containing 50 mL of liquid GYM *Streptomyces* medium (2 g of glucose, 4 g of yeast extract, 4 g of malt extract, and 2 g of $CaCO_3$ in 1 L at pH 7). The bacterium culture was grown at 25 °C on a rotary shaker incubator at 120 rpm for seven days. After seven days for pre-culture, 42 mL of bacterium culture was transferred into 14 Erlenmeyer flasks (500 mL), each containing 300 mL of liquid GYM *Streptomyces* medium. The fermentation culture was then incubated at 25 °C with 120-rpm shaking for seven days. After seven days of culture, the fermentation broth was collected and centrifuged at 3500 rpm, at 4 °C for 15 min. After removal of the pellet, sterilized XAD-7-HP resin (40 g/L) was added to the supernatant to absorb the organic products from the culture, and the resin was then shaken at 220 rpm for 4 h. The resin was filtered and de-adsorbed by a mixture of solvent acetone/MeOH (50/50, *v*/*v*). This mixture of solvent was removed under reduced pressure; the resulting aqueous layer was extracted with ethyl acetate (EtOAc; 3 × 300 mL). The EtOAc/solute extract was dried under vacuum to yield 439.5 mg of organic extract from 4.0 L of the culture.

Supplementation assays: BHA was supplemented with a quantity of 0.1 mg per 25 mL of liquid GYM *Streptomyces* medium at day zero of the culture to check the origin of isolated compounds.

3.4. Purification and Isolation

The organic extract (439.5 mg) from strain PC-GYM-TO, after biological assays, was subjected to flash chromatography with a 50-g SiOH Chromabond® Flash column, using a sequential mixture of solvent with increasing polarity from cyclohexane to dichloromethane, EtOAc, and MeOH for 4 h to furnish 14 fractions. Guided by HPLC analysis, the first fraction containing Compound **2** (FA, 39.6 mg) and the second fraction containing Compound **1** (FB, 46.6 mg) were purified with semi-preparative HPLC (using a Prevail® C_{18} column with a gradient of 0% to 100% CH_3OH in H_2O for 60 min, and a flow rate of 2.5 mL/min) and preparative TLC to afford Compound **1** (5.0 mg) and Compound **2** (5.9 mg), with yields of 1.25 mg/L and 1.5 mg/L, respectively.

LC–MS was applied using a Prevail® C_{18} column, with a gradient of 0% to 100% CH_3CN in H_2O for 60 min, a flow rate of 0.8 mL/min, a sample concentration of 1 mg/mL, and an MS full range from 100–1200.

*5,5'-Thiobis(2-*tert*-butyl-4-methylphenol)* (Compound **1**): white amorphous powder, retention time = 35.7 min; R_f = 0.45 (chloroform 100%). ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) are described in Table 1. HRESIMS *m*/*z* 357.1983 $[M - H]^-$ (calculated for $C_{22}H_{29}O_2S$, Δ = 0 ppm).

*Octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate* (Compound **2**): while solid, retention time = 26.7 min. ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) are described in Table 2. HRESIMS *m*/*z* 553.4592 $[M + Na]^+$ (calculated for $C_{35}H_{32}O_3Na$).

3.5. Molecular Models and Dynamic Simulations

The structures of Compound **1** and Santonox were built using the Yasara program and were parameterized for the Yamber3 force field following the automated AutoSMILE procedure [20]. Both geometries were optimized through the standardized minimization protocol of Yasara. Finally, to enhance the conformational space exploration available to the structures, molecular models were used as an initial point for molecular dynamics (MD) simulations. Each isomer was placed in an explicit chloroform solvent box and simulated under periodic boundary conditions at a constant temperature of 300 K. Structures were relaxed during a 2-ns MD simulation and trajectories were collected at 1-ps intervals. Analyses of the MD trajectories (root-mean-square deviation (RMSD) and clustering) was performed using Gromacs tools [21].

3.6. Cytotoxicity Assays

The cytotoxic assays were performed on pure compounds (with a concentration for each sample as 40 mg/mL) against HaCaT human keratinocytes and B16 murine melanoma cell lines as described in the literature [19]. HaCaT (2000 cells/well) and B16 (1800 cells/well) were cultivated in Roswell Park Memorial Institute RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and antibiotics in an atmosphere of 5% CO_2 at 37 °C. After a 24-h culture, the samples were added at different concentrations (1, 10, 50, 100, and 200 μg/mL) and each 96-well plate was continuously incubated at the same temperature and atmosphere as above. After a 48-h culture, cell growth and viability were then measured at 540 nm using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Doxorubicin was used as a positive control. Each experiment was repeated three times.

3.7. DNA Damage Assays

U2OS cells were cultivated in Dulbecco's Modified Eagle's medium DMEM supplemented with 10% fetal calf serum and antibiotics in an atmosphere of 5% CO_2 at 37 °C. The γH2AX staining was performed as previously described [22]. Images were acquired on an ArrayScan VTI high-content screening reader with a 320 lens (Thermo Scientific, Villebon sur Yvette, France). The images were analyzed using the Cell Profiler software (http://www.cellprofiler.org, Broad Institute). For all analyses, raw data files were obtained with the total

amount of Hoechst fluorescence and the total amount of γH2AX fluorescence. The number of γH2AX foci per nucleus is indicated for each condition in Table 4 with more than 3000 cells counted except for the 1 μM concentration because of the high cytotoxicity.

4. Conclusions

In summary, two *tert*-butylphenol compounds were firstly isolated from the culture of a bacterium, *P. odorifer*, associated with the lichen, *Rhizocarpon geographicum*. Compound **1** displayed a symmetric structure including two units of BHA linked by a sulfur bond. This point can be explained by the fact that Compound **1** was putatively formed via the bioaccumulation of BHA from the culture flask used in the culture process, followed by the biotransformation of BHA into Compound **1**. Therefore, a putative biosynthesis pathway was proposed for this compound, and involved an iron–sulfur cluster protein with cysteine as a sulfur donor. Compound **1** exhibited a moderate cytotoxicity, making it promising for further investigation to determine its mechanism. The results also highlighted *P. odorifer* as a new case of *tert*-butylphenol-utilizing bacterium.

Supplementary Materials: Supplementary Materials are available online.

Author Contributions: S.T. and T.-B.-L.N. conceived and designed the experiments; T.-B.-L.N. performed the experiments; T.-B.-L.N. analyzed the data; I.R. realized the biological assays on HaCaT and B16; R.P. and L.C. designed and realized the assays on DNA damage; O.D. designed and realized the NOE calculations; S.F. ran NMR; T.-B.-L.N and S.T. wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the Compounds **1, 2** are available from the authors.

4.4. THE ISOLATION PROCESS OF A NOVEL ALKALOID

4.4.1. General presentation of alkaloids

Alkaloids are a large and structurally diverse group of natural products which could be isolated from distinct sources as plants, fungi and bacteria or even animals. Many alkaloids have been developed like therapeutic agents to treat various diseases including malaria, diabetis, cancer, cardiac dysfunctions etc (see in Ain et al., 2016). To date, more than 18000 alkaloids have been found (Dembitsky el al., 2005) and this number will continuously increase in the future. Alkaloids bearing nitrogen atoms were identified as compounds containing either indole, indolizidine, quinoline, isoquinoline, piperazine, indole-quinoline... etc (Figure 2.4.3) moities.

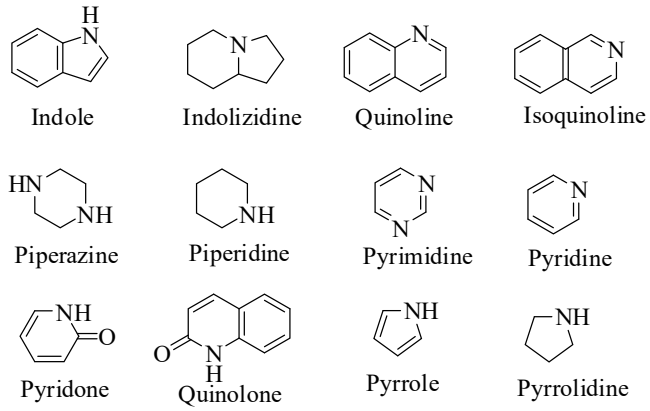
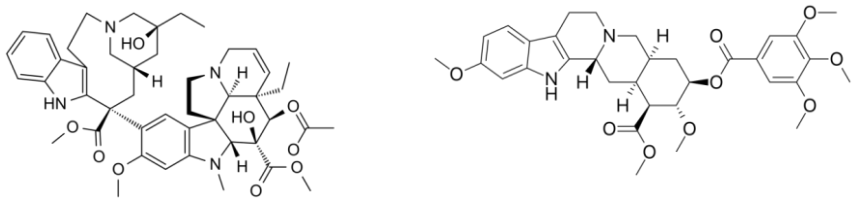


Figure 2.4.3 Some structural features found in alkaloids

A vast majority of alkaloids were frequently found in plant kingdom. These compounds exhibited pharmacological properties including analgesic (e.g. codeine) (Honig et al., 1984), antihypertensive (e.g. reserpine) (see in Yu et al., 2013), antipyretic (e.g. quinine) (Santos et al., 1998), anticholinergic (e.g. atropine) (Braun et al., 1993), antitumor (e.g. vinblastine) (Fodstad et al., 1996), antimalarial (e.g. quinine) (see in Achan et a., 2011) The structures of some compounds were shown in Figure 2.4.4. Moreover some new alkaloids with significant biological activities are continuously reported in many studies..



Vinblastine - anticancer
Isolated from *Madagascar periwinkle*

Reserpine- antihypertensive
Isolated from *Rauwolfia canescens*

Figure 2.4.4 Structures of some drugs as natural alkaloids

However, when bacteria became a new source of exploitation of active natural products, alkaloids were also produced by these organisms. These compounds showed interesting biological properties. Some of them recently isolated are reported in Figure 2.4.5 including an antibacterial dihydroquinoline from *Pseudomonas aeruginosa* (Uzair et al., 2006), a cytotoxic ammosamide D from *Streptomyces variabilis* (Pan et al., 2012), an antibiotic hunanamycin D from *Bacillus hunanensis* (Hu et al., 2013), a potent inhibitor of the developement of human neuroblastoma streptonigrin from *Micromonospora* sp. (Wang et al., 2002) and also active alkaloids isolated from lichen-associated bacteria such as an antibiotic and cytotoxic uncialamycin (Davies et al., 2005), an anti-HIV derivative unciaphenol (Williams et al., 2015) and cytotoxic cladoniamides from *Streptomyces uncialis* (Williams et al., 2008).

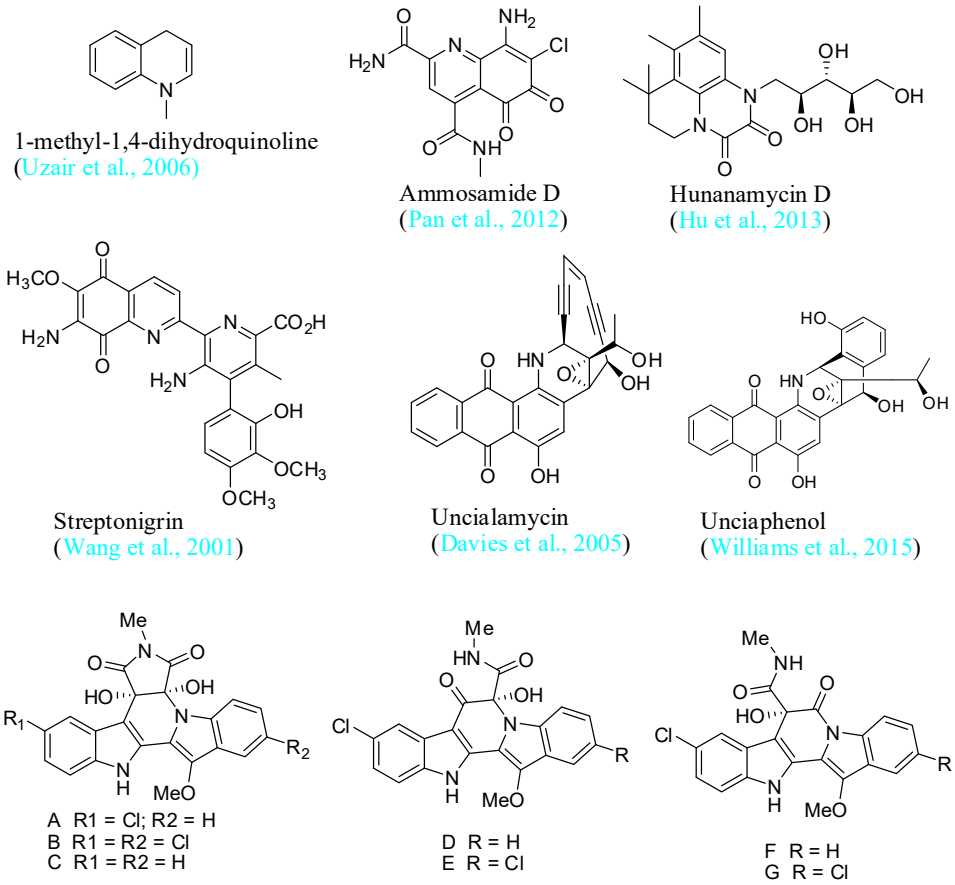


Figure 2.4.5 Some cytotoxic alkaloids recently isolated from bacteria

4.4.2 Isolation of alkaloid

In our study, a new cytotoxic alkaloid was also isolated. Its structure was a combination of dihydronaphthalene moiety fused to a pyrrolooxazine unit. Moreover, it exhibited a cytotoxicity against B16 murine melanoma and HaCaT human keratinocyte cell lines with micromolar IC_{50} values. This property promised it as a potent agent for further pharmaceutical researches. The isolation, structural elucidation and bioactive evaluation of this alkaloid were displayed as an article. The supporting information of the publication will be reported in ANNEXE 2.

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Article

Paeniloxazine, a new alkaloid isolated from *Paenibacillus odorifer* a lichen-associated bacterium

Le Thi Bach Nguyen, Isabelle Rouaud, Solenn Ferron, Sophie Tomasi*

Univ Rennes, CNRS, ISCR – UMR 6226, F-35000 Rennes, France ; thi-bach-le.nguyen@univ-rennes1.fr (L.N); isabelle.rouaud@univ-rennes1.fr (I.R.); solenn.ferron@univ-rennes1.fr (S.F);
*Correspondence: sophie.tomasi@univ-rennes1.fr (S.T.); Tel.: +33-223-234-817

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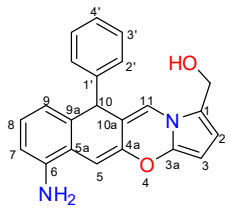
Abstract: A new tetracyclic alkaloid skeleton named paeniloxazine consisting of a dihydronaphthalene part fused to a pyrrolooxazine unit was isolated from the culture broth of *Paenibacillus odorifer*, a bacterium associated to the lichen *Rhizocarpon geographicum*. Its structure was elucidated as (6-amino-10-phenyl-10*H*-naphtho[2,3-e]pyrrolo[2,1-b][1,3]oxazin-1-yl)methanol by spectroscopic data. It exhibited weak cytotoxic effects against B16 murine melanoma and HaCaT human keratinocyte cell lines by MTT assay with micromolar IC_{50} values of 76.0 µM and 78.9 µM, respectively. Moreover, a putative biosynthetic pathway was proposed for this compound. It is the first example of a dihydronaphthalene-pyrrolooxazine alkaloid produced by a bacterium

Keywords: dihydronaphtalene; pyrrolooxazine; alkaloid; *Paenibacillus odorifer*; *Rhizocarpon geographicum*.

1. Introduction

Lichens were admitted harboring an important bacterial community [1] from which many bioactive compounds have been already isolated [2]. For instance, uncialamycin, a compound showing a high cytotoxic effect, was isolated from *Streptomyces uncialis*, a bacterial strain associated with the lichen *Cladonia uncialis* [3, 4].

In our ongoing search on discovery novel and bioactive natural products from lichen-associated bacteria, we investigated the production of metabolites by *Paenibacillus odorifer*, one of the strains collected from the lichen *Rhizocarpon geographicum*. In this report, we described the isolation and structural elucidation of a novel cytotoxic alkaloid **1** formed by a dihydronaphthalene part fused to a pyrrolooxazine unit produced by *P. odorifer*. Pyrrole unit was often present as a partially structure of alkaloids containing tetracyclic ring systems [5]. These derivatives exhibited many significant bioactivities such as lamellerins which have exhibited antitumor [6] or anti-HIV [7] activities; or halitulin an interesting cytotoxic compound [8]. However, pyrrolooxazine skeleton was uncommon in the structure of alkaloids. To date, this structure was only found in those of formoxazine, a metabolite isolated from a fungus *Paecilomyces formosus* [9].



Paeniloxazine

2. Results

2.1. Structural Elucidation

After the fermentation (40 L) of *P. odorifer* cultured at 25°C and with stirring of 120 rpm, the obtained crude extract (2.8 g) was fractionated by normal phase column chromatography leading to several fractions. Guided by HPLC analysis and biological screening, an interesting cytotoxic fraction (244 mg , IC_{50} values of 23 ± 0.5 µg/mL and 22.5 ± 1.5 µg/mL for HaCaT and B16, respectively) was purified by reverse phase semi-preparative HPLC using a gradient elution with water and CH_3CN to afford Paeniloxazine (**1)** (18 mg).

This compound was isolated as an orange-red solid and it has according to HRESIMS data a $C_{22}H_{18}O_2N_2$ molecular formula suggesting 15 degrees of unsaturation. The ^{1}H-NMR spectrum recorded in $CDCl_3$ exhibited two protons of an oxygenated methylene group at δ 4.38 ppm, twelve aromatic protons at δ from 5.96 - to 7.46 ppm and two exchangable protons at δ 7.82 ppm . Jmod and HSQC data of **1** revealed the presence of signals corresponding to aromatic carbons, some of them as quaternary carbons, together with oxygenated carbon at δ 57.5 ppm (**Table 1**) and two carbon atoms at downfield shifts δ 152.7 and 157.3 ppm. The combination of data between molecular formula and number of unsaturations displayed that compound **1** possessed at least three aromatic rings.

Careful analysis of 2D NMR spectra of **1** (COSY, HSQC and HMBC) led to the identification of two structural parts named (a) and (b) (Figure 1). The 1,2-disubstituted aromatic ring in the first part (a) was highlighted by the presence of four protons at δ 7.03 (H-8/H-9), 7.22 (H-7) and 6.65 (H-5), and their ^{1}H-^{1}H COSY correlations were shown in Table 1 and Figure 2. The correlations observed in the HMBC spectrum between all the aromatic protons mentioned above with carbon at δ 136.5 led to the assignment of C-6. Its downfield shift proved that it was connected to a nitrogen atom. These data suggested the partial structure (a) corresponded to a dihydronaphthalene unit bearing an amino group at C-6 with their correlations illustrated in Table 1 and Figure 2. The position of this amino group has been confirmed by the HMBC correlations between these exchangeable protons (not visible in CD_3OD) and C-5 (δ 123.4), C-5a (δ 116.8), C-6 (δ 135.6) and C-9a (δ 126.7), This dihydronaphthalene unit was connected with a mono-substituted phenyl nucleus shown by HMBC correlations from the proton at δ 7.03 (H-9) to the carbon at δ 136.5 indicating that it corresponds to C-1′, from H-2′/H-6′ (δ 7.46) of the mono-substituted phenyl group to C-9a (δ 126.7) of the unit (a). The substitution patterns in (a) were confirmed by COSY, HMBC data and the measured coupling constants.

The structure of the other part (b) presented three aromatic protons consisting of two coupled protons at δ 5.96 (H-3) and 6.13 (H-2) and one proton corresponding to a doublet at δ 6.65 (H-6). The carbons at δ 107.5 (C-1) and 108.8 (C-2) correlated to the two coupled protons showed lower chemical shifts than those of aromatic carbons on the benzene ring. Combined to the measured coupling constant (J = 3.1 Hz), these data highlighted that these carbons belonged to a pyrrole ring. In addition, the HMBC correlations (**Figure 1**) from H-1 and H-2 to downfield shift carbons (δ 152.7 (C-3a) and 157.3 (C-4a)) demonstrated a linkage between pyrrole ring and another ring.

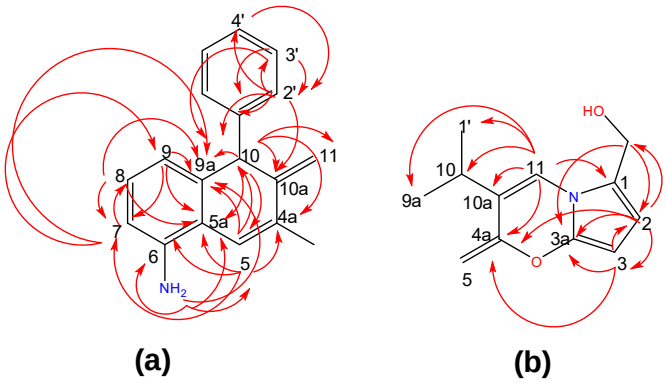


Figure 1: Key HMBC (H → C) correlations between the parts (a) and (b) of Paeniloxazine (**1**).

On the other hand, the presence of an oxygenated methylene group at δ 4.38 and δ 57.5 and the HMBC correlations of this proton to C-2 indicated the linkage of this group on pyrrole nucleus at C-1. All these data elucidated part (b) as a pyrrolooxazine moiety with an oxygenated methylene group at C-1.

Moreover, the connection of the dihydronaphthalene unit (part a) to the pyrrolooxazine moiety (part b) was revealed via HMBC correlations from H-10 (on part a) to C- 4a, C-10a, and C-11 of part b, from H-2′ to C-10a, and from H-11 (part b) to C-9a, C-10 and C-1′ of part a (**Figure 2**). Indeed, the structure of **1** was formed by the two units (a) and (b) fused via two junction carbon atoms at position 4a and 10a. The entire structure of **1** with the combination of the two parts was finally confirmed via NOESY experiment (**Figure 2**). The NOESY spectrum of **1** revealed several NOEs between proton aromatics of the dihydronaphthalene unit, phenyl group and pyrrolooxazine part. Among them, the best important correlations which supported the junction between the partial structures (a) and (b) were NOEs between the protons H-2′ and H-10 part (a) and H-11 of the pyrrolooxazine ring (b).

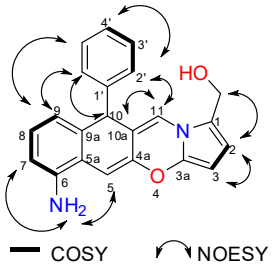


Figure 2: Selected ^{1}H-^{1}H COSY and NOESY correlations for compound **1**

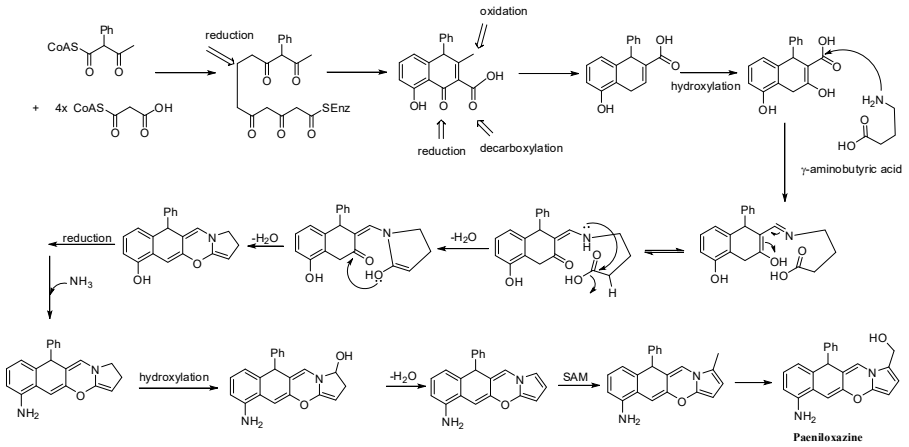
Table 1. 1D and 2D-NMR data of **1** (300 and 75 MHz, $CDCl_3$, δ ppm)

Position	δC	Type	δH mult (J in Hz)	HMBC (H→ C)	COSY	NOESY
1	126.7	qC				
2	108.8	CH	6.13, d (3.1)	3, 3a, 4a, OCH_2	3, OCH_2	3, OCH_2
3	107.5	CH	5.96, d (3.1)	2, 3a, 4a	2	2
3a	152.7	qC				
4a	157.3	qC				
5	123.4	CH	6.65, d, (1.8)	4a, 5a, 6, 7, 9a, 10	10, NH_2	10, NH_2
5a	116.8	qC				
6	136.5	qC				
7	111.4	CH	7.22, d, (8.0)	8, 9, 9a	8, 9	NH_2
8	119.4	CH	7.03, m	5a, 7, 9a	7	2′/6′
9	119.4	CH	7.03, m	7, 5a, 9a, 1′	7, 2′/6′	2′/6′
9a	126.7	qC				
10	34.2	CH	5.86, s	4a, 5a/10a, 5/11, 9a	5/11	11, 2′/6′
10a	116.8	qC				
11	123.4	CH	6.65, d, (1.8)	1/9a, 4a, 10, 10a, 1′	10	10, 2′/6′
1′	136.5	qC				
2′/ 6′	119.6	CH	7.46, d (8.0)	1′, 3′/5′, 4′, 9a, 10a	3′/5′, 9	9, 10, 11
3′/ 5′	122.0	CH	7.14, m	1′, 2′/6′, 9a	2′/6′, 4′	9
4′	111.4	CH	7.22, d (8.0)	2′/6′	3′/5′, 2′/6′	2′/6′
OCH_2	57.5	CH_2	4.38, s	2, 3a	2	2
NH_2			7.82, s	5, 5a, 6, 9a		5, 7

Therefore, all data confirmed that compound **1,** produced by this bacterium *P. odorifer*, could be a tetracyclic alkaloid skeleton consisting of a dihydronaphthalene unit fused to a pyrrolooxazine moiety and was

named Paeniloxozine. To our knowledge, it is the first report of this skeleton from natural sources. The new alkaloid **1** is probably biosynthesized, according to Dewick [10], via an amino acid pathway (Scheme 1) combined to a polyketide pathway. After the formation of a dihydronaphtalene part coming from the condensation between 4 unit of malonyl CoA and one phenylmalonylCoA derivative, the obtained bicyclic compound was condensed to a gamma-butyric acid unit to form the pyrroloxazine part. The last steps could involve various reactions from hydroxylation, dehydration, C-methylation to lead the substitution of the pyrrole group by an oxygenated methylene.

Scheme 1: Putative biosynthetic pathway for compound **1**



2.2. Cytotoxic Activity

Paeniloxazine was also investigated in vitro for its cytotoxic effects against B16 murine melanoma and HaCaT human keratinocyte cell lines by MTT assay [11] with doxorubicin as a positive control. The compound **1** exhibited a weak activity with micromolar IC_{50} values (76.0 1 and 78.9 µM for B16 and HaCaT cell lines, respectively) which could explain the activity of the fraction.

3. Materials and Methods

3.1. General Experimental Procedures.

1D and 2D NMR spectroscopic data were recorded in MeOH-d_4 solution containing Me_4Si as internal standard on Bruker DMX 300 spectrometry [300 MHz (^1H) and 75 MHz (^{13}C)]. NMR spectroscopic data were processed using the MestReNoVa version 10.0 software. HRMS measurements for exact mass determination were performed with a Q-Extractive Focus at CRMPO (Centre Regional de Measure Physique de l'Ouest), Rennes, France. Analytical HPLC (High Resolution Analysis Performed) and semi preparative HPLC were performed on Prevail C_{18} column 5µm (250 mm x 4.6 mm for the former and 250 mm x 10 mm for the later), GRACE, Columbia, MD, USA. The chiral HPLC was set up using a chiral AD-H column. Optical rotations were determined at 589 nm (sodium D line) using PerkinElmer-343 polarimeter. Infrared spectra were recorded on Perkin Elmer apparatus. Wavelengths of maximum absorbance (ν_{max}) are quoted in wave numbers (cm^{-1}).UV spectrum was recorded on SPECORD 205-222A190 in MeOH.

3.2. Collection and Analysis of PC-GYM-TO Strain

PC-GYM-TO Strain was isolated from the crustose lichen *Rhizocarpon geographicum*-collected in Brittany, France in February 2015. The strain has been identified as *Paenibacillus odorifer* based on its 16S rRNA gene sequence analysis (GenBank accession number AJ223990) (L. Intertaglia, Banyuls/mer Platform). Comparative Blast similarities search of the 16S rRNA gene sequence gave a 98.46 % similarity to that of *P. odorifer* (Gene bank entry PODO_RS03805). After culture in Gym *streptomyces* medium (containing 4 g Glucose (Sigma-Aldrich, St Louis, MO, USA), 4 g yeast extract (Sigma-Aldrich, St Louis, MO, USA), 10 g malt extract (Sigma-Aldrich, St Louis, MO, USA), 2 g $CaCO_3$ (Merck, Germany), 12 g agar (Sigma-Aldrich, St Louis, MO, USA) in 1L), the bacterium was stored in mixture of 47.5% (v/v) glycerol, 47.5% (v/v) H_2O and 5%(v/v) DMSO at -80°C with reference as PC-GYM-TO (CORINT collection).

3.3. Cultivation and Extraction.

P. odorifer (strain PC-GYM-TO) was cultured on Gym *streptomyces* medium agar (2 g glucose, 4 g yeast extract, 4 g malt extract, 2 g $CaCO_3$ and 12 g agar in 1 L at pH 7). The inoculum was prepared by transferring one loop full of culture (PC-GYM-TO) from medium agar to culture flask (250 mL) consisted of 50 mL of liquid Gym *streptomyces* medium (2 g glucose, 4 g yeast extract, 4 g malt extract and 2 g $CaCO_3$ in 1 L at pH 7). The bacterium culture was grown at 25°C on a rotary shaker incubator at 120 rpm for 7 days. After 7 days for pre-culture, 400 mL of bacterium culture was put into 140 Erlenmeyer flasks (500 mL), each containing 300 mL of liquid Gym *streptomyces* medium (1% inoculum). The fermentation culture was then performed at 25°C with 120 rpm of stirring for 7 days. After 7 days of culture, the broth was collected and centrifuged at 3500 rpm, 4°C for 15 min. After removal of the pellet, sterilized XAD-7-HP resin (40 g/L) was added into supernatant to absorb the organic products from the culture. The mixture then was shaken at 220 rpm for 4h. The resin was filtered and de-adsorbed by mixture of solvent acetone/MeOH (50/50, v/v). This mixture of solvent was removed under reduce pressure; the resulting aqueous layer was extracted with ethyl acetate (EtOAc) (3 x 300 mL). The EtOAc-solute extract was dried under vacuum to yield 3.0 g of organic extract from 40.0 L of the culture.

3.4. Extraction and Isolation

The organic extract (2.9 g) from strain PC-GYM-TO, used after biological assay, was subjected to normal phase chromatography column using silica gel 0.06-0.2 mm, 60 A° (Acros Organics, Germany) and a sequential mixture of solvent elution with increasing polarity from cyclohexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) to furnish 18 fractions. Guided by HPLC analysis and bioactivities for each fraction, the bioactive fraction containing Paeniloxazine (F8, 244.0 mg, IC_{50} 23 ± 0.5 µg/mL and 22.5 ± 1.5µg/mL using MTT assay against HaCaT and B16, respectively) was purified by semi-preparative HPLC (using Prevail® C_{18} column with gradient of 0% to 100% CH_3OH in H_2O for 60 min, a flow rate of 2.0 mL/min) and preparative TLC to afford Paeniloxazine (18.0 mg).

Paeniloxazine: (6-amino-10-phenyl-10*H*-naphtho[2,3-e]pyrrolo[2,1-b][1,3]oxazin-1-yl)methanol: orange-red solid, R_f = 0.45 (in $CHCl_3$/EtOAc = 2/1)); HR-MS-ESI at *m/z* 365.1261 of $[M+Na]^+$ ion, calcul. $C_{22}H_{18}N_2O_2$ molecular formula; FT-IR ν_{max} (cm^{-1}): 3400, 2921, 1705, 1610, 1415; UV-vis (MeOH,) λ_{max} (log ε): 224 (3.27), 281(2.47); ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) see Table 1. $[\alpha]_D^{20}$ was not measurable at the operating wavelength of the polarimeter (589 nm) at c, 0.01 in EtOH.

3.5. Cytotoxic evaluation

The cytotoxic effects were evaluated on crude extract and pure compounds (with mother concentration for each sample as 40 mg/mL) against HaCaT human keratinocytes and B16 murine melanoma cell line described in literature [11]. HaCaT (2000 cells/well) and B16 (1800 cells/well) were cultivated in RMPI 1640 medium supplemented with 5% of fetal calf serum (FCS) and antibiotic in atmosphere of 5% CO_2 at 37°C. After 24h culture, the samples were added at different concentrations (1, 10, 50, 100 and 200 µg/mL) and each 96-well plate was continuously incubated at the same temperature and atmosphere as above. After 48 culture, cell growth and viability were then measured at 540 nm using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Doxorubicin was used as a positive control. Each experiment was repeated three times.

4. Conclusions

In summary, a new tetracyclic alkaloid skeleton was firstly isolated from *P. odorifer* associated with the lichen *Rhizocarpon geographicum*. Its structure consists of dihydronaphthalene unit that is a well-known group of natural products, fused to a rare pyrrolooxazine moiety. This compound exhibited a weak cytotoxicity against HaCaT and B16 cells. Further experiments will be done to determine its activity on a larger panel of cancer cell lines and its eventual mechanism of action.

