ENHANCEMENT OF CHEMICAL DIVERSITY IN FUNGAL ENDOPHYTES FROM ARID PLANTS OF ANDALUSIA

Víctor M. González Menéndez

PhD Thesis

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Universidad de Granada-Fundación MEDINA

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La presente Tesis Doctoral ha sido realizada en los Departamentos de Microbiología, Química y Screening de la Fundación MEDINA bajo la línea de investigación de Nuevas Dianas Terapéuticas del Programa Oficial de Postgrado en Farmacia de la Universidad de Granada. El trabajo desarrollado fue cofinanciado por la Fundación MEDINA y por el proyecto de Excelencia de la Junta de Andalucía RMN-7987 "*Uso sostenible de plantas y de sus parásitos fúngicos para la obtención de nuevas moléculas útiles en terapias antifúngicas y neuroprotectoras".*

Para la obtención de la Mención Internacional, durante el transcurso de 4 meses, se realizó una estancia en la Universidad de Reims bajo la supervisión del profesor Jean-Hugues Renault y en colaboración con la empresa SOLIANCE, donde se llevaron a cabo estudios para el escalado de hongos dimórficos en biorreactores de 30 Litros.

Los resultados de esta Tesis Doctoral han sido publicados o están en vías de publicación en las siguientes revistas científicas:

1. González-Menéndez V, Crespo G, de Pedro N, Diaz C, Martín J, Serrano R, Mackenzie TA, Justicia C, González-Tejero MR, Casares M, Vicente F, Reyes F, Tormo JR, Genilloud O. (2018). Fungal endophytes from arid areas of Andalusia: high potential sources for antifungal and antitumoral agents. Scientific Reports 8(1):9729. doi: 10.1038/s41598-018-28192-5. JCR: 4.1222, Q1, total journal citations: 192,481

2. González-Menéndez V, Martín J, Siles JA, González-Tejero MR, Reyes F, Platas Gonzalo, Tormo JR, Genilloud O. (2017). Biodiversity and chemotaxonomy of *Preussia* isolates from Iberian Peninsula. Mycological Progress 16:713–728. doi: 10.1007/s11557-017-1305-1. JCR: 1.914, Q1, total journal citations 1,474.

3. González-Menéndez V, Asensio F, Moreno C, de Pedro N, Monteiro MC, de la Cruz 1, Vicente F, Bills GF, Reyes F, Genilloud O, Tormo JR. (2014). Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity. Mycology 5:179–191. doi: 10.1080/21501203.2014.942406. JCR: No included, Q2, SJR: 0,45

4. González-Menéndez, V, Pérez-Bonilla M, Pérez-Victoria I, Martín J, Muñoz F, Reyes F, Tormo JR, Genilloud O. (2016) Multicomponent Analysis of the Differential Induction of Secondary Metabolite Profiles in Fungal Endophytes. *Molecules (Basel*, *Switzerland)* 21, doi: 10.3390/molecules21020234. JCR 3.098, Q1, total journal citations 31,047

5. González-Menéndez, V, Martínez G, Serrano R, Muñoz F Martín J, Genilloud O, Tormo JR (in press). Ultraviolet (IUV) and mass spectrometry (IMS) imaging for the deconvolution of microbial interactions. BMC System Biology doi: 10.1186/s12918-018-0617-3. JCR 2.050, Q1, total journal citations 4,313

Además, parte de los resultados derivados de la presente Tesis Doctoral han sido publicados y/o presentados en las siguientes aportaciones científicas:

Comunicaciones Orales

Victor González-Menéndez. Inducción de la producción de metabolitos secundarios en hongos filamentosos. Ponencia en 14 Congreso nacional Micología 2018, Tarragona 19-21 Septiembre, 2018

Victor González-Menéndez. Endophytes as biocontrol agents against fungal phytopathogens. Biopesticides 2018, Amsterdam, 6-8 Junio, 2018.

Victor González-Menéndez, Gloria Crespo, Clara Toro, Francisca Muñoz, Jesus Martin, Fernando Reyes, Olga Genilloud and José R. Tormo. *Enhancement of the Production of Cerulenin by New Fungal Producer Strain*. BIOTEC 2017, Murcia, 18-21de Junio, 2017.

Victor González-Menéndez, Gloria Crespo, Clara Toro, Francisca Muñoz, Jesus Martin, Fernando Reyes, Olga Genilloud and José R. Tormo. Fungal endophytes isolated from arid plants of Andalucía: production of new antitumor and antifungal activities by the addition of adsorptive polymeric resins. XIII Reunión de la Red Nacional de Microorganismos Extremófilos, celebrada los días 21 y 22 de octubre 2016 en Alicante.

Victor González-Menéndez, Nuria de Pedro, Caridad Díaz, Gloria Crespo, Jesus Martin, Clara Toro, Francisca Muñoz, Carlos Justicia, Francisca Vicente, Fernando Reyes, Olga Genilloud and José R. Tormo. Fungal endophytes isolated from arid plants of Andalucía: Induction of exclusive antitumoral and antifungal activities by the addition adsorptive polymeric resins. 13 Congreso Nacional de Micología. Lérida. Septiembre 2016.

Victor González-Menéndez, Jesus Martin, Caridad Diaz, Clara Toro, Catalina Moreno, Francisca Vicente, Fernando Reyes, Jose R. Tormo y Olga Genilloud. Quimiotaxonomía de especies de *Preussia* aisladas en la península Ibérica. XVI Reunión Taxonomía, Filogenia y Biodiversidad. Santiago de Compostela. Junio 2016.

Victor González-Menéndez, F. Muñoz, C. Toro, I. Perez-Victoria, J. Martin, F. Reyes, O. Genilloud, J. R. Tormo. Metabolomics on the Differential Secondary Metabolite Profiles Induced by Epigenetic Modifiers in Fungal Endophytes, a Case Study. BIOIBEROAMERICA 2016, Salamanca, 5-9 de junio, 2016.

Victor González-Menéndez, Jose A Siles, Gerald F Bills, Jose R. Tormo, Gonzalo Platas y Olga Genilloud. Diversidad de la familia *Sporormiacea*e en la Península Ibérica. XV Reunión Taxonomía, Filogenia y Biodiversidad. Alcalá de Henares. Julio 2014. Por la que se obtuvo premio a la mejor comunicación oral.

Comunicaciones Escritas

Victor González-Menéndez, Gloria Crespo, Clara Toro, Francisca Muñoz, Jesus Martin, Fernando Reyes, Olga Genilloud and José R. Tormo. Enhanced production of cerulenin by a new fungal species of *Phoma* following improved fermentation conditions. FEMS 2017. Valencia 9-13 Julio.

Victor González-Menéndez, Gloria Crespo, Clara Toro, Francisca Muñoz, Jesus Martin, Fernando Reyes, Olga Genilloud and José R. Tormo. Fungal endophytes isolated from arid plants of Andalucía: production of new antitumor and antifungal activities by the addition of adsorptive polymeric resins. FEMS 2017. Valencia 9-13 Julio.

Victor González-Menéndez, German Martinez, Rachel Serrano, Francisca Muñoz, Jesus Martin, Olga Genilloud and Jose R Tormo. Ultraviolet (IUV) and Mass Spectrometry (IMS) Imaging for the Deconvolution of Microbial Interactions. FEMS 2017. Valencia 9-13 Julio.

Victor González-Menéndez, German Martinez, Rachel Serrano, Francisca Muñoz, Jesus Martin, Olga Genilloud and Jose R Tormo. Ultraviolet (IUV) and Mass Spectrometry (IMS) Imaging for the Deconvolution of Microbial Interactions. International Work-Conference on Bioinformatics and Biomedical Engineering IWBBIO 2017. Abril, 2017. Granada (SPAIN)

Victor González-Menéndez, Jesus Martín, Clara Toro, Rachel Serrano, Catalina Moreno, Jose R. Tormo y Olga Genilloud. Nuevos biomarcadores quimio-taxonómicos de especies de *Preussia* (*Sporormiaceae*) aisladas en la península Ibérica. 13 Congreso Nacional de Micología. Lérida. Septiembre 2016

Victor González-Menéndez, F. Muñoz, C. Toro, I. Perez-Victoria, J. Martin, F. Reyes, O. Genilloud, J. R. Tormo. Metabolomics on the Differential Secondary Metabolite Profiles Induced by Epigenetic Modifiers in Fungal Endophytes, a Case Study. ASM Microbe 2016. Boston (USA) Junio 2016

Victor González-Menéndez, Caridad Díaz, Mercedes Perez, M. Reyes Gonzalez, Joaquín Molero, Manuel Casares, Francisca Vicente, Fernando Reyes, José R. Tormo and Olga Genilloud. Fungal endophytes and epiphytes isolated from arid plants of Andalucía: valuable sources of antifungal compounds. COST FA1103 Workshop Vienna. Nov 2015

Victor González-Menéndez, B. García, F. Muñoz, JR Tormo and O. Genilloud. Production of new secondary metabolites upon addition of small-molecule epigenetic elicitors to fungal endophytes fermentations. VAAM-Jahrestagung 2015 | 01. –04.03.2015 – Marburg (DE)

Victor González-Menéndez, R Serrano, F Muñoz, F Reyes, O Genilloud, JR Tormo: Differential induction of secondary metabolite profiles in endophyte fungi by the addition of epigenetic modifiers. ASP2015. Colorado (USA). Julio de 2015

Publicaciones

Germán Martínez, Víctor González-Menéndez, Jesús Martín, Fernando Reyes, Olga Genilloud, José R. Tormo: *MASS Studio: A Novel Software Utility to Simplify LC-MS Analyses of Large Sets of Samples for Metabolomics*. Bioinformatics and Biomedical Engineering, 04/2017: pp 230-244; doi:10.1007/978-3-319-56148-6_20

Mercedes Pérez-Bonilla, Victor González-Menéndez, Ignacio Perez-Victoria, Nuria de Pedro, Jesus Martin, Joaquin Molero-Mesa, Manuel Casares-Porcel, M Reyes González-Tejero, Francisca Vicente, Olga Genilloud, Fernando Reyes. (2017) Hormonemate Derivatives from *Dothiora* sp., an Endophytic Fungus. J Nat Prod. 28;80(4):845-853. doi: 10.1021/acs.jnatprod.6b00680.

Victor González-Menéndez, Rachel Serrano, Francisca Muñoz, Fernando Reyes, Olga Genilloud, Jose R Tormo. (2015) Differential induction of secondary metabolite profiles in endophyte fungi by the addition of epigenetic modifiers. Planta Medica 81, (11), 924-924.

¨Sorprendernos por algo es el primer paso de la mente hacia el descubrimiento¨

Louis Pasteur (1822-1895)

A mis padres

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RESUMEN

Ha sido ampliamente descrito que los hongos representan una de las fuentes más prolíficas de nuevos productos naturales, pero los bajos rendimientos de producción y la falta de expresión de genes crípticos en condiciones estándar de laboratorio son factores limitantes clave para poder explotar al máximo el potencial metabólico de los hongos *in vitro*. Las comunidades nativas de plantas asociadas a zonas áridas presentan características distintivas para sobrevivir en tales condiciones extremas. El gran número de plantas endémicas muy poco estudiadas representa una fuente potencial única para el descubrimiento de nuevos hongos simbiontes, así como de endófitos específicos de su hospedador aún no descritos. Este trabajo representa el primer estudio donde se lleva a cabo el cultivo y la caracterización de hongos asociados a un extenso número de especímenes de plantas halófitas y xerofíticas de zonas áridas de Andalucía. Además, una vez caracterizada taxonómicamente la población de aislados fúngicos, el objetivo del estudio fue estimular y caracterizar su diversidad química en cuanto a producción de metabolitos secundarios aplicando diversas metodologías basadas en el cultivo, como la adición de resinas de adsorción polimérica, la utilización de modificadores epigenéticos de origen químico o mediante el co-cultivo de microrganismos provenientes del mismo nicho ecológico.