Supplementary Materials: Supplementary Materials are available online

Author Contributions: S.T. and L.N. conceived of and designed the experiments; L.N. performed the experiments; L.N. analyzed the data; I.R. realized the biological assays; S.F. helps for UV measurement; L.N. and S.T. wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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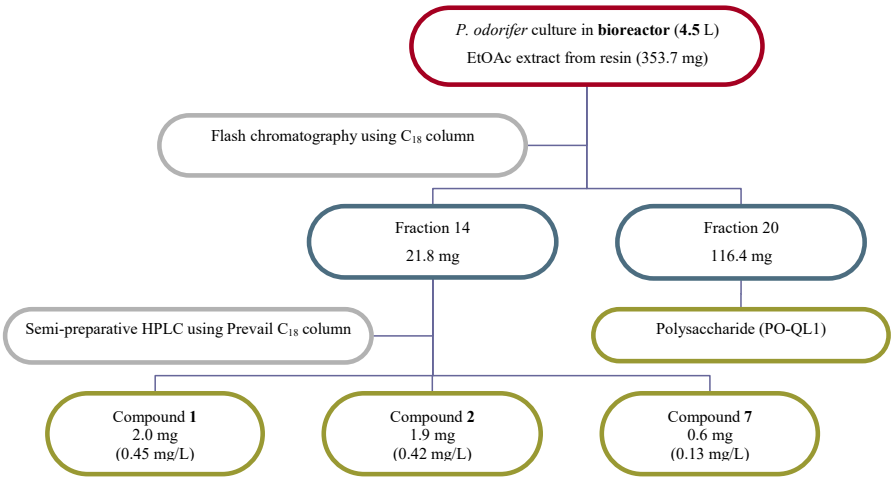
Sample Availability: Samples of the compounds **1** is available from the authors.

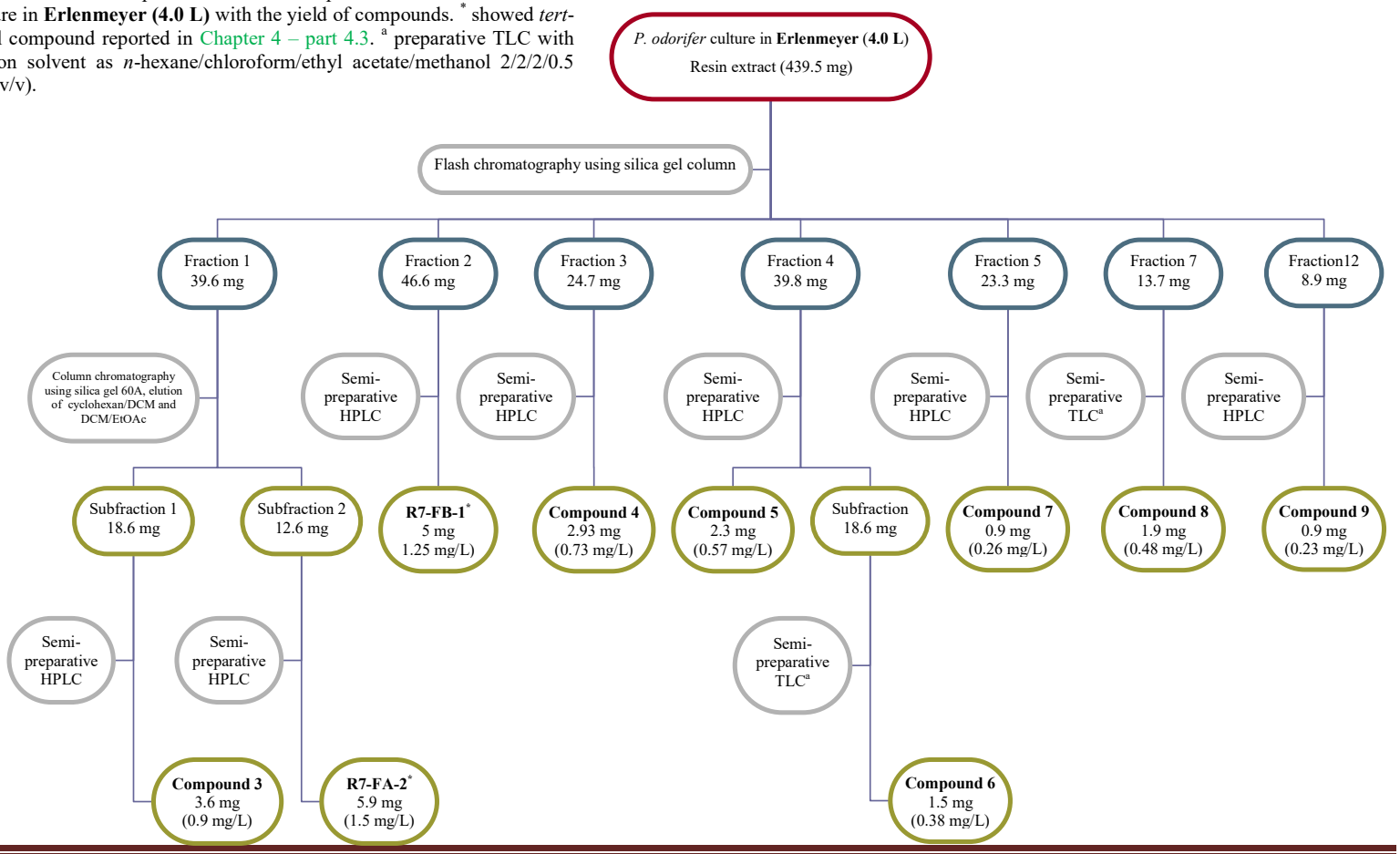
4.5. DESCRIPTION OF THE OTHER ISOLATED METABOLITES

Along with cytotoxic compounds reported above, other known and no cytotoxic metabolites were also isolated from the different cultures carried out during this work including two diol compounds (**1, 2**) (found from sawdust of Oak wood reported in Rukachaisirikul et al., 2012), an aliphatic ester (**3**), two furfural derivatives (**4, 5**) a butyrolactone (**6**), an acrylate derivative cyaneodimycin (**7**) (isolated from *Streptomyces cyaneofuscatus* - Parrot et al., 2016), a cyclopentene (**8**), a salicylate ester (**9**) (Green et al., 2008), a diphenol (**10**) and an indole compound (**11**) (found from *Nocardia ignorata* Noël et al 2017) (Figure 2.4.6). The physical and chemical properties of these compounds will be introduced in Chapter 6 – 6.3 and their NMR spectra will be provided in ANNEXE 3. Among them, the two diols and (**1, 2**) and cyaneodimycin (**7**) were found in both of the resin extracts from the culture performed using bioreactor or Erlenmeyer flasks. The other compounds were isolated from extracts obtained by culture in either bioreactor or Erlenmeyer with too small yield. The Schemes 2.4.1 to 2.4.3 described the process of isolation of these compounds. The isolation process is currently in progress from the Erlenmeyer 40L culture.

Scheme 2.4.1: Description of the isolated process on resin extract from culture in bioreactor with the yield of compounds in mg/L.

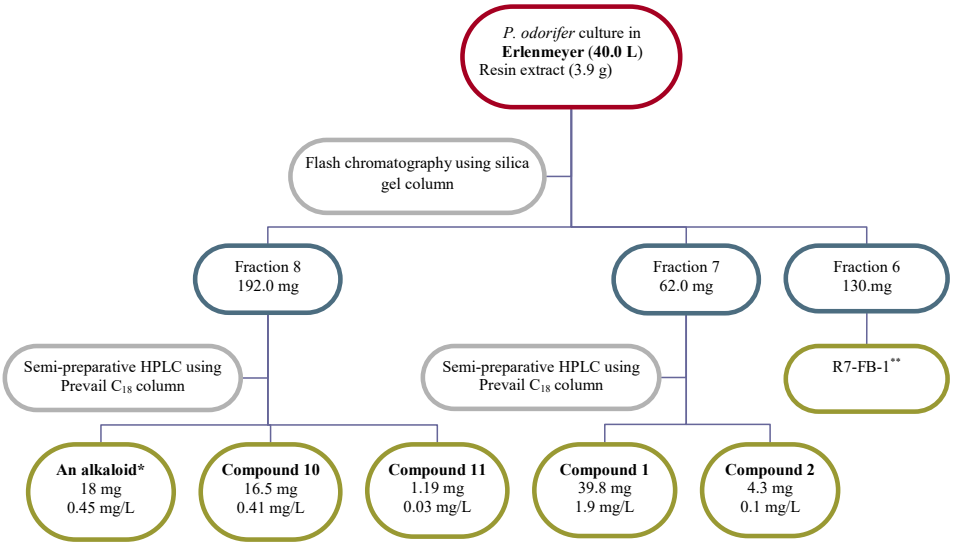


Scheme 2.4.2: Description of the isolation process on resin extract from culture in **Erlenmeyer (4.0 L)** with the yield of compounds. * showed *tert*-butyl compound reported in Chapter 4 – part 4.3. [a] preparative TLC with elution solvent as *n*-hexane/chloroform/ethyl acetate/methanol 2/2/2/0.5 (v/v/v/v).



Scheme 2.4.3: Description of the isolation process on resin extract from culture in **Erlenmeyer (40.0 L)** with the production yield of compounds (mg/L). * An alkaloid reported in Chapter 4 – part 4.4, ** *tert*-butylphenols found via HPLC-MS analysis, reported in Chapter 4 – part 4.3

Comment [ST3]: In scheme the yield is indicated in % to change



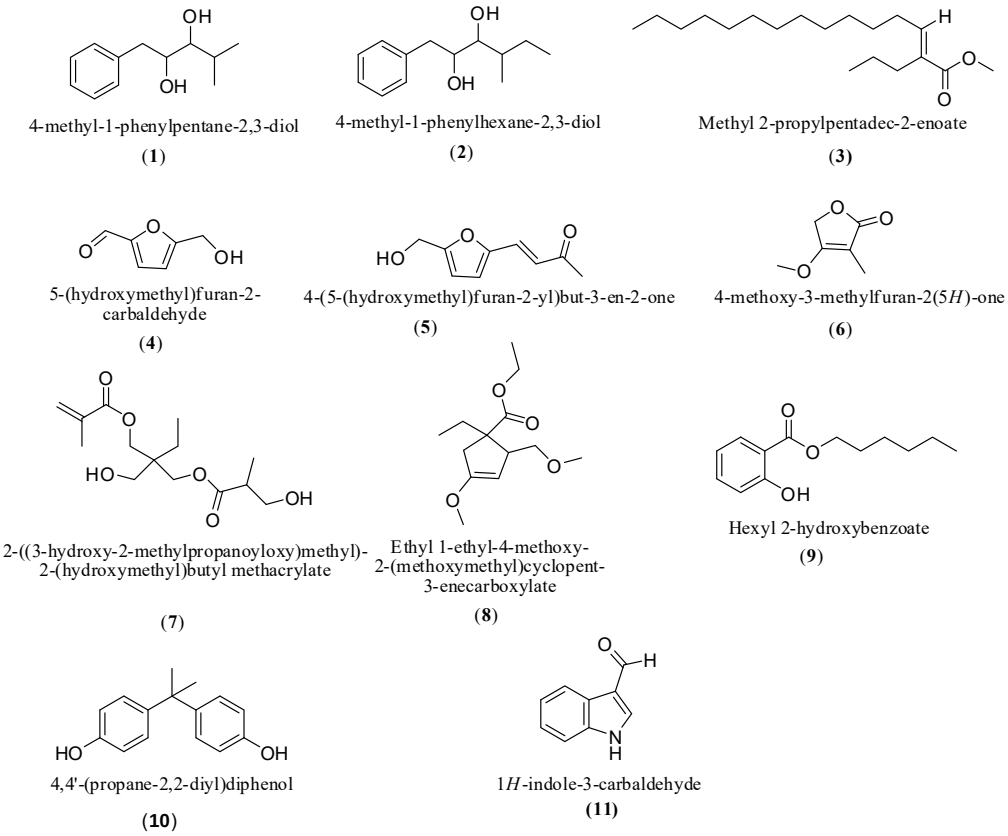


Figure 2.4.6 Structures of no cytotoxic or well-known compounds isolated during this work

4.5.1. Structural elucidation of compounds

Compound **1** was isolated as a crystal. Its molecular formula was determined to be $C_{12}H_{18}O_2$ (requiring 4 degrees of unsaturation) from positive-ion HRESIMS with a $[M+Na]^+$ ion at *m/z* 217.1199. The Jmod spectrum suggested the presence of a monosubstituted phenyl nucleus with carbons at δ_C 127.2 (C-4'), 129.4 (C-2', C-6'), 130.6 (C-3', C-5') and 140.8 (C-1'); of three methine (two of them oxygenated) at δ_C 73.9 (C-2), 79.2 (C-3) and 31.8 (C-4); one methylene at δ_C 41.8 (C-1); two methyls at δ_C 19.9 (C-5) and 19.2 (C-6) (Table 2.4.1). The ^{1}H NMR spectrum corroborated this data with protons resonating at δ_H 7.19 (1H, m, H-4') and 7.27 (4H, m, H-2', H-3', H-5', H-6') for

monosubstituted phenyl ring, two oxygenated methine protons at δ_H 3.85 (1H, ddd, J = 2.8, 6.1, 7.6 Hz, H-2), 2.99 (1H, dd, J = 2.8, 7.6, H-3) and one aliphatic methine at at δ_H 1.87 (1H, m, H-4) and two deshielded methyl group at δ_H 0.88 (3H, d, J = 6.7 Hz, H-5) and 0.97 (3h, d, J = 6.7 Hz, H-6). Analysis of 2D NMR spectra of **1** (COSY, HSQC and HMBC) led to the identification of two partial structure (a-b) (Figure 2.4.7). The first moiety (a) was a monosubstituted phenyl nucleus that was connected with another part (b) via HMBC correlations from H-1 to C-2'/C-6', and from H-2'/H-6' to C-2 to give the full structure of **1**.

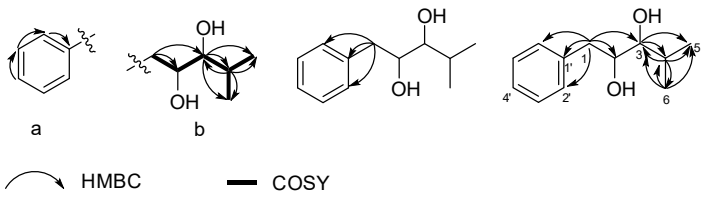


Figure 2.4.7 Key correlations for the structural assignment of **1**

The stereochemistry of H-2 and H-3 in compound **1** can be assigned by the analysis of coupling constants of H-2, H-3. According to literature (Silverstein and Webster, 1996), if $J_{2,3}$ is smaller than 7.0 Hz, the two protons H-2 and H-3 are in a trans-configuration, otherwise, if $J_{2,3}$ is more than 7.0 Hz, they are in *cis* configuration. In the case of **1**, the *trans* configuration between H-2 and H-3 was attributed by their small coupling content ($J_{2,3}$ = 2.8 Hz). Moreover, the absolute configuration of **1** could be determined by Mosher's method. The determination of the absolute configuration of **1** will be further realized. Compound **2** was obtained as a crystal. Its molecular formula was assigned to be $C_{13}H_{20}O_2$ based on the HRESIMS data which corresponded to a supplementar 14 mass unit (one CH_2 group) in comparison with **1**. Analysis of ^1H, ^{13}C NMR spectra revealed that the structure of **2** was similar to **1**. The major difference was the presence of one methylene group at $\delta_{C/H}$ 26.1(C-5)/1.15 (2H, m, H-5) (Table 2.4.1). Furthermore, the COSY and HMBC correlations also confirmed this structure (Figure 2.4.8). The stereochemistry at C-2 and C-3 of **2** was also determined as described earlier for compound **1**. It was suggested as *trans* configuration due to a small coupling constant of $J_{2,3}$ as 2.3 Hz. Likewise, the absolute configuration of **2** could be determined in further work.

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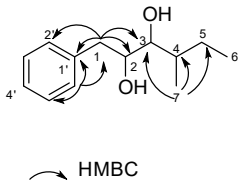


Figure 2.4.8 Key correlations for the structural assignment of **2**

Table 2.4.1 Comparison of ^{1}H NMR (500MHz, CD_3OD) and ^{13}C NMR (75MHz, CD_3OD) spectroscopic data of compounds **1** and **2**

	δ_H, mult. (*J* in Hz)		δ_C	
	Compound 1	Compound 2	Compound 1	Compound 2
1a 1b	2.80, dd (7.6, 13.5) 2.87, dd (6.1, 13.5)	2.85, dd (6.6, 5.5)	41.8	41.8
2	3.85, ddd (7.6, 6.1, 2.8)	3.87, ddd (7.4, 6.6, 2.3)	73.9	73.5
3	2.99, dd (2.8, 7.7)	3.07, dd (8.2, 2.3)	79.2	77.3
4	1.87, m	1.67, m	31.8	38.2
5	0.88, d (6.7)	1.15, m	19.9	26.1
6	0.97, d (6.7)	0.88, t (7.5)	19.2	11.4
7	-	0.85, d (6.8)		15.6
1'	-	-	140.8	140.6
2'/6'	7.27, m	7.27, m	129.4	129.3
3'/5'			130.6	130.5
4'	7.19, m	7.19, m	127.2	127.1

The compounds **1** and **2** were already found in *Mangrove Rhizophora* (Rukachaisirikul et al., 2012) but their spectroscopic data were not shown.

Compound **3** was obtained as colorless oil. Its molecular formula was determined to be $C_{19}H_{36}O_2$ from the $[M+Na]^+$ ion at *m/z* 319.26130 (calcd for $C_{19}H_{36}O_2Na$ 319.26075) observed in positive ion HR-ESI-MS spectrum.

The ^{1}H-NMR spectrum of **3** exhibited a methoxy group at δ 3.62 (H-19), two methylene groups linked to an alkene at δ 2.26 and 1.97 (H-16, H-4), a chain of aliphatic protons at δ 1.24-1.54 (H-5-14, H-17) and two methyl groups at δ 0.86 (H-15, H-18). The presence of only one olefinic proton at δ 5.38 (H-3) highlighted the presence of a monosubstituted alkene. The ^{13}C-NMR data of **3**, which were

assigned by Jmod, HSQC and HMBC analysis, displayed signals for a carbonyl at δ 173.5 (C-1), two olefinic carbons at δ 135.4 (C-2) and 129.8 (C-3), a oxygenated carbon at δ 50.6 (C-19) and a chain of aliphatic carbons at δ 28.8 to 32.0. The positions of these groups were confirmed by HMBC correlations from H-4 to C-3, from H-3 and H-5 to C-4, H-18 to C-17, C-16, H-17 to C-16, C-2 and H-16 to C-1 (shown in Chapter 5 - 5.3.2).

Moreover, the ^{1}H-^{1}H COSY experiments highlighted correlations between protons H-4 with H-3 and H-5, H16-and H17 as well as H14 and H15 (Figure 2.4.9).

Additionally, the HMBC data exhibited the correlation from protons of methoxy to carbonyl carbon that formed an ester group. On the other hand, protons of methylene group attached to alkene (H-16, δ 2.26) also presented HMBC correlations with carbon of the carbonyl group. This observation suggested that the ester group was one of the substituted groups in the double bond. Therefore, with above data, **3** presented an entire structure as methyl 2-propylpentadec-2-enoate. To our knowlegede, this compound was an aliphatic ester found for the first time from bacteria. However, it exhibited no cytotoxicity on HaCaT and B16 cell lines

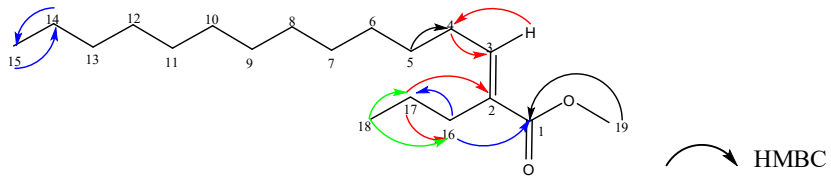


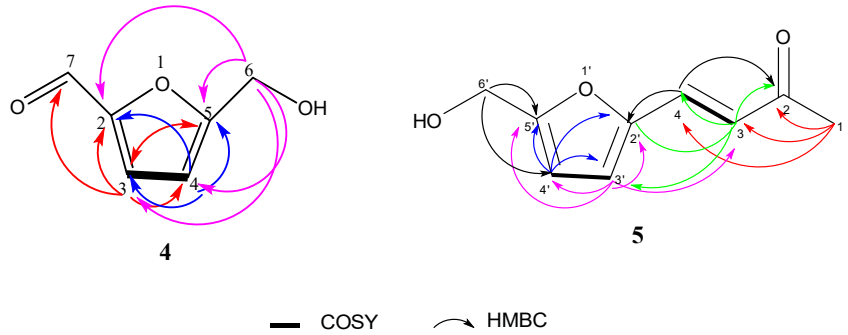
Figure 2.4.9 Key HMBC correlations in **3**

Compound **4** and **5** were assigned as furfural derivatives. Compound **4** was collected as colorless oil while compound **5** was found as yellow oil. Their molecular formula was determined to be $C_6H_6O_3$ for **4** and $C_9H_{10}O_3$ for **5**

The ^{1}H NMR spectrum of **4** exhibited a symmetrical structure with three proton signals including a methylene group at δ 4.70 (H-6) joined to an oxygen, two aromatic protons at δ 7.22 (H-3) and 6.52 (H-4) and one proton of carbonyl group at δ 9.59 (H-7). The presence of a small coupling constant (3.5 Hz) between the two aromatic protons supported an *ortho* relationship of them on furan ring. The *J*mod and HSQC spectra of **4** showed carbon signals including carboxyl groups at δ 178.1,

aromatic carbons at δ 123.0 and 110.6 and methylene group at δ 58.7. The assignements of these groups were confirmed by HMBC correlations (Figure 2.4.10).

The ^{1}H and ^{13}C NMR data of compound **5** revealed four signals at downfield shifts as methine groups at δ_C 131.8 (δ_H 7.41, H-3), δ_C 124.7 (δ_H 6.58, H-4), δ_C 118.6 (δ_H 6.80, H-3') and δ_C 111.3 (δ_H 6.46, H-4'). It simultaneously presented a carbonyl group at δ_C 200.9; a methyl group at δ_C 27.2 (δ_H 2.34, H-2), an oxygenated methylene moiety at δ_C 57.5 (δ_H 4.56, H-6') and two quaternary carbons at δ_C 151.9 (C-2') and δ_C 159.6 (C-5'). The COSY correlations established two spin systems from these four methine groups as H-3 and H-4, H3'and H4' whereas, the HMBC correlations from protons of methyl group from H-1 to C-2, C-3, C-4 ; from H-3 to C-2, C-4, C-2' and C-3'; from H-4 to C-2, C-2'; from H-3' to C-3, C-2', C-4', C-5'; from H-3' to C-1', C-2', C-4'; and from H-6' to C-4' C-5' demonstrated the structure of **5** (Figure 2.4.10).



4 **5**

Figure 2.4.10 Key HMBC correlations in **4** and **5**

Although two compounds **4** and **5** were well- known synthetic compounds (Rigal and Gaset et al.,1983), it was the first example in nature. Both compound **4** and **5** showed no cytotoxicity on HaCaT and B16 cell lines.

Compound **7** was isolated as a white amorphous powder, it has $C_{10}H_{18}O_4$ molecular formula from the $[M+Na]^+$ ion at *m/z* 225.1100 according to HRESIMS data. Its 1D and 2D NMR were analyzed in $CDCl_3$. The NMR data analysis of **7** was assigned to cyaneodimycin which has been already reported in the literature from *S. cyaneofuscatus* associated to *Lichina pygmeae* (Delphine Parrot et al., 2016).The NNR data are characteristic and exhibited two germinal protons at δ_H 6.12,

5.63 corresponded to a methylene group with δ_C 126.5, one carbonyl carbon atom at δ_C 166.5 and one methyl group at δ_C 18.0, δ_H 1.94. All of them displayed a methacrylate moiety - a rare group in natural products (See in Table 2.4.2). Moreover, **7** also possessed one carbonyl carbon (at δ_C 175.2), four oxygenated methylene group (at δ_C 64.7, 66.2, 68.1, 60.5 corresponding with δ_H 4.28, 3.68, 3.65, 3.72, respectively), two methyl groups (at δ_C 7.6, 14.4 and δ_H 0.88, 1.30, respectively) and a quaternary carbon at δ_C 43.2. Interestingly, HRESIMS data of **7** supported a $[M+Na]^+$ ion at *m/z* 225.1100 related to molecular formula as $C_{10}H_{18}O_4Na$ (instead of $C_{14}H_{24}O_6$) which corresponds to a loss of a $C_4H_6O_2$ unit. This observation was fully explained by Delphine Parrot and it confirmed again that this kind of compound was easily hydrolyzed during the analysis process.

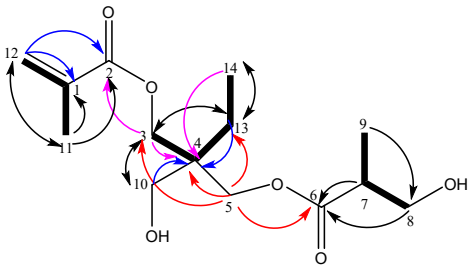


Figure 2.4.11 Key COSY (black line) and HMBC (arrows H to C) correlations for compound **7**

Table 2.4.2 Comparison of NMR data between compound 7 and literature

Compound 7 (in $CDCl_3$)			**Reference** (Parrot et al., 2016) in $CDCl_3$	
Position	**δ_H, mul, (*J*[Hz])**	**δ_C**	**δ_H, mul, (*J*[Hz])**	**δ_C**
1	-	131.0	-	136.7
2	-	166.5		167.3
3	4.28, s	64.7	4.06,s	64.9
4	-	43.2	-	42.2
5	3.65	68.1	3.60-4.10	71.5
6	-	175.2	-	176.1
7	2.03	31.1	2.69-2.73, m	39.9
8	3.73	60.5	3.46-3.56	73.4
9	1.23, m	14.4	1.12, d, (10)	13.6
10	3.68	66.2	4.06, s	64.9
11	1.94	18.5	1.90, br. s	18.4
12a	6.12, s	126.5	6.04, br. s	125.6
12b	5.63, m		5.54, br. s	
13	1.30, q, (7.6)	22.9	1.48, q, (7.5)	23.6
14	0.88, t, 7.6	7.6	0.87, t, (7.5)	7.6

Compound **8** was obtained as colorless oil. Its molecular formula was determined to be $C_{13}H_{22}O_4$ from the $[M-H]^-$ peak at *m/z* 241.1448 (calcd. for $C_{13}H_{21}O_4$ 241.14453) in negative ion HR-ESI-MS spectrum. The ^{1}H-NMR spectrum of **8** showed signals of methine proton at δ_H 6.17 (s, H-3), two oxygenated methylene protons at δ_H 4.47 (d, *J* = 5.9 Hz, H-9) and 4.05 (q, *J* = 7.1 Hz, H-7). Additionally, it displayed two methyl groups at δ_H 1.19 (t, J = 7.1 Hz, H-8) and 0.87 (t, *J* = 6.7 Hz, H-12), two methylene groups at δ_H 1.27 (br, H-5 and H-11), two methoxyl groups at δ_H 3.31 (s, H-13) and 3.30 (s, H-10). The ^{13}C-NMR data highlighted also the presence of two carbons sp^2 at δ_C 107.3 (C-3) and 155.0 (C-4), two oxygenated methylene carbons at δ_C 59.7 (C-7) and 56.4 (C-9), two methoxy carbons at δ_C 48.9 (C-13 and C-10), two methylene carbons at δ_C 31.4 (C-5) and 22.4 (C-11) and two methyl carbon atoms at δ_C 13.8 (C-8) and 13.6 (C-12). Moreover, the presence of a carbonyl resonance at δ_C 163.9 (C-6) and one quaternary carbon at δ_C 78.3 (C-1) together with HMBC correlations (Table in Chapter 6- 6.3.8) supported a fully structure for **8**.reported in Figure 2.4.12. To date, in our knowledge, no report exists about this compound **8**, but this novel compound exhibited no cytotoxic effect on HaCaT and B16 cell lines

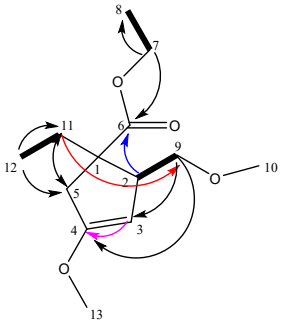


Figure 2.4.12 Key COSY (black lines) and HMBC (arrows H to C) correlations for compound **8**

Compound **9** was isolated as white powder. Its molecular formula was determined to be $C_{13}H_{18}O_3$ from the $[M+Na]^+$ peak at *m/z* 245.1152 (calcd. for $C_{13}H_{18}O_3Na$ 245.11481) in positive ion HR-ESI-MS spectrum. The ^{1}H-NMR spectrum of **9** presented signals of four aromatic protons at δ_H 7.87 (dd, *J* = 7.9 and 1.7 Hz, H-6), 6.89 (m, H-5), 7.48 (m, H-4) and 7.01 (dd, *J* = 8.4 and 0.9 Hz, H-3); five methylene groups (one oxygenated at δ_H 4.37 (t, *J* = 6.7 Hz, H-8), 1.79 (m, H-9), 1.65 (m, H-10) and 1.30 (m, H-11, H-12) and one methyl group at δ_H 0.91 (m, H-13). Additionally, these functional groups were confirmed in ^{13}C-NMR spectrum that showed six aromatic carbons (two of

them as a quaternary carbon and one is an oxygenated aromatic carbon) at δ_C 115.3 (C-1), 161.9 (C-2), 117.9 (C-3), 135.9 (C-4), 119.4 (C-5) and 130.2 (C-6); one carbonyl carbon at δ_C 170.5 (C-7); five methylene carbons at δ_C 65.8 (C-8) (oxygenated carbon), 28.5 (C-9), 25.0 (C-10), 23.0-33.8 (C-11, C-12) and one methyl group at δ_C 14.4. Furthermore, the combination of the all correlations highlighted in 2D-NMR spectra (HSQC, HMBC, COSY) (Figure 2.4.13) supported the assignment of the full structure of **9** as 1-hexylsalicylate.

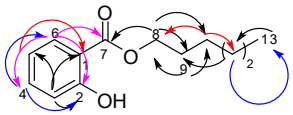


Figure 2.4.13 Key HMBC correlations for compound **9**

Compound **9** was reported as a synthetic compound (Green et al., 2008) but its NMR data was not shown. It was, thus, the first report in nature. Compound **10** has a commercial name as bisphenol A that was synthetic compound and was ingredient of plastic. However, its derivative (bisphenol A propyl ether analogue) was found from *Streptomyces* sp. (Qin et al., 2018) and it was confirmed to not be an artifact product. Compound **10** exhibited a moderate cytotoxic effect oh HaCaT and B16 cell lines with IC_{50} values of 34 ± 2 and 74 ± 4 µg/mL, respectively.

Compound **11** was isolated as a yellow crystal with a C_9H_7ON molecular formula. The ^1H NMR spectrum presented five aromatic protons and a carbonyl proton. This data and its molecular formula provided the assignment of this compound to be indole carbadehyde which was already reported from *Nocardia* sp isolated from *Collema auriforme* (Noël et al 2017), and from plant (Shankar et al., 2009), from marine sponge (McKay et al., 2005) and from bacteria belonging to Actinomyces (Wang et al., 2014). The HMBC correlations of compound **11** were shown in Figure 2.4.13.

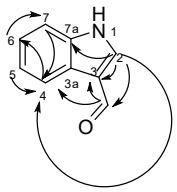


Figure 2.4.14 Key HMBC correlations for compound **11**

Table 2.4.3 Comparison of NMR data between compound **11** and reference

	Compound 11				**Reference** (Noël et al., 2017)	
Position	**δ_H, mul, (*J*[Hz])**	**δ_C**	**HMBC**	**COSY**	**δ_H, mul, (*J*[Hz])**	**δ_C**
1-NH	-	-			-	-
2-CH	8.12, s	138.3	C-3, C-4, C-7a, C-8	-	8.10, s	139.7
3	-	118.6	-	-	-	120.1
3a	-	126.5	-	-	-	125.6
4-CH	7.28, m	123.6	C-6	H-5	7.25, dt (7.6; 1.2)	123.6
5-CH	8.17, m	121.0	C-4	H-4/H-6	8.16, dt (7.6; 0.9)	122.3
6-CH	7.28, m	122.2	C-7	H-5, H-7	7.28, td (7.6; 7.8; 1.2)	125.0
7-CH	7.50, m	111.7	C-4	H-6	7.48, dt (8.0; 0.8)	113.1
7a	-	137.8	-	-	-	139.1
8-CHO	9.90, s	190.0	C-3, C-4	-	9.89, s	187.2

Conclusions

The compounds isolated from the culture of *P. odorifer* showed a variety of skeleton from primary metabolite (polysaccharide) to secondary metabolites (alkaloid, *tert*-butylphenol compounds, diol compounds, furfural derivatives, ester salicylate, indole carbaldehyde...). Although polysaccharides were reported in many cultures of bacteria, it was firstly found in *P. odorifer* species. Excepted the alkaloid, most compounds isolated from *P. odorifer* were either rare as natural products (e.g. *tert*-butylphenol compounds) or were synthetic compounds (furfural derivative, ester salicylate). A novel compound but no cytotoxic has been also described. Besides, indole carbaldehyde, a well-known compound that was found from many bacteria, was isolated from this bacterial species. However, no diketopiperazines which act as chemical signals (Brelles-Mariño and Bedmar, 2001) were found from *P. odorifer*.

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CHAPTER 5: CONCLUSIONS AND PERSPECTIVES

CHAPTER 5: CONCLUSIONS AND PERSPECTIVES

Lichens are complex organisms which can grow on many different supports. They can be found on trees, soil, rock surfaces and even inside rocks as well as on glass ceramics, metal objects... Lichens are also perfect self-supply mini-ecosystems growing very slowly and formed by the association of three partners such as fungi (mycobiont), algae or/and cyanobacteria (photobiont) and bacterial communities. Each partner plays distinct roles and participates together to permit to this organism to survive under drastic conditions of light, temperature and water supply, from the poles to the tropics, from the intertidal zones to peaks of the mountains (Brodo et al., 2001).

Among partners of the lichen, the third partner corresponding to the bacterial communities became a new interesting source for chemists to study due to their ability to produce many active compounds. In our efforts, using a culture-based strategy, in discovery and study these communities found on *Rhizocarpon geographicum*, one of the crustose lichens living on the rock surface, 13 pure strains including 10 of bacteria, one cyanobacterium and one fungus (Chapter 1) have been collected. The ten bacteria strains belonged to the two phyla of Firmicutes and Proteobacteria. Indeed, unlike to the other lichens, Proteobacteria was not the predominant class of bacteria among all the symbiotic microorganisms of *R. geographicum*. Instead, Firmicutes phylum took place in this dominance among these bacterial communities. Moreover, in this phylum, the genus which was present with the highest percentage (33%) was *Paenibacillus* genus. Throughout the studies of literature already published on the strains of this genus, *Paenibacillus odorifer* was selected to isolate its metabolites due to its potential production of active compounds (Chapter 2).

The next step of this work was to determine the optimal parameters of culture of *P. odorifer* in liquid medium. The first optimization (Chapter 3 – 3.1) carrying out with small scale (25 mL) resulted that *P. odorifer* grew the best in Gym *Streptomyces* medium supplemented with $CaCO_3$ at pH =7 and 25°C. These parameters chosen from the first optimization step were applied for the culture by bioreactor and yielded polysaccharide (Chapter 4 – 4.2) and two diol compounds (Chapter 4 – 4.5).

- The polysaccharide, firstly identified by IR and NMR data, was formed by three sugar units of glucuronic acid, fructose and fucose with an average molecular ratio of 4/2/1, respectively. The results were determined from the comparison of retention time observed in

HPLC chromatograms between sugar units derived from the hydrolysis of polysaccharide and those of standard monosaccharides. Then, a series of reactions involving methylation, hydrolysis, sodium borohydride reduction, acetylation by acetic anhydride and final GC-MS analysis of alditol acetates led to a hypothesis of the structure of this polysaccharide fraction as →2)- β-D-GlcA*p*-(1→ 2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(1→2)-β-D-GlcA*p*-(2→2)-β-D-Fru*f*-(2→4)-β-D-Fru*f*-(2→4)- β-L-Fuc*p*-(1→. This polysaccharide also possessed a significant cytotoxicity measured by MTT assay with IC_{50} value of 19µg/mL and 27 µg/mL on HaCaT human keratinocyte and B16 murine melanoma cell lines respectively. This may provide a potential source of antitumor agents as microbial polysaccharide fraction produced from *P. odorifer*.

- The two diol compounds (named 4-methyl-1-phenylpentane-2,3-diol (**1**) and 4-methyl-1-phenylhexane-2,3-diol (**2**)) harvested from culture in bioreactor, did not show any cytotoxic activity. Although these two compounds were identified by Rukachaisirikul and co-workers (2011), their spectroscopic data was not shown. To our knowledge it is the first report of these compounds from *P. odorifer* culture.

The disadvantage of the culture performed in the bioreactor (4.5 L) is the presence of other metabolites in a too scale amount. As a result, to increase the amount of secondary metabolites produced, a secondary optimization process was established using Erlenmeyer flasks (Chapter 3 – 3.2). The difference of production following the vessels used could be explained by the different mode of aeration and the stirring system inducing different stress conditions for the strain. In fact, the stirring in the bioreactor is realized by agitation blade submerged in the culture broth which implicates hyphal breaks, while in shaken flasks the stirring is performed with an orbital shaker allowing the hyphal formation. By the same way while in shaken flasks there is no specific system for aeration those in bioreactor consisted in a tube submerged in the culture broth which conducts oxygen into the medium causing the formation of bubbles.

Based on the results of the first optimization, the second one was carried out using Erlenmeyer flasks (4.0 L) leading to the selection of the best conditions for the production of metabolites from *P. odorifer* as medium Gym *Streptomyces* supplemented with $CaCO_3$ at pH 7, 25°C, and 120 rpm stirring as well as 1% inoculum. The resin extract obtained using the best conditions of the culture was subjected to chromatography approaches to give some compounds as described here. Among them, two *tert*-butylphenols were isolated and one of them exhibited

significant cytotoxic activity against HaCaT and B16 cell lines (Chapter 4 – 4.3). These two compounds were rare in nature. Their presence in the broth of the culture could be explained by the bioaccumulation and the biotransformation from *tert*-butyl derivatives such as BHA (benzohydroxyanisole). Besides, *P. odorifer* has also produced some other compounds either no cytotoxic or well-known (Chapter 4 – 4.5) such as methyl 2-propylpentadec-2-enoate (**3**); 5-(hydroxymethyl)furan-2-carbaldehyde (**4**); 4-(5-(hydroxymethyl)furan-2-yl)but-3-en-2-one (**5**); 4-methoxy-3-methylfuran-2(5H)-one (**6**); 2-((3-hydroxy-2-methylpropanoyloxy)methyl)-2-(hydroxymethyl)butyl methacrylate (**7**); Ethyl 1-ethyl-4-methoxy-2-(methoxymethyl)cyclopent-3-enecarboxylate (**8**); hexyl 2-hydroxybenzoate (**9**).

Further, the culture with a large volume (40 L in Erlenmeyer) using the best conditions selected following the results of the second optimization was carried out to find bioactive metabolites with higher amounts. A new alkaloid was found from a cytotoxic fraction (fraction 8). Its structure consists of a dihydronaphthalene moiety, which is a well-known structural group in natural products, fused to a rare pyrrolooxazine unit (Chapter 4 – 4.4). This new compound was the first example of this skeleton produced by any living organisms (and especially *P. odorifer*). This compound also presented week cytotoxicity with IC_{50} values of 76.0 µM and 78.9 µM on B16 and HaCaT cell lines, respectively. Moreover, two well- known compounds were also found from the fraction 8 such as 4,4'-(propane-2,2-diyl)diphenol (**10**) and 1*H*-indole-3-carbaldehyde (**11**) which were no active.

By comparing the report of all compounds isolated from each culture of *P. odorifer* using different means as bioreator and Erlenmeyer, it seems that the polysaccharide was only found from the culture using bioreactor. This can be explained by the fact that for culture in bioreator, the inoculum ratio was not controlled and the stirring was fixed at 150 rpm during the culture. These factors are different to the optimal parameters selected from optimization stages whereas these factors seem to play an important role for the production of active metabolites (Chapter 3 – 3.2.2). Moreover, the difference in the aeration method used could be also one of the reasons for this difference in production. Therefore, the realization of a culture in the bioreactor following the best conditions found during the second optimization could afford an accurate conclusion for the production of this polysaccharide fraction by *P. odorifer*. Endeavors will be done to control in a better way all the parameters of the culture in bioreactor (aeration rate, stirring, inoculum ratio

etc…).

In the same way, for the other metabolites, the two *tert*-butylphenols were found from the culture in Erlenmeyer but were absent in culture using bioreactor. The origin of this kind of compounds must be studied and especially their putative biosynthetic pathway. We envisaged realizing the entire genome sequencing in order to emphasize some interesting metabolic pathways.

Unlike, the diol compounds **1** and **2** were reported from the culture in both using bioreactor and Erlenmeyer flask. Thus, we could conclude that they are one of the major compounds produced by *P. odorifer*. Interestingly, when the volume of culture increased the mass of these compounds also increased (see in Scheme 2.3.1 and 2.4.2). In the same manner, to increase the mass of cytotoxic compounds, it is better to cultivate this strain in a large volume of medium.

Besides, two furfural derivatives (**4**, **5**) were also found in the cultures using Erlenmeyer flask. The medium used for the culture of *P. odorifer* contained malt extract which is a mixture of cereal seeds. It is important to note that furfural was reported to be formed from the treatment of cereals at high temperature (Mesias et al., 2017). Correspondingly, we could suggest that the furfural derivatives (**4**, **5**) were formed by the bioaccumulation of furfural which was present in medium after sterilization step and following by biotransformation by P. *odorifer.* Therefore, the bioaccumulation and biotransformation seem to be a characteristic behavior of *P. odorifer*. As discussed above this particular behavior must be studied in further experiments. We also suggest submitting this interesting strain in the biotransformation studies of lichen compounds.

Finally, *P. odorifer* is an interesting producer as this strain could produce original compounds such as a new alkaloid, some diol compounds in a large amount, some furfural derivatives, and some *tert*-butylphenols which could be derived from the biotransformation of particular precursors. Interestingly, while a significant amount of diketopiperazines have already been isolated from the culture of various lichen-associated bacteria, none of these compounds, classically found from cultures of microorganisms, were found herein. These observations highlight that this bacterium could be an interesting strain for biotransformation or producer of novel active compounds.

The isolation of other potential active metabolites is in progress from other active fractions obtained from our culture in large volume. Other evaluation of activities such as antibiotic or antifungal properties will be undertaken to valorize these compounds particularly the diol compounds obtained in large amount. We also envisage studying the other extracts as supernatant of from pellets which could contain potentially active intracellular compounds.

Moreover, the co-culture between *P. odorifer* and the fungus which has already been isolated from *R. geographicum* should be done to give some hypothesis about the ability of this bacterium to produce defensive weapons in the presence of this fungus. These further experiments will give us some argues to highlight the competition between various microorganisms which can be appeared inside this complex micro-ecosystem which could be the lichen.

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CHAPTER 6: MATERIALS AND METHODS

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6.1. MATERIALS

All solvents, reagents and materials used in all experiments were purchased from Sigma-Aldrich (Lyon-France), Carlo Erba Reactif (Val de Reuil, France), VWR (Fontenay-sous-bois, France), Acros Organics (Halluin, France). The solvent used for analysis or separation in HPLC were the quality of HPLC. The water was used from the EasyPure system (Barnstead^{TM}, ThermoFisher Waltham, MA, USA).

The Table 2.6.1 describes the ingredients composing of the media used to isolate the strains from *R. geographicum*.

Table 2.6.1 Ingredient of media used to isolate bacterial strains from *R. geographicum*

Elements (g/liter sterile water)	Bacillus acido	Gym	Gym + 10%NaCl	Thiobacillus	Marine agar
Yeast extract (Sigma-Aldrich, France)	1.0	4.0	4.0		
Malt extract (Sigma-Aldrich, France)		10.0	10.0		
Glucose (Sigma-Aldrich, Germany)	1.0	4.0	4.0	10.0	
Marine broth (Difco^{TM})					37.4
Agar	20.0	15.0	15.0	15	15.0
$(NH_4)_2SO_4$	0.2			3.0	
$MgSO_4.7H_2O$	0.5			1.0	
$CaCl_2.2H_2O$	0.25				
KH_2PO_4	0.6			0.5	
$CaCO_3$		2.0	2.0		
KCl				0.1	
$Ca(NO_3)_2.4H_2O$				$1.8.10^{-2}$	
$FeSO_4.7H_2O$				10^{-5}	
NaCl			100.0		
pH	3.5	7.2	7.2	4.5	

6.2. METHODS

6.2.1. The process for the production of crude extracts from fermentation

After the culture at selected parameters, the supernatant and the bacterial cells (pellets) were separated by the centrifugation at 3500 rpm at 4°C during 15 minutes. A XAD-7HP resin was added to the supernatant (40 g of resin per 1 L of supernatant) to adsorb organic compounds during 4 hours at 240 rpm of orbital rotation. After 4 hours, resin and supernatant were separated by filtration. The desorption from the resin was practiced 3 times with 800 mL of methanol:acetone mixture (50:50, v:v) for 15 minutes at 180 rpm of stirring. After the filtration step, the solution containing organic compounds was adjusted pH up to 7, concentrated under vacuum and extracted three times with ethyl acetate solvent. The resulting organic phase was dried by magnesium sulfate before evaporation under vacuum to afford a crude extract named resin extract (Scheme 2.4.1 chapter 4, part 4.1).

Similarly, the supernatant was also extracted 3 times by ethyl acetate solvent and the resulting organic phase was dried by $MgSO_4$ before evaporation under vacuum to give the supernatant extract.

6.2.2. Analytical methods used for isolation steps

6.2.2.1. Thin layer chromatography (TLC)

The TLC was performed on Aluminum sheets (20 x 20 cm) (silica gel 60 F254, Merck, Germany) for normal phase and using the solvent of elution as several mixtures of $CHCl_3$, EtOAc, MeOH with different ratios. The substances on silica gel sheets were observed under UV light at 254 nm, 312 nm or 365 nm and often visualized by anisaldehyde (ANS) reagent.

The ANS reagent was prepared by 3mL of ANS dissolved in 40 mL of glacial acetic acid and 90 mL of ethanol. 2% H_2SO_4 was then added to the resulting solution before the use.

6.2.2.2. Classical column chromatography

The separation using classical column chromatography was performed by a column with silica gel 60A (0.06 – 0.2 mm) (ACROS-ORGANICS). The elution solvents were solvents with increasing polarity from cyclohexane, dichloromethane (DCM), EtOAc to MeOH. The process was checked by TLC on normal phase.

6.2.2.3. Flash chromatography

The fractionation of crude extracts from fermentation was carried out by Flash chromatography (PuriFlash, Interchim, Montlucon, France) using a packaged column either as normal phase (40g SiOH Chromabond®Flash column) or reverse phase (40g, C18, Reveleris, Grace), flow rate of 15 mL/min, collection of 10 mL, UV detector at 272 nm. The elution solvent for normal phase was a sequential mixture of solvents with increasing polarity from cyclohexane, dichloromethane, EtOAc to MeOH (see Figure 2.6.1) during 4 hours. The gradient of elution for reverse phase was a mixture of H_2O and acetonitrile (ACN) during 75 min, beginning from ACN at 2% to 10% in 6 min, increasing to 15% at 15 min, 30% at 30 min, 40% at 45 min, 65% at 60 min and 100% at 75 min (See Figure 2.6.2).

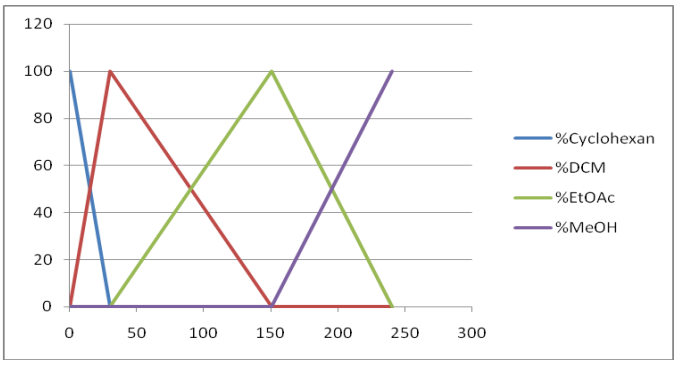


Figure 2.6.1 Gradient of elution for the separation of extracts by flash chromatography using a 40g SiOH Chromabond column

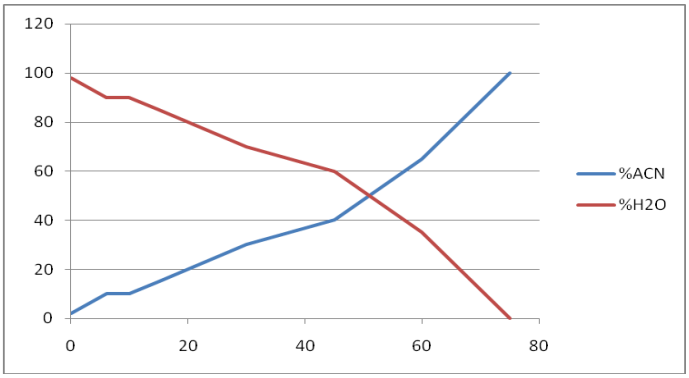


Figure 2.6.2 Gradient of elution in flash chromatography using a reverse phase C_{18} Reveleris (Grace) column

6.2.2.4. HPLC-UV/MS analysis

The chemical profiles of extracts or of some fractions were analyzed by HPLC using Prevail C18 column (5 µm, 250 x 4.6 mm, Grace, Columbia, MD, USA), with a flow rate of 0.8 mL/min, concentration of sample as 1 mg/mL, volume injection of 20 µL, with elution solvents as gradient of a mixture of H_2O and Acetonitrile during 60 min, beginning at 0% of ACN in 5 min, then increasing up to 100% of ACN at 35 min and maintaining this concentration in next 10 min, then decreasing to 0% at 50 min (Figure 2.6.3). The mass spectrometry detector was connected on-line with HPLC. The analysis on MS was performed by either positive or negative mode ESI (Electrospray) on CMS of Advion (Ithaca, NY, CA). The data were analyzed by the software Labsolution for HPLC and by Advion for MS.

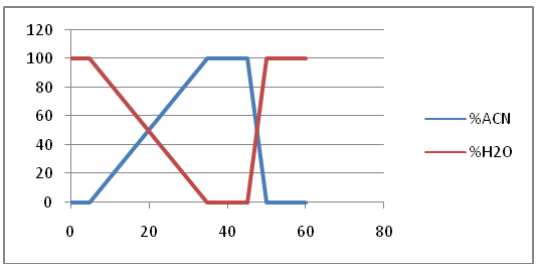


Figure 2.6.3 Gradient of solvent in HPLC analysis using Prevail C_{18} column

6.2.2.5. Semi-preparative HPLC

The purification was performed by semi-preparative HPLC (Shimadzu, Marne-la-Vallée, France) with a diode detector, using Prevail C18 column (5 µm, 250 x 10 mm, Grace, Columbia, MD, USA). The gradients of elution were various mixtures of H_2O and ACN as shown in Figure 2.6.4

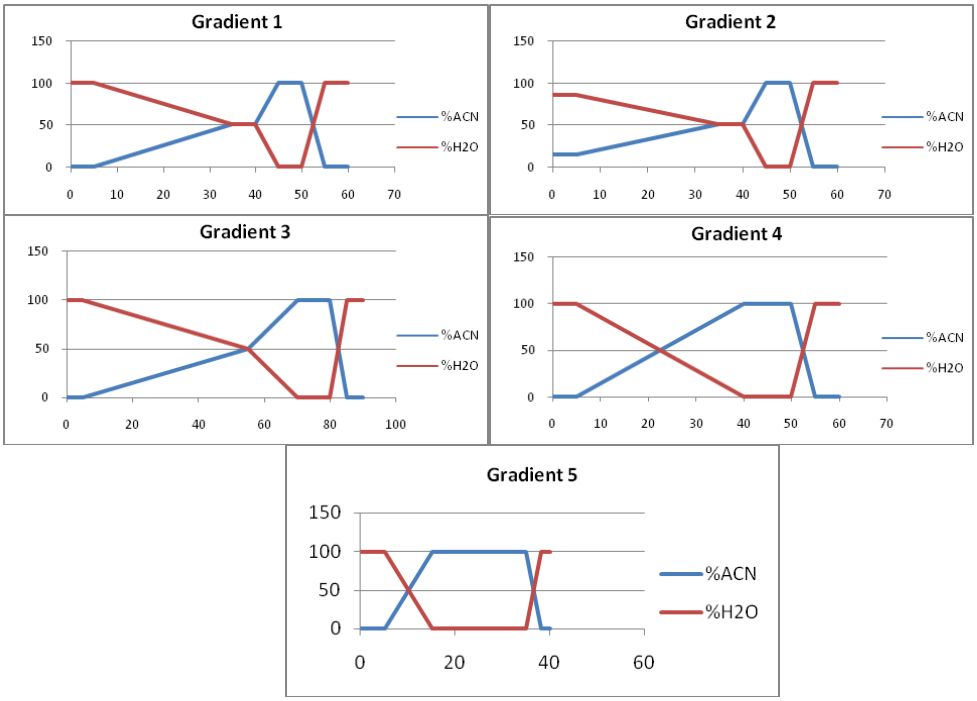


Figure 2.6.4 Gradients of elution used in semi-preparative HPLC (using Prevail C_{18} column)

6.2.2.6. GC-MS (Gas Chromatography- Mass Spectrometry)

The GC-MS system spectrometry (Agilent Technologies GC-MS 7820A-GC, Agilent, Waldbronn, Germany) was set up with nitrogen as carrier gas, using a DB-5 column (30 M x 0.25 mm x 0.25 µm), the injection volume of 1 µL at injection temperature 260°C and detector temperature 270°C during 105.05 min. The initial 37°C oven temperature was set for 6s following injection, raised to 140°C for 30 min at a rate of 20°C/min, then to 180°C for 40 min at a rate of 40°C/min, and finally maintained at 230°C for 30 min (Table 2.6.2).

Table 2.6.2 Parameters for GC-MS process

Over ramps	Rate (^{o}C/min)	Temperature	Hold time (min)
Initial	--	37	0.10
Ramp 1	20.0	140	30.00
Ramp 2	20.0	180	40.00
Ramp	30.0	230	30.00

6.2.3. HRMS (High Resolution Mass Spectrometry)

The HRMS analysis was performed using Q-TOF 6510 (quadrupole-time of flight) (Agilent, Santa Clara, CA, USA) or Q-Extractive (ThermoFisher, Waltham, MA, USA) at CRMPO (Centre Régional de Mesures Physiques de l'Ouest, Rennes, France).

6.2.4. NMR (Nuclear Magnetic Resonance) spectroscopy

The pure compounds were launched in NMR spectrometers: Fourier BRUKER DMX 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) (CORINT team, Rennes, France) and Fourier BRUKER 500 and cryo500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) (Plateforme PRISM, Rennes, France).

The spectra were performed in different deuterium solvents as $CDCl_3$, CD_3OD. DMSO-d_6. The chemical shift (δ) and coupling constant (J) were determined in parts-per-million (ppm) and in Hertz (Hz), respectively. The multiple signals were indicated as s (singlet), d (doublet), t (triplet), dd (double doublet), m (multiplet)… The NMR data were analyzed by MestRenova software.

6.2.5. Optical rotation

The optical rotation was measured by a polarimeter system (Perkin Elmer Model 341) at wavelength of the sodium light (598 nm), 20^{o}C. The value of optical rotation was determined by the formula cited below:

$$[\alpha]_D^{20} = [\alpha]/(l \times C)$$

Where

- $[\alpha]_D^{20}$ is the specific rotation in degrees cm^3 dm^{-1} g^{-1}

- [α] is the measured angle of rotation of a substance.
- l is the path length in decimeter (dm)
- C is the concentration in g/mL

6.2.6. Fourier transform infra-red (FT-IR) spectroscopy

The Infra-red spectrum was recorded on Fourier transform infrared (FTIR) spectrometer (Thermo Fisher, Germany) using Attenuated Total Reflectance (ATR). The sample of one milligram was pressed and scanned in the frequency range of 4000 – 400 cm^{-1}. The vibration bands were indicated at ν in cm^{-1}.

6.2.7. Biological assays

6.2.7.1. Cytotoxicity evaluation using MTT assay

The cytotoxic effects were evaluated on extracts and pure compounds against HaCaT human keratinocytes and B16 murine melanoma cell lines. HaCaT (10 000 cells/well) and B16 (6000 cells/well) were cultivated in RMPI 1640 medium supplemented with 5% of foetal calf serum (FCS) and antibiotics (penicillin/streptomycin 1%) in atmosphere of 5% CO_2 at 37°C. After 24h culture, the samples were added at different concentrations (1, 10, 50, 100 and 200 µg/mL) and each 96-well plate was continuously incubated at the same temperature and atmosphere as above. After 48 culture, cell growth and viability were added 200 µL of DMSO and were then measured at 540 nm using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Doxorubicin was used as a positive control. Each experiment was repeated three times.

6.2.7.2. Antioxidant evaluation

a) DPPH assay

The free radical scavenging activity was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl radical) method with quercetin and gallic acid as positive controls. 100 µL of DPPH methanol solution (freshly prepared at a concentration of 1 mg/mL) were mixed with 10 µL of substance at different concentrations (4.00; 11.1; 33.3; 100 µg/mL) in DMSO. After 15 min culture in the dark, the absorbance was measured at 540 nm using a UV-visible spectrophotometer.

The DPPH scavenging percentage activity was calculated as follow:

$$\text{Scavenging abilty \%} = [1- (A_s – A_b)/A_c] \times 100$$

Where A_s is the absorbance of sample with DPPH, A_b is the absorbance of the sample without DPPH (blank), A_c is the absorbance of negative control.

b) NBT assay

Phenazine methosulphate in the presence of NADH and under aerobic conditions (+ O_2) produces superoxide anions whose formation is determined by a dye nitro blue tetrazolium (NBT). In the solution containing 40 µL of tris hydrochloride buffer (16 mM, pH = 8), 50 µL nicotinamide adenine dinucleotide (NADH) (78 µM), 50 µL NBT (50 µM) and 50 µL phenazine methosulfate (910 µM) were added to test different concentrations of the substance (0.25; 1.85; 2.5; 5.5; 16.0; 25.0; 50 mg/mL in DMSO). The color reaction between superoxide radical and NBT was monitored by measurement of the absorbance at 560 nm. Vitamin C was used as positive control. The experiment was repeated three times. The inhibition percentage was calculated as follows:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{Sample at 560 nm}}/A_{\text{Control at 560 nm}}) \times 100$$

6.2.8. CFU (Colony-forming unit)

At the same time of the measurement of the optical density (OD) of the bacterial growth, 100 µL of the broth was diluted several times following demonstration of Figure 2.6.5 before cultured on the agar plates. The counting of the colonies appearing on the agar was carried out the next days.

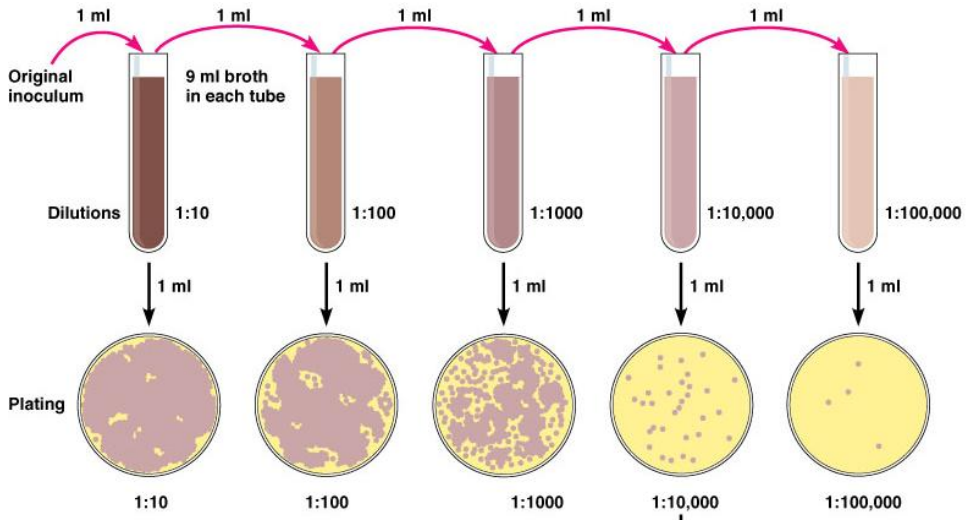


Figure 2.6.5 The process of viable plate counts

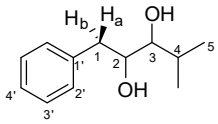
The colony forming unit (CFU) per milliliter was calculated by equal as following:

CFU/ml = number of colony x (1/ total of dilution)

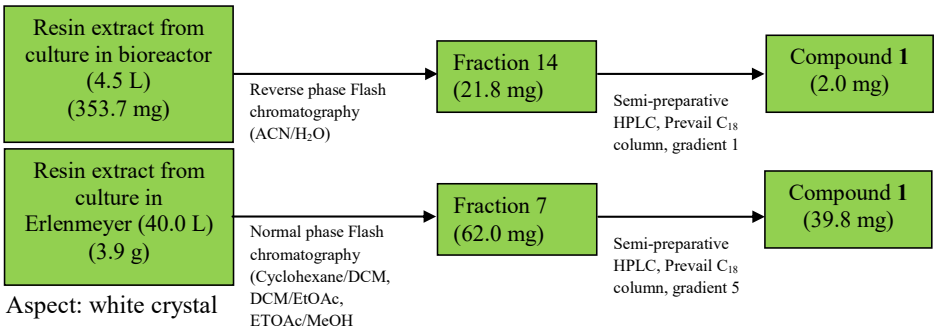
6.3. DESCRIPTIONS OF ISOLATED COMPOUNDS

The metabolites isolated from all the culture processes in either bioreactor or Erlenmeyer will be presented in this part and their spectroscopic data will be reported in Annexe 3.

6.3.1. 4-methyl-1-phenylpentane-2,3-diol (1)



The process of isolation for compound **1**



Aspect: white crystal

Molecular formula: $C_{12}H_{18}O_2$ (MW: 194 $g.mol^{-1}$)

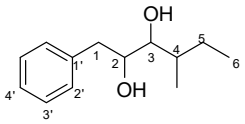
$[\alpha]^{20}_D$ = - 3.4 (c, 10^{-3}, MeOH) ($dm^{-1}g^{-1}.cm^3$).

HRESIMS: *m/z* $[M+Na]^+$ found : 217.1202 , calculated: 217.1199

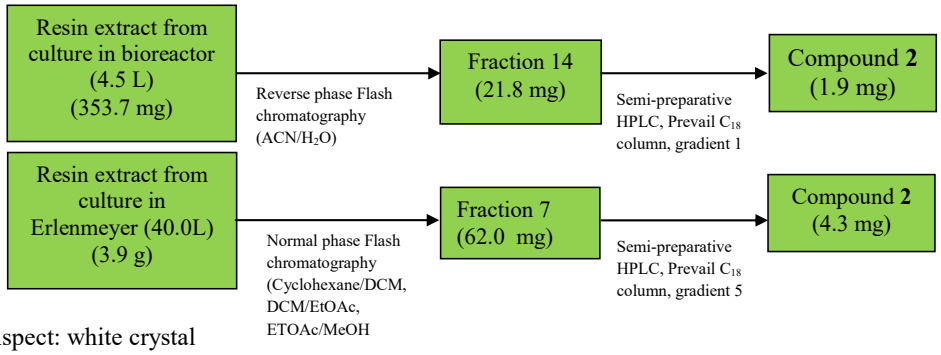
NMR spectra recorded in CD_3OD at 300 MHz or 75 MHz

Position	δ_C	δ_H, mult. (*J* in Hz)	COSY	HMBC
1a-CH	41.8	2.80, dd (7.6, 13.5)	2	1', 2', 6', 2, 3
1b-CH		2.87, dd (6.1, 13.5)	2	
2-CH	73.9	3.85, ddd (2.8, 6.1, 7.6)	1, 3	
3-CH	79.2	2.99, dd (2.8, 7.6)	2, 4	4, 5, 6
4-CH	31.8	1.87, m	3, 5, 6	3, 5, 6
5-CH_3	19.9	0.88, d (6.7)	4	3, 4, 6
6-CH_3	19.2	0.97, d (6.7)	4	3, 4, 5
1'	140.8	-		
2'/6'-CH	129.4	7.20 - 7.30, m	4'	4'
3'/5'-CH	130.6			
4'-CH	127.2	7.19, m	3'/ 5'	

6.3.2. 4-methyl-1-phenylhexane-2,3-diol (2)



The process of isolation for compound **2**



Aspect: white crystal

Molecular formula: $C_{13}H_{20}O_2$ (MW: 208 g.mol^{-1})

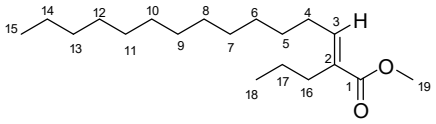
$[\alpha]^{20}_D$ = - 8 (c, 10^{-3}, MeOH) (dm^{-1}g^{-1}.cm^3).