Para el presente trabajo se aislaron un total de 349 cepas fúngicas a partir de las 63 especies de plantas recolectadas en ecosistemas áridos ubicados en el sureste de Andalucía, caracterizándose tanto morfológicamente, como en base a sus secuencias ribosomales ITS y 28S. La comunidad de aislados fúngicos se distribuyó a lo largo de 19 órdenes taxonómicos, incluyendo basidiomicetes y ascomicetes, siendo *Pleosporales* el orden más abundante entre todos los aislados. Identificándose así un total de 107 géneros diferentes, siendo *Neocamarosporium* el género más frecuentemente aislado, seguido de *Preussia* y *Alternaria*. Para los aislados pertenecientes al género *Preussia*, se llevó a cabo, además, un estudio específico para revisar su taxonomía y caracterizar quimiotipos dentro de las especies de *Preussia* aisladas a partir de muestras de zonas áridas y otras muestras ambientales recolectadas dentro de toda la Península Ibérica. Se identificaron 16 biomarcadores y mediante un análisis combinado, se infirieron 11 quimiotipos que pueden ser usados para diferenciar especies de *Preussia* estrechamente relacionadas.

En lo referente a su capacidad de producir diversidad química mediante fermentación, las cepas se crecieron en primer lugar en cuatro medios de cultivo diferentes, tanto en presencia como en ausencia de resinas de adsorción seleccionadas para promover la generación de nuevos metabolitos secundarios. Los extractos obtenidos a partir de las fermentaciones se caracterizaron químicamente y se evaluaron en busca de nuevas actividades antifúngicas contra patógenos de plantas y humanos, así como actividades citotóxicas contra la línea celular de cáncer de hígado humano HepG2. De los 349 aislados analizados, 126 (36%) fueron capaces de generar extractos con actividades biológicas significativas, 58 con propiedades antifúngicas específicas y 33 cepas con actividades específicas en la línea celular de carcinoma hepatocelular HepG2. Tras analizar los

Resumen

extractos activos mediante LC-MS de baja y alta resolución, se pudieron identificar 68 metabolitos secundarios con actividad biológica conocía producidos por 96 de las cepas, además de 12 metabolitos secundarios potencialmente nuevos en un subconjunto de 14 de los hongos endofíticos.

Los inhibidores de histona desacetilasas (HDAC) y de ADN metiltransferasas (DNMT) son comúnmente utilizados con el fin de perturbar la producción de metabolitos fúngicos mediante la inducción de la expresión de rutas biosintéticas crípticas. Por lo tanto, se desarrolló una metodología sistemática para evaluar e identificar los posibles efectos de un batería de inhibidores de HDAC y DNMT en los perfiles metabólicos de los hongos del estudio. Además, análisis metabolómicos basados en datos de LCMS y representados mediante "Volcano-plots" fueron aplicados con el fin de determinar moléculas producidas diferencialmente entre condiciones de interés a comparar. Se llevo a cabo de esta manera la identificación y caracterización química de los metabolitos secundarios más significativos inducidos por el uso de modificadores epigenéticos para un subconjunto de los aislaos fúngicos.

El uso de co-cultivos también fue evaluado como una metodología para detectar relaciones antagonistas entre los aislados a parir de plantas de zonas áridas de Andalucía. Para este propósito, se realizó un cribado en busca de interacciones antagonistas entre la población de aislados mediante el co-cultivo de parejas de cepas sobre placas de agar y, posteriormente, se diseñó y desarrolló un método de extracción automatizado para determinar si en estos efectos antagónicos observados entre los aislados se inducían nuevos metabolitos secundarios o si estos se acumulaban espacialmente en la zona de la interacción. Se desarrollaron así técnicas de imagen por espectrometría de masas (MS) y ultravioleta (UV) para visualizar la distribución espacial de los metabolitos y facilitar la identificación de aquellos metabolitos secundarios inducidos gracias a las interacciones microbianas.

Además de caracterizar los perfiles químicos diferenciales de producción de metabolitos secundarios después de aplicar cada uno de estos tres enfoques basados en el cultivo de los aislados, y como prueba de concepto de la inducción potencial de nuevas estructuras químicas, se purificaron e identificaron 14 metabolitos secundarios inducidos únicamente tras aplicar estas aproximaciones. Las estructuras de seis de estos compuestos, algunos bioactivos, resultaron nuevas y se elucidaron en el presente trabajo.

Los resultados obtenidos confirman que la explotación de la riqueza microbiana asociada a las zonas áridas de Andalucía, combinada con la aplicación de métodos integrados de cultivo puede favorecer la activación de rutas metabólicas silentes en ellos y conducir al descubrimiento de nuevos productos naturales con actividad biológica y potencial para convertirse en cabezas de serie para el desarrollo de futuros agentes terapéuticos.

ABSTRACT

It has been widely described that fungi represent one of the most prolific sources of novel NPs, but low production yields and the lack of expression of cryptic gene clusters in laboratory conditions are frequently key limiting factors for exploiting the full secondary metabolite potential of fungi *in vitro.* Native plant communities from arid areas present distinctive characteristics to survive in extreme conditions. The large number of poorly studied endemic plants represents a unique potential source for the discovery of novel fungal symbionts as well as host-specific endophytes not yet described. This work represents the first study culturing and characterizing the fungal symbiont community of an extensive number of halophytic and xerophytic plant specimens from arid areas of Andalusia. Moreover, once the populations of isolates were characterized, the aim of the study has been to stimulate and characterize their chemical diversity on secondary metabolites production by applying diverse culture-based methodologies: adding adsorptive polymeric resins, using small-molecule epigenetic elicitors and/or co-culturing microorganisms isolated from the same ecological niche.

A total of 349 fungal strains isolated from 63 selected plant species from arid ecosystems located in the southeast of Andalusia, were characterized morphologically as well as based on their ITS/28S ribosomal gene sequences. The fungal communities isolated were distributed among 19 orders including Basidiomycetes and Ascomycetes, with *Pleosporales* as the most abundant order. In total, 107 different genera were identified being *Neocamarosporium* the most frequently isolated genus, followed by *Preussia* and *Alternaria*. In the case of the *Preussia* isolates, a specific study was carried out to review their taxonomy and to characterize occurring chemotypes of *Preussia* species from arid and other environmental samples of the Iberian Peninsula. Sixteen natural compounds were identified and based on combined analyses, 11 chemotypes were inferred and could be used to resolve groups of closely related *Preussia* species.

Initially, strains were grown in four different media in presence and absence of selected resins to promote chemical diversity in the generation of new secondary metabolites. Fermentation extracts were chemically characterized and evaluated looking for new antifungal activities against plant and human fungal pathogens, as well as, cytotoxic activities against the human liver cancer cell line HepG2. From the 349 isolates tested, 126 (36%) exhibited significant bioactivities including 58 strains with exclusive antifungal properties and 33 strains with exclusive activity against the HepG2 hepatocellular carcinoma cell line. After LCMS analysis of the active extracts, 68 known bioactive secondary metabolites could be identified as produced by 96 strains, and 12 likely unknown compounds were found in a subset of 14 fungal endophytes.

Small molecule histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors are commonly used to perturb the production of fungal metabolites leading to the induction of the expression of silent biosynthetic pathways. Thus, a systematic approach to evaluate and identify the possible effects of HDAC and DNMT inhibitors on

Abstract

the metabolic profiles of wild type fungal endophytes was developed. Metabolomics by volcano plot representations based on LC/MS was used to determine significant differences between two sets of samples from two different conditions of interest. Chemical identification and characterization of the most significant SMs induced by these epigenetic modifiers was performed with part of the isolates obtained.

Co-culture approach was also tested as a third way of inducing antagonist relationships between the isolates from plants of arid zones of Andalusia. For this purpose, a screening looking for antagonistic interactions among the population of isolates was performed by co-culturing the strains on agar plates in pairs. An automated extraction method was designed and developed to determine if these antagonistic effects observed between the isolates were induced by co-culturing and if the antifungal compounds were accumulating spatially in the interaction zone. Ultraviolet (UV) and Mass Spectrometry (MS) imaging techniques were developed to visualize the spatial distribution of metabolites and facilitated the identification of induced secondary metabolites upon the microbial interactions.

The chemical profiles of the differential expression after applications of these three culture-based approaches were compared. As proof of concept of induced new chemistry, fourteen induced secondary metabolites only produced under these approaches were purified and identified. The structures of six of these compounds, some of them bioactives, were new and herein elucidated.

Results confirmed that the exploitation of the microbial richness associated to arid areas of Andalusia, combined with the application of integrated culturing methods, can favour the activation of fungal cryptic metabolic pathways towards the discovery of new bioactive natural products with the potential to become new leads for the development of future therapeutic agents.

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LIST OF ABBREVIATIONS

28S	28S ribosomal RNA
A-2058	Human melanoma cell line
A-549	Human lung cancer cell line
ADP	Adenosine Diphosphate
DAD	Diode Array Detector
DESI	Desortion Electrospray Ionization
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
ED50	Effective dose for 50% of the population
ESI	Electrospray Ionization
HAT	Histone Acetyl Transferase
HCA	Hierarchical Cluster Analysis
HDAC	Histone Deacetilase
HepG-2	Human hepatocellular carcinoma cell line
HPLC	High-Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HT-29	Human colon cancer cell line
HTS	High-Throughput Screening
ICA	Independent Component Analysis
IMS	Mass Spectrometry Imaging
ITS	Internal Transcribed Spacer
IUV	Ultraviolet Imaging
LC-MS	Liquid Chromatography Mass Spectrometry
m/z	Mass to charge ratio
MALDI	Matrix Assisted Laser Desorption Ionization
MCF-7	Human breast cancer cell line
MF	Molecular Formula
Miapaca-2	Human pancreas cancer cell line
MS	Mass Spectrometry
MVSA	Multicomponent Variable Statistical Analysis
NAD	Nicotinamide Adenine Dinucleotide
NMR	Nuclear Magnetic Resonance
NP	Natural Product
NRPS	Non-Ribosomal Peptide Synthetase
OSMAC	One Strain Many Compounds
PCA	Principal Component Analysis

List of abbreviations

PKS	Polyketide Synthase
PLS	Partial Least Square
rDNA	Ribosomal Deoxyribonucleic Acid
RT	Retencion Time
SAHA	Suberoylanilide Hydroxamic Acid
SBHA	Suberohydroxamic Acid
SIMCA	Soft-Independent Method of Class Analogy
SIMS	Secondary Ion Mass Spectrometry
SM	Secondary Metabolite
SOM	Self-Organizing Map
UPGMA	Unweighted Pair Group Method using Arithmetic Averages
UPLC	Ultra-High-Performance Liquid Chromatography
UV	Ultraviolet
VIS	Visible
VP	Volcano Plot

I. INTRODUCTION

1. ANTIFUNGAL AND ANTITUMORAL NATURAL PRODUCTS DRUG DISCOVERY

Natural Products (NPs) are defined as small organic molecules synthesized by an organism that are not essential for its own growth, development and reproduction. They have been a prolific and continued source for new lead compounds in drug discovery. Approximately 35% of the new chemical entities approved as drugs over the past 30 years are derived from NPs and a significant proportion of synthetic drugs have also been inspired by natural structures (Newman and Cragg, 2016).