HRESIMS: *m/z* $[M+ Na]^+$ found : 231.1356 , calculated: 231.13555

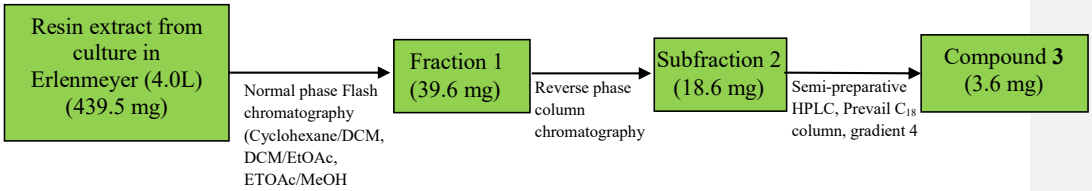
NMR spectra recorded in CD_3OD at 300 MHz or 75 MHz

Position	δ_C	δ_H, mult.(*J* in Hz)	COSY	HMBC
1-CH_2	41.8	2.85, dd (6.8, 5.5)	2	1', 2', 6', 2, 3
2-CH	73.5	3.87, ddd (7.4, 6.6, 2.3)	1, 3	
3-CH	77.3	3.07, dd (8.2, 2.3)	2, 4	
4-CH	38.2	1.67, m	3, 5, 7	
5-CH_2	26.1	1.15, m	4, 6	
6-CH_3	11.4	0.88, t (7.5)	5	4, 5
7-CH_3	15.6	0.85, d (6.8)	4	3, 4, 6
1'	140.6	-		
2'/6'-CH	129.3	7.20 – 7.30, m	4'	4', 1'
3'/5'-CH	130.5			
4'-CH	127.1	7.19, m	3' 5'	

6.3.3. Methyl 2-propylpentadec-2-enoate (3)



The process of isolation for compound **3**



Aspect: white powder

Molecular formula: $C_{19}H_{36}O_2$ (MW: 296 $g.mol^{-1}$)

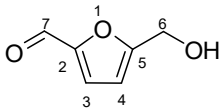
R_f = 0. 565 (*n*-Hexane/Chloroform = 1/1 (v/v))

HRESIMS: *m/z* $[M+ Na]^+$ found : 319.26075 , calculated: 319.2613

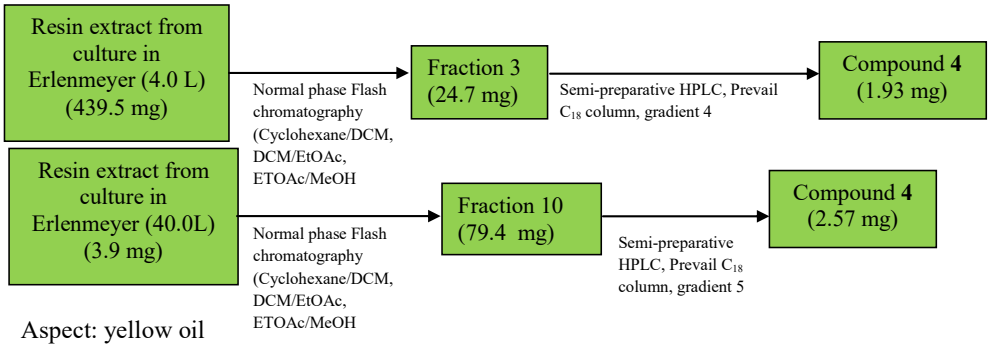
NMR spectra recorded in CD_2Cl_2, 300 MHz or 75 MHz

Position	δ_H , mul, (*J*[Hz])	δ_C (Jmod, HSQC)	HMBC	COSY
1	-	173.5	-	-
2	-	135.4	-	-
3-CH	5.38, m	129.8		H-4
4-CH_2	1.97, m	31.4	C-3	H-3, H-5
5-14-CH_2	1.24, m	28.5-32.0	C-4, C-15	H-4, H-15
15/18-CH_3	0.86, m	13.3	C-14/C16, C-17	H-4
16-CH_2	2.26, t, *7.5*	33.4	C-17, C-1	H-17
17-CH_2	1.54, m	24.3	C-16, C-2	H-16
19-OCH_3	3.62, s	50.6	C-1	-

6.3.4. 5-(hydroxymethyl) furan-2-carbaldehyde (4)



The process of isolation for compound **4**



Aspect: yellow oil

Molecular formula: $C_6H_6O_3$ (MW: 126 g.mol^{-1})

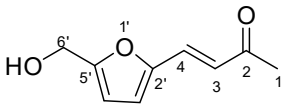
R_f = 0.41 (Chloroform/Methanol (9/1) (v/v))

HRESIMS: *m/z* $[M+Na]^+$ found : 149.0208 , calculated: 149.02091

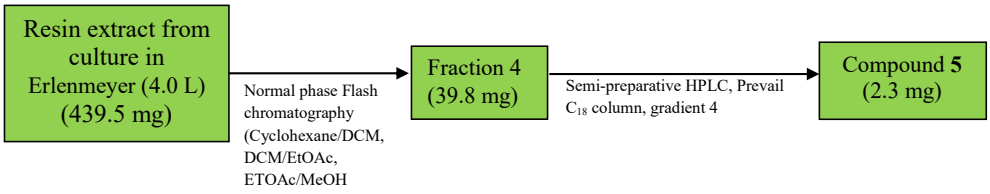
NMR spectra recorded in $CDCl_3$, 300 MHz or 75 MHz

Position	δ_H , mul, (*J*[Hz])	δ_C	HMBC[a]	COSY
2	-	153.3	-	-
3-CH	7.22, d (3.5)	123.0	C2,C4,C5,C7	H-4
4-CH	6.52, d (3.5)	110.6	C2, C3, C5	H-3
5	-	161.0	-	-
6-OCH_2	4.70, s	58.7	C2, C3,C4, C5	-
7-CHO	9.59, s	178.1	-	-

6.3.5. 4-(5-(hydroxymethyl) furan-2-yl)but-3-en-2-one (5)



The process of isolation for compound **5**



Aspect: yellow oil

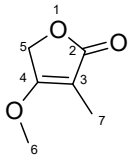
Molecular formula: $C_9H_{10}O_3$ (MW: 166 $g.mol^{-1}$)

HRESIMS: *m/z* $[M+ Na]^+$ found : 189.0522 , calculated: 189.05221

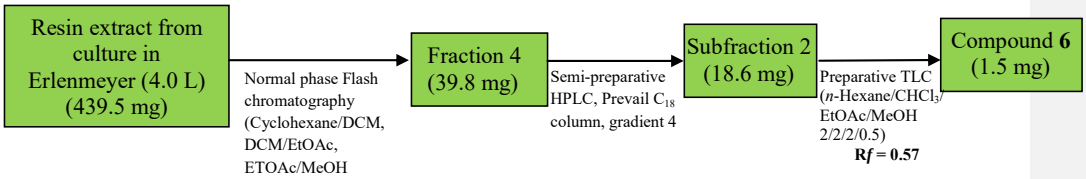
NMR spectra recorded in CD_3OD, 300 MHz or 75 MHz

Position	δ_H , mul, (*J* [Hz])	δ_C	HMBC	COSY
1-CH_3	2.34, s	27.2	C-2, C-3, C-4	
2	-	200.9		
3-CH	7.41, d, (16.0)	131.8	C-2, C-4, C2', C3'	H-4
4-CH	6.58, d, (16.0)	124.7	C-2', C-2	H-3
2'	-	151.9		
3'-CH	6.80, d (3.4)	118.6	C-3, C-2', C-4', C-5'	H-4'
4'-CH	6.46, d (3.4)	111.3	C-2', C-3', C-5'	H-3'
5'	-	159.6		
6'-OCH_2	4.56, s	57.5	C-4', C-5'	

6.3.6. 4-methoxy-3-methylfuran-2(5*H*)-one (6)



The process of isolation for compound **6**



Aspect: colorless crystal

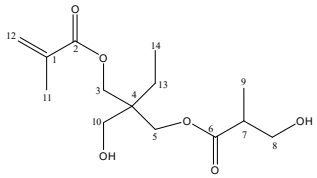
Molecular formula: $C_6H_8O_3$ (MW: 128 g.mol^{-1})

HRESIMS: *m/z* $[M+Na]^+$ found : 151.0366 , calculated: 151.03656

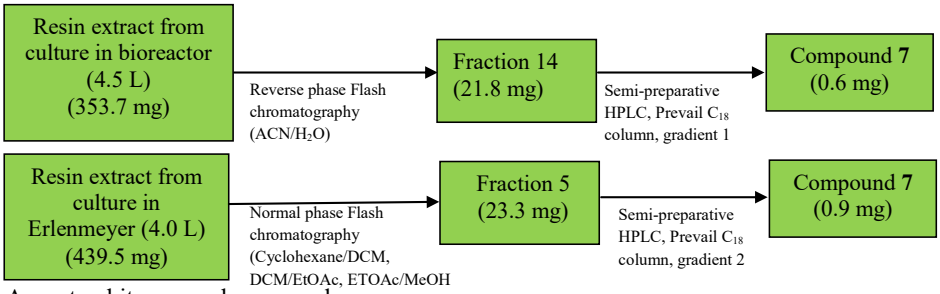
NMR spectra recorded in $CDCl_3$, 300 MHz or 75 MHz

Position	δ_H , mul, (*J* [Hz])	δ_C	HMBC
1	-	-	
2	-	170.2	
3	-	128.7	
4	-	129.4	
5-CH_2	4.14, q (7.0)	60.4	C-2
6-OCH_3	3.51, s	50.9	
7-CH_3	2.07, s	21.1	C-2

6.3.7. 2-((3-hydroxy-2-methylpropanoyloxy)methyl)-2-(hydroxymethyl)butyl methacrylate (7)



The process of isolation for compound **7**



Aspect: white, amorphous powder

Molecular formula: $C_{14}H_{24}O_6$ (MW: 256 $g.mol^{-1}$)

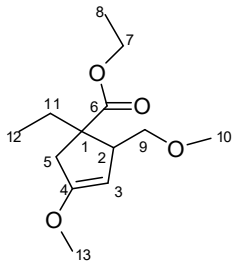
R_f = 0.13 (*n*-Hexane/Chloroform/Ethyl acetate/ Methanol (2/2/2/0.5), (v/v))

HRESIMS: *m/z* $[M+Na]^+$ ($C_{10}H_{18}O_4Na$) found : 225.11 , calculated: 225.10973 (lost of a $C_4H_6O_2$ unit due to hydrolysis)

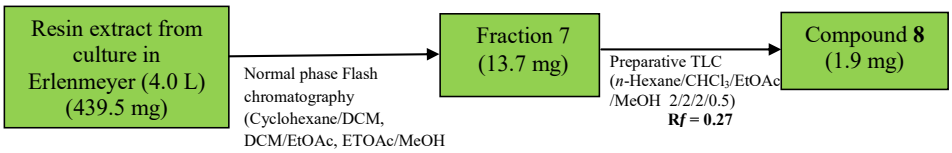
NMR spectra recorded in $CDCl_3$, 500 MHz or 125 MHz

	Compound 7 (in $CDCl_3$)				Reference (Parrot et al., 2016) in $CDCl_3$	
Position	**δ_H, mul, (*J*[Hz])**	**δ_C**	**HMBC[b]**	**COSY**	**δ_H, mul, (*J*[Hz])**	**δ_C**
1	-	131.0	-	-	-	136.7
2	-	166.5	-	-		167.3
3	4.28, s	64.7	C2,C4,C10	H-13	4.06,s	64.9
4	-	43.2			-	42.2
5	3.65	68.1	C3, C4, C13	H13	3.6-4.1	71.5
6	-	175.2			-	176.1
7	2.03	31.1	C6		2.69-2.73, m	39.9
8	3.73	60.5	C6	H-9	3.46-3.56	73.4
9	1.23, m	14.4	C8	H-8	1.12, d, (10)	13.6
10	3.68	66.2	C3, C4, C13		4.06, s	64.9
11	1.94	18.5	C1, C2, C12	H-12	1.90, br. s	18.4
12a 12b	6.12, s 5.63, m	126.5	C1, C2, C11	H-11	6.04, br. s 5.54, br. s	125.6
13	1.30, q, (7.6)	22.9	C3, C4, C14	H-14	1.48, q, (7.5)	23.6
14	0.88, t, (7.6)	7.6	C4, C13	H-13	0.87, t, (7.5)	7.6

6.3.8 Ethyl 1-ethyl-4-methoxy-2-(methoxymethyl)cyclopent-3-enecarboxylate (8)



The process of isolation for compound **8**



Aspect: colorless oil

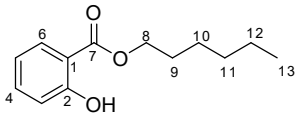
Molecular formula: $C_{13}H_{22}O_4$ (MW: 242 $g.mol^{-1}$)

HRESIMS: *m/z* $[M+H]^-$ found : 241.1448 , calculated: 241.14453

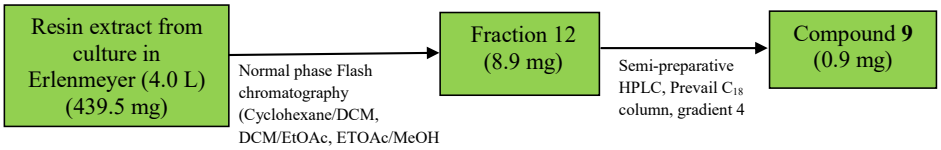
NMR spectra: recorded in CD_3COCD_3, 300 MHz or 75 MHz

Position	δ_H, mul, (*J*[Hz])	δ_C	HMBC	COSY
1	-	78.3	-	-
2-CH	1.96, s	19.9	C6	H-9
3-CH	6.17, s	107.3	C4	-
4	-	155.0		
5-CH_2	1.27, br	31.4	C11	
6	-	163.9		
7-OCH_2	4.05, q (7.1)	59.7	C6, C8	H-8
8-CH_3	1.19, t (7.1)	13.5	C6	H-7
9-OCH_2	4.47, d (5.9)	56.4	C3, C4, C6	H-2
10-OCH_3	3.30, s	48.9		
11-CH_2	1.27, br	22.4	C5, C9	H-12
12-CH_3	0.87, t (6.7)	13.6	C5, C11	H-11
13-OCH_3	3.31, s	48.9		

6.3.9. Hexyl 2-hydroxybenzoate (9)



The process of isolation for compound **9**



Aspect: white powder

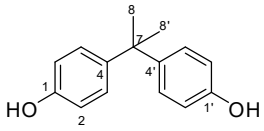
Molecular formula: $C_{13}H_{18}O_3$ (MW: 222 $g.mol^{-1}$)

HRESIMS: *m/z* $[M+ Na]^+$ found : 245.1152 , calculated: 245.11481

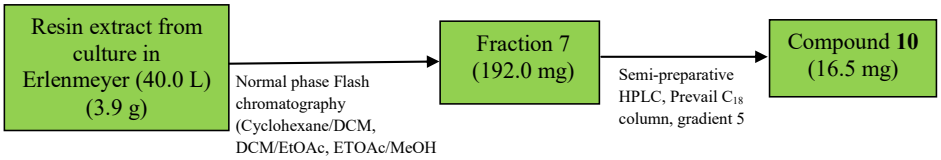
NMR spectra: recorded in $CDCl_3$, 500 MHz or 125 MHz

Position	**δ_H, mul, (*J* [Hz])**	**δ_C**	**HMBC**	**COSY**
1	-	115.3	-	-
2	-	161.9	-	-
3-CH	7.01, dd (8.4 and 0.9)	117.9	C-1, C-2, C-5	H-4
4-CH	7.48, m	135.9	C-2, C-6	H-3, H-5
5-CH	6.89, m	119.4	C-1	H-6, H-6
6-CH	7.87, dd (7.9 and 1.7)	130.2	C-2, C-4, C-7	H-5
7	-	170.5		
8-CH_2	4.37, t (6.7)	65.8	C-7, C-9, C-10	H-9
9-CH_2	1.79, m	28.5	C-8, C-10	H-8, H-10
10-CH_2	1.65, m	25.0	C-9, C-11/12	H-9, H-11/12
11, 12-CH_2	1.30, m	23.0-33.8	C-10, C-13	H-10, H-13
13-CH_3	0.91, m	14.4	C-11/12	H-11/12

6.3.10. 4,4'-(propane-2,2-diyl)diphenol (10)



The process of isolation for compound **10**



Aspect: yellow oil

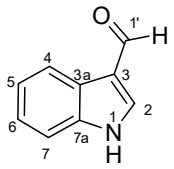
Molecular formula: $C_{15}H_{16}O_2$ (MW: 228 $g.mol^{-1}$)

HRESIMS: *m/z* $[M-H]^-$ found : 227.1080 , calculated: 227.10775

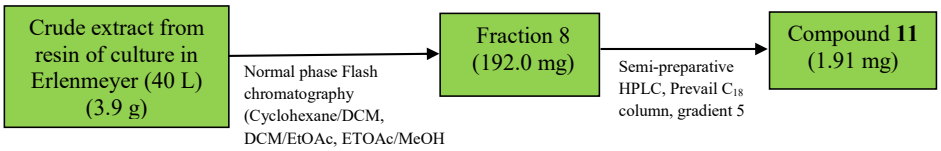
NMR spectra: recorded in $CDCl_3$, 300 MHz or 75 MHz

Position	δ_H, mul, (*J*[Hz])	δ_C	HMBC	COSY
1, 1'	-	153.4	-	-
2, 2'-CH	6.73, d, (8.8 Hz)	114.8	C-1, C-4, C-6/C-1', C-4', C-6'	H-3/H-3'
3, 3'-CH	7.09, d, (8.8 Hz)	128.1	C-1, C-5, C-7/C-1', C-5', C-7	H-2/H-2'
4, 4'	-	143.5	-	-
5, 5'-CH	7.09, d, (8.8 Hz)	128.1	C-1, C-3, C-7/C-1', C-3', C-7	H-6/H-6'
6, 6'-CH	6.73, d, (8.8 Hz)	114.8	C-1, C-4, C-2/C-1', C-4', C-2'	H-5/H-5'
7	-	41.5	-	-
8, 8'-CH_3	1.62, s	31.2	C-4, C-7, C-8'/C-4', C-7, C-8	

6.3.11. 1*H*-indole-3-carbaldehyde (11)



The process of isolation for compound **11**



Aspect: yellow crystal

Molecular formula: C_9H_7ON (MW: 145 g.mol^{-1})

HRESIMS: *m/z* $[M + Na]^+$ found : 168.0420 , calculated: 168.04198

NMR spectra: recorded in CD_3OD, 300 MHz or 75 MHz

Position	δ_H, mul, (*J*[Hz])	δ_C	HMBC	COSY
1-NH	-	-		
2-CH	8.12, s	138.3	C-3, C-4, C-7a, C-8	-
3	-	118.6	-	-
3a	-	126.5	-	-
4-CH	7.28, m	123.6	C-6	H-5
5-CH	8.17, m	121.0	C-4	H-4/H-6
6-CH	7.28, m	122.2	C-7	H-5, H-7
7-CH	7.50, m	111.7	C-4	H-6
7a	-	137.8	-	-
8-CHO	9.90, s	190.0	C-3, C-4	-

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ANNEXES

ANNEXE 1: Supporting information for the article of *tert*-butylphenol compounds

Supporting information

tert-Butylphenolic Derivatives from *Paenibacillus odorifer*—A Case of Bioconversion

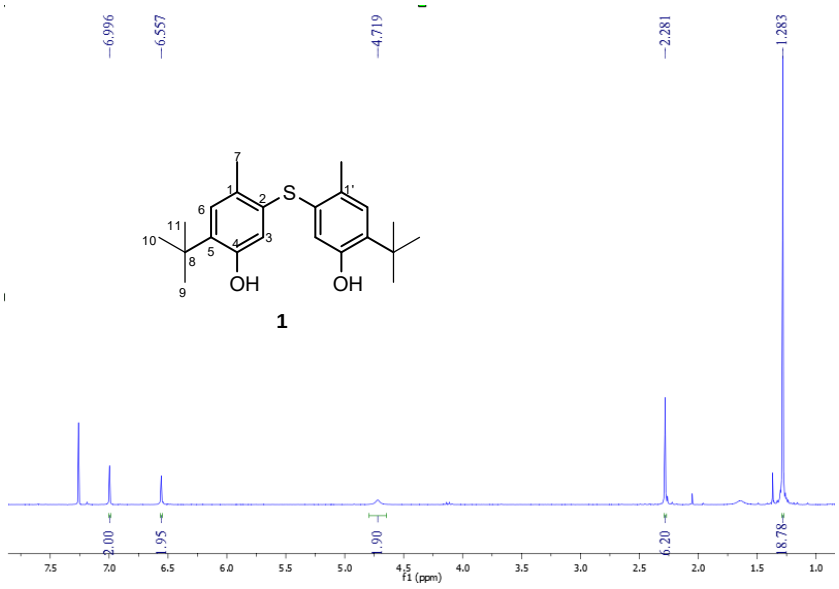
Thi-Bach-Le Nguyen [1], Olivier Delalande [2], Isabelle Rouaud [1], Solenn Ferron [1], Laura Chaillot [3], Rémy Pedeux [3] and Sophie Tomasi [1,*]

[1] University of Rennes 1, CNRS, ISCR—UMR 6226, F-35000 Rennes, France; nguyen.bachle@yahoo.com (T.-B.-L.N.); isabelle.rouaud@univ-rennes1.fr (I.R.); solenn.ferron@univ-rennes1.fr (S.F.)

[2] University of Rennes 1, CNRS, IGDR—UMR 6290, F-35000 Rennes, France; olivier.delalande@univ-rennes1.fr

[3] Chemistry, Oncogenesis, Stress, Signaling, Centre Eugène Marquis, Université de Rennes 1, INSERM U1242, 35000 Rennes, France; laura.chaillot@univ-rennes1.fr (L.C.); remy.pedeux@univ-rennes1.fr (R.P.)

* Correspondence: sophie.tomasi@univ-rennes1.fr; Tel.: +33-223-234-817



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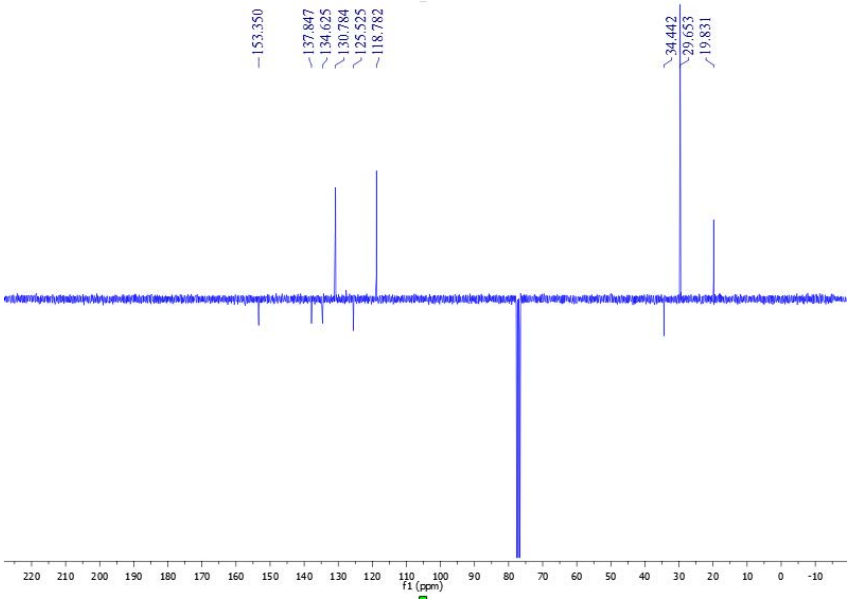
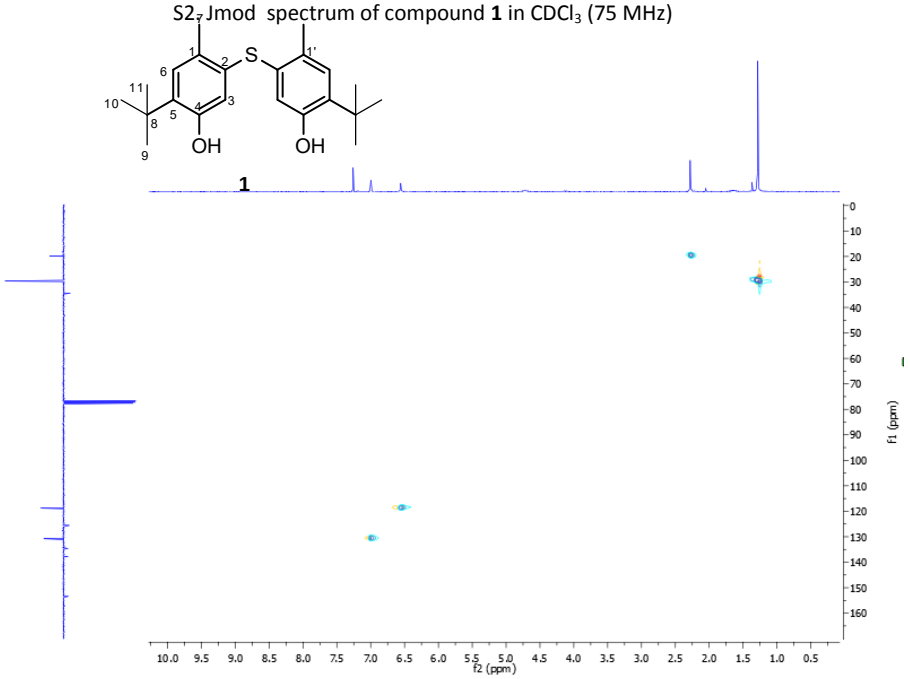
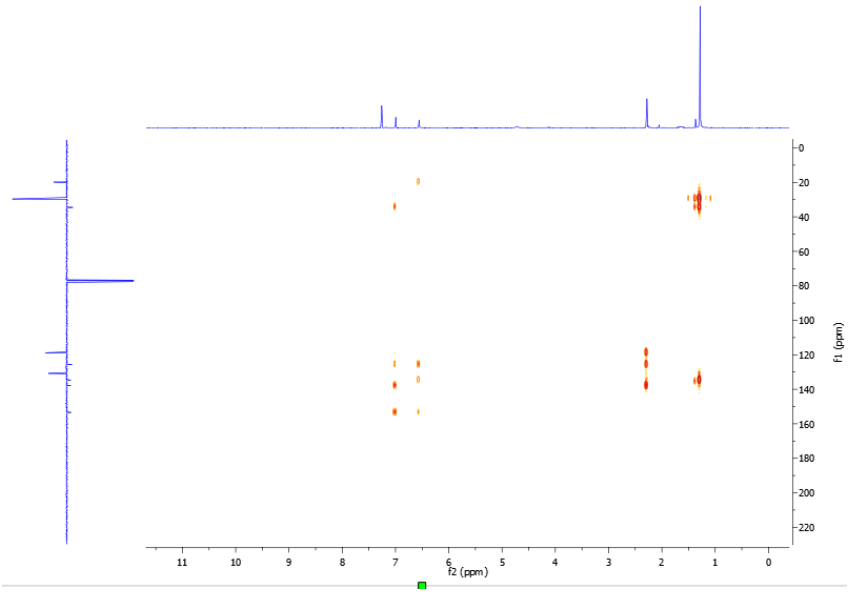
S1. ^{1}H-NMR spectrum of compound **1** in $CDCl_3$ (300 MHz)



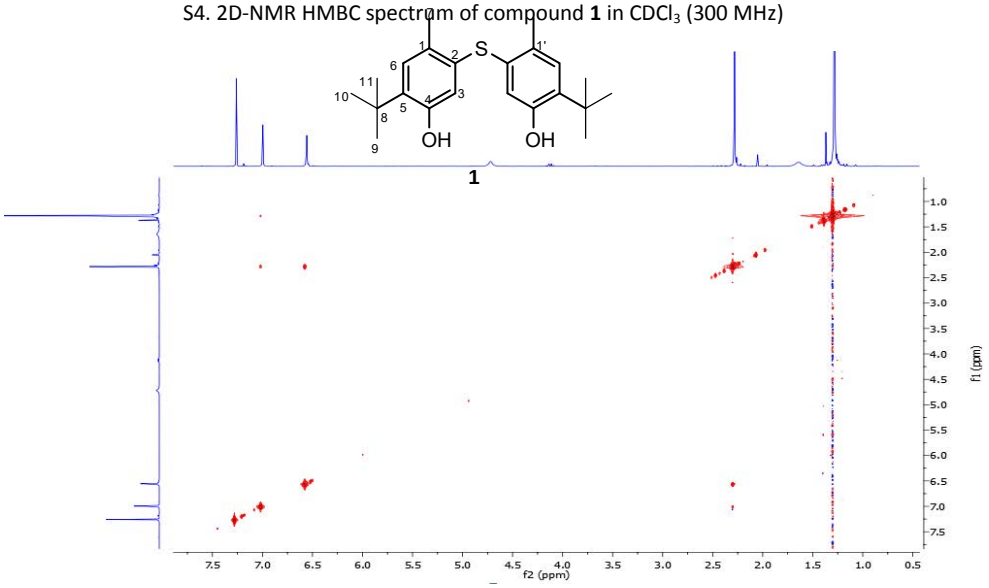
S2_7Jmod spectrum of compound **1** in $CDCl_3$ (75 MHz)



S3. 2D-NMR HSQCedit spectrum of compound **1** in $CDCl_3$ (300 MHz)



S4. 2D-NMR HMBC spectrum of compound **1** in $CDCl_3$ (300 MHz)



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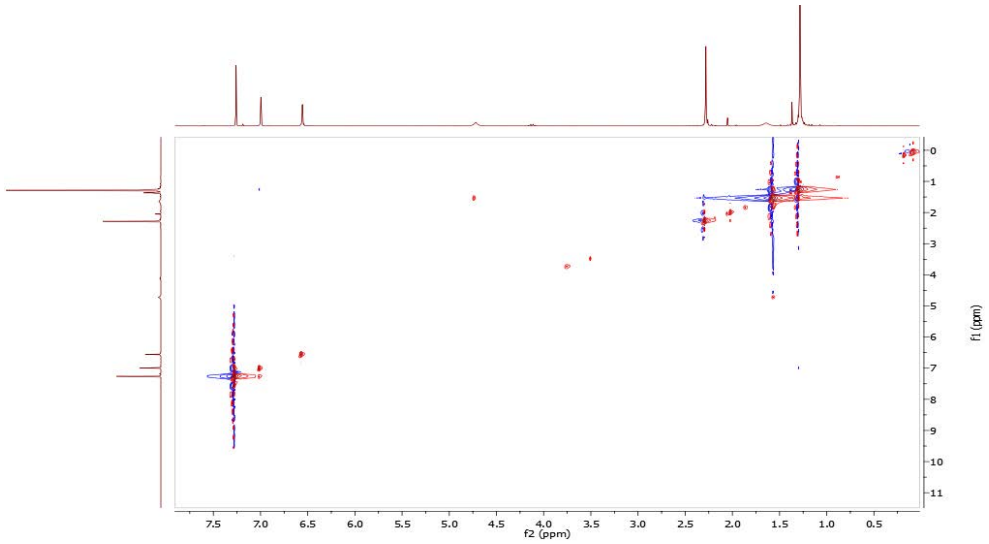
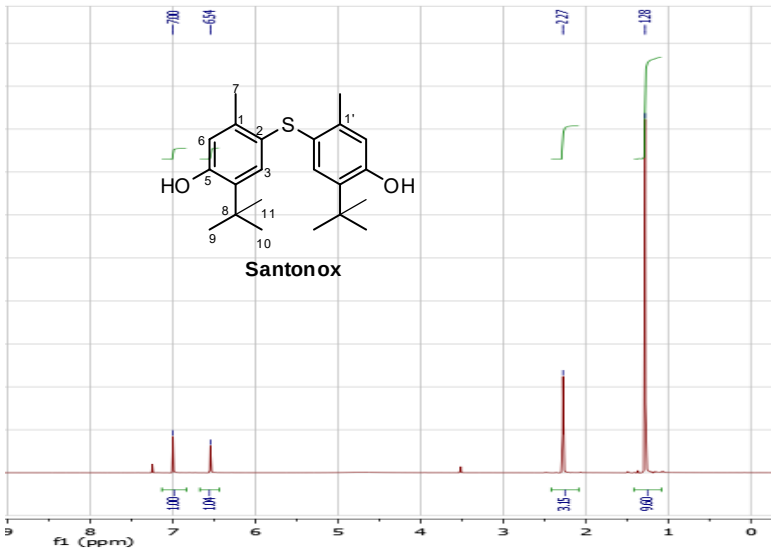
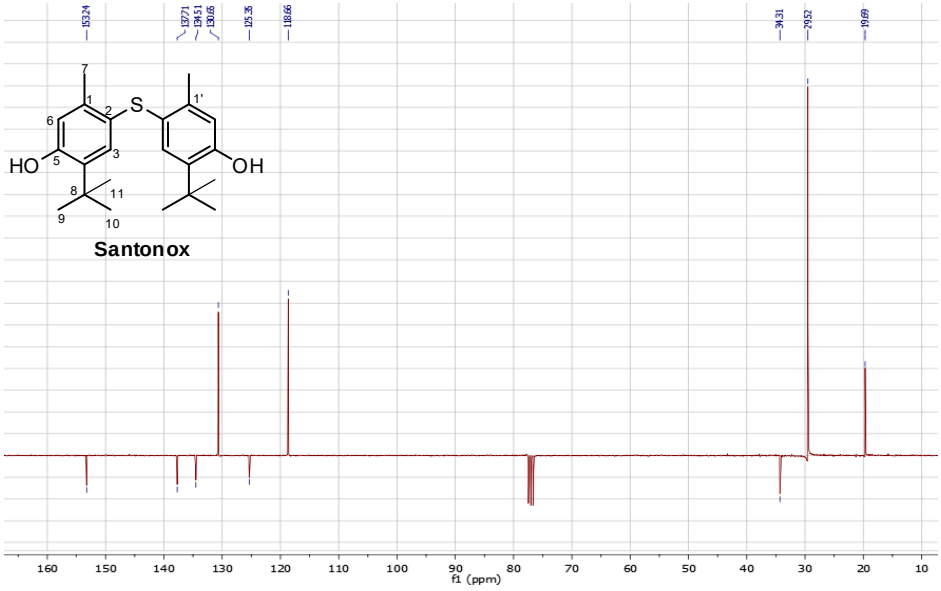
S5. 2D-NMR COSY spectrum of compound **1** in $CDCl_3$ (300 MHz)



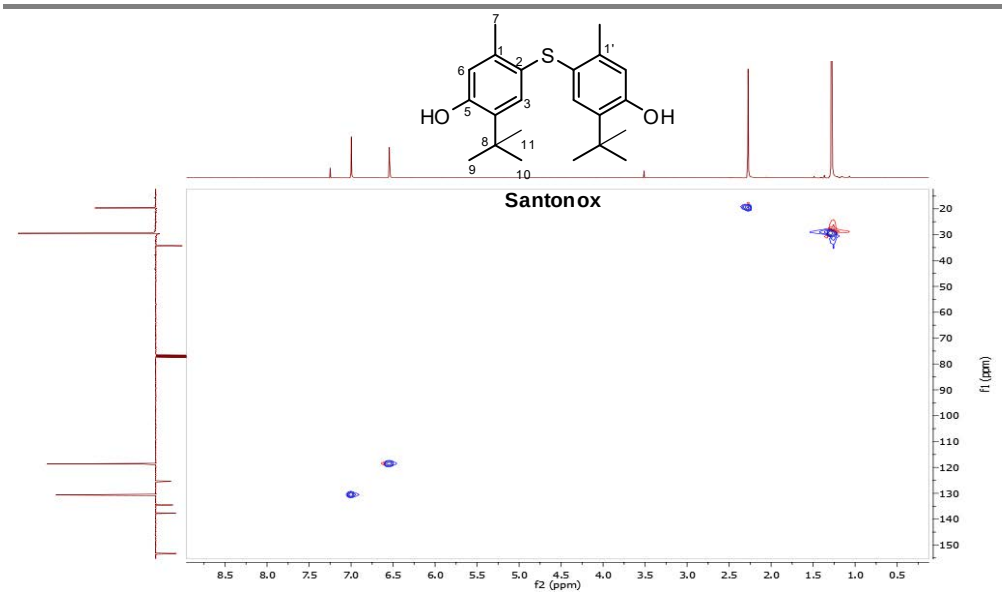
S6. 2D-NMR NOESY spectrum of compound **1** in $CDCl_3$ (300 MHz)



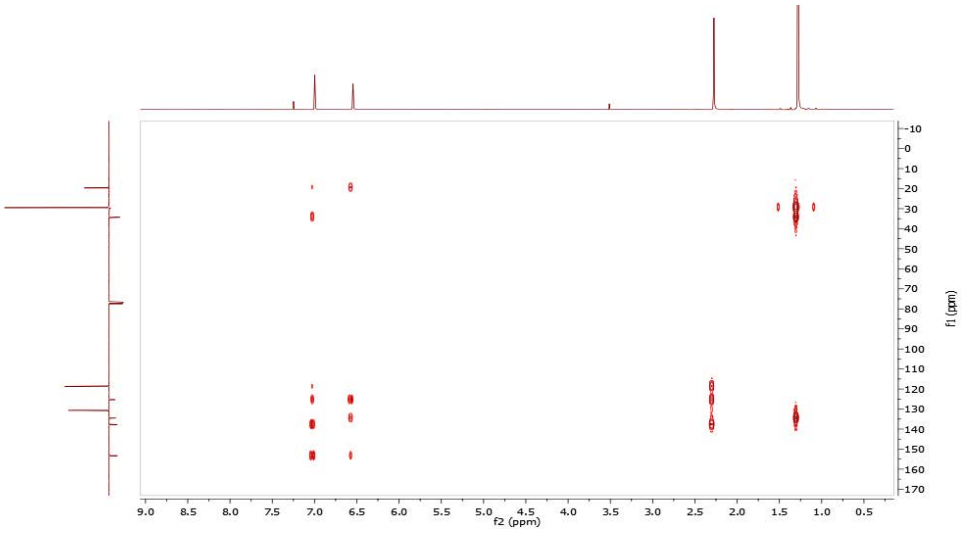
S7. ^{1}H- NMR spectrum of compound Santonox in $CDCl_3$ (300 MHz)



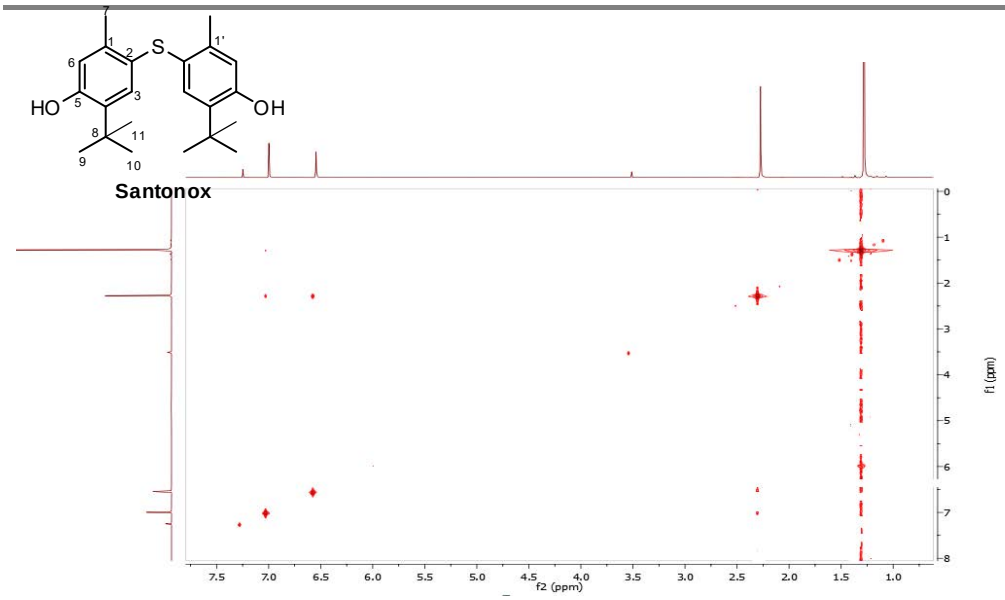
S8. Jmod-spectrum of compound Santonox in $CDCl_3$ (75 MHz)



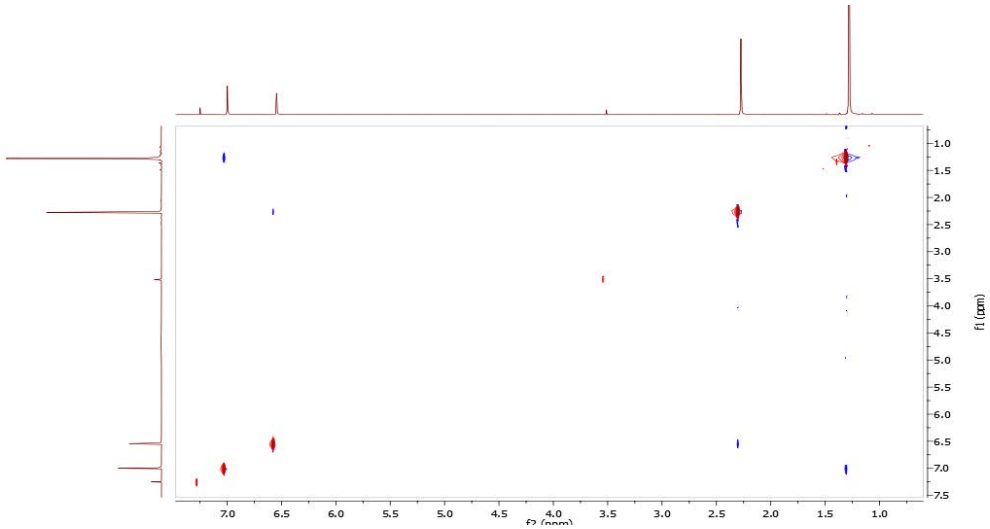
S9. 2D-NMR HSQC spectrum of Santonox in $CDCl_3$ (300 MHz)



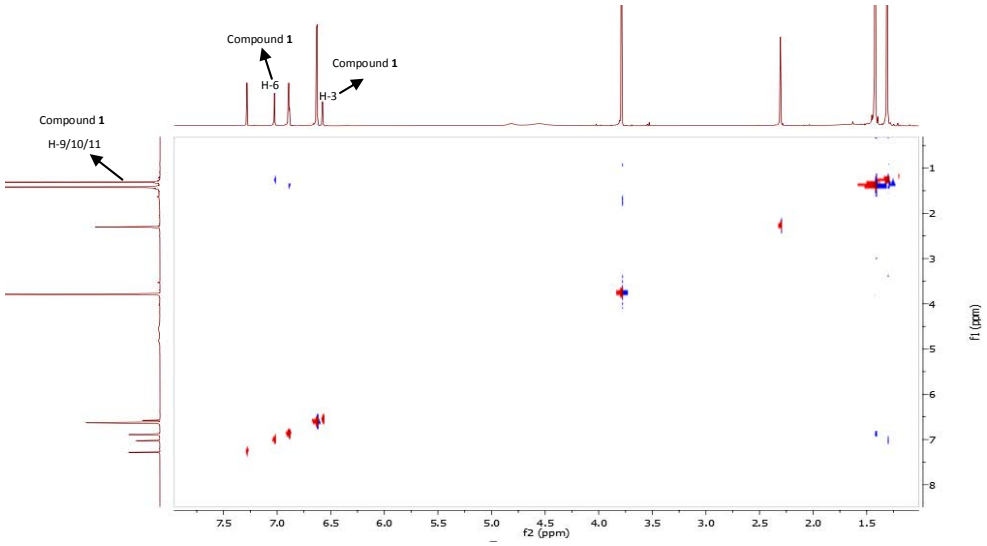
S10. 2D-NMR HMBC spectrum of Santonox in $CDCl_3$ (300 MHz)



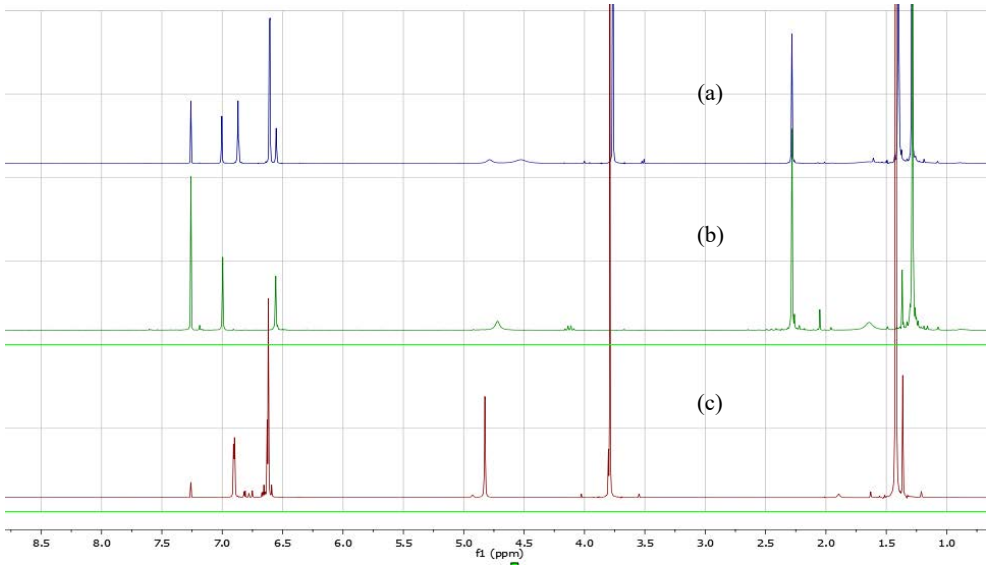
S11. 2D-NMR COSY spectrum of Santonox in $CDCl_3$ (300 MHz)



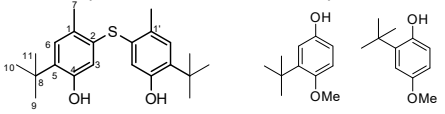
S12. 2D-NMR NOESY spectrum of Santonox in $CDCl_3$ (300 MHz)



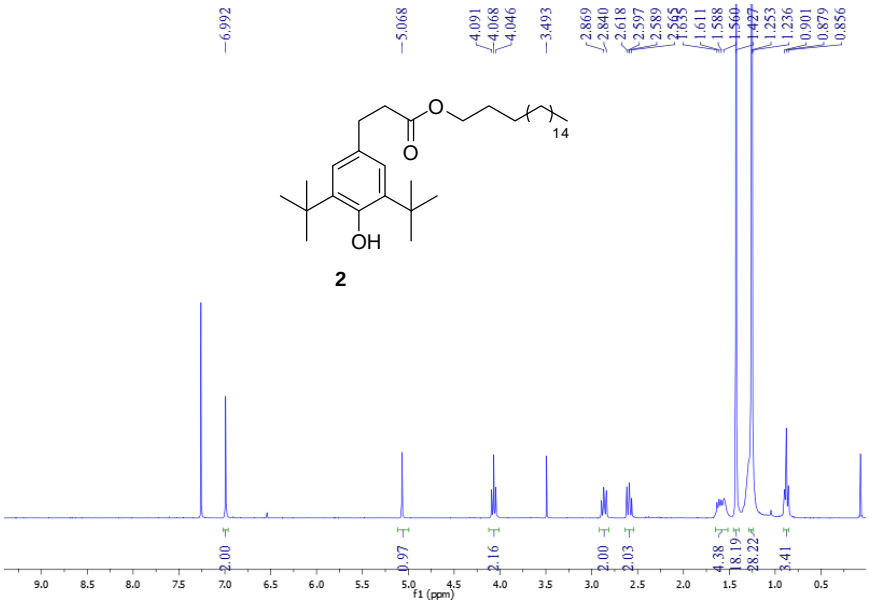
S13: NOESY spectrum of compound **1'** (mixture of BHA and compound **1**) from the extract of the culture supplemented with BHA in Erlenmeyer flask



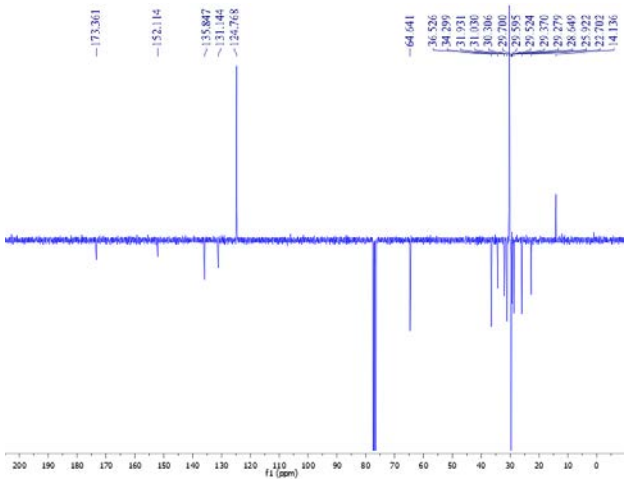
S14: ^{1}H – NMR spectra of compound **1'** (mixture of compound **1** and BHA) (a); compound **1** (b) and BHA (c)



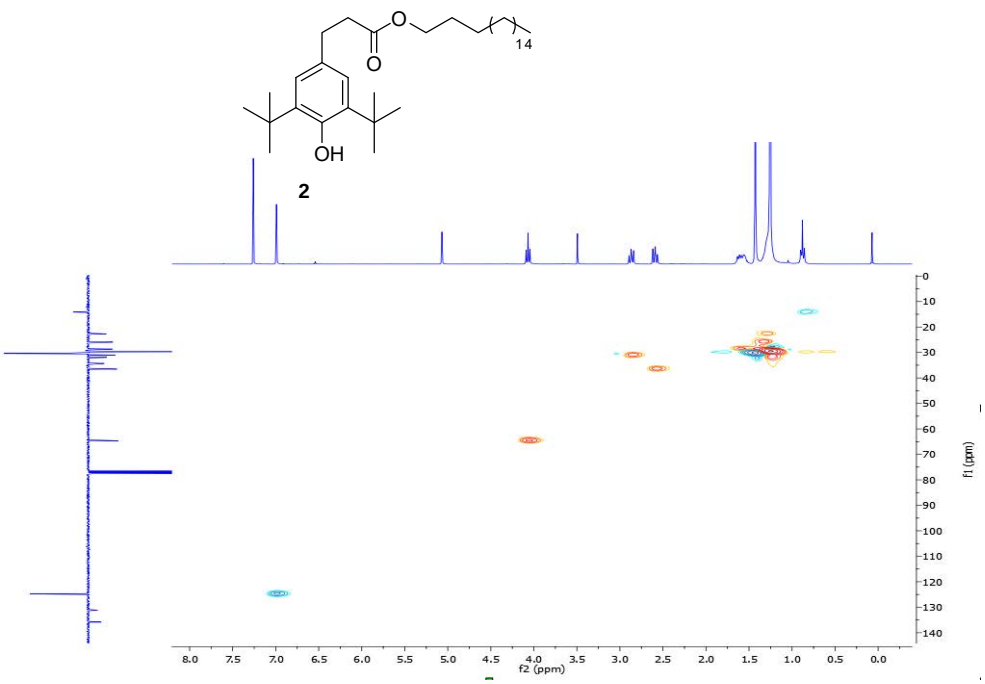
1 **BHA**



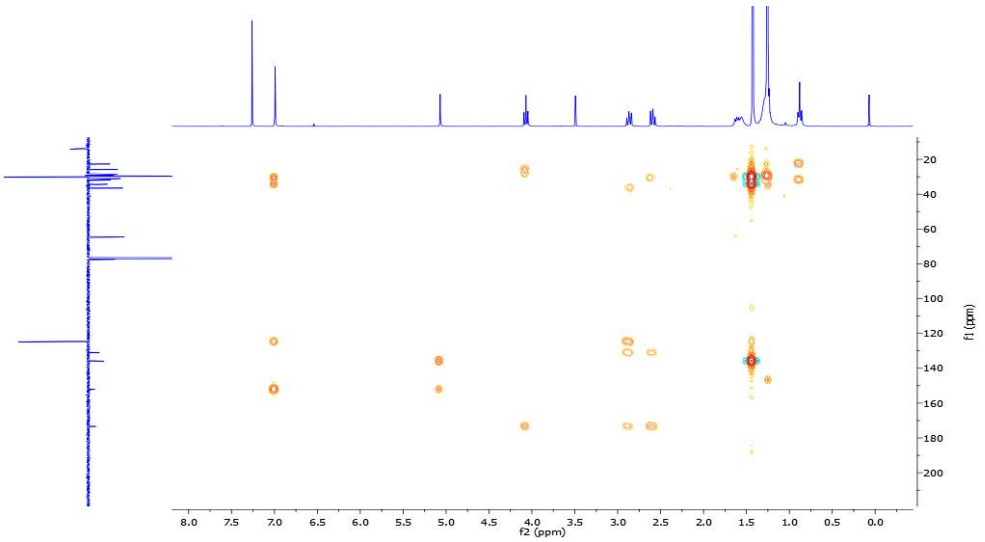
S15. ^{1}H-NMR spectrum of compound **2** in $CDCl_3$ (300 MHz)



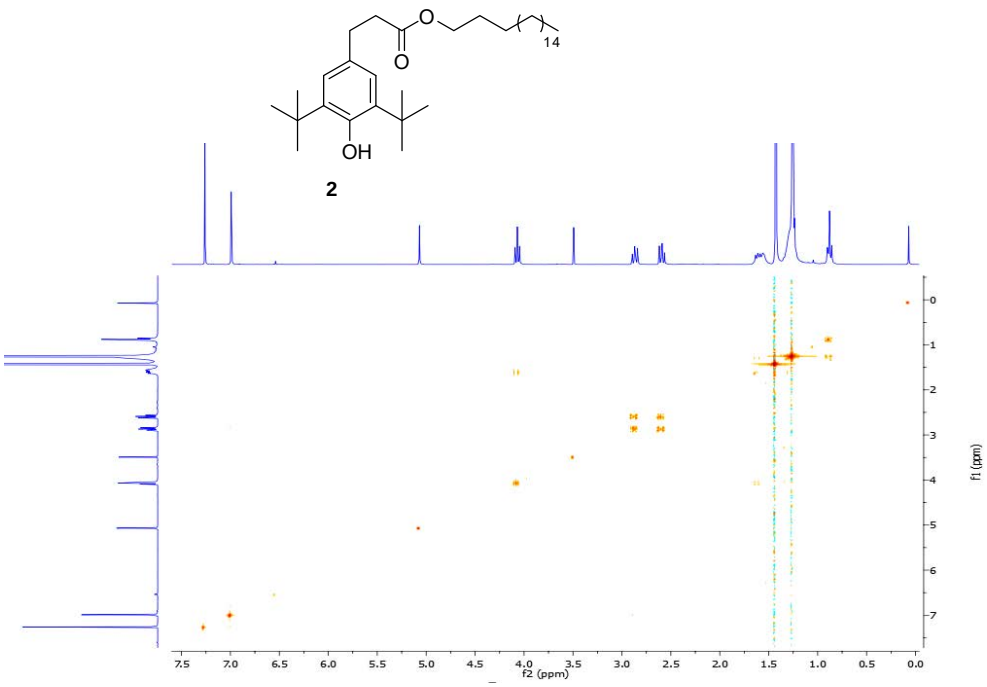
S16. Jmod-NMR spectra of compound **2** in $CDCl_3$ (75 MHz)



S17. 2D-NMR HSQCedit spectrum of compound **2** in $CDCl_3$ (300 MHz)



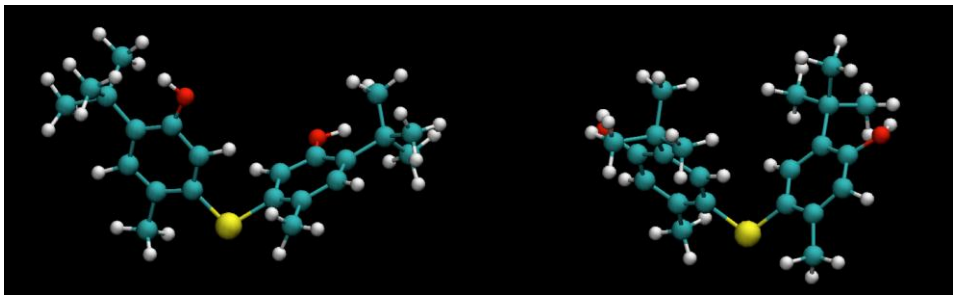
S18. 2D-NMR HMBC spectrum of compound **2** in $CDCl_3$ (300 MHz)



S19. 2D-NMR COSY of compound **2** in $CDCl_3$ (300 MHz)

Table 1: Predicted inter-protons distances on the base of molecular models corresponding to the major conformers of compound 1 and santonox extracted from the molecular dynamics simulation (see S20).

	Santonox	Santonox Isoform
H3-tBut	3,58	5,66
H6-tBut	5,83	3,87



S20: Major conformers for the compound **1** (0.34) and santonox (0.46) extracted from the molecular dynamics simulations performed in chloroform.

ANNEXE 2: Supporting information for the article of alkaloid compound

Paeniloxazine, a new alkaloid isolated from *Paenibacillus odorifer* a lichen-associated bacterium.

Le Thi Bach Nguyen, Isabelle Rouaud, Solenn Ferron, Sophie Tomasi*,[†]

Univ Rennes, CNRS, ISCR – UMR 6226, F-35000 Rennes, France

*Correspondence: sophie.tomasi@univ-rennes1.fr

[†] *Univ Rennes, CNRS, ISCR – UMR 6226, F-35000 Rennes, France*

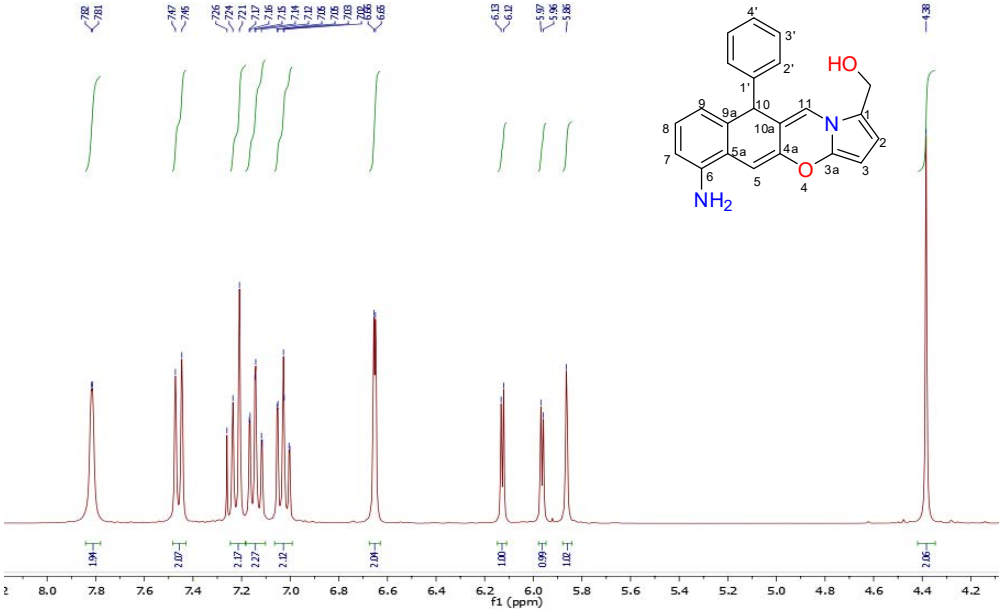


Figure1: ^{1}H-NMR spectrum of compound **1** in $CDCl_3$ (300 MHz)

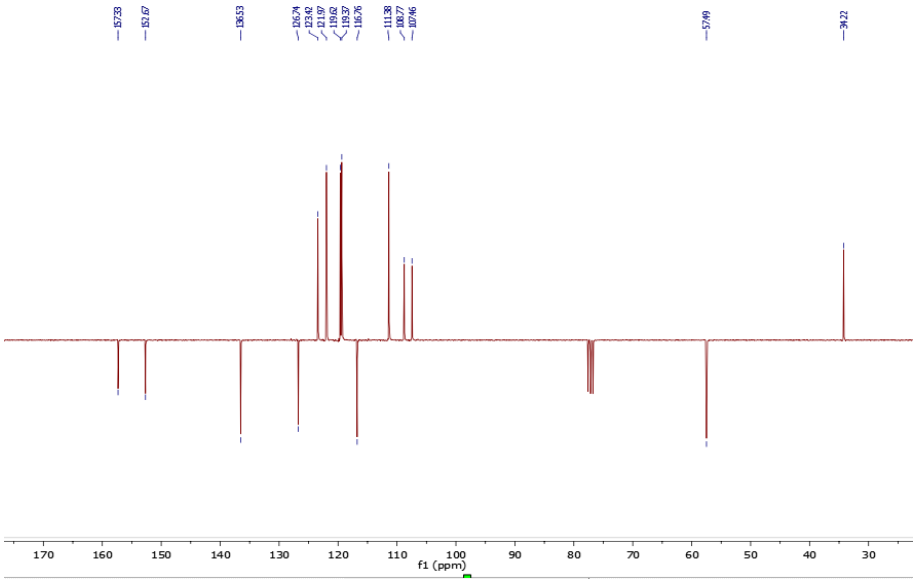


Figure 2: 1D-NMR Jmod spectrum of compound **1** in $CDCl_3$ (75 MHz)

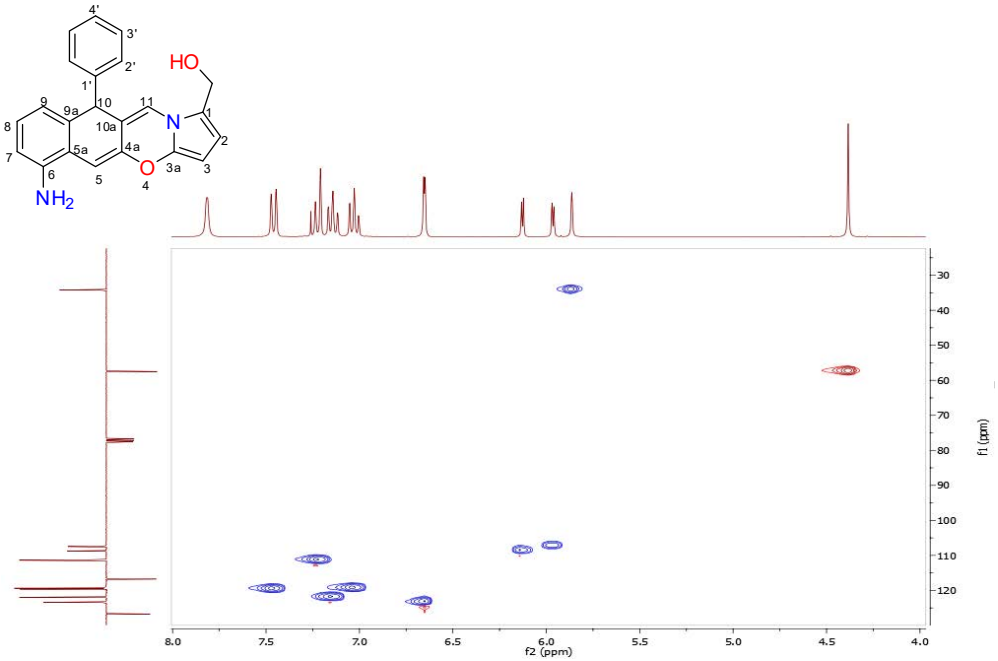


Figure 3: 2D-NMR HSQCedit spectrum of compound **1** in $CDCl_3$ (300 MHz)

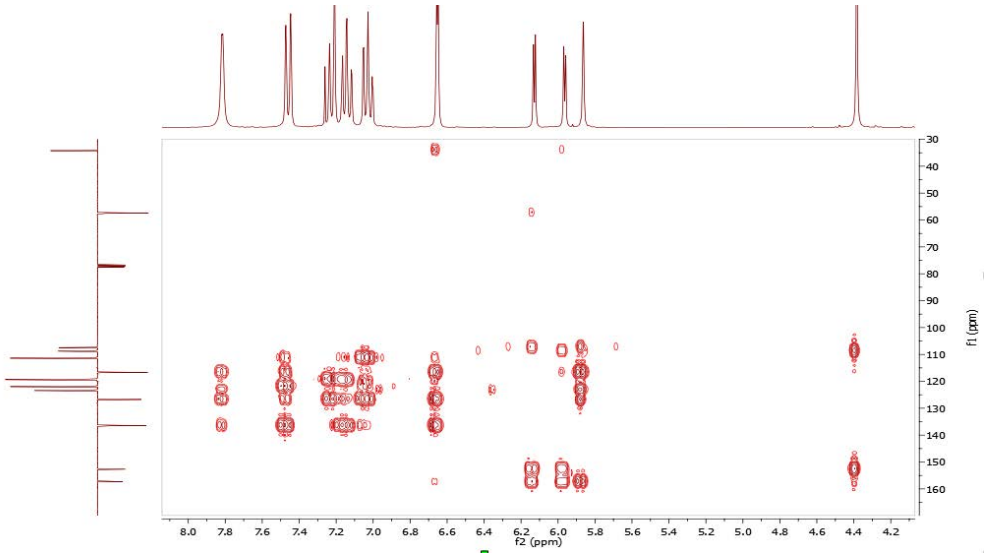


Figure 4: 2D-NMR HMBC spectrum of compound **1** in $CDCl_3$ (300MHz)

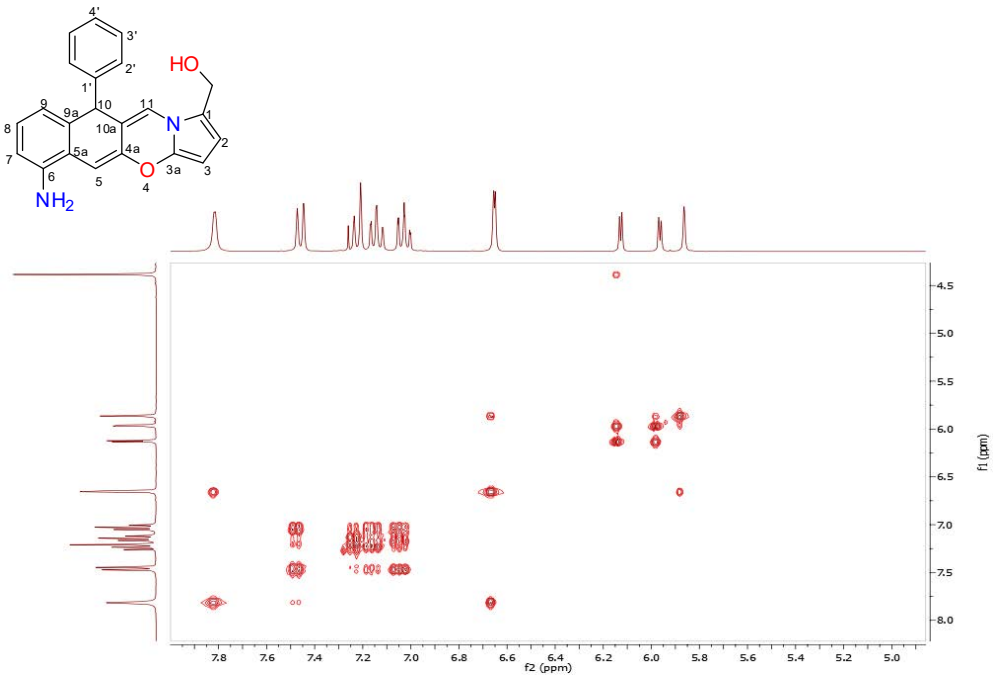


Figure 5: 2D-NMR COSY spectrum of compound **1** in $CDCl_3$ (300MHz)

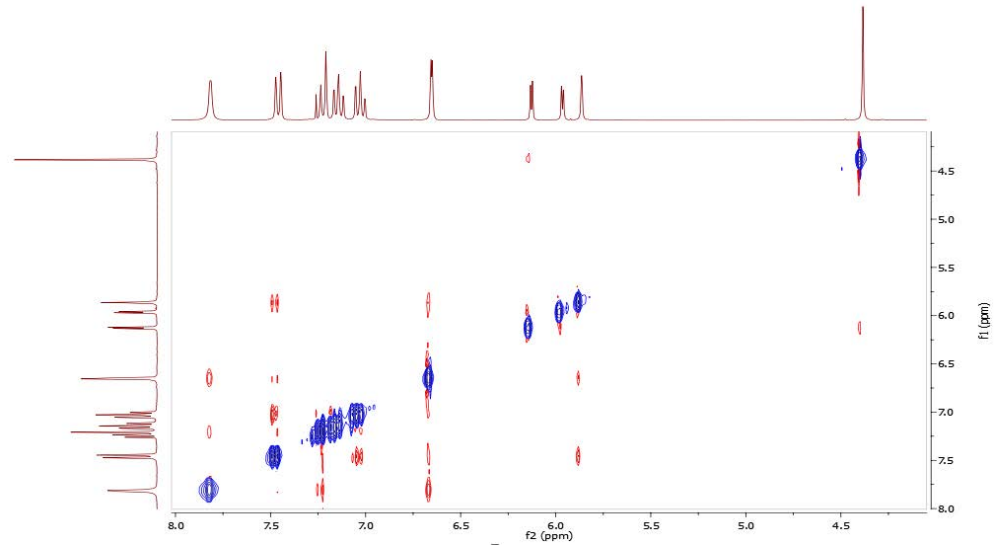


Figure 6: 2D-NMR NOESY spectrum of compound **1** in $CDCl_3$ (300MHz)

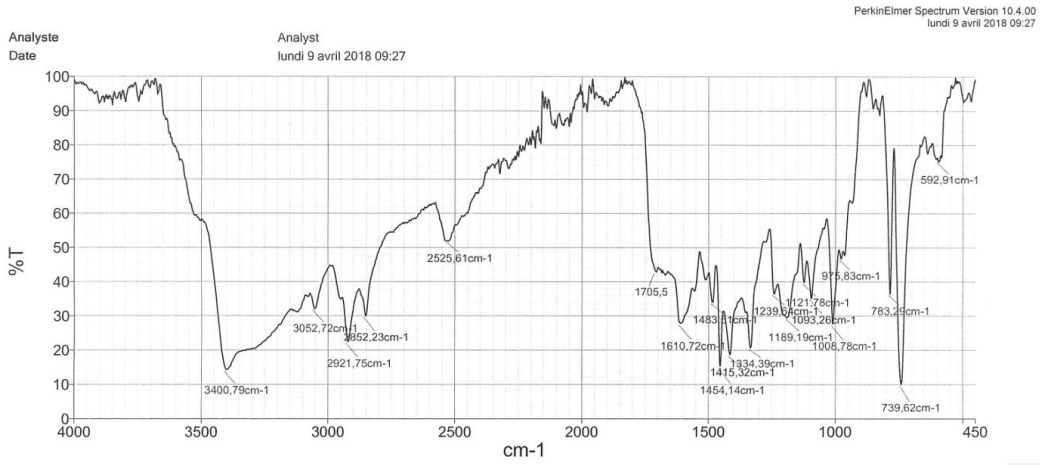


Figure 7: FT-IR spectrum of compound **1**

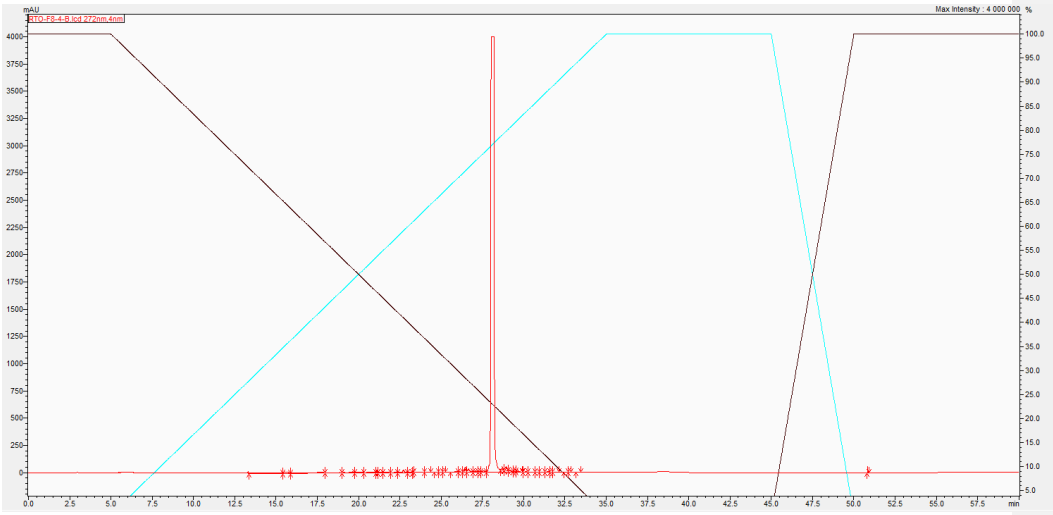


Figure 8: HPLC chromatogram of compound **1** at 272 nm using Prevail C18 column, elution solvent as gradient of H_2O/Acetonitrile; flow rate 0.8 mL/min.