Drug discovery from microbial NPs involves a long and complex process (Figure 1), that traditionally starts with the collection of different environmental samples from various sources, including, plants, soil, feces, invertebrates, sediments, lichens, rocks, bird nests, water or plant materials. New resources and technologies have been developed to enhance this step of the process. Improvements in sampling methods now provide opportunities to isolate previously uncultured microorganisms, including the adaptation of the dilution to extinction technique for a high-throughput culturing of fungi (Collado *et al*., 2007), the use of Ichip for the cultivation of new microbial species (Nichols *et al*., 2010), or the access to extreme environments (Chavez *et al*., 2015). The combination of improved isolation and cultivation methods with culture-independent techniques (based on molecular biology) have revealed that microbial ecosystems possess unexpectedly high levels of diversity and complexity (Rampelotto, 2013). Microbial isolates are selected on the basis of their taxonomic diversity and fermented in different conditions, obtaining extracts which will then undergo a screening process against different targets of therapeutic interest. Once the extract activity is confirmed, bioassay-guided isolation and structural elucidation is attempted. The most potent and selectively active compounds must also pass *in vitro* toxicological and pharmacological studies, as well as an optimization process that assures the safety requirements for the lead compound (Peláez, 2006).

Despite the success stories of NPs as direct and indirect sources of drugs and unequivocal structural diversity (Newman and Cragg, 2016), large pharmaceutical companies have discontinued their NP screening programs. Several factors have contributed to this situation. With the popularization of the high-throughput screening (HTS) programs among the pharmaceutical companies in the late 90s, classical approaches used in NP discovery programs requiring multiple bioactivity-guided purification steps of hit compounds, could not compete with the tight deadlines of the HTS based programs and the use of combinatorial chemistry libraries of compound. The continued rediscovery of already known structures and screening interferences, due to the complex mixture of chemistries in the extracts, represented additional limitations (Potterat and Hamburger, 2013).

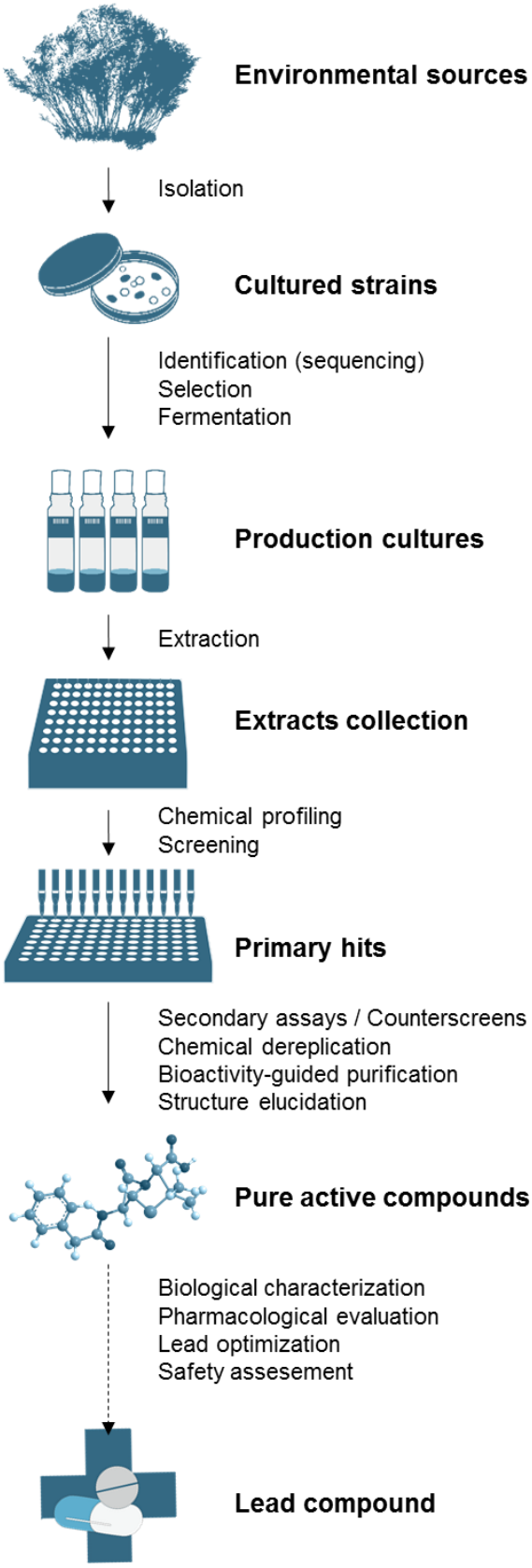

Figure 1. *Early stages of the drug discovery process from microbial NPs.*

New challenges in NP research have been a driving force for the development of technological advances in the field of NP drug discovery. Specifically, the contribution of HPLC chromatography coupled to mass spectroscopy detection has been enormous, providing structural information on the chemical composition of a complex mixture with high sensitivity, maximizing resources during bioactivity-guided fractionation. Nowadays, molecular formula prediction can be obtained from very small amounts of material (from pictograms to micrograms), allowing an efficient identification of already known molecules. Furthermore, laboratory automation has facilitated, the rapid and efficient generation of large libraries of low complexity mixtures and pure NPs more suitable for some HTS assays.

Despite achievements in the detection of novel active compounds, there remains an urgent need for faster and more efficient strategies to generate and track new chemical diversity. The most important challenges, in this context, are the generation of high quality sample collections representing the broadest chemical diversity and the correlation of biological and chemical data during the recurring purification and identification steps of the active components (Potterat and Hamburger, 2013).

Continuous advances in microbial genomics and metagenomics have definitively revealed that the earth's overwhelming biodiversity is still under-explored for NP discovery (Charlop-Powers *et al*., 2014). Innovations in microbial cultivation and synthetic biology have provided a mechanism to access the potential chemical space of NPs through the engineered production of NPs from uncultivated or difficult to grow species in heterologous host models (Milshteyn *et al*., 2014). Improved knowledge on NP biosynthesis has facilitated the expression of cryptic pathways through the rational manipulation of the biosynthetic machinery to produce NPs and their engineered variants. Finally, the combination of microbial engineering, bioinformatic approaches, analytical technologies, fast detection and isolation tools of active compounds, and structural prediction have fostered a new paradigm in NP research (Shen, 2015).

Historically, microorganisms are one of the most important sources of antifungal and anti-cancer drugs. Over a span of ten years, between 2006-2015, a total of 142 NPs with antifungal activity were reported, 16 of them confirmed by means of *in vivo* infection models (Zida *et al*., 2017). Antifungal modes of action of commercial fungicide drugs include: the interaction with ergosterol biosynthesis (e.g. polyenes), the inhibition of 1,3-β-d-glucan synthase (e.g. echinocandins), the inhibition of the synthesis of cell wall components (e.g. chitin and mannoproteins), the inhibition of sphingolipid synthesis (e.g. serine palmitoyltransferase, ceramide synthase, inositol phosphoceramide synthase), and the inhibition of protein synthesis (e.g. sordarins).

Introduction

Fungal diseases kill more than 1.5 million people and affect over a billion. However, they are still a neglected topic by public health authorities even though most deaths from fungal diseases are avoidable (Bongomin *et al*., 2017). Skin infections caused by fungal pathogens are common and can be successfully treated in most cases with current drugs (Garber, 2001). Nevertheless, more severe types of infections (i.e phaeohyphomycosis, aspergillosis, candidiasis, cryptococcosis and mucormycosis) are becoming a serious problem to human health as a result of the advance of other medical treatments that affect immunocompromised patients such as HIV infected patients, transplant recipients, cancer patients with chemotherapy treatments, diabetics or patients treated with steroids or antibiotics (Raman Sharma, 2010).

Moreover, the resistance of clinical isolates to treatment with antifungal agents has increased alarmingly in the last decade reducing the efficacy of traditional therapies (Bongomin *et al*., 2017). Amphotericin B, a broad-spectrum antifungal drug, has been the last line of defence for 30 years. It is an excellent fungicide, but it has important nephrotoxicity based on tubular abnormalities and a mild increase in serum creatinine (Degray, 2002). Imidazoles and triazoles, a breakthrough discovery in the 80s and 90s as inhibitors of the ergosterol biosynthesis in fungi, frequently develop resistance, giving rise to recurrent infections. Echinocandins, developed in the 90´s are the most recent class of commercial antifungal agents that act on the fungal cell wall by inhibiting glucan synthesis. Caspofungin (Cancidas®), the first member of this class, as well as micafungin (Mycamine®) and anidulafungin (Eraxis®), have addressed many of the issues to treat severe fungal infections, but emerging resistances against these drugs require a continuous pursuit of new antifungal molecules (Arendrup and Perlin, 2014).

A continued importance exists for the application of NPs as therapeutic agents for cancer treatment, due to the increasing recurrence of mammalian tumours, the reduction of clinical efficacy of current anticancer treatments and their severe side-effects, highlighting the need to develop alternative molecules or potentiators for reducing doses and side-effects (Ali *et al.,* 2012). In the last decades, NPs have played an important role as sources of chemotherapeutic agents with the discovery of new antitumor agents from microbes such as doxorubicin, actinomycin and epothilones (Kinghorn *et al*., 2009). Marine organisms, including both invertebrates and microorganisms, have provided either classical non-specific cytotoxic moieties (e.g. DNA, enzymes, microtubules) or new compounds targeting macromolecules specifically expressed in cancer cells (e.g. oncogenic signal transduction pathways). Some high-profile examples of new compounds include the microtubule interfering agents Tasidotin (ILX-651) and Soblidotin (TZT 1027) produced by bacterial strains or the vascular disrupting agent Plinabulin (NPI-2358) obtained from a marine *Aspergillus* sp. isolate (Bhatnagar and Kim, 2010). Similar to marine organisms, fungal endophytes have the capacity to produce effective antitumoral agents such as vincristine, vinblastine or camptothecin (Chandra, 2012) and for this

reason, similar to marine organisms, fungal endophytes will continue to be explored as a relevant source for the discovery of new anticancer agents.

2. FUNGI AS A SOURCE OF BIOACTIVE SECONDARY METABOLITES

Fungi are known to produce a wide range of NPs often called secondary metabolites (SMs), some of them are deleterious to humankind like mycotoxins while others have beneficial applications, like antibiotics or immunosuppressants (Demain and Fang, 2000). These SMs represent a high chemical diversity and multiple functions, such as, communication signals during coexistence with other microorganisms and virulence factors during pathogenic interactions with plants and animals (Macheleidt *et al.,* 2016). Since the discovery of penicillin from *Penicillium notatum* by Fleming in 1928, many bioactive molecules produced by fungi have been described. Many of the filamentous fungi known to produce a wide variety of SMs are included in the division *Ascomycota* subdivision *Pezizomycotina* (Cox, 2007).

The orders *Chaetothryales, Diaporthales, Dothideales, Eurotiales, Helotiales, Hypocreales* and *Pleosporales* are known to produce a chemically diverse range of molecules. ***Chaetothryales*** comprises morphologically diverse dematiaceous fungi that cause skin infections in humans and animals and can be found in a wide variety of habitats. Members of this order are *Capronia, Cyphellophora, Exophiala, Metulocladosporiella* or *Phialophora* (Réblová *et al*., 2013*)*. These genera include producers of biologically active SMs such as fellutamides or ascochlorin (Xu *et al*., 2011). The order ***Diaporthales*** contains important phytopathogenic and endophytic fungi being *Diaporthe, Phomopsis, Gnomonia* and *Sydowiella* some of which produce bioactive compounds like cytochalasins (Chapla *et al.,* 2014) and diaportheones (Bungihan *et al*., 2011). ***Eurotiales*** are also known as 'green and blue molds' and include some of the most studied genera: *Aspergillus, Emericella, Penicillium* or *Monascus.* These filamentous fungi are producers of molecules with proven biological activities and an effective clinical use as antimicrobials, antifungals and/or cytotoxic agents, such as various penicillins, echinocandin B, brefeldin A, mycophenolic acid or sterigmatocystin (Bills *et al*., 2009). ***Helotiales*** is a diverse order that includes many ericaceous and grass endophytes (Zijistra *et al*., 2005), as well as several saprobic fungi from the genera *Pezicula, Cryptosporiopsis, Glarea, Botrytis, Mollisia* and *Lachnum,* (encompassing species that produce antifungal compounds such as pneumocandin Ao (Bills *et al*., 1999) and virgineone (Ondeyka *et al*.,2009)). ***Hypocreales*** contains important entomopathogen, endophytic and saprobic like *Acremonium, Beauveria***,** *Calonectria, Cylindrocladium, Hypocrea, Fusarium, Cladobotryum, Tolypocladium* and *Trichoderma,* with species that produce the active biomolecules cyclosporin, apicidin, equisetin and the enniatins among others (Bills *et al.*, 2009).