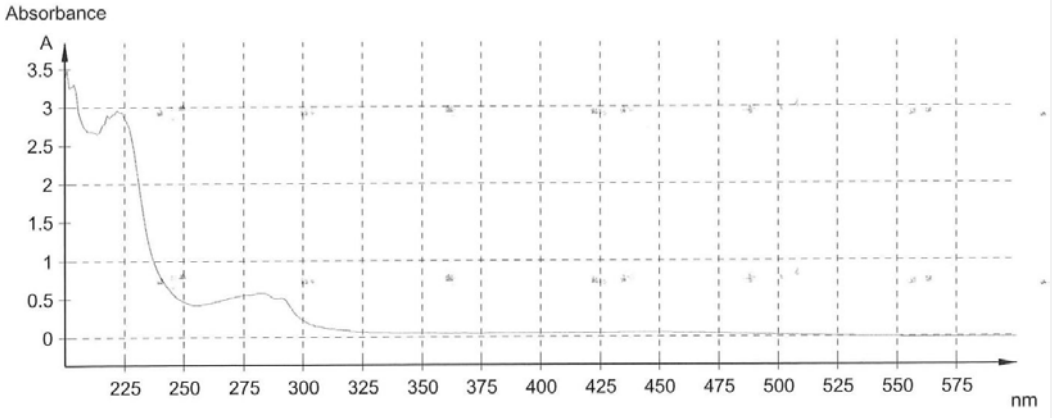


Figure 9: UV spectrum of compound **1**

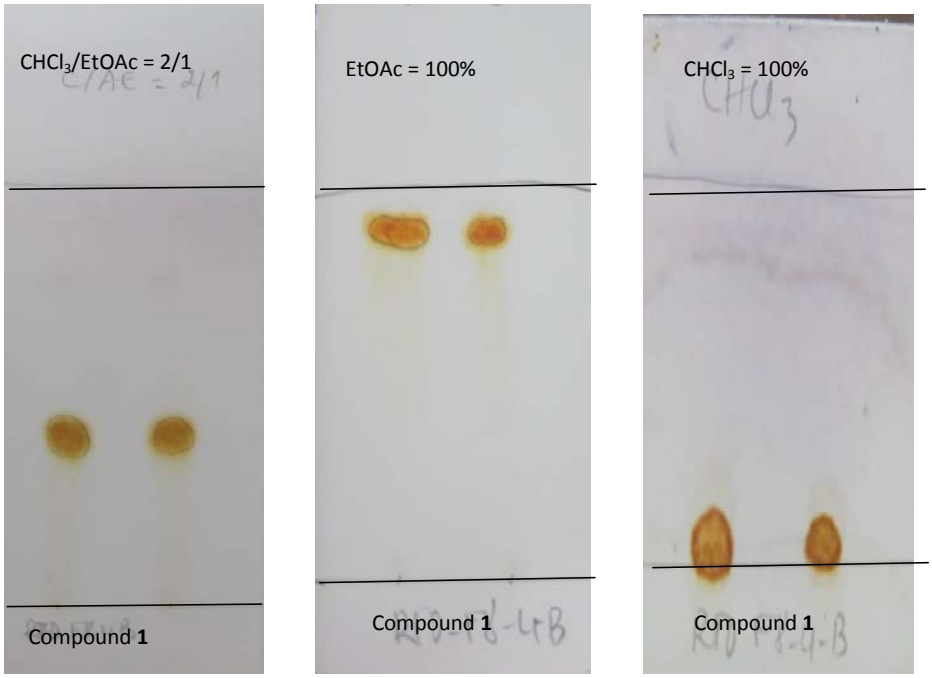
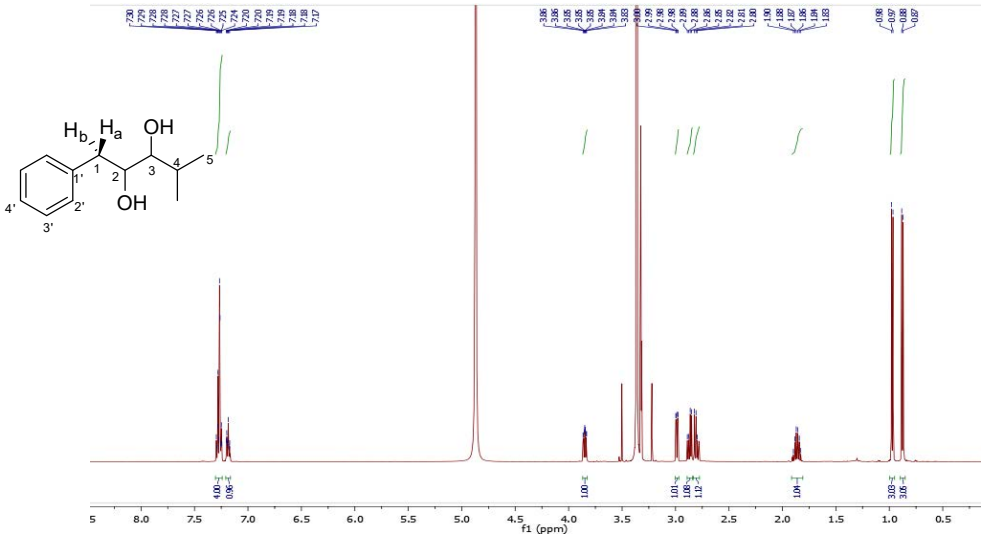


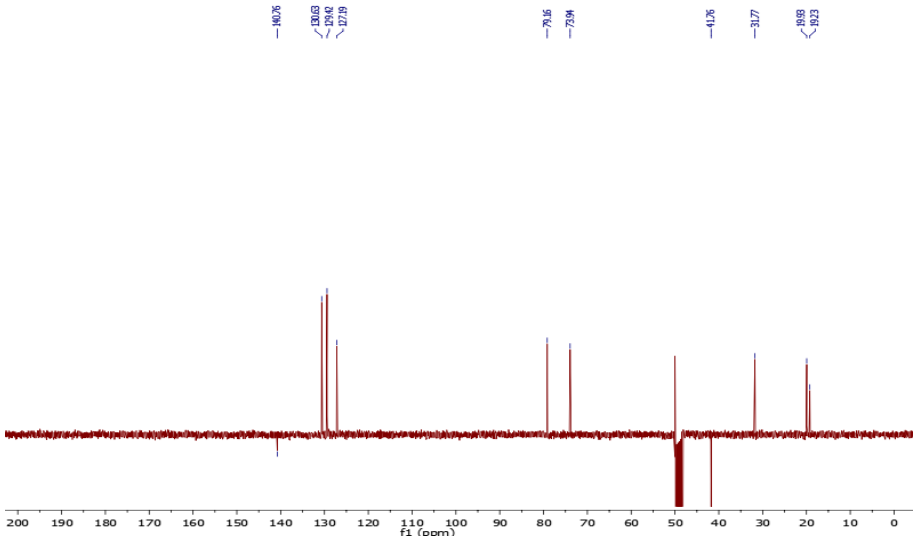
Figure 10: Thin layer chromatography (TLC) of compound **1,** visualization by anisaldehyde (ANS), R_f = 0.45; 0.72 and 0.05 in elution solvents of $CHCl_3$/EtOAc (2/1); EtOAc (100%) and $CHCl_3$ (100%), respectively.

ANNEXE 3: NMR spectra of the metabolites from *P. odorifer*

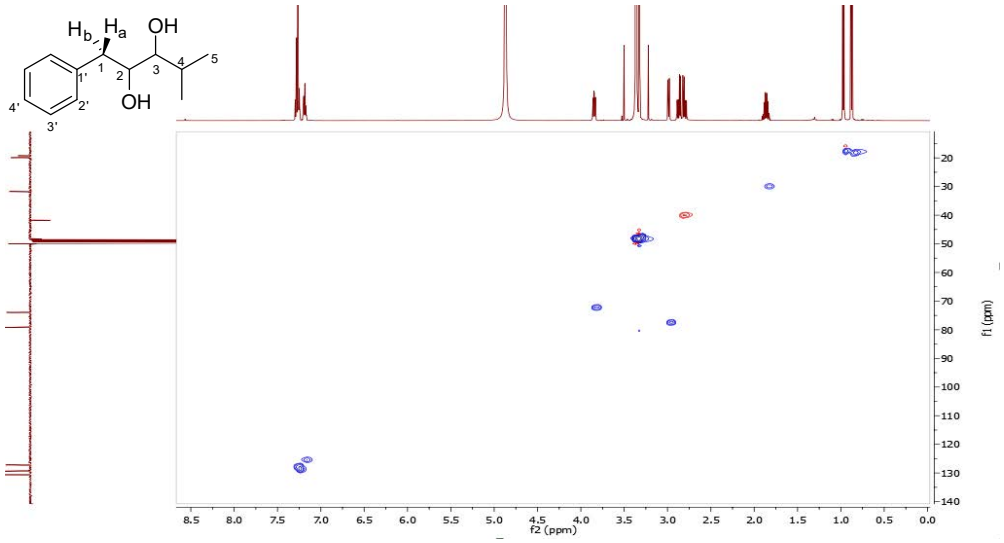
NMR spectra (500 MHz) of compound **1** (4-methyl-1-phenylpentane-2,3-diol) in CD_3OD



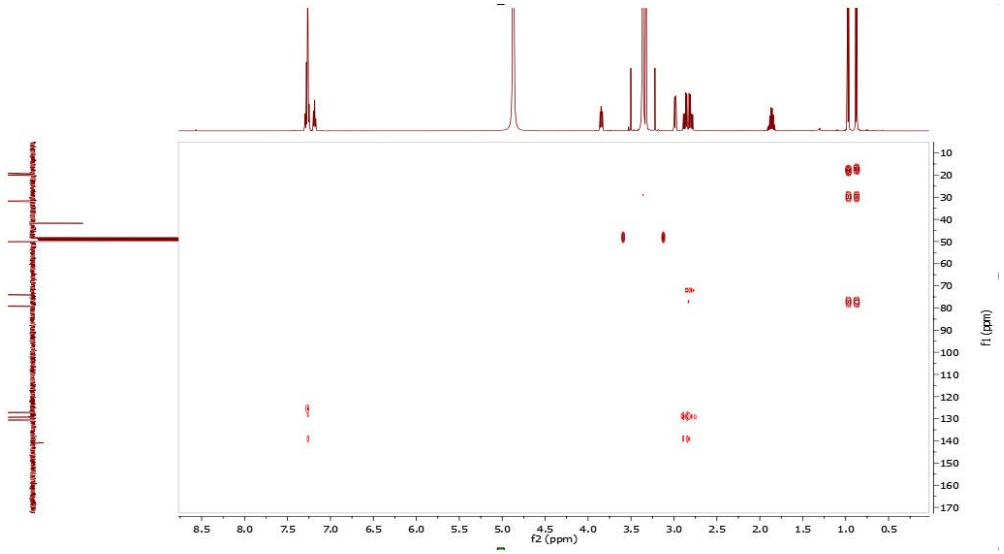
^{1}H-NMR spectrum of compound **1**



^{13}C-NMR spectrum of compound **1**

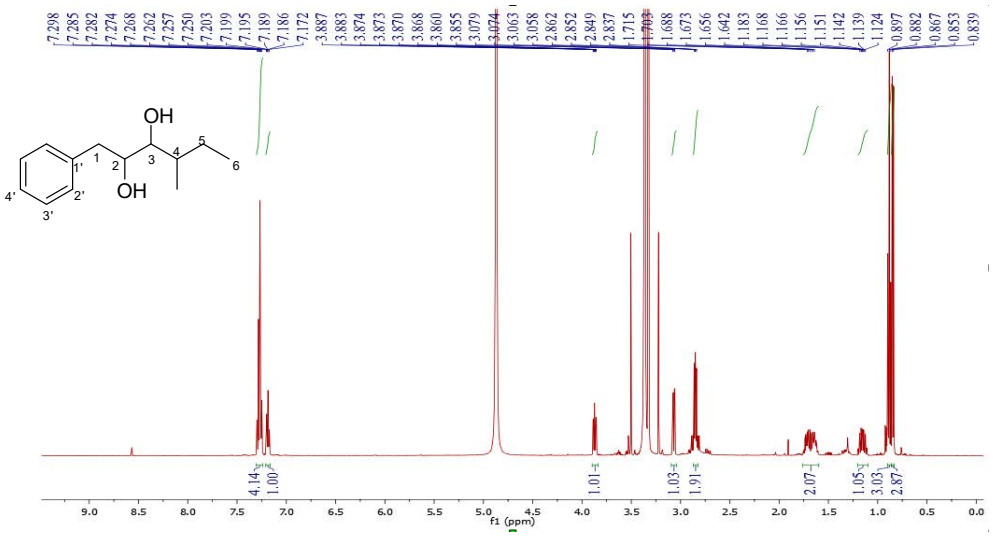


HSQCedit spectrum of compound **1**

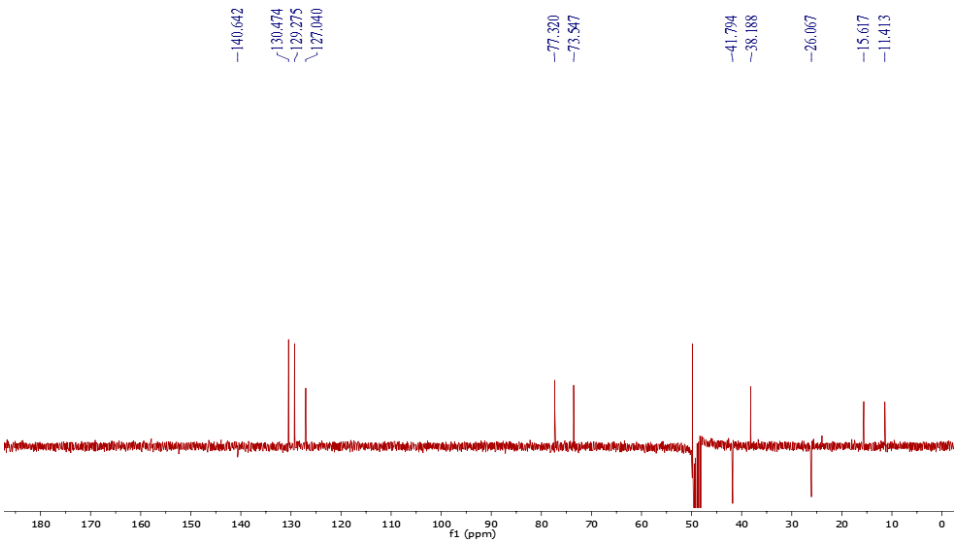


HMBC spectrum of compound **1**

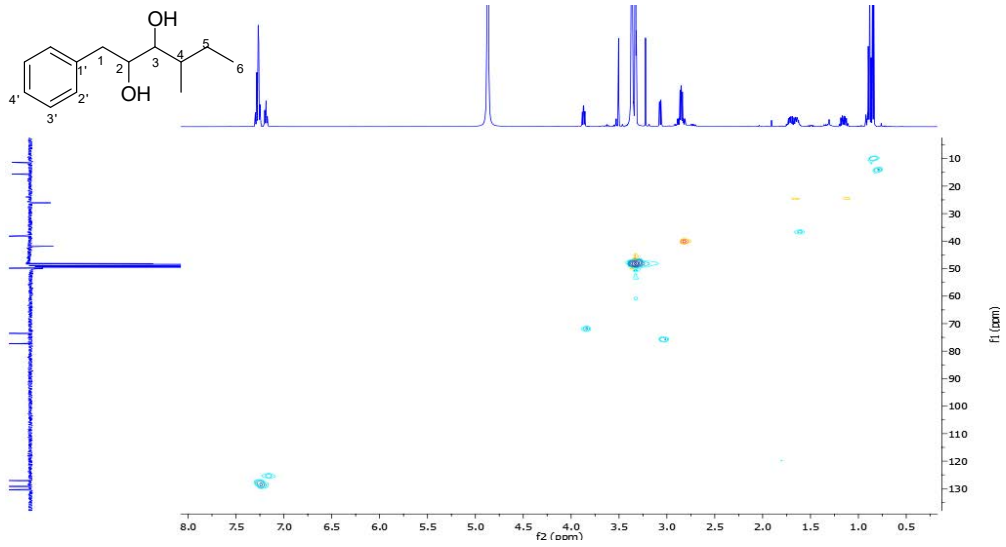
NMR spectra (500 MHz) of compound 2 (4-methyl-1-phenylhexane-2,3-diol) in CD_3OD



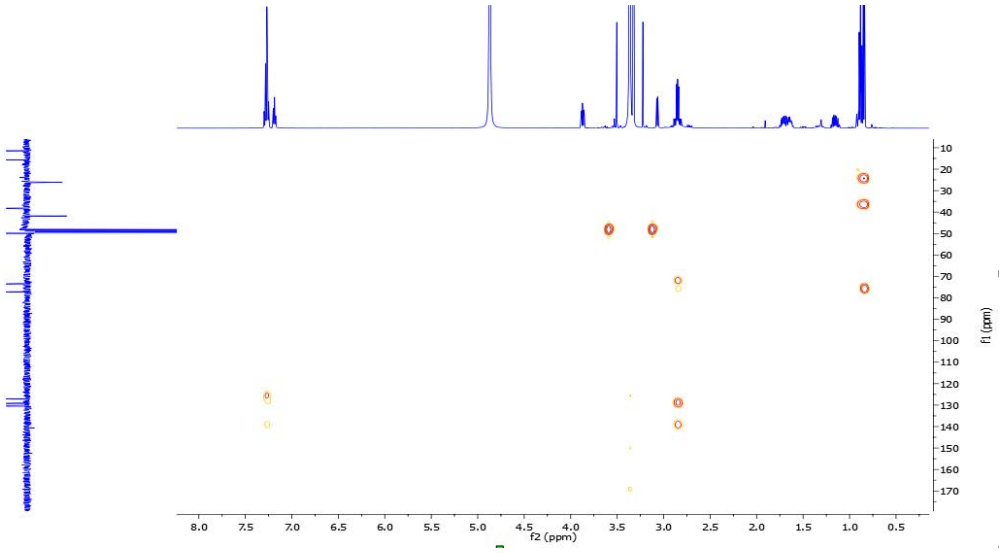
^{1}H-NMR spectrum of compound **2**



Jmod spectrum of compound **2**

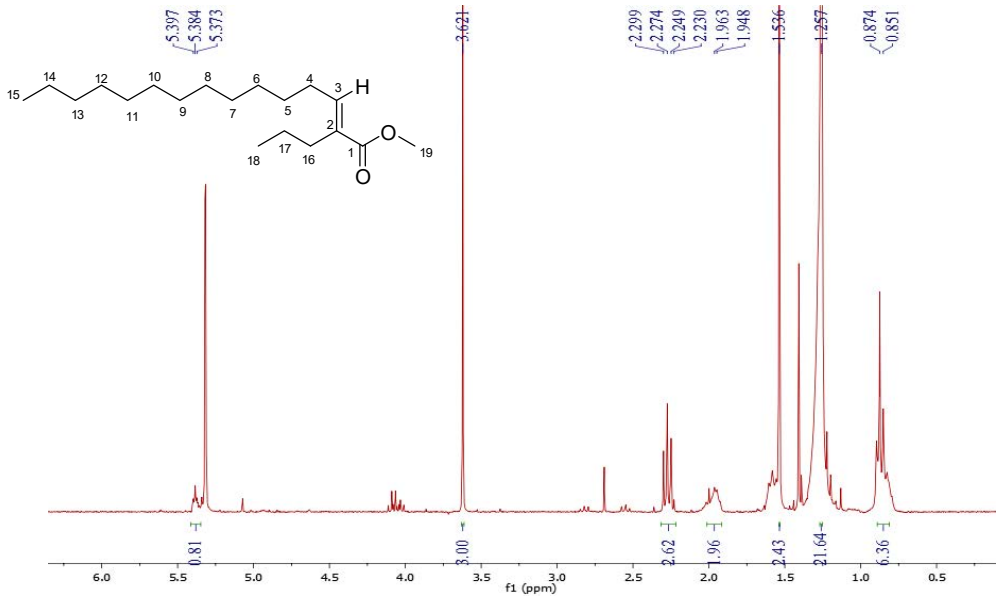


HSQCedit spectrum of compound **2**

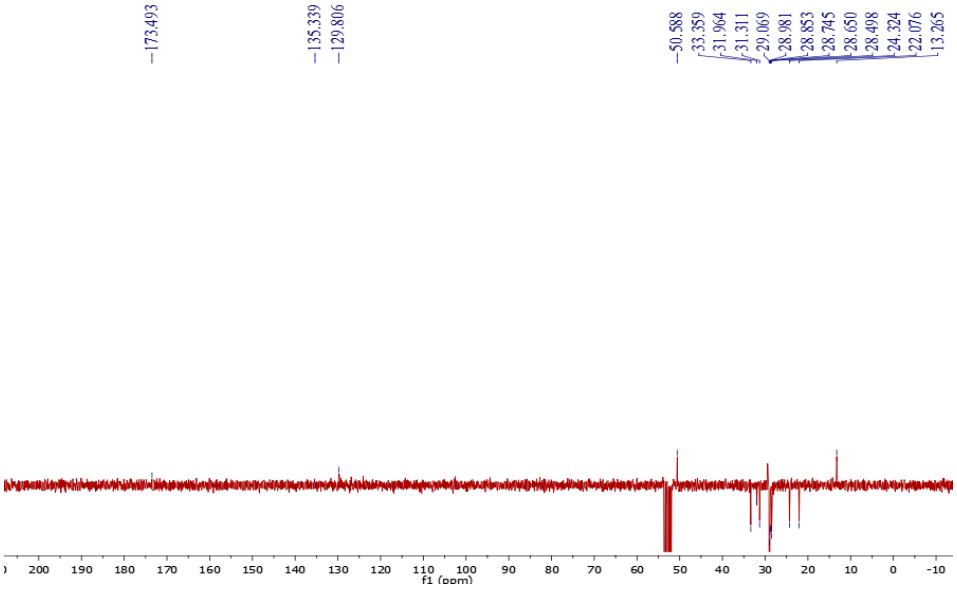


HMBC spectrum of compound **2**

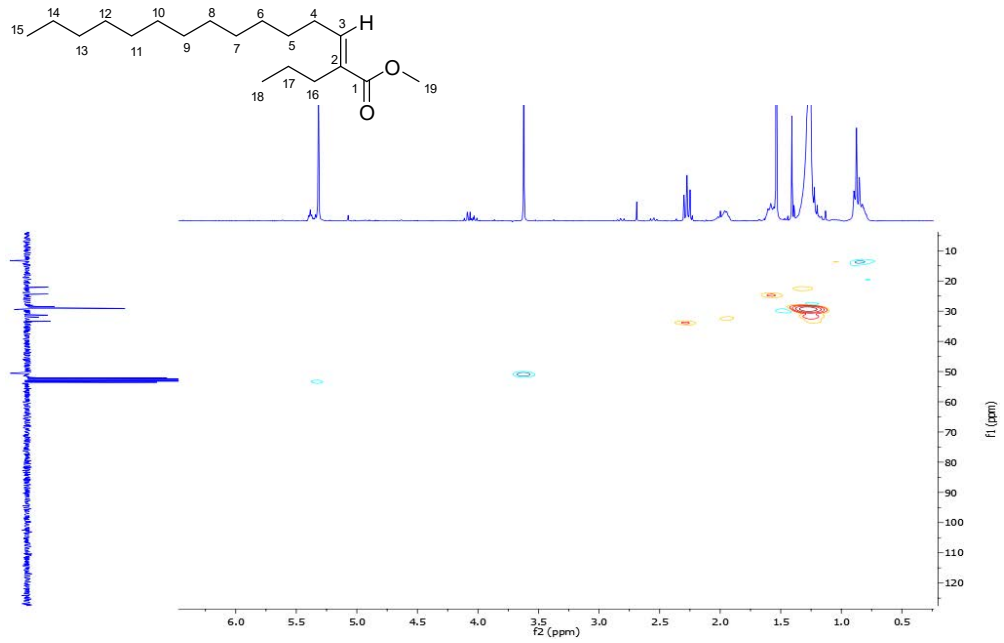
NMR spectra (300 MHz) of compound **3 (**Methyl 2-propylpentadec-2-enoate) in CD_2Cl_2



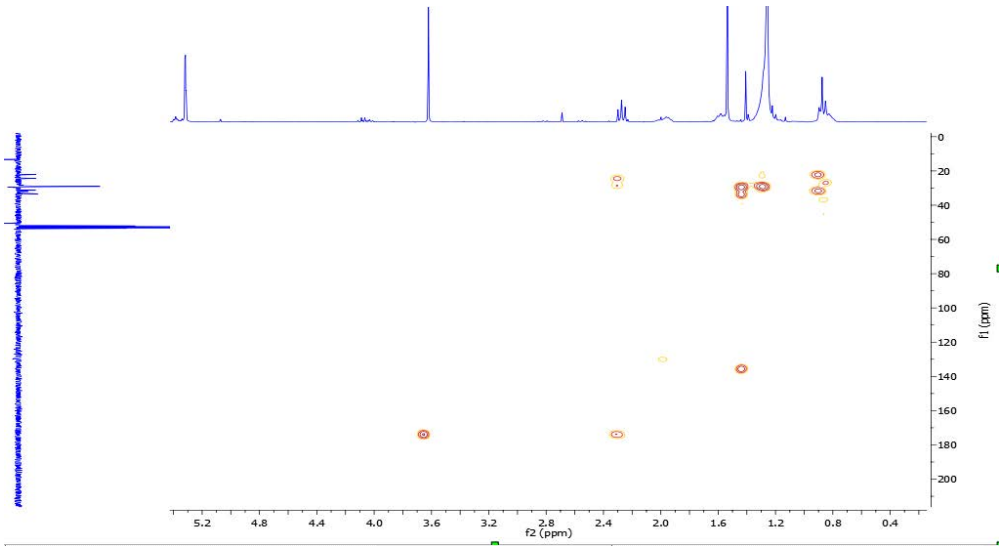
[1] H-NMR spectrum of compound **3**



Jmod spectrum of compound **3**

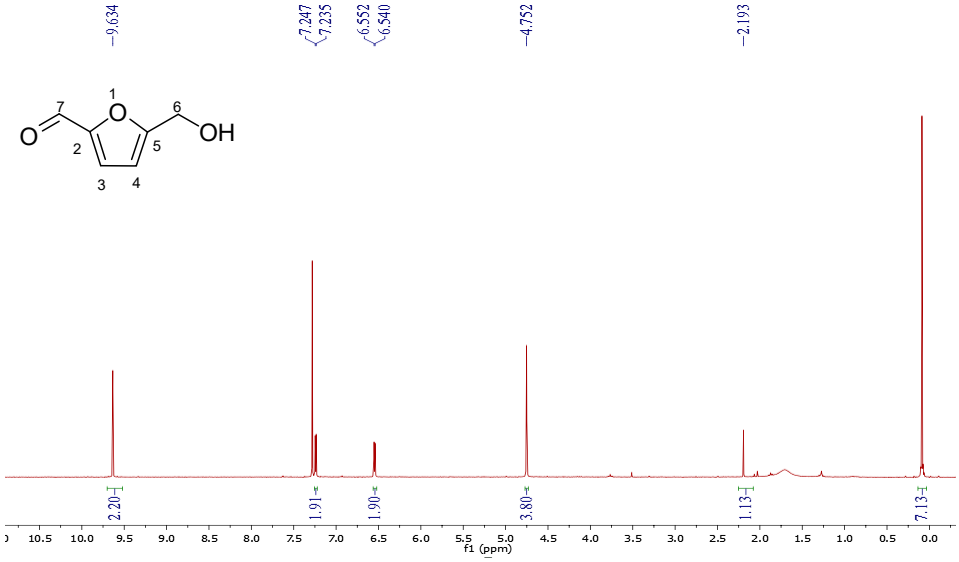


HSQCedit spectrum of compound **3**

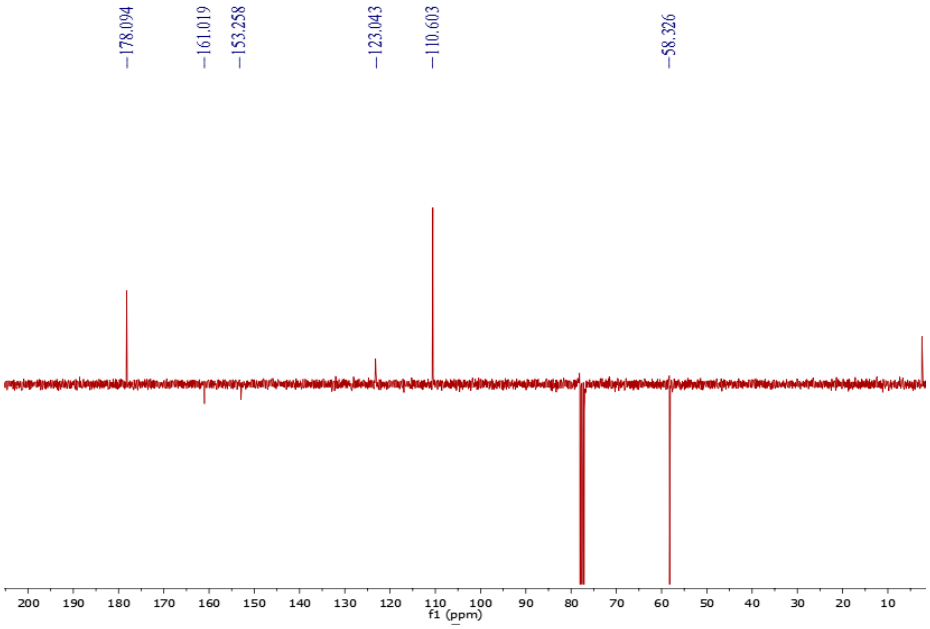


HMBC spectrum of compound **3**

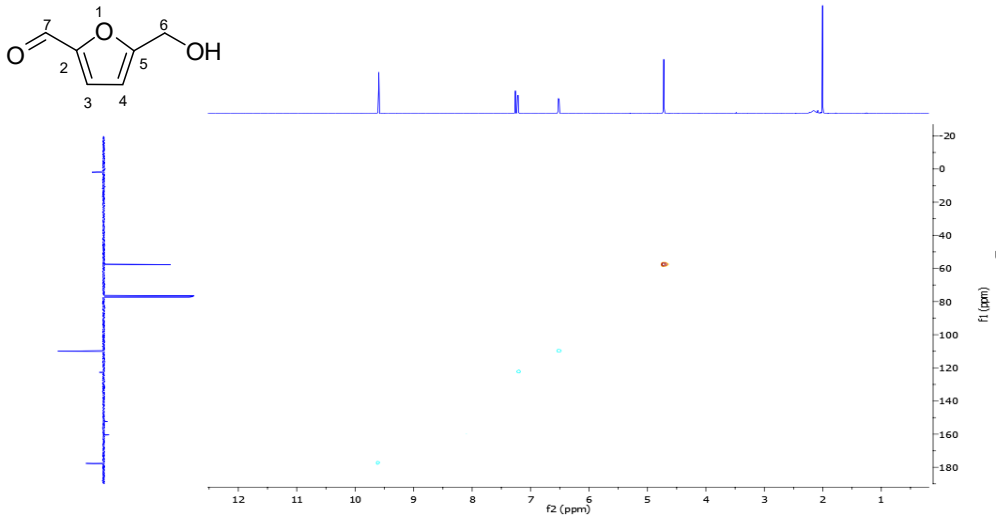
NMR spectra (300 MHz) of compound **4** (5-(hydroxymethyl)furan-2-carbaldehyde) in $CDCl_3$



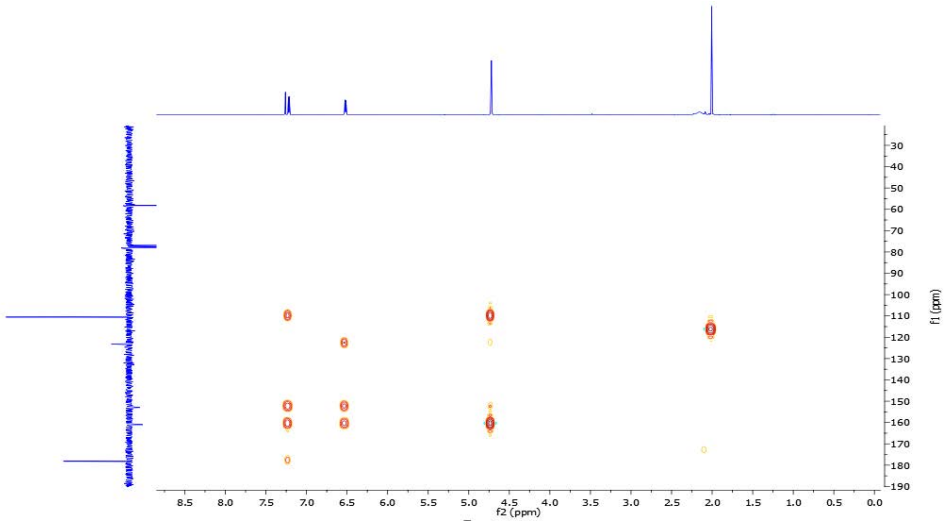
^{1}H-NMR spectrum of compound **4**



^{13}C –NMR spectrum of compound **4**

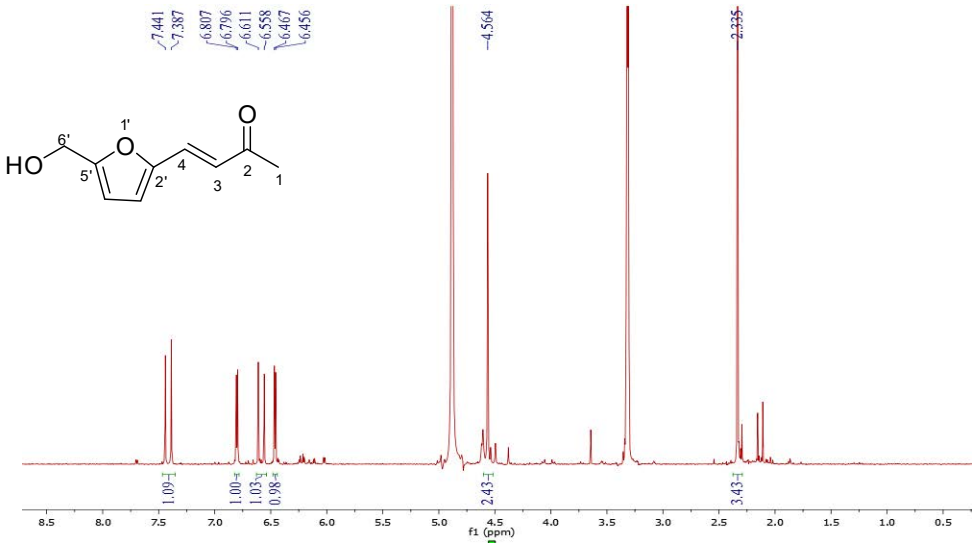


HSQCedit spectrum of compound **4**

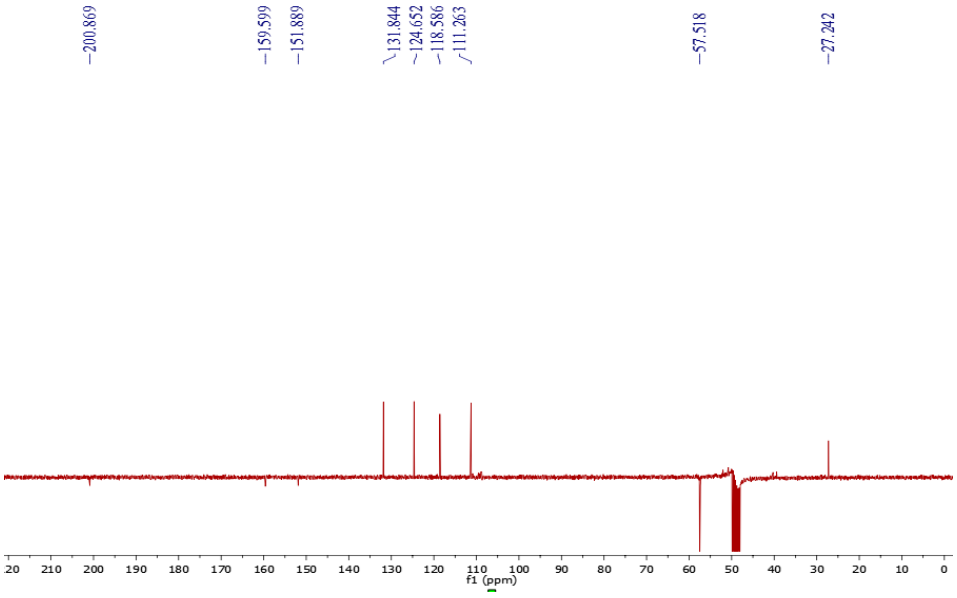


HMBC spectrum of compound **4**

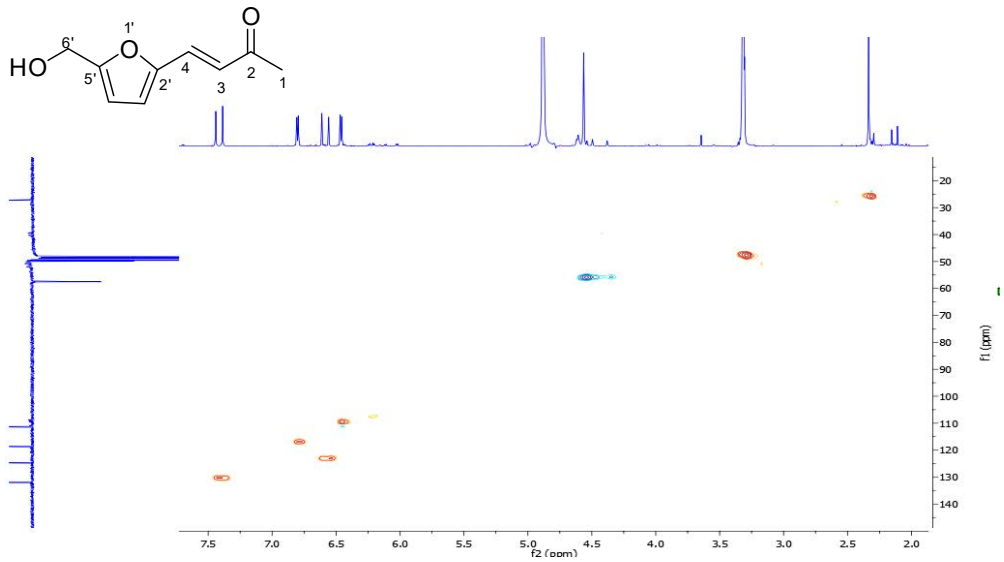
NMR spectra (300 MHz) of compound **5** (4-(5-(hydroxymethyl)furan-2-yl)but-3-en-2-one) in CD_3OD



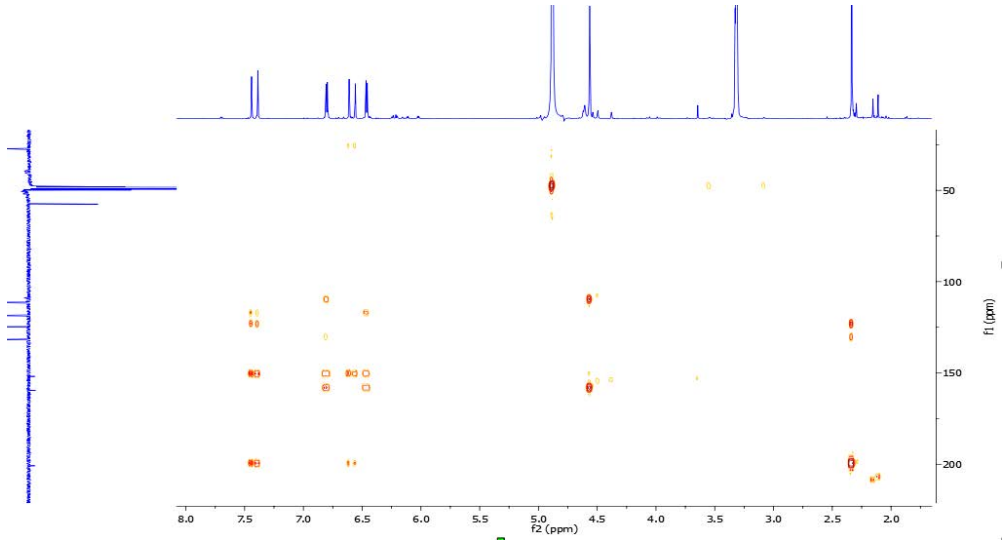
^{1}H-NMR spectrum of compound **5**



Jmod spectrum of compound **5**

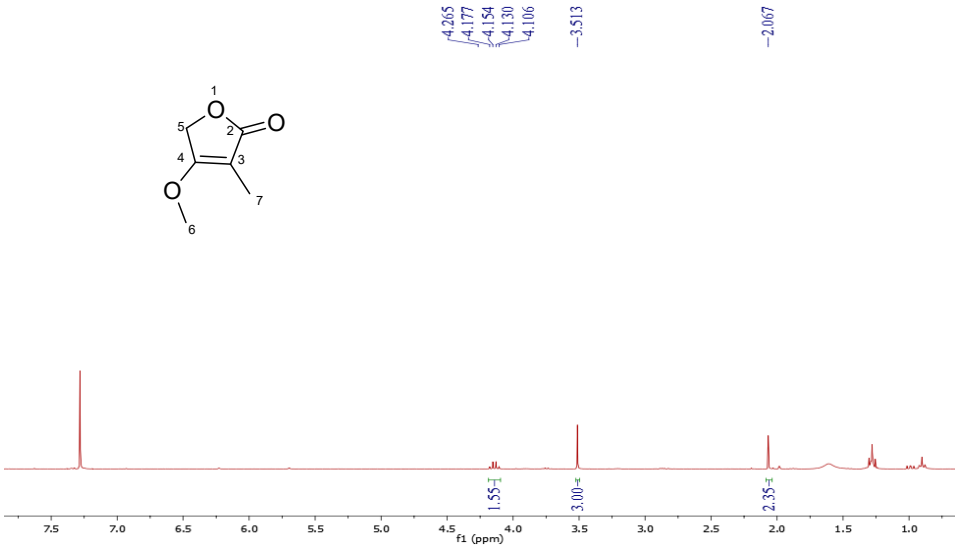


HSQCedit spectrum of compound **5**

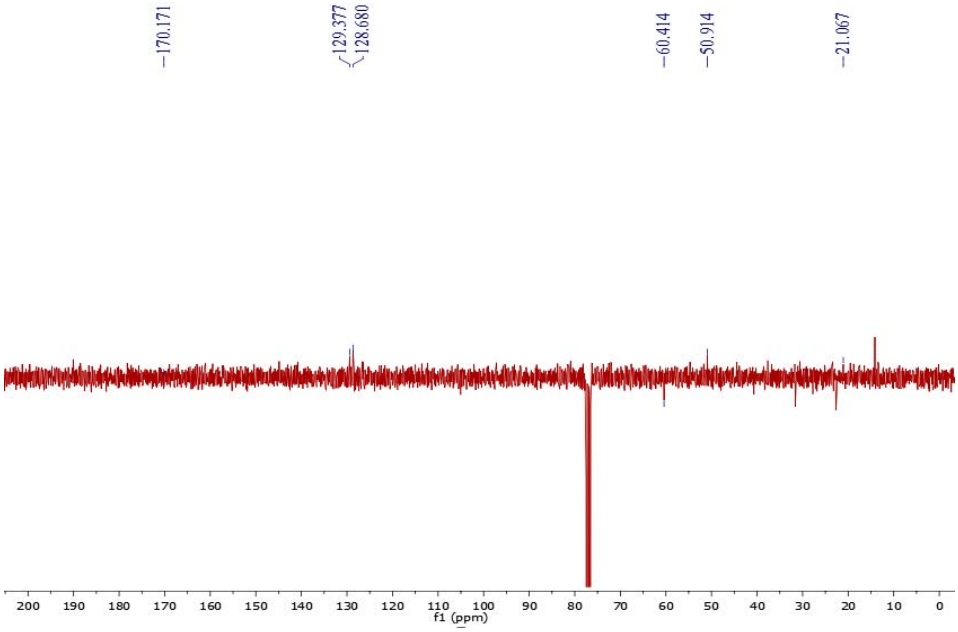


HMBC spectrum of compound **5**

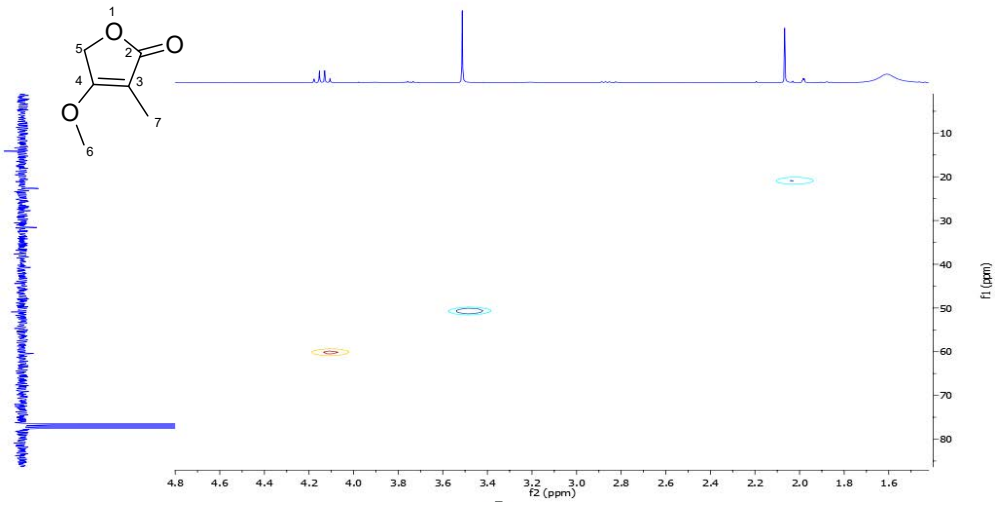
NMR spectra (300 MHz) of compound **6** (4-methoxy-3-methylfuran-2(5H)-one) in $CDCl_3$



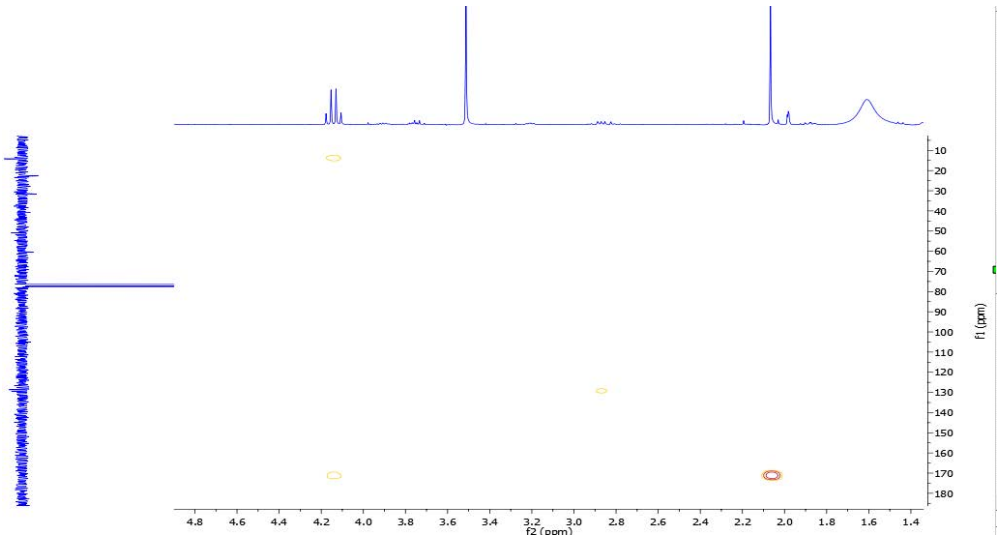
^{1}H-NMR spectrum compound **6**



Jmod spectrum compound **6**

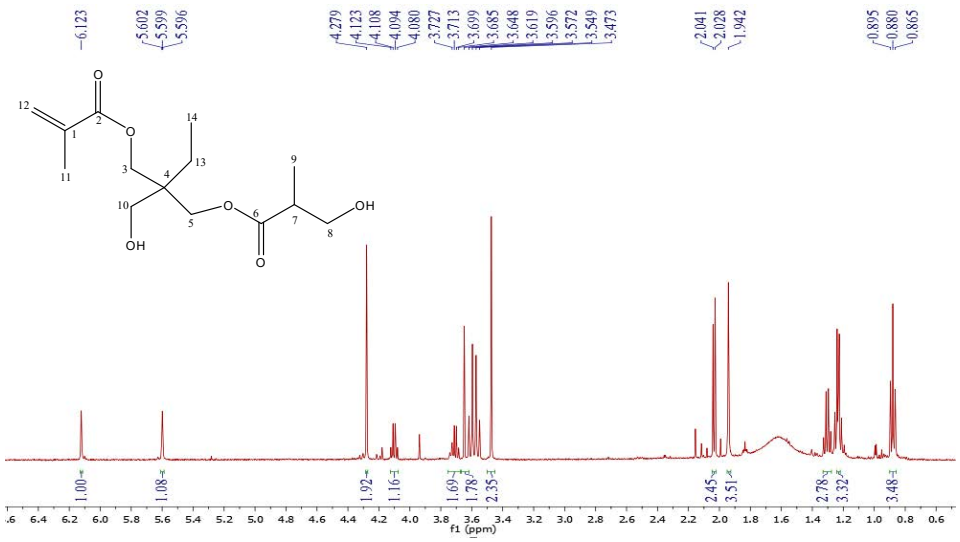


HSQCedit spectrum of compound **6**

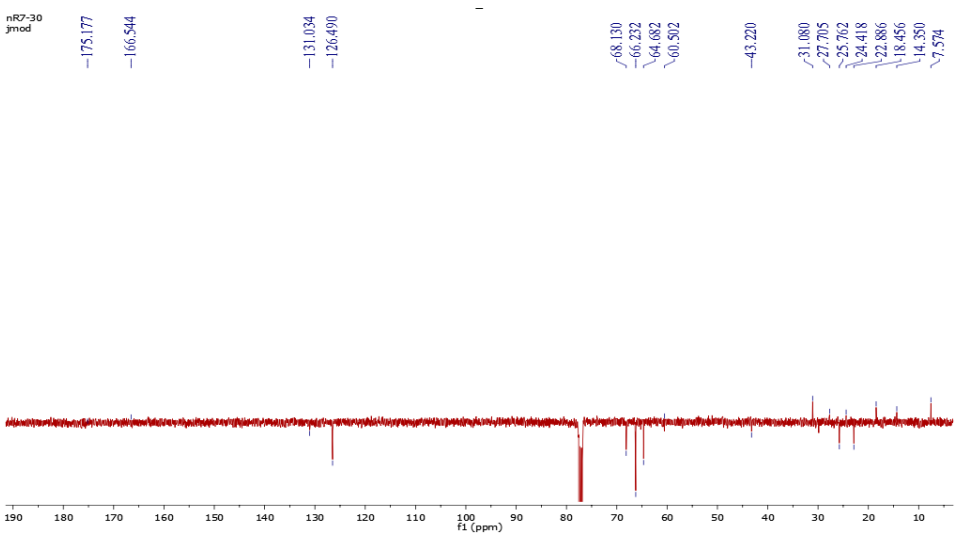


HMBC spectrum of compound **6**

NMR spectra (500 MHz) of compound **7** (2-((3-hydroxy-2-methylpropanoyloxy)methyl)-2-(hydroxymethyl)butyl methacrylate) in $CDCl_3$

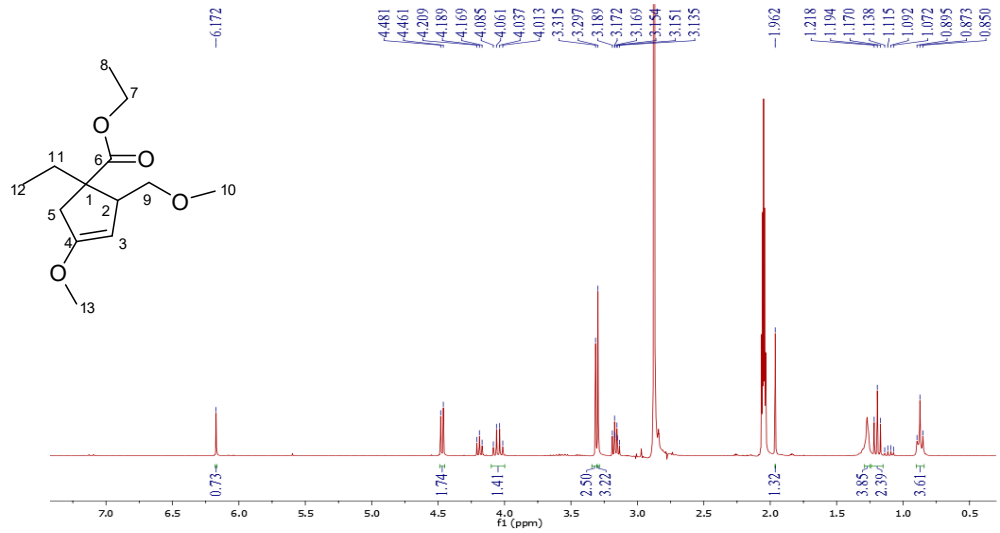


^{1}H-NMR spectrum compound **7**

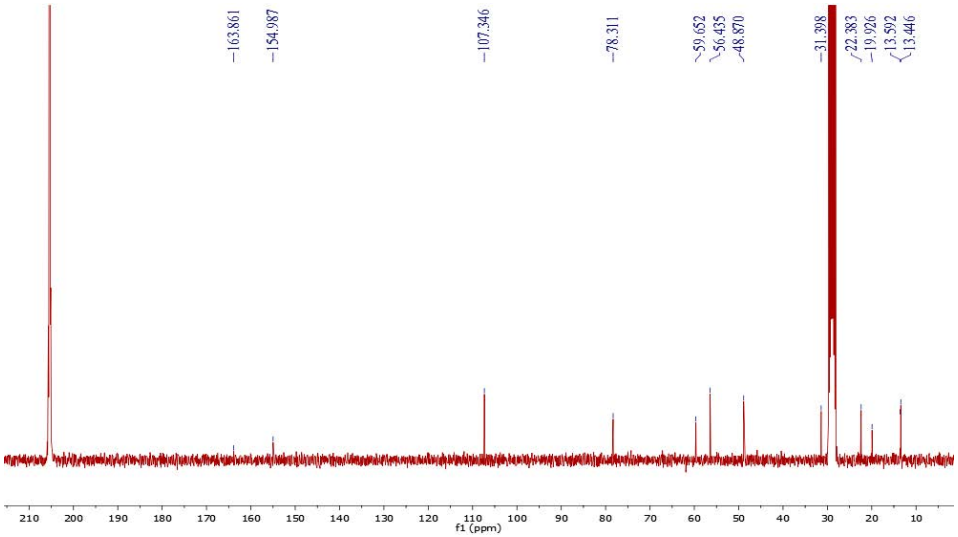


Jmod (125 MHz) spectrum of compound **7**

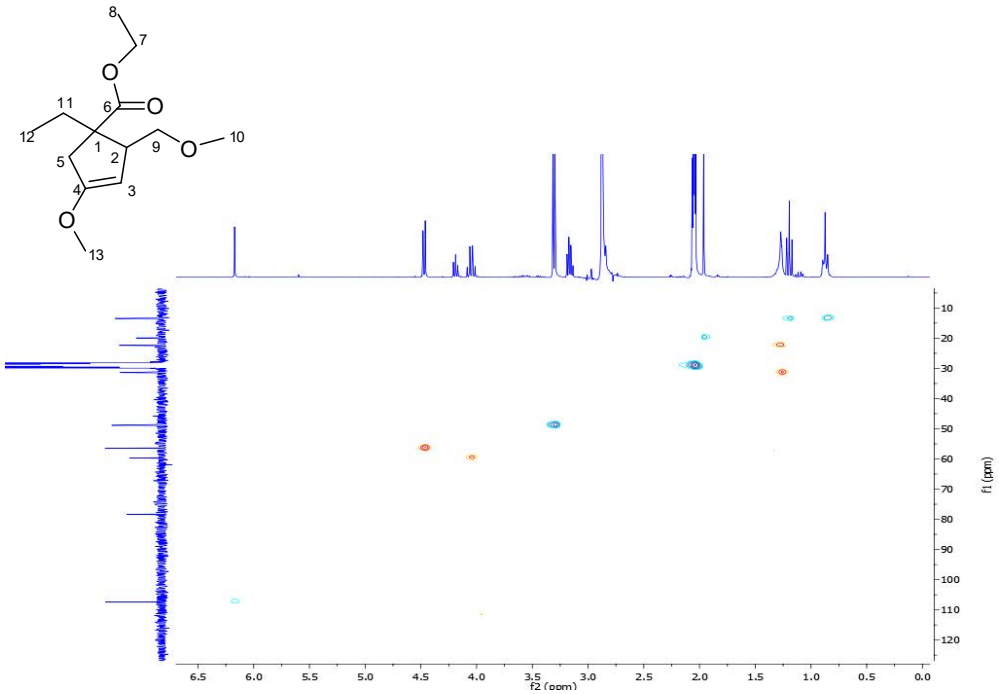
NMR spectra (300 MHz) of compound **8** (Ethyl 1-ethyl-4-methoxy-2-(methoxymethyl)cyclopent-3-enecarboxylate) in CD_3COCD_3



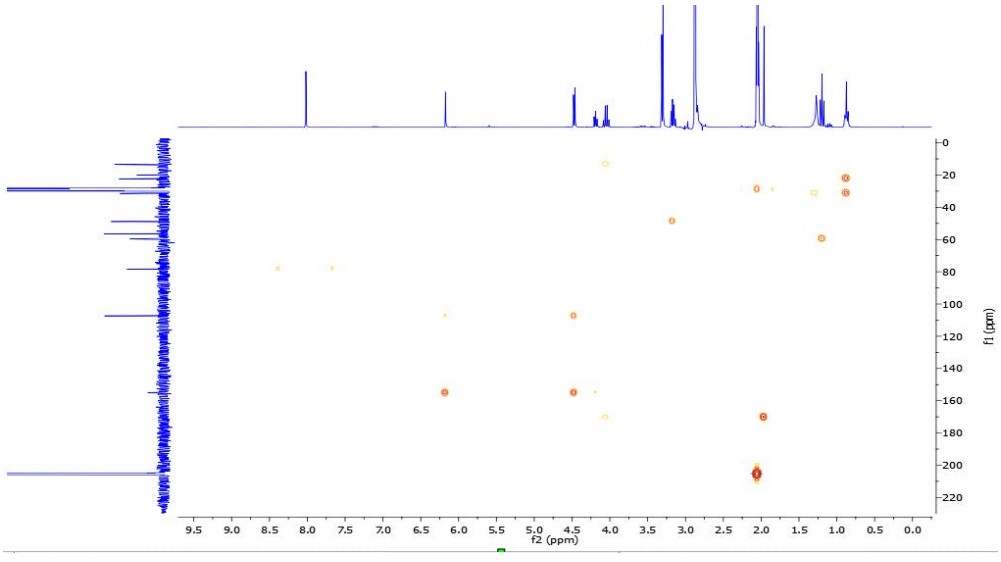
^{1}H-NMR spectrum of compound **8**



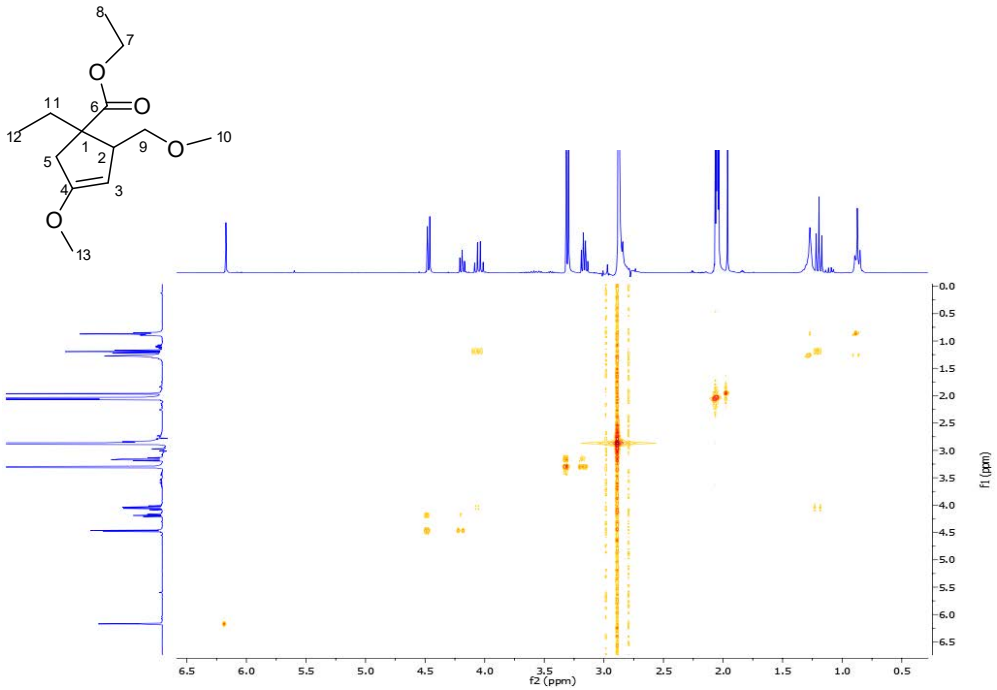
^{13}C-NMR spectrum of compound **8**



HSQCedit spectrum of compound **8**

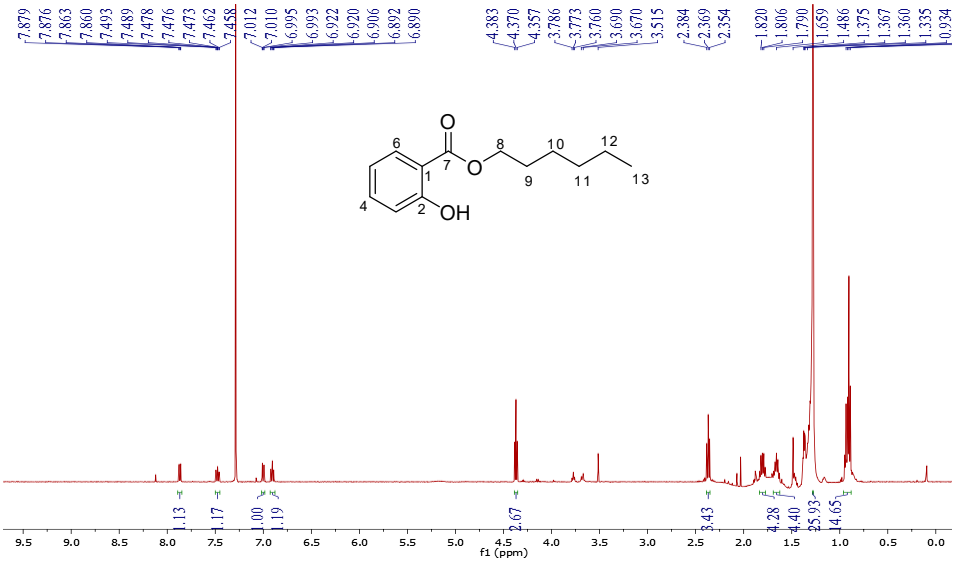


HMBC spectrum of compound **8**

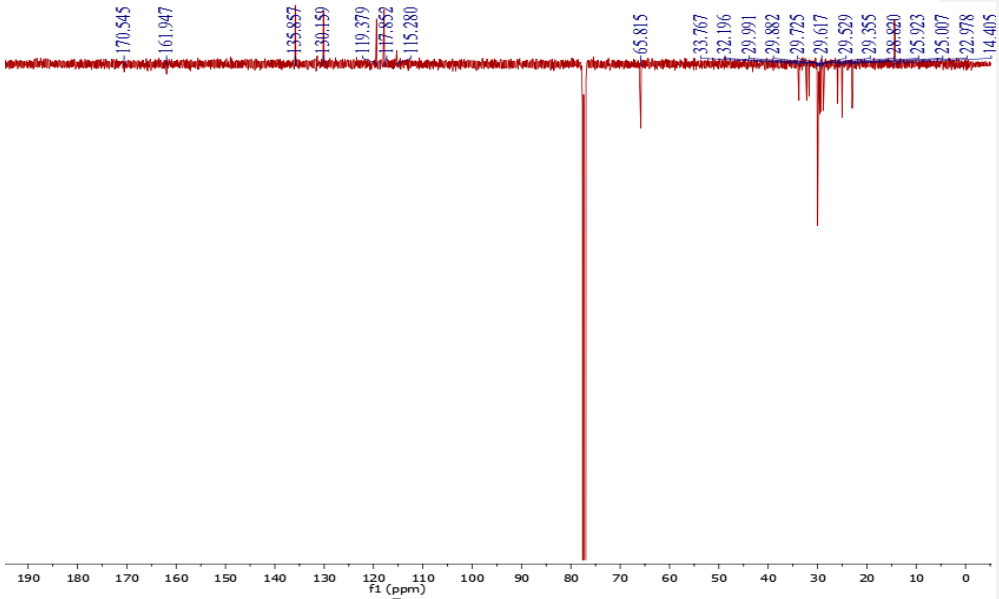


^{1}H-^{1}H COSY correlations of compound **8**

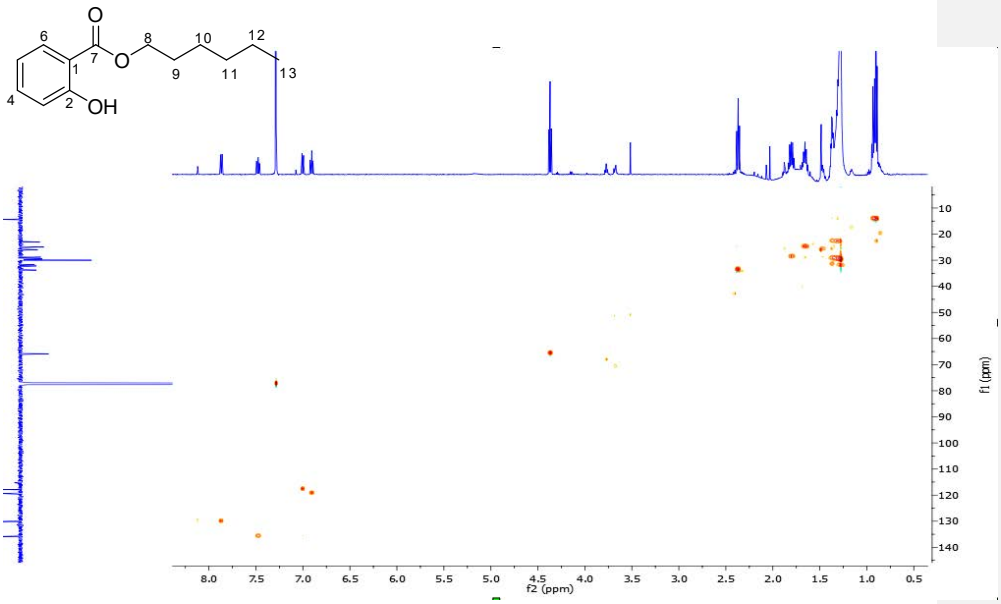
NMR spectra (500 MHz) of compound **9** (Hexyl 2-hydroxybenzoate) in $CDCl_3$