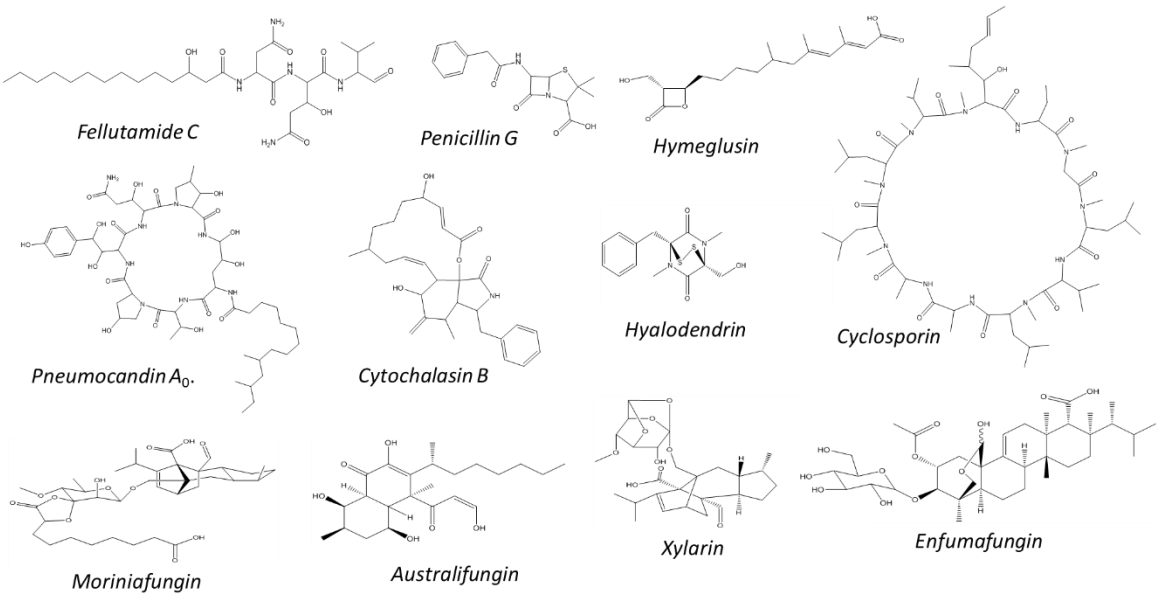

***Figure 2**. Chemical structure of relevant bioactive SMs.*

The order ***Pleosporales*** includes over 332 distinct genera of filamentous fungi, many of which are endophytes, epiphytes and parasites. Some genera can be noted such as *Alternaria, Ascochyta, Curvularia, Lophiostoma, Pleospora, Phaeosphaeria, Phoma* and *Preussia* because they include species that produce the antifungal compounds phomafungin (Herath *et al*., 2009), phaeofungin (Singh *et al*., 2013), australifungin (Mandala *et al*., 1995) and phomasetin (Hazuda *et al*., 1999).

Many antifungal compounds have also been isolated from other fungal orders or even phyla. Enfumafungin produced by *Hormonema carpetanum* ***(Dothideales),*** morniniafungin by *Morina pestalozioides* (***Amphisphaeriales)***, or Xylarin by *Xylaria* sp. (***Xylariales)*** are some highlighted examples (see Table 1). Interestingly, several **Basidiomycota** strains have been reported as antimicrobial compound producers with important applications in the biocontrol of phytopathogens, e.g. Strobilurins isolated from *Strobilurus tenacellus* and Coprinol from *Coprinus* sp. (Sivanandhan *et al*., 2017). Studies that included a wide taxonomic diversity of fungal strains have confirmed that the production of bioactive SMs is widespread among the fungal kingdom (Bills *et al*., 2009).

Table 1. Relevant bioactive SMs produced by a wide taxonomical diversity of fungal strains.

Producer strain	Order	Compound	Activity	MoA	Reference
Coprinus spp	*Agaricales*	Lagopodin A and B	Antimicrobial	unknown	Agger *et al.*, 2009
Coprinus sp.	*Agaricales*	Coprinol	Antimicrobial	unknown	Johansson *et al.*, 2001
Strobilurus tenacellus	*Agaricales*	Strobilurins A & B	Antifungal	respiration inhibitors	Anke *et al.*, 1977
Morinia pestalozzioides	*Amphisphaeriales*	Moriniafungin	Antifungal	Elongation Factor 2	Basilio *et al.*, 2006
Metulocladosporiella sp.	*Chaetothyriales*	Fellutamides C and B	Antifungal	20S Proteosome	Xu *et al.*,2011
Sirococcus conorum	*Diaporthales*	Conocandin	Antifungal	TCA cycle	Muller *et al.*, 1976
Hormonema carpetanum	*Dothideales*	Enfumafungin	Antifungal	Cell wall	Pelaez *et al.*, 2000
Neosartorya sp.	*Eurotiales*	11-desacetoxywortmannin	Antifungal	Phosphoribosyl complex	Haefliger and Hauser, 1973
Aspergillus sydowi	*Eurotiales*	Mulundocandin	Antifungal	RHO1, PTP3	Roy *et al*.,1987
Paecilomyces inflatus	*Eurotiales*	YW 3548, BE-49385	Antifungal	Cell wall	Kushida *et al.*, 1999
Penicillium spp	*Eurotiales*	Penicillin G	Antibacterial	β lactam antibiotic	Yocum *et al.*, 1980
Aspergillus nidulans	*Eurotiales*	Echinocandin B	Antifungal	glucan synthase inhibitors	Nyfeler and Keller 1974
Aspergillus oryzae	*Eurotiales*	Aspirochlorine	Antifungal	Elongation factor 3	Sakata *et al.*, 1983
Lachnum virgineum	*Helotiales*	Virgineone	Antifungal	unknown	Ondeyka *et al.*,2009
Glarea lozoyensis	*Helotiales*	Pneumodandin Ao	Antifungal	glucan synthase inhibitors	Bills *et al.*, 1999
Trichoderma longibrachiatum	*Hypocreales*	Desulphated ergokonin A	Antifungal	Cell wall	Vicente *et al.*, 2001
Trochothecium sp.	*Hypocreales*	Furanocandin	Antifungal	Cell wall	Magome *et al.*, 1996
Fusarium larvarum	*Hypocreales*	Parnafungins	Antifungal	mRNA polyadenylation	Bills *et al.*, 2009
Trichoderma viride	*Hypocreales*	Viridiofungin A	Antifungal	Sphingolipid biosynthesis	Harris *et al.*, 1993
Tolypocladium inflatum	*Hypocreales*	Cyclosporine	Immunosuppressant	calcineurin–phosphatase pathway	Laupacies *et al.,* 1982
Fusarium oxysporum	*Hypocreales*	Vincristine	Antitumoral	binding to tubulin, induced apoptosis	Kumar *et al.*, 2013
Trichoderma atroviride	*Hypocreales*	Camptothecin	Antitumoral	Topoisomerase inhibitor	Pu *et al.*, 2013
Colispora cavincola	*Incertae sedis*	Colisporifungin, cavinafungins	Antifungal	IRE1, SPC3, SEC11, SEC63	Ortiz-Lopez *et al.*, 2015
Scopulariopsis candida	*Microascales*	Hymeglusin, (Antibiotic 1233A)	Antifungal	Ergosterol pathway, ERG 13	Omura *et al.* 1987
Preusia australis	*Pleosporales*	Australifungin	Antifungal	Ceramide synthase inhibitor	Mandala *et al.*, 1995
Phaeosphaeria sp.	*Pleosporales*	Phaeofungin	Antifungal	Potentiates caspofungin,cause ATP leakage	Singh *et al.*, 2013
Ganoderma lucidum	*Polyporales*	Ganodermin	Antifungal	unknown	Wang and Ng, 2006
Chaetomium sp.	*Sordariales*	new papulacandin	Antifungal	Cell wall	Roemer *et al.*, 2011
Hyalodendron sp.	*Trichosporonales*	Hyalodendrin	Antimicrobial	zinc homeostasis	Stiwell *et al.*, 1974
Xylaria sp.	*Xylariales*	Xylarin	Antifungal	Elongation Factor 2	Schneider *et al.* 1995

3. FUNGAL ENDOPHYTES FROM ARID REGIONS

The combination of different geo-climatic factors (e.g. altitude, average environment curvature and distance to the sea) that affect the Iberian Peninsula have shaped a variety of habitats in it, such as deserts, salt marshes, wetlands, Eurosiberian and Mediterranean forests (Riera, 2006). These privileged areas for biodiversity studies, have a great richness in flora and fauna and a number of unique endemic plants. Specifically, the Iberian southeast is one of the most arid regions in Europe, characterized by a hot and dry Mediterranean climate, with gypsum and saline soils. Despite its desolate appearance, with vegetal coverings that do not frequently surpass 20% of their surface, arid areas of Andalusia are a territory with high biodiversity, in which more than 1000 taxa have been registered (Mota *et al*., 2004). Large part of the arid and semi-arid zones of Andalusia are found in the province of Almería (e.g. Tabernas desert) and the 'Hoya of Guadix-Baza' in Granada, as well as the important salt marsh of El Margen, also in Granada.

Unique ecological conditions have influenced the development and diversification of a wide range of endemic plant species in these arid areas where specimens of the *Aizoaceae*, *Chenopodiaceae* or *Plumbaginaceae* families are frequently found, yet they are rarely encountered outside these environments (Meyer, 1986; Parsons, 1976). Thus, exclusive endemisms such as the shrub *Euzomodendrom bourgaeanum (Brassicaceae)*, are present in the Tabernas desert of Almería, or *Salsola papillosa (Chenopodiaceae)*, distributed exclusively in the Almería and Murcia provinces. Furthermore, the vegetation that has evolved in the isolated salt marsh of El Margen consist of a wide number or endemic halophytic plants such as *Limonium insigne, Limonium majus (Plumbaginaceae), Centaurea dracunculifolia (Asteraceae)* or *Gypsophila tomentosa* (*Caryophyllaceae*) (Lendínez *et al*., 2011)

Symbiosis is an intimate, long-term, and specific association between organisms of two or more species. The concept of symbiosis was first proposed by Anton de Bary in 1879 and can be broadly applied to a multitude of relationships of beneficial, neutral or harmful nature. The diversification of Ascomycota (the most species-rich fungal phylum) is reflected in its multiple symbiotic strategies with vascular plants, with several symbiotic lifestyles: mutualistic, commensalistic, parasitic and pathogenic; in response to host genotype and environmental factors (Leavitt *et al*., 2012). Fungal endophytes are mostly known to infect plants without causing symptoms. Latent pathogens represent a relatively small proportion of endophytic partnerships, also including latent saprophytes and mutualistic species (Zabalgogeazcoa, 2008). However, there are some exceptions to this situation as some reports confirm that plants from arid ecosystems can be heavily colonized by dark septate fungi possessing melanized hyphae (Porras-Alfaro *et al*., 2007, 2008).

In addition to the specific anatomical adaptations (small leaves called microphylly, well developed root systems, waxy coating on surfaces) to arid conditions, gypsophilous plants are also known to form specific associations with mycorrhizal fungi (Porras-Alfaro *et al*., 2014). Mycorrhizal colonization of arid plants could be essential when nutrients and water are limited, conditions that are common in gypsum soils where their colonization capacity increases from 26 to 96% (Palacio *et al*., 2012). Recently, the abundance, diversity and host affiliations of endophytes in leaves and stems of representative plants from the Arizona Desert have been examined confirming that, despite their low isolation frequency, endophytes are species-rich and phylogenetically-diverse within *Preussia*, the most common genus isolated (Massimo *et al*., 2015).