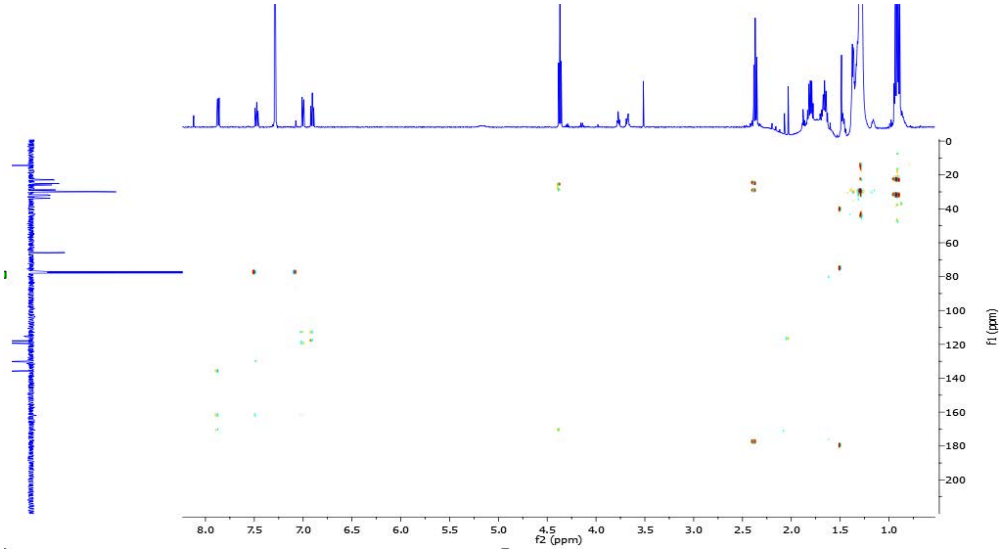
^{1}H-NMR spectrum of compound **9**



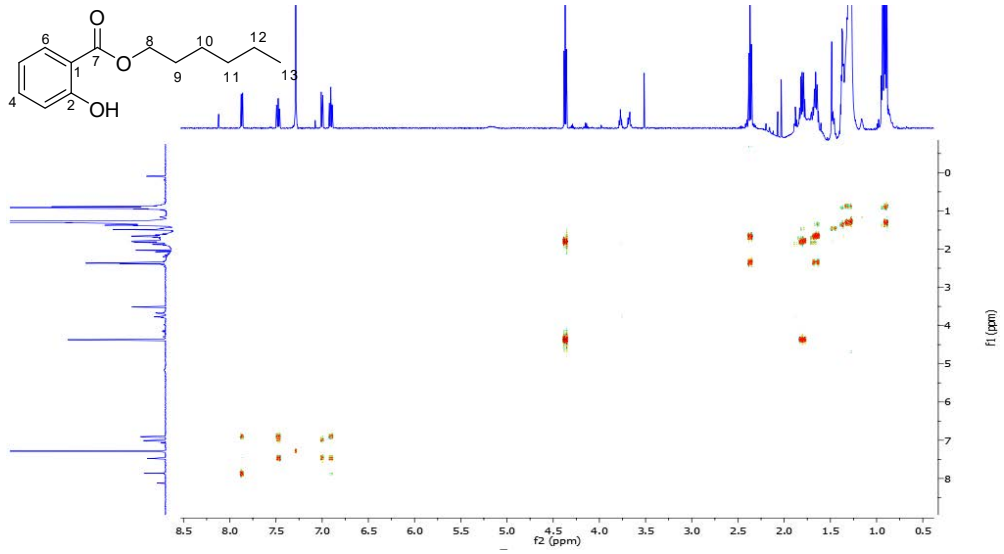
Jmod spectrum of compound **9**



HSQCedit spectrum of compound **9**

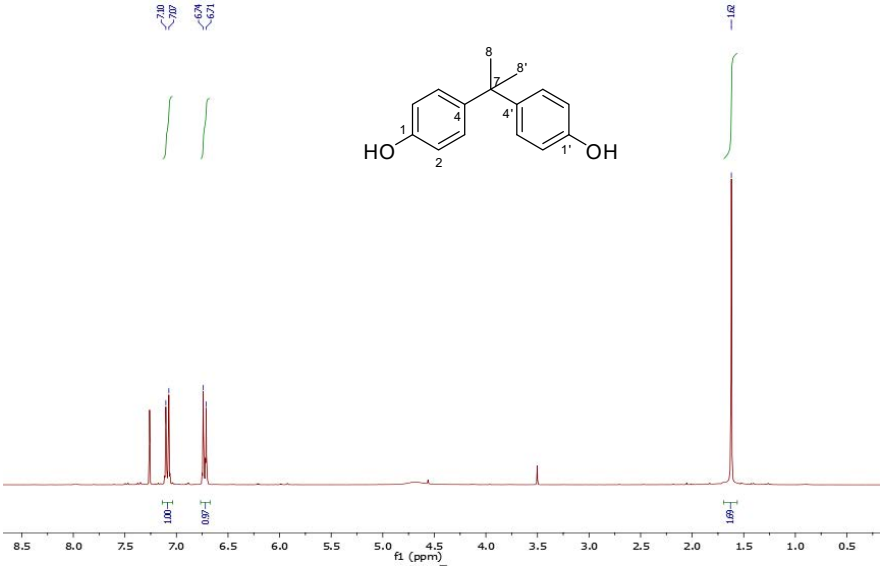


HMBC spectrum of compound **9**

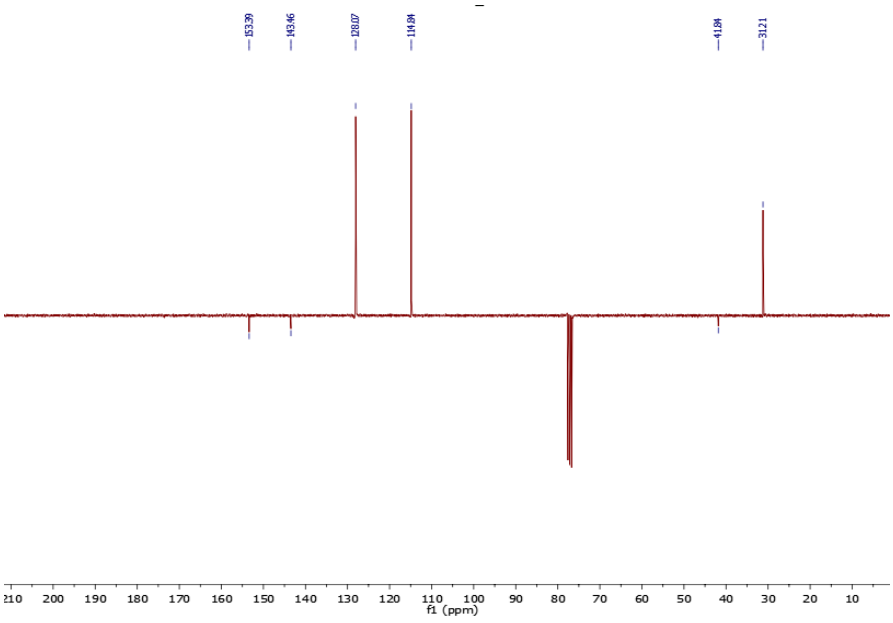


^{1}H-^{1}H- COSY spectrum of compound **9**

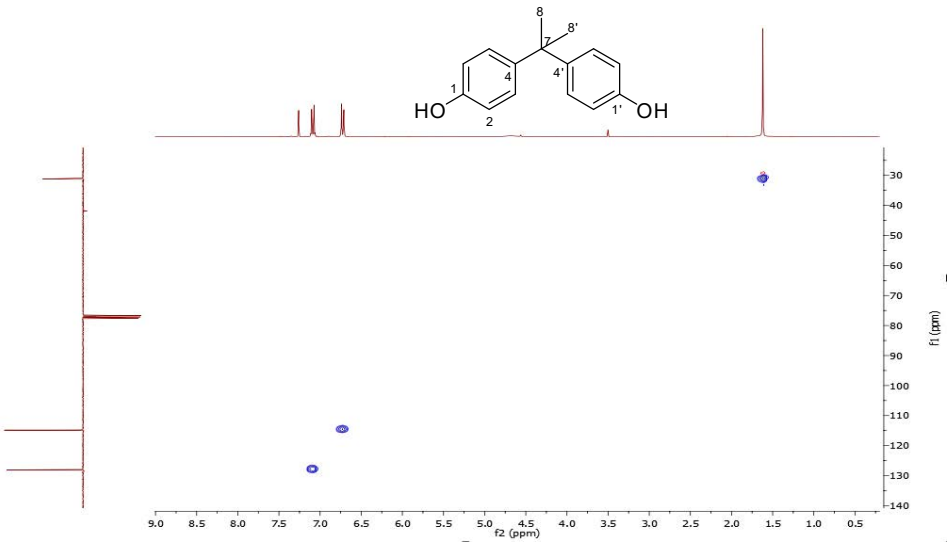
NMR spectra (300 MHz) of compound **10** (4,4'-(propane-2,2-diyl)diphenol) in $CDCl_3$



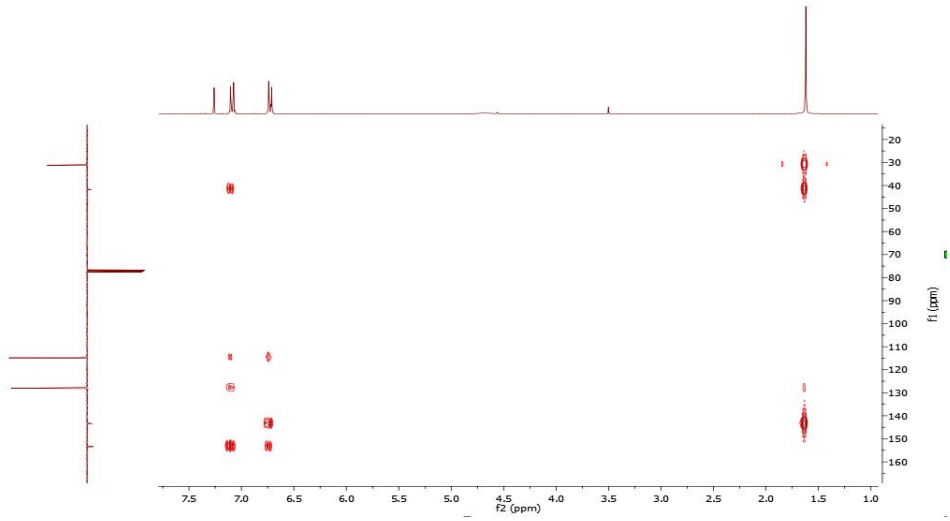
^{1}H-NMR spectrum of compound **10**



Jmod spectrum of compound **10**

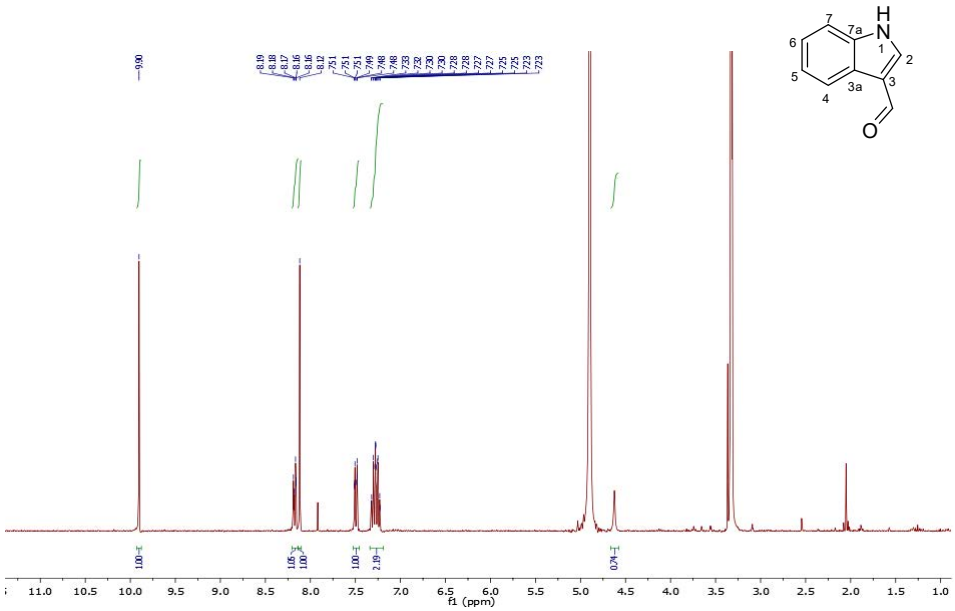


HSQCedit spectrum of compound **10**

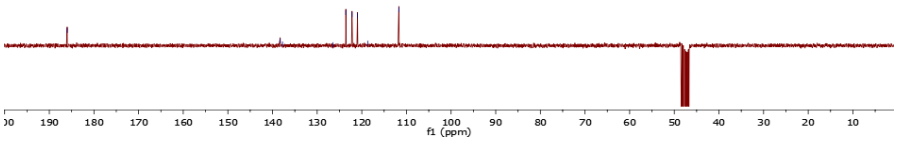


HMBC spectrum of compound **10**

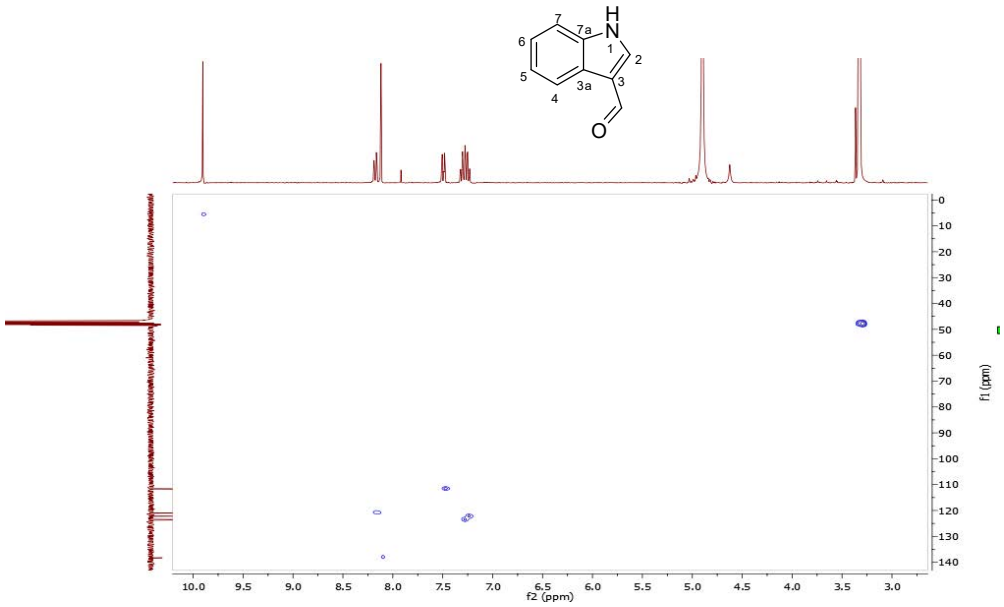
NMR spectra (300 MHz) of compound **11** (1*H*-indole-3-carbaldehyde) in CD_3OD



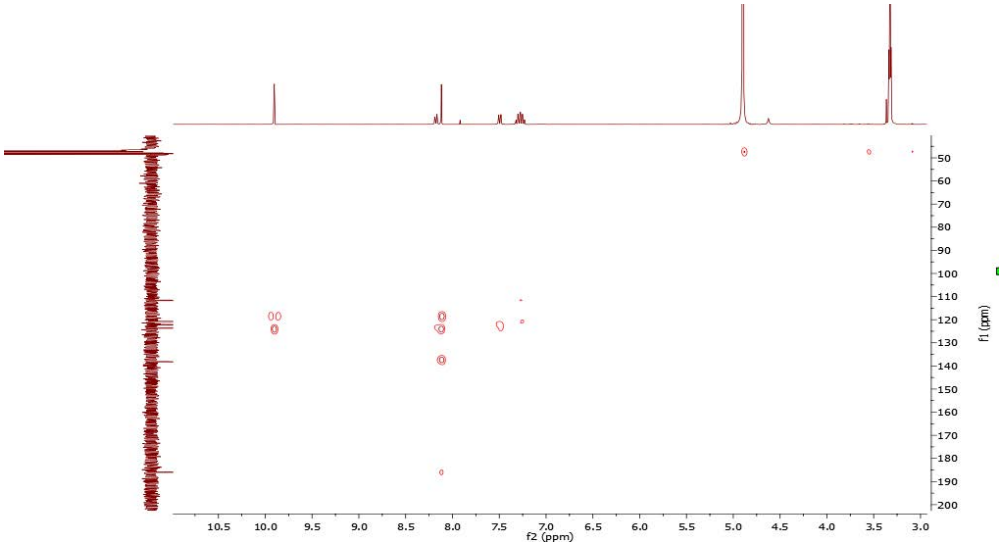
^{1}H-NMR spectrum of compound **11**



Jmod spectrum of compound **11**



HSQCedit spectrum of compound **11**



HMBC spectrum of compound

Titre : Découverte des métabolites secondaires actifs de *Paenibacillus odorifer*, une bactérie associée à un lichen

Mots clés : *Paenibacillus odorifer*, *Rhizocarpon geographicum*, bacterial compounds, alkaloid, *tert*-butylphenols

Résumé : Les bactéries qui sont des sources prolifiques d'antibiotiques et des fournisseurs importants d'agents pharmaceutiques peuvent produire une grande variété de métabolites. Ainsi, la découverte de métabolites issus bactéries est un nouveau challenge pour les chimistes. Parmi ces sources, les lichens sont admis comme niches intéressantes de nouvelles bactéries et de nouveaux composés bactériens. Par conséquent, les communautés de micro-organismes associées aux lichens sont devenues des sources prometteuses pour la production de composés naturels actifs.

Dans cette thèse, nous avons concentré notre travail sur l'isolement des bactéries de *Rhizocarpon geogaphicum*, l'un des lichens crustacés les plus populaires vivant sur la roche. Parmi les souches isolées, *Paenibacillus odorifer* a été sélectionnée pour poursuivre les travaux visant à produire des composés actifs.

Après des étapes d'optimisation de culture, l'étude des extraits issus des cultures de *P. odorifer* soit par le bioréacteur soit en Erlenmeyer a permis l'isolement des métabolites : un polysaccharide antioxydant, deux dérivés *tert*-butylphénoliques cytotoxiques issus de la bioaccumulation et de la biotransformation de précurseurs, d'un nouvel alcaloïde cytotoxique, de deux diols, de deux dérivés de type furfural et quelques autres composés connus. Des hypothèses de biosynthèse ont pu être proposés pour certains composés.

La diversité des métabolites isolés de *P. odorifer* indique que cette espèce possède un grand potentiel de production des composés actifs et est une bactérie utilisatrice de substrats *tert*-butyl phénoliques.

Title: **Discovery of active secondary metabolites from *Paenibacillus odorifer*, a lichen-associated bacterium**

Keywords : *Paenibacillus odorifer*, *Rhizocarpon geographicum*, bacterial compounds, alkaloid, *tert*-butylphenols

Abstract: Bacteria which are prolific sources of antibiotics and important suppliers to the pharmaceutical agents can produce a wide variety of metabolites. Thus finding metabolites from the bacterial lineages represented new interests for chemists. Among that, lichens are admitted as a rich source of new bacterial lineages and novel bacterial compounds. Therefore, microorganism communities associated with lichens became significant subjects as great potential for the production of active natural compounds.

In this thesis, we focus our work on the isolation of bacterial lineages from the lichen *Rhizocarpon geogaphicum*, one of the most popular crustose lichens dwelling on the rock. Among the strains isolated, *Paenibacillus odorifer* was selected for further work to produce active compounds.

After the culture optimization steps, the study of extracts from the *P. odorifer* cultures either in the bioreactor or in Erlenmeyer flask led to the production of metabolites: an antioxidant polysaccharide, two cytotoxic *tert*-butylphenol derivatives which came from the bioaccumulation and biotransformation of precursors, a novel and cytotoxic alkaloid compound, two diol compounds, two furfural derivatives and some other known compounds. Putative biosynthetic pathways have been proposed for some compounds.

The diversity of metabolites isolated from *P. odorifer* highlighted that this species possessed a great potential of the production active compounds and were a new case of *tert*-butyl phenol utilizing bacterium.

LE RÉSUMÉ DE LA THÈSE par Thi Bach Le NGUYEN

Discovery of active secondary metabolites from *Paenibacillus odorife*r, a lichen-associated bacterium

Les lichens sont des organismes complexes qui peuvent se développer sur de nombreux supports différents. On les trouve sur les arbres, les sols, les surfaces rocheuses et même à l'intérieur des roches, ainsi que sur la vitrocéramique, les objets métalliques… Les lichens sont également des mini-écosystèmes auto-approvisionnements parfaits qui se développent très lentement et sont formés par l'association de trois partenaires tels que champignons (mycobionte), algues et / ou cyanobactéries (photobionte) et communautés bactériennes. Chaque partenaire joue un rôle distinct et participe ensemble pour permettre à cet organisme de survivre dans des conditions extrêmes de lumière, de température et d'eau, des pôles aux tropiques, des zones intertidales aux sommets des montagnes. (Brodo et al., 2001).

Parmi les partenaires du lichen, le troisième partenaire correspondant aux communautés bactériennes est devenu une nouvelle source intéressante à étudier pour les chimistes en raison de leur capacité à produire de nombreux composés actifs. Dans nos efforts, en utilisant une stratégie basée sur la culture, pour découvrir et étudier ces communautés trouvées sur *Rhizocarpon Geographicum*, l'un des lichens crustacés vivant à la surface de la roche, 13 souches pures comprenant 10 bactéries, une cyanobactérie et un champignon ont été collectés. Les dix souches de bactéries appartenaient aux deux phylums de *Firmicutes* et *Proteobacteria*. En effet, contrairement aux autres lichens, les protéobactéries ne constituaient pas la classe prédominante de bactéries parmi tous les microorganismes symbiotiques de *R. geographycum*. Au lieu de cela, le phylum *Firmicutes* a eu lieu dans cette domination parmi ces communautés bactériennes. De plus, dans ce phylum, le genre *Paenibacillus* présentait le pourcentage le plus élevé (33%). Tout au long des études de la littérature déjà publiées sur les souches de ce genre, *Paenibacillus odorifer* a été sélectionné pour isoler ses métabolites en raison de la production potentielle de composés actifs.

La prochaine étape de ce travail consistait à déterminer les paramètres optimaux de la culture de *P. odorifer* en milieu liquide. La première optimisation effectuée à petite échelle (25 ml) a permis à *P. odorifer* de bénéficier de la meilleure croissance dans le milieu Gym Streptomyces supplémenté en $CaCO_3$ à pH 7 et à 25°C. Ces paramètres choisis dans la

première étape d'optimisation ont été appliqués à la culture par bioréacteur et ont donné le polysaccharide et deux composés diols.

- Le polysaccharide, identifié premièrement par les données IR et RMN, était formé de trois unités de sucre d'acide glucuronique, de fructose et de fucose avec un rapport moléculaire moyen de 4/2/1, respectivement. Les résultats ont été déterminés à partir de la comparaison du temps de rétention observé dans les chromatogrammes HPLC entre les unités de sucre dérivées de l'hydrolyse de polysaccharide et celles de monosaccharides classiques. Ensuite, une série de réactions impliquant la méthylation, l'hydrolyse, la réduction du borohydrure de sodium, l'acétylation par l'anhydride acétique et l'analyse finale par GC-MS des acétates d'alditol ont conduit à une hypothèse de la structure de cette fraction de polysaccharide telle que → 2) - β-D-GlcAp- (1 → 2) -β-D-GlcAp- (1 → 2) -β-D-GlcAp- (1 → 2) -β-D-GlcAp- (2 → 2) -β-D-Fruf- (2 → 4) -β-D-Fruf- (2 → 4) - β-L-Fucp- (1 →. Ce polysaccharide possédait également une cytotoxicité significative mesurée par titrage au MTT avec une valeur de CI50 de 19 µg / mL et de 27 µg / mL HaCaT et des lignées cellulaires de mélanomes murins B16, respectivement, ce qui pourrait constituer une source potentielle d'agents antitumoraux sous forme de fraction polysaccharidique microbienne produite à partir de *P. odorifer*.
- Les deux composés de diol (nommés 4-méthyl-1-phénylpentane-2,3-diol (**1**) et 4-méthyl-1-phénylhexane-2,3-diol (**2**)) récoltés de la culture dans un bioréacteur ne montrent pas toute activité cytotoxique. Bien que ces deux composés aient été identifiés par Rukachaisirikul et ses collaborateurs (2011), leurs données spectroscopiques n'ont pas été présentées. A notre connaissance, il s'agit du premier signalement de ces composés issus de la culture de *P. odorifer*.

L'inconvénient de la culture réalisée dans le bioréacteur (4,5 L) est la présence d'autres métabolites en quantité trop importante. En conséquence, afin d'augmenter la quantité de métabolites secondaires produits, un processus d'optimisation secondaire a été établi à l'aide des flacons Erlenmeyer. La différence de production suivant les récipients utilisés pourrait s'expliquer par le mode d'aération différent et par le système d'agitation induisant des conditions de contrainte différentes pour la souche. En fait, l'agitation dans le bioréacteur est réalisée par une lame d'agitation immergée dans le bouillon de culture qui implique des pauses hypha, tandis que dans les flacons agités, l'agitation est réalisée avec un agitateur orbital permettant la formation de l'hyphale. De même, dans les flacons agités, il n'existe pas

de système spécifique d'aération; ceux du bioréacteur consistent en un tube immergé dans le bouillon de culture qui conduit l'oxygène dans le milieu, ce qui provoque la formation de bulles.

A la base des résultats de la première optimisation, la seconde a été réalisée à l'aide de fioles Erlenmeyer (4,0 L) permettant de sélectionner les meilleures conditions de production de métabolites à partir de *P. odorifer* sous forme de *Gym Streptomyces* moyen additionné de $CaCO_3$ à pH 7, Sous agitation à 25°C et à 120 tr/min, ainsi qu'à 1% d'inoculum. L'extrait de résine obtenu en utilisant les meilleures conditions de la culture a été soumis à des approches de chromatographie pour donner certains composés qui seront décrits suivant. Parmi eux, deux tert-butylphénols ont été isolés et l'un d'eux a montré une activité cytotoxique significative contre les lignées de cellules HaCaT et B16. Ces deux composés étaient rares dans la nature. Leur présence dans le bouillon de culture pourrait s'expliquer par la bioaccumulation et la biotransformation à partir de dérivés de tert-butyle tels que le BHA (benzohydroxyanisole). Par ailleurs, *P. odorifer* a également produit d'autres composés non cytotoxiques ou bien connus, tels que le 2-propylpentadec-2-énoate de méthyle (**3**); Le 5-(hydroxyméthyl) furanne-2-carbaldéhyde (**4**); 4- (5- (hydroxyméthyl) furanne-2-yl) but-3-én-2-one (**5**); 4-méthoxy-3-méthylfurane-2 (5H) -one (**6**); Méthacrylate de 2 - ((3-hydroxy-2-méthylpropanoyloxy) méthyl) -2- (hydroxyméthyl) butyle (**7**); 1-éthyl-4-méthoxy-2-(méthoxyméthyl) cyclopent-3-énécarboxylate d'éthyle (**8**); le 2-hydroxybenzoate d'hexyle (**9**).

De plus, la culture avec un volume important (40 L dans l'Erlenmeyer flask) en utilisant les meilleures conditions choisies à la suite des résultats de la seconde optimisation a été réalisée pour trouver des métabolites bioactifs en plus grande quantité. Un nouvel alcaloïde a été trouvé dans une fraction cytotoxique (fraction 8). Sa structure consiste à une fraction dihydronaphtalène, qui est un groupe structurel bien connu dans les produits naturels, fusionnée à une unité rare de pyrrolooxazine. Ce nouveau composé était le premier exemple de ce squelette produit par des organismes vivants (notamment *P. odorifer*). Ce composé présentait également une cytotoxicité hebdomadaire avec des valeurs de CI_{50} de 76,0 µM et 78,9 µM sur les lignées cellulaires B16 et HaCaT, respectivement. De plus, deux composés bien connus ont également été trouvés dans la fraction 8, tels que le 4,4 '- (propane-2,2-diyl) diphénol (**10**) et le 1H-indole-3-carbaldéhyde (**11**) qui n'étaient pas actifs.

Par comparer le compte-rendu de tous les composés isolés de chaque culture de *P. odorifer* en utilisant différents moyens comme bioréacteur et Erlenmeyer flasks, il semble que le polysaccharide n'ait été trouvé que dans la culture utilisant le bioréacteur. Ceci s'explique que, pour la culture en bioréacteur, le rapport d'inoculum n'était pas contrôlé et l'agitation était fixée à 150 tr / min pendant la culture. Ces facteurs diffèrent des paramètres optimaux choisis parmi les étapes d'optimisation, alors qu'ils semblent jouer un rôle important dans la production de métabolites actifs. De plus, la différence de méthode d'aération utilisée pourrait également être l'une des raisons de cette différence de production. Par conséquent, la réalisation d'une culture dans le bioréacteur en respectant les meilleures conditions trouvées lors de la deuxième optimisation pourrait permettre de conclure avec précision pour la production de cette fraction de polysaccharide par *P. odorifer*. Ces efforts seront faits pour mieux contrôler tous les paramètres de la culture dans le bioréacteur (taux d'aération, brassage, rapport d'inoculum, etc.).

De la même manière, pour les autres métabolites, les deux tert-butylphénols ont été trouvés dans la culture à Erlenmeyer flasks mais étaient absents dans la culture au bioréacteur. L'origine de ce type de composés doit être étudiée particulièrement par leur voie de biosynthèse supposée. Nous avons envisagé de réaliser le séquençage complet du génome afin de mettre en évidence certaines voies métaboliques intéressantes.

Contrairement aux composés **1** et **2** du diol, ils ont été rapportés de la culture à la fois par utiliser un bioréacteur et une fiole Erlenmeyer flask. Nous pourrions donc conclure qu'ils constituent l'un des principaux composés produits par *P. odorifer*. Fait intéressant, lorsque le volume de culture a augmenté, la masse de ces composés a également augmenté. De la même manière, pour augmenter la masse de composés cytotoxiques, il est préférable de cultiver cette souche dans un grand volume de milieu.

En outre, deux dérivés du furfural (**4**, **5**) ont également été trouvés dans les cultures en utilisant un erlenmeyer. Le milieu utilisé pour la culture de *P. odorifer* contenait de l'extrait de malt, qui est un mélange de graines de céréales. Il est important de noter que le furfural aurait été formé par le traitement des céréales à haute température (Mesias et al., 2017). De manière correspondante, nous pourrions suggérer que les dérivés de furfural (**4**, **5**) ont été formés par la bioaccumulation de furfural présent dans le milieu après l'étape de stérilisation et ensuite par biotransformation par *P. odorifer*. Par conséquent, la bioaccumulation et la

biotransformation semblent être un comportement caractéristique de *P. odorifer*. Comme discuté ci-dessus, ce comportement particulier doit être étudié dans des expériences ultérieures. Nous suggérons également de soumettre cette souche intéressante dans les études de biotransformation de composés du lichen.

Enfin, *P. odorifer* est un producteur intéressant car cette souche pourrait produire des composés originaux tels qu'un nouvel alcaloïde, certains composés de diol en grande quantité, certains dérivés du furfural et certains tert-butylphénols qui pourraient être dérivés de la biotransformation de précurseurs particuliers. Fait intéressant, bien qu'une quantité importante de dicétopipérazines ait déjà été isolée de la culture de diverses bactéries associées au lichen, aucun de ces composés, classiquement trouvé dans des cultures de microorganismes, n'a été trouvé ici. Ces observations montrent que cette bactérie pourrait constituer une souche intéressante pour la biotransformation ou produire de nouveaux composés actifs.

L'isolement d'autres métabolites actifs potentiels est en cour traiter sur les autres fractions actives obtenues de notre grand volume de culture. D'autres évaluations d'activités telles que les propriétés antibiotiques ou antifongiques seront entreprises pour valoriser ces composés, particulièrement une grande quantité de ces composées diols a été obtenue. Nous envisageons également d'étudier les autres extraits en tant que surnageant de pellets pouvant contenir des composés intracellulaires potentiellement actifs.

De plus, la co-culture entre *P. odorifer* et le champignon qui a déjà été isolé de *R. geographicum* devrait être réalisée afin de donner une hypothèse sur la capacité de cette bactérie à produire des armes défensives sur la présence de ce champignon. Ces expériences ultérieures nous donneront quelques arguments pour mettre en évidence la compétition parmi les divers microorganismes pouvant apparaître dans ce micro-écosystème complexe qui pourrait être le lichen.

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