Moreover, a partial characterization of Ascomycotina from Spain indicates that fungi associated to plants of arid areas of Almeria and Bética Mountains remain to be discovered (Bills *et al*., 2012). Some reports also support the hypothesis that the region harbours many other undescribed fungi. Novel beneficial endomycorrhizal fungus-host relationships were observed from the rhizospheres of endemic plants from Sierra de Baza and Sierra Nevada (Palenzuela *et al.,* 2008, 2010) and the discovery of the endophyte *Kabatiella bupleuri* sp. *nov*, recently described on the endemic plant *Bupleurum gibraltaricum* (Figure. 3; Bills *et al*., 2012) support this hypothesis. From fore mentioned discoveries we have hypothesized that plants from arid zones of Andalusia could also be an important source of unexplored fungal biodiversity.

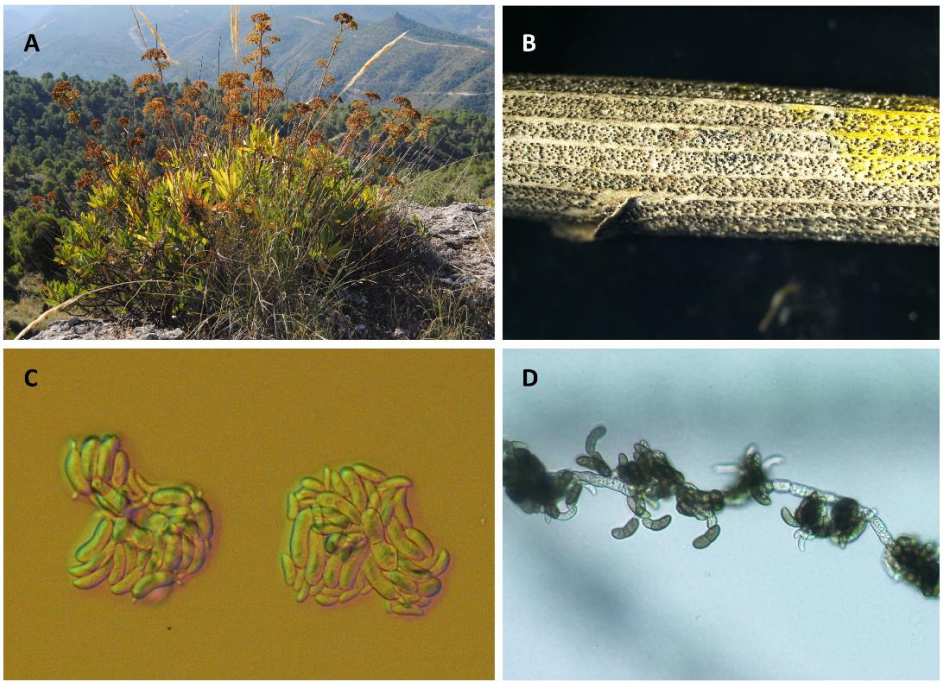

Figure 3. *Bupleurum gibralatarum and Kabatiella bupleuri. A) B. gibraltarum. Mesa de Fornes, Granada, Spain. Habit. B) K. bupleuri. Acervular conidiomata on dead flower rachis. C) Germinating K. bupleuri conidia from conidiomata. D) K. bupleuri in culture developed hyphae, conidiogenesis, and conidia (Bills et al., 2012).*

4. CULTURE-BASED APPROACHES TO TRIGGER THE PRODUCTION OF NEW SECONDARY METABOLITES

Recent studies in the field of genome mining have revealed the poorly studied biosynthetic potential of filamentous fungi. The number of predicted gene clusters coding for the production of SMs in several fungi were far larger than the number of SMs reported by these microorganisms (Cichewiz *et al.*, 2010). Genes encoding the enzymes responsible for the biosynthesis of SMs are often clustered in fungal chromosomes reminiscent of bacterial operons (Sarikaya-Bayram *et al*., 2015). SM gene clusters tend to be transcriptionally co-regulated by a wide variety of genetic mechanisms that range from specific regulations by DNA binding transcription factors to global regulations *via* changes in the chromatin structure (Palmer and Keller, 2010; Gacek and Strauss, 2012). Interestingly, global regulatory protein complexes involved in fungal differentiation in response to environmental signals (including light, nutrient deprivation or pH), have also been shown to regulate SM gene clusters; narrowing the link between the induction of secondary metabolism and fungal development (Brakhage, 2013).

4.1 Influence of cultivation conditions and the OSMAC approach

The chemical composition of the culture media (carbon, nitrogen, phosphorus, sulphur or iron sources) is one of the most important factors that influence the SM profiles of extracts obtained following the fungal fermentation process. The nutritional requirements that microorganisms need to produce certain SMs are complex, varied, and specific for each strain. One of the earliest examples where this phenomenon was observed was the suppression of the biosynthesis of penicillin in the presence of lysine (Demain, 1957).

Besides the nutrient composition of the fermentation medium used, abiotic culture conditions (e.g. static or shaken conditions) influence the growth rate of fungi and, consequently, the final SMs produced. One of the key abiotic factors influencing fungal growth in liquid medium is agitation, as it is directly related to the availability and accessibility of oxygen in solution. Not only can small changes in the culture medium formulation or in a physical-chemical parameter of the fermentation affect the amount of a specific compound, but they also influence the diversity of SMs produced by a microorganism (Figure 4; Scherlach and Hertweck, 2009). The term “OSMAC approach” (One Strain Many Compounds) was introduced to describe an array of different conditions, which included variations of the media composition, aeration, shape of culturing flasks and temperature, in which a strain (*Aspergillus ochraceus* in Bode *et al*., 2002) increased its SM diversity by producing additional compounds in response to perturbations in environmental parameters

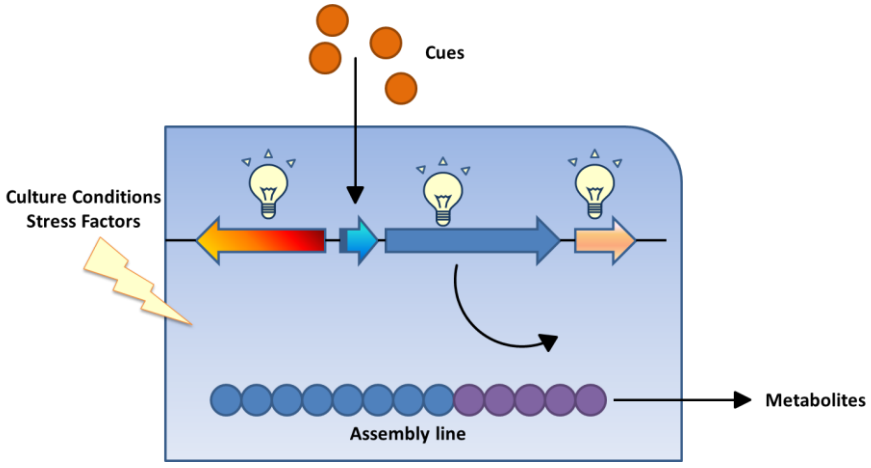

***Figure 4**. Influence of culture conditions and stress in the biosynthesis of NPs (Scherlach and Hertweck, 2009)*

One approach is to use inert supports as substrates for fermentations in static conditions, which also affects the growth and morphology, and in turn, the production of SMs (Bigelis *et al*., 2006). This was confirmed when the production of echinocandin B was evaluated in the anamorphic state of *Aspergillus* strains, and an increase in the production was observed when fermented on an inert vermiculite substrate in static conditions (NPF2-verm), (see Figure 5; de la Cruz *et al.,* 2012).

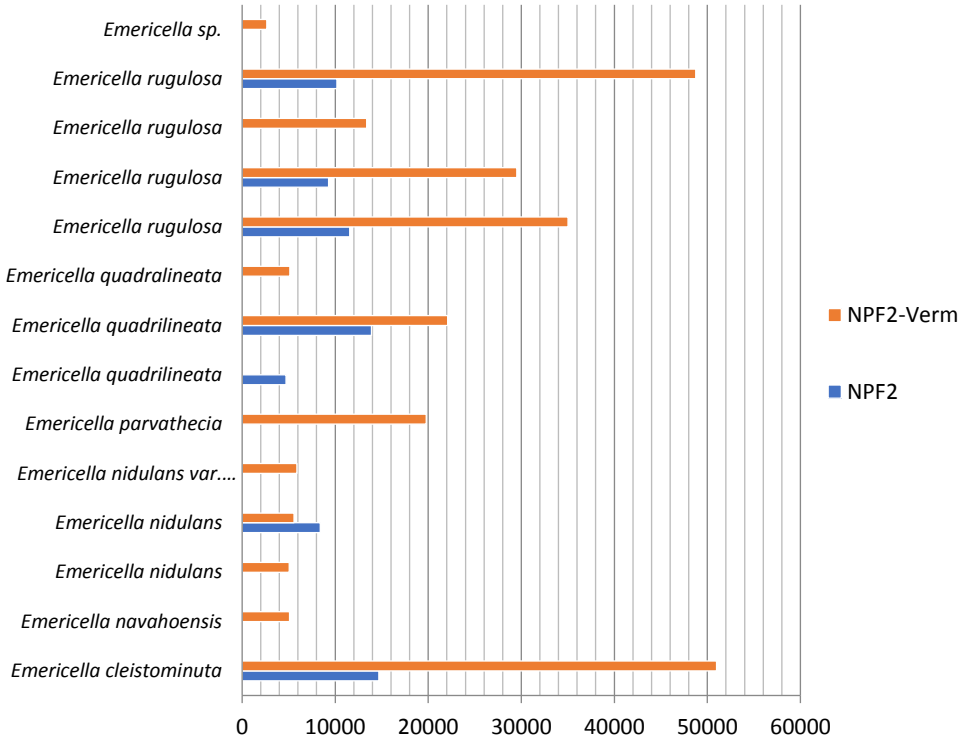

***Figure 5**. Production of the echinocandin B among Emericella species grown in NPF2 liquid medium versus vermiculite solid fermentations (de la Cruz et al., 2012).*

Another factor influencing metabolite production profiles is the differentiation stage of the fungal culture during its growth phase and especially conidiation. Some SMs are only produced during sporulation. Different genes and regulatory molecules play a major role in the expression of SMs. Small hormone-like molecules, such as conidiogenone that induces conidiation, may also induce changes in SM profiles (Roncal *et al*., 2002).

4.2 Adsorptive polymeric resins

Some SMs may undergo biotransformation processes during cultivation or even suppress their biosynthesis. A strategy to avoid such issues is the addition of adsorptive polymeric resins to capture the products of interest *in situ*, removing the SMs from the culture broth once they are excreted, thus reducing negative feedback repression to production or preventing post-modifications. Furthermore, in culture conditions, fungi can produce a wide variety of antifungal compounds, sometimes up to the maximum concentrations that their tolerance/resistance mechanisms allow. Under such circumstances, production levels of specific bioactive SMs can be below the detection level, and therefore will not be detected in screening programs or can cause problems in industrial production optimizations based on highly mutated overproducer strains (Singh *et al*., 2010). The use of adsorptive resins to increase the production titers of different bioactive metabolites have been frequently used in the fermentation of myxobacteria and actinomycetes but have rarely been reported in fungi (Frykman *et al*., 2006).

Some examples of SMs obtained by addition of resins to submerged cultures of different microorganisms include, among others, the production of the antibiotic rubradirin by *Streptomyces achromogenes* subsp*. rubradiris* in which the addition of resins XAD-2, XAD-7, XAD-16, HP-20 and HP-21 led to an increase of 2-4 times of rubradirin in 3-4 days of fermentation (Marshall *et al*., 1990).The controlled production of Paulomicin where the resin prevents the conversion of paulomicin to paulomenol during the fermentation process (Marshall *et al*., 1987). The production of BMS-182123 by *Penicillium chrysogenum* an inhibitor of tumour necrosis factor alpha (TNF-α), whose production is increased 5.5 times when adding XAD-8 resin to the fermentation medium (Warr *et al*., 1996). The generation of migrastatin and isomigrastatin, potential anticancer agents, produced by *Streptomyces platensis* (Woo *et al*., 2002), whose production is enhanced in presence of XAD-16 resin. The production of ambruticin and jerangolides, new antifungal compounds produced by the myxobacterium, *Sorangium cellulosum* which achieved an improved production when fermented in the presence of XAD-16 resin (Gerth *et al*., 1996) or the generation of noamicine, a new antimicrobial active polyketide isolated from an actinomycete of the genus *Actinomadura* when fermented in the presence of the HP-20 resin (Igarashi *et al*., 2012). A recent study has also described the positive effect of adsorptive polymeric resins such as Amberlite® XAD-7, on the production titers of certain SMs during fungal fermentation (Figure 6).

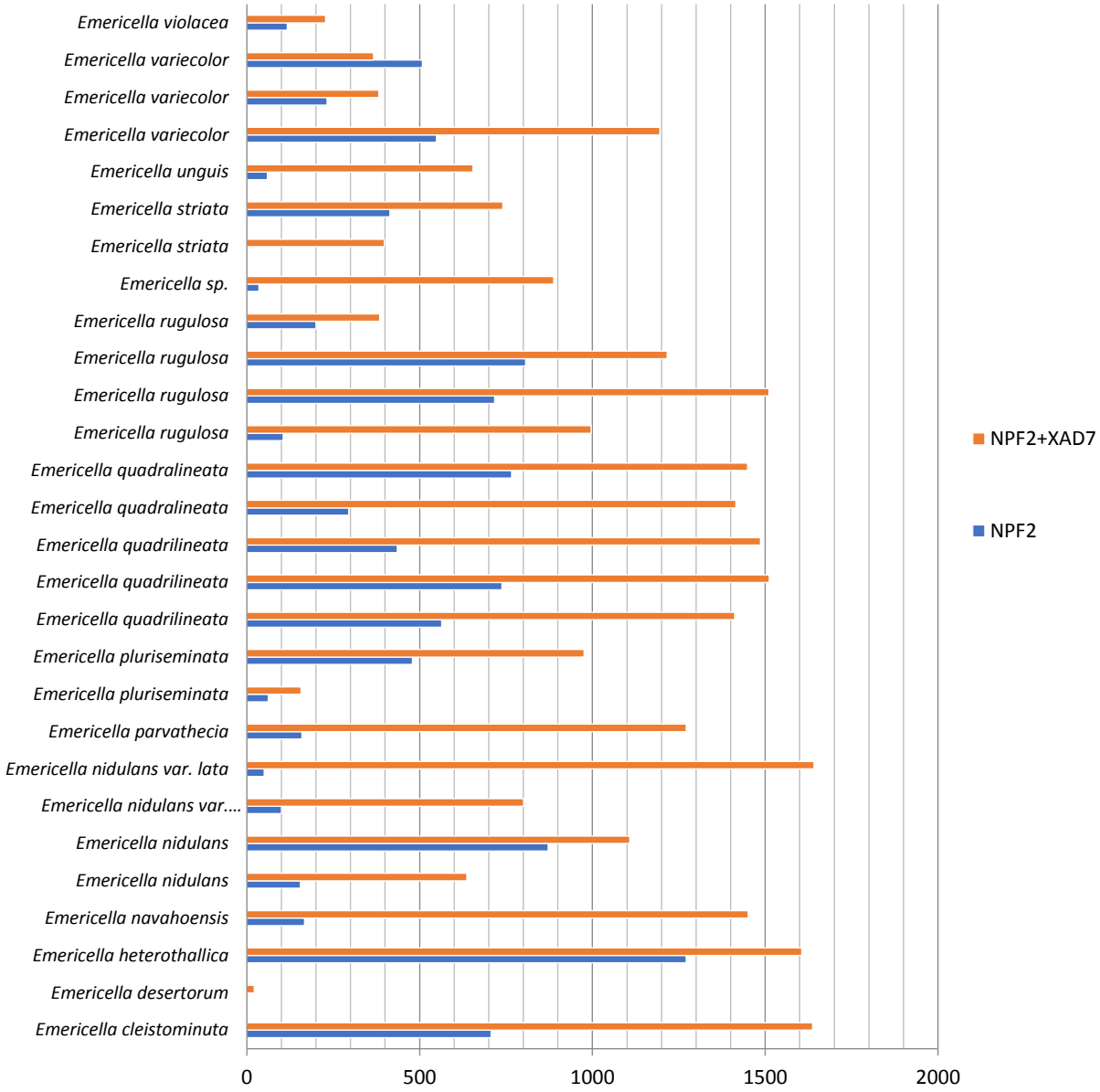

***Figure 6**. Effects of adsorptive resins on the production of desferritriacetylfusigen (UV 218 nm; with and without XAD-7 resin; de la Cruz et al., 2012)*

4.3 Epigenetic modifiers

Gene expression in fungi can be regulated by DNA methylation and chromatin remodelling through histone modification by acetylation, methylation, phosphorylation, and ADP-ribosylation (Chiang *et al*., 2011; Cichewicz, 2012). Combinations of different histones and DNA modifications may result in different states of chromatin compaction and/or transcription levels, for open structures with high transcriptional areas (euchromatin), or more condensed structures, with less transcriptionally active areas (heterochromatin) (Figure 7), (Grewal and Jia, 2007). Euchromatin has few histones present so that "house-

keeping" genes are readily transcribed. However, epigenetic factors will still act on histones in euchromatin to expose wound DNA for transcription. Heterochromatin is so tightly packed with histones that it is not readily transcribed. Many stress related genes are located in heterochromatin, where transcription will occur by the unravelling of associated histones by epigenetic factors. Indeed, many chromatin signalling mechanisms have been found to regulate the expression of SMs (Strahl and Allis, 2000).

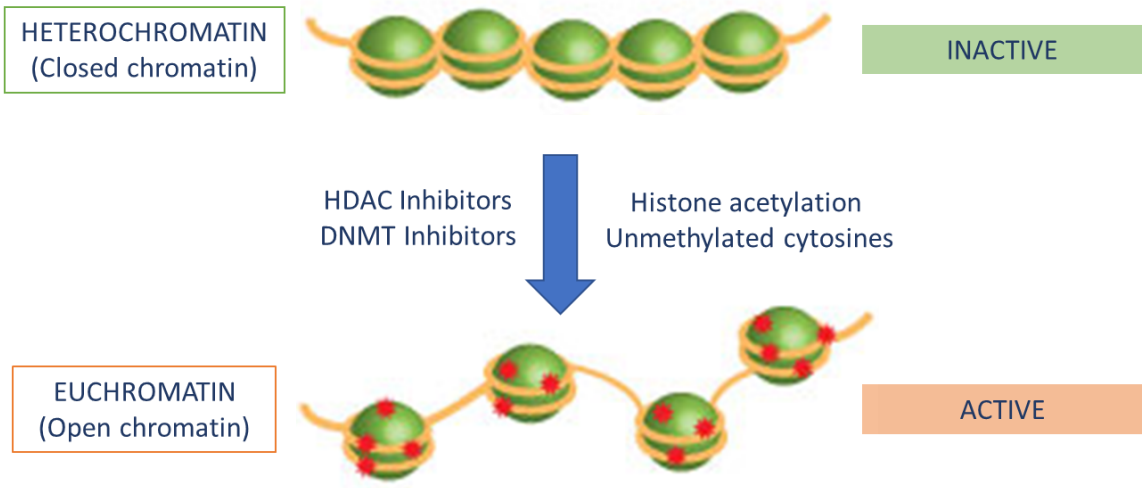

***Figure 7**. Alteration of chromatin compaction by deacetylation/methylation of histones regulated by HDAC and DNMT Inhibitors.*

Moreover, several factors have been indicated to contribute to the coordinated expression or repression of gene clusters involved in the production of fungal SMs. One of the few known conserved global regulators of SMs in fungi is the putative methyltransferase LaeA (Bok and Keller, 2004). LaeA has been shown to control a large percentage of SM clusters as well as several aspects of fungal development in several genera (Kamerewerd *et al*., 2011; Butchko *et al*., 2012; Karimi-Aghcheh *et al*., 2013). Furthermore, LaeA forms a heterotrimeric protein complex with the two members of the fungal-specific velvet family transcription factors (VeA and VelB) that coordinate fungal development and SM production (Bayram et al., 2008). Interestingly, in addition to LaeA, three other methyltransferases have recently been discovered to interact with VeA (Palmer *et al*., 2013; Sarikaya-Bayram *et al*., 2015). Thus, the epigenetic modifiers may affect the VelB/VeA/LaeA complex inducing the production of SMs in fungi.

There are two classes of chemical epigenetic effectors commonly described in literature to modify the chromatin compaction and transcription levels: DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors (Figure 8). Both effectors can lead to increase cluster specific gene expression of genes encoding fungal SMs (Soukup and Keller, 2012).

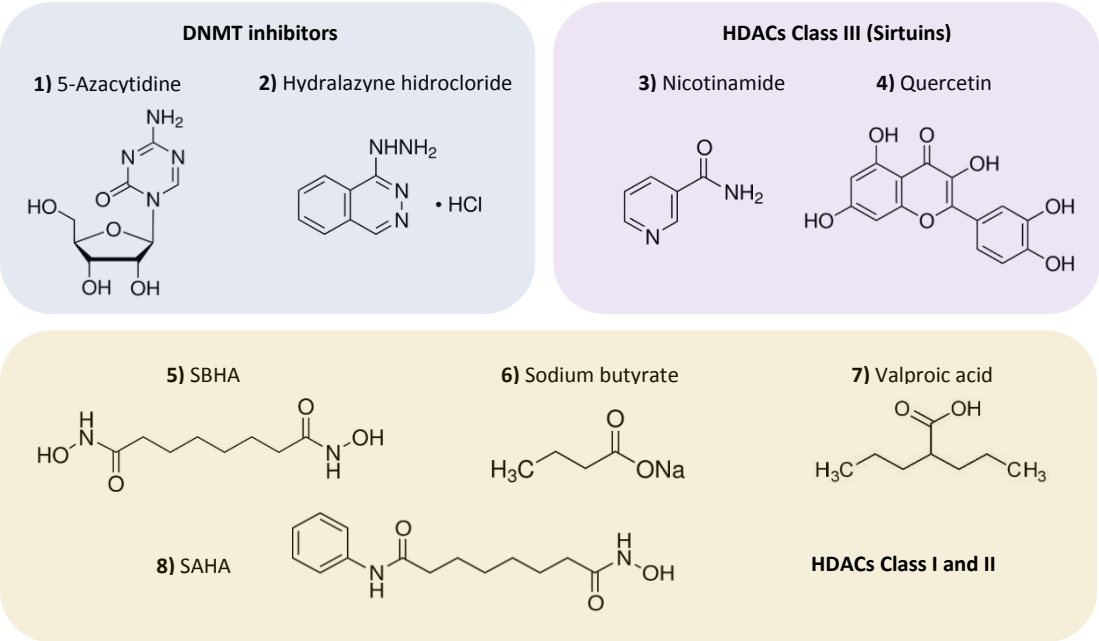

***Figure 8**. DNMT and HDACs inhibitors*

DNA methylation occurrence in fungi is still unclear as it is not universally distributed. In some fungi, DNA methylation occurs in cytosine residues and has often been associated with a genome defence process (Montanini *et al*., 2014). The cytosine methylation of DNA is involved in maintaining a repressive chromatin state in eukaryote organisms (Suzuki and Bird, 2008). DNMT inhibitors such as 5-azacitidine and hydralazine hydrochloride have demonstrated their ability to reduce DNA methylation by silencing different resistance genes and cellular processes in a wide variety of fungal species (Cichewicz, 2012; Aghcheh and Kubicek, 2015).

Histone acetylation is a reversible process mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Both are correlated with histone acetylation in chromatin and gene expression (Wang *et al*., 2009). In fact, HDAC inhibitors prevent the "repacking" of DNA into histones; causing the "unpacked" DNA to remain exposed for transcription and are the most commonly used *in vitro*. There are three classes of HDACs identified in fungi namely I, II and III (Brosch *et al*., 2008), with sirtuins (HDAC class III) being the most studied. Sirtuins are NAD-dependent protein deacetylases involved in a wide variety of biological processes, including transcriptional silencing, regulation of apoptosis, fat mobilization, and stress resistance (Preyat and Leo, 2013). The activities of sirtuins are regulated by nicotinamide, a non-competitive inhibitor that promotes a base-exchange reaction at the expense of deacetylation (Avalos *et al*., 2005), and quercetin, a NP belonging to the flavonoid family, that acts as an activator of their activities (Moore *et al*., 2012). Quercetin antioxidant properties modulate the expression of specific enzymes by its effect on protein kinases, inhibiting DNA topoisomerases and regulating gene

expression (Moskaug *et al*., 2004). The addition of nicotinamide to culture medium has resulted in the induction of the newly described SMs chaetophenol G and cancrolides A and B in *Chaetomium cancroideum* (Asai *et al*., 2016).

Several reports have described the variable effects of HDAC class I and II inhibitors on fungi (Table 2). Suberoylanilide hydroxamic acid (SAHA), suberohydroxamic acid (SBHA), sodium butyrate and valproic acid are potent inhibitors of HDACs of these classes. The majority of reported epigenetic studies have used SAHA, a hydroxamic-acid compound with similar HDAC inhibitory effects. Examples of the stimulation of SMs with the addition of SAHA include: the production of a new fungal biosynthetic cyclodepsipeptide (EMG-556) by *Microascus* sp. when cultured with SAHA (Vervoort *et al*., 2011), the new anti-infective cytosporones by the marine fungus *Leucostoma persoonii* (Beau *et al*., 2012) and novel fusaric acid derivatives by the endophyte *Fusarium oxysporum* when SBHA was added to the production medium (Chen *et al*., 2013).

Table 2. Examples of induced SMs by the addition of epigenetic modifiers during fermentation processes.

Compound	Epigenetic modifier	Microorganism	Reference
Lunalides A and B	5-Azacytidine	*Diatrype* sp.	Williams *et al*., 2008
Oxylipins	5-Azacytidine	*Cladosporium cladosporoides*	Williams *et al*., 2008
Novel prenyldepsides	5-Azacytidine + SBHA	*Pestalotiopsis acaciae*	Yang *et al*., 2013
Novel methycoumarins	5-Azacytidine + SBHA	*Pestalotiopsis acaciae*	Yang *et al*., 2013
Chaetoglobosins (510,540 and 542)	Jasplakinolide	*Phomospis asparagi*	Christian *et al*., 2005
Cancrolides A and B	Nicotinamide	*Chaetomium cancroideum*	Asai *et al*., 2016
Chaetophenol G	Nicotinamide	*Chaetomium cancroideum*	Asai *et al*.,2016
Phenolic compounds	Nicotinamide	*Penicillium brevicompactum*	El-Hawary *et al*., 2018
Novel cyclodepsipeptide EMG-556	SAHA	*Microascus* sp.	Vervoort *et al*., 2011
Novel cytosporone	SAHA	*Leucostoma persoonii*	Beau *et al*., 2012
Cladochromes F and G	SAHA	*Cladosporium cladosporoides*	Williams *et al*., 2008
Nygerone A	SAHA	*Aspergillus niger*	Henrikson *et al*., 2009
Novel Fusaric acid derivates	SBHA	*Fusarium oxysporum*	Chen *et al*., 2013
Anthranilic acid	Sodium butyrate	*Penicillium brevicompactum*	El-Hawary *et al*., 2018
Xylarolide A and B	Valproic acid	*Diaporthe* sp.	Sharma *et al*., 2018
Diportharine A	Valproic acid	*Diaporthe* sp.	Sharma *et al*., 2018

Positive effects of both classes of epigenetic modifiers (DNMTs and HDACs) on fungal gene expression have been proven for *Aspergillus niger* where the addition of 5-azacytidine and SAHA promoted changes in the expression of PKS, NRPS, and HPN pathways and increased the transcriptional rates of 47 genes clusters (Fisch *et al*., 2009). Also, in *Aspergillus nidulans* the addition of SAHA activated the *ors*A gene cluster and induced the production of orsellinic acid (Nutzmann *et al*., 2011). Thus, the use of epigenetic modifiers could lead to the development of new techniques, targeting gene expression stimulation, with the hope of inducing the generation of new potentially active NPs.

4.3 Co-cultivation

Microorganisms are in constant interaction with their natural environment, living in close contact and competing for resources with other microorganisms. Their ability to adapt their physiology to match environmental conditions is the reason for the failure of many *in vitro* experiments that do not mimic these phenotypes correctly (Brakhage *et al*., 2008; Brizuela *et al*., 1998, Combès *et al*., 2012). Microbial interactions with different combinations of microorganisms have been extensively reported to modulate fungal metabolism up to the point of inducing the production of new SMs (Table 3). These interactions may lead to the activation of complex regulatory mechanisms, and finally induce the biosynthesis of a broad diversity of, otherwise silent, NPs (Bertrand *et al*., 2014).

Co-culturing has proven to be an effective approach to simulate part of the physiological conditions that occur during fungal interactions in their natural environment and may have a potential for the discovery of new molecules (Bader *et al*., 2010; Brakhage, 2013; Netzker *et al*., 2015). Whereas traditional screening processes involve culturing a single microbial strain to discover new bioactive compounds, the use of co-cultures presents new opportunities (and challenges) for the potential activation of cryptic biosynthetic pathways. Microorganisms can sense the presence of other microorganisms, this may trigger responses derived from the interaction, that sometimes may promote changes in the morphology of the microorganisms involved in the interaction coupled to a possible induction of different SMs, enzymes, or other components (Hynes *et al*., 2007).

Such culture conditions have facilitated the detection of compounds which are not produced when individual fungi are cultured alone (Hynes *et al*., 2007). Analytical methods have allowed the detection of changes in their metabolite profiles depending on the interacting fungi (Peiris *et al*., 2008; Rodrıguez-Estrada *et al*., 2011). Different co-culturing techniques have been developed for this purpose including the use of liquid or solid media, but all approaches consist on culturing two or more microorganisms in a single confined environment to facilitate interactions with the intent to induce the expression of further chemical diversity (Bertrand *et al*., 2013). Some examples of compounds derived from these interactions include a long-distance growth inhibitor

reported between *Trichophyton rubrum* and *Bionectria ochroleuca* (Bertrand *et al*., 2013), the production of acremostatins A-C, by *Acremonium* sp. when cultivated in a mixed culture with *Mycogone rosea* (Degenkolb *et al*., 2002), the production of aspergicin by a co-culture of two *Aspergillus* species (Zhu *et al*., 2011) or the production of cyclo-(l-leucyl-trans-4-hydroxy-l-prolyl-d-leucyl-trans-4-hydroxy-l-proline), when two mangrove-derived strains of *Phomopsis* sp. and *Alternaria* sp. were co-cultured in liquid media (Li *et al*., 2014).

More examples of the induction of new SMs during microbial interactions were the interaction between the phytopathogen *Fusarium verticillioides* and the endophyte *Ustilago maydis* (Rodriguez-Estrada *et al*., 2011), the increase in production of certain SMs during the interaction of *Penicillium pinophilum* and *Trichoderma harzianum* (Nonaka *et al*., 2011), the induction of 12 metabolites by *Paraconiothyrium variable* when co-cultured with *Fusarium oxysporum* (Combés *et al*., 2012), as well as many other cases detailed in Table 3.

Table 3. Examples of induced SMs by the co-culturing of two microorganisms (A and B).

Compound	Microorganism (A)	Microorganism (B)	Reference
11-O-methylpseurotin A2	*Aspergillus fumigatus*	Streptomyces bulli	Rateb *et al*., 2013
4-hydroxysulfoxy-2-dimethyltielavin P	*Trichophyton rubrum*	*Bionectria ochroleuca*	Bertrand *et al*., 2013
8-Hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methylether	unidentified fungus	unidentified fungus	Li *et al*., 2011
acremostatins A–C	*Acremonium* sp.	*Mycogone rosea*	Degenkolb *et al*., 2002
aspergicin, noaspergillic acid	*Aspergillus* sp.	*Aspergillus* sp.	Zhu *et al*., 2011
autramide	*Aspergillus austroafricanus*	*Bacillus subtillis*	Ebrahim *et al*., 2016
cyclo-proline	*Phomopsis* sp.	*Alternaria* sp.	Li *et al*., 2014.
disulphide glionitrin A	*Sphingomonas* sp.	*Aspergillus fumigatus*	Park *et al*., 2009
emericellamide A and B	*Emericella* sp.	*Salinospora arenicola*	Oh *et al*., 2007
enacyloxin	*Gluconobacter* sp.	*Aspergillus oryzae*	Watanabe *et al*., 1982
libertellenones A-D	*Libertella* sp.	α-proteobacterium	Oh *et al*., 2005
marinamide	unidentified fungus	unidentified fungus	Zu *et al*., 2006
pentalenic acid and nocardamine	*Aspergillus fumigatus*	*Streptomyces leeuwenhoekii*	Wakefield *et al*., 2017
pestalone	*Pestalotia* sp.	α-proteobacterium	Cueto *et al.,* 2001
secopenicillide C	*Penicillium pinophilum*	*Trichoderma harzianum*	Nonaka *et al*., 2011

5. EVALUATION OF THE CHEMICAL DIVERSITY IN MICROBIAL FERMENTATIONS

Comparisons of the chemical profiles of microorganisms have been used as a valuable method for determining and prioritizing fermentation conditions that produce a higher chemical diversity for a strain of interest (Tormo *et al.,* 2003). The combination of HPLC-UV(-DAD) in gradient mode is a widely used technique in the last decades to obtain SM profiles of complex extracts from microbial fermentations. This technique allows a good separation of a large range of SMs with different polarities, and UV or DAD detection for small amounts of NPs. HPLC Studio, a software developed for comparing SM profiles, has made possible the unattended computer analyses of HPLC-UV chromatograms of extracts to then evaluate the best set of fermentation conditions for obtaining the maximum chemical diversity, in terms of polarity, of SMs and production titers (Garcia and Tormo, 2003). This software determines the number of different peaks and the amount of detectable material in a given extract by HPLC-UV compared to its control fermentation medium. It also, automatically, prioritizes the different fermentation conditions tested for each microorganism. The application of this software enabled the selection of the best production media with the most diverse and least overlapping chemical profiles for taxonomically diverse actinomycetes in the study (Tormo *et al*., 2003).

Ultra-high-pressure liquid chromatography (UPLC) coupled to electrospray mass spectroscopy (ESI-MS) is currently the most frequently used technique for the qualitative and quantitative analysis of microbial SM profiles. In contrast to UV detection, MS has a higher sensitivity and specificity and facilitates the identification of components present in complex samples by database matching (Songsermsakul and Razzazi-Fazeli, 2008; Villas-Bôas *et al*., 2005). Media components can interfere with the analytes of interest and exhibit similar UV signals, whereas the principle of MS is based on the ionization fingerprints of molecules and their fragments in a sample, their separation according to mass-to-charge ratios (*m/z*) and further detection by their respective *m/z* abundance of mass fragments (Gross, 2011). A variety of ionization modes and types of mass analyzers are currently available, but the two most commonly used metabolomic approaches are targeted or untargeted depending on the kind of data obtained from a sample (Dettmer & Hammock, 2004) (see Figure 9).

Introduction

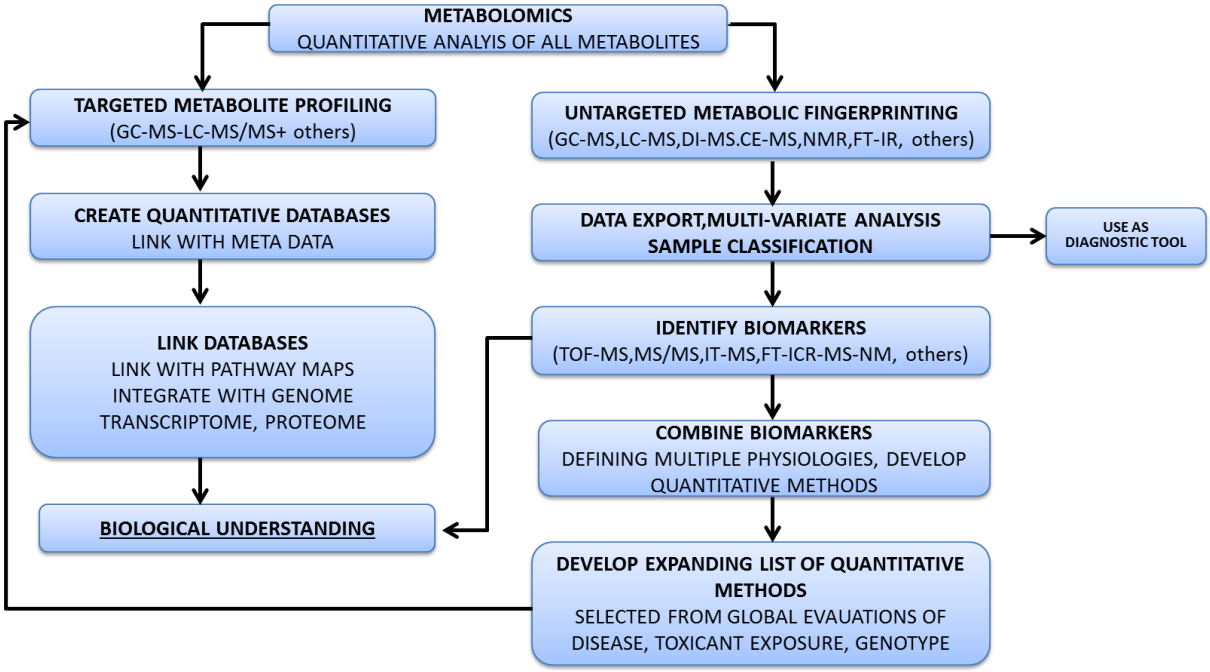

***Figure 9**. Strategies for metabolomic studies based on LCMS data (based on Dettmer et al., 2007).*

Targeted metabolic profiling focuses on the analysis of a group of metabolites either related to a specific metabolic pathway or a class of compounds, while untargeted metabolic fingerprinting compares patterns or "fingerprints" of metabolites as a whole, that change in response to disease, toxin exposure, environmental or genetic alterations (Dettmer *et al*., 2007). In untargeted metabolic fingerprinting studies where a HPLC-MS equipment is used, the m/z values, retention times, and intensities represent the metabolic fingerprint of the analysed sample and this data is exported for sample classification using multivariate data analysis. Metabolomic software is required for raw data pre-treatment with the aim to reduce background noise, the alignment of mass spectra and mass chromatograms, the automated picking and annotation of mass spectrometric data, and the reduction of the complexity of three-dimensional matrixes into bi-dimensional ones to facilitate the statistical treatment of the information (Dettmer *et al*., 2007).

In addition, different statistical methods are required for metabolomic data analysis and their selection depends on the knowledge of the samples and the aim of the study. For classification studies where the identity of the SMs is unknown, the initial data analysis tool used to infer a sense of similarity among the samples is Principal Component Analysis (PCA), but Hierarchical Cluster Analysis (HCA) is used as a second stage for detailed classification studies. For example, PCA and HCA applied to the untargeted metabolite profiles of four *Suillus* species (Basidiomycetes) revealed several biomarkers useful for the identification and classification of these species (Heinke *et al*., 2014). Supervised methods,

where you have training populations of samples whose data is already known, and when looking for biomarkers, partial least square (PLS) or soft-independent method of class analogy (SIMCA) are the most recommended methods (Jonsson *et al*., 2005). The different mass spectrometry-based untargeted metabolomics for microbial SMs with their applications and disadvantages have been reviewed recently by Covington *et al*., 2017 (Table 4):

Table 4. Overview of methods for metabolomic data analysis in the context of SMs (Covington *et al*., 2017).

Method	Description	Applications	Disadvantages
PCA and projections to latent structures	MVSA to identify significant covariance within data	• Identifying data outliers • Strain prioritization • Grouping samples • Compound prioritization	• Less effective with large datasets
Self-organizing maps	Organizes features into a 2-dimensional map based on feature response experimental conditions	• Grouping samples • Compound prioritization • Comparing large number of experimental conditions	• Less effective with small numbers of conditions
Molecular networking	Organizes features into a connectivity network based on similarities in molecular fragmentation patterns	• Compound prioritization • Compound dereplication	• Fragmentation can vary with instrument parameters

PCA and PLS are the most commonly used methods of multivariate statistics applied to SM discovery. The PCA makes no assumptions about the data (unsupervised) and identifies the sources of the highest variance across the samples with the aim to distinguish them (Bro and Smilde, 2014). The PLS method assumes that changes within the data are largely driven by a subset of latent variables, which are not themselves measured within the data, such as experimental conditions. This method identifies latent vectors, within the data, that describe the maximal covariance between groups defined by the user (supervised) (Covington *et al*., 2017). In both cases, samples are represented via score plots where the data describes sample covariances, hence similar samples will be grouped nearby in principle component space while dissimilar ones will be far away according to their most significantly varying features.

The PCA score plot provides a visual representation of variances between m/z ions, retention times and intensities of microbial SM profiles, which has been proven to be useful for strain differentiation studies in NP discovery (Hou *et al*., 2012). But cluster analysis and heatmaps on their own are more powerful tools for chemotaxonomical studies when widely diverse strains need to be classified. Fungal taxonomy based on SM profiling has successfully been used in a large number of ascomycete and some

basidiomycete genera (Frisvad *et al*., 2008; Heinke *et al*., 2014). For example, the analysis of *Ascochyta* and *Phoma* strains (21), based on similarity of metabolic profiles, resulted in species-specific clusters consistent among the strains of the same species isolated from different geographical regions and/or host plants (Figure 10, (Kim *et al*., 2016)).The observed metabolic features were highly conserved among strains in genetically differentiated populations and therefore were used as a species-specific taxonomic characters/biomarkers (Kim *et al*., 2016).

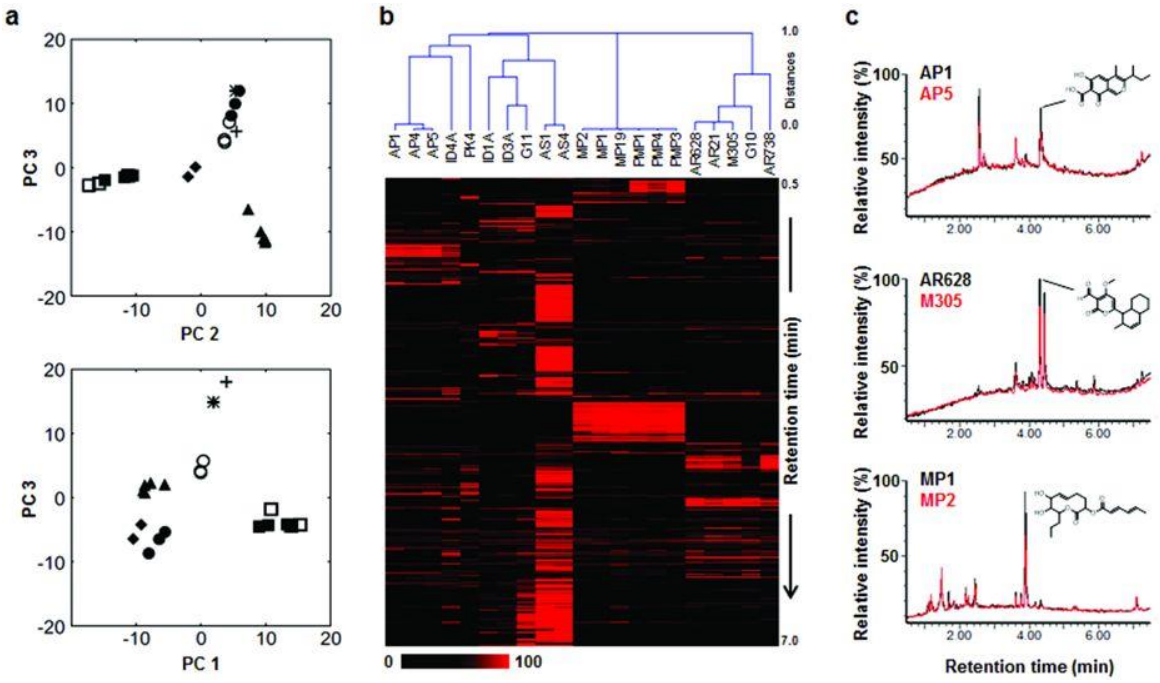

Figure 10*. Example of the application of PLS supervised multivariate analysis using metabolic profiles obtained for 21 Ascochyta and Phoma strains to achieve the chemotaxonomical study (**a**) PCA scores plots showing the variation in the metabolic profiles from 21 fungal strains. (**b**) Dendrogram of 21 strains relying on chemical similarity, based on UPGMA clustering method. The heatmap of respective metabolites corresponding to each strain is presented. (**c**) Total ion chromatograms and chemical structure of distinct biomarkers for different Ascochyta species (Kim et al., 2016).*

Another technique used for data clustering and visualization is the self-organizing map (SOM) (Kohonen, 2001), which facilitates processing a variety of biological data. Fungal images, DNA and MS data have been analyzed using this methodology (Marique *et al*., 2012; Delgado *et al*., 2015). SOM analyses the raw data using an artificial neuronal network in a 2-dimensional grid based on feature response patterns across all experimental conditions. This methodology can be more effective for representing experiments with a high number and diversity of samples than PCA. The application of this methodology has enabled the identification of several SMs produced in response to a battery of stimuli in *Streptomyces coelicolor* (Goodwin et al., 2015).

Volcano plots (VPs) have recently been used for the visualization of statistical results of omics data, such as, differential expression of genes measured through microarrays (Li, 2012). It is the most important application of the univariate analysis by a pairwise comparison of individual variables from two different classes in a data set (modified and control conditions). They typically represent the significances from a statistical t-test (as a p-value) on the y-axis and fold-quantity changes on the x-axis. Thus, metabolites that have a relatively low fold-change between the two sample classes in the volcano plot appear near the centre of the chart, whereas the metabolites that have significant p-values are found in the upper-right or upper-left corners (Hur *et al*., 2013). This plot provides a visual representation of both, the fold changes in expression between two sample classes, as well as, the confidence level of that measurement, for each metabolite produced among the two different fermentation conditions compared. This approach has successfully been applied to identify induced SMs in response to epigenetic perturbation in *Aspergillus nidulans,* where the VP showed an induced metabolite that could be annotated by accurate mass and verified by MS fragmentation (Figure 11; Albrigh *et al*., 2015).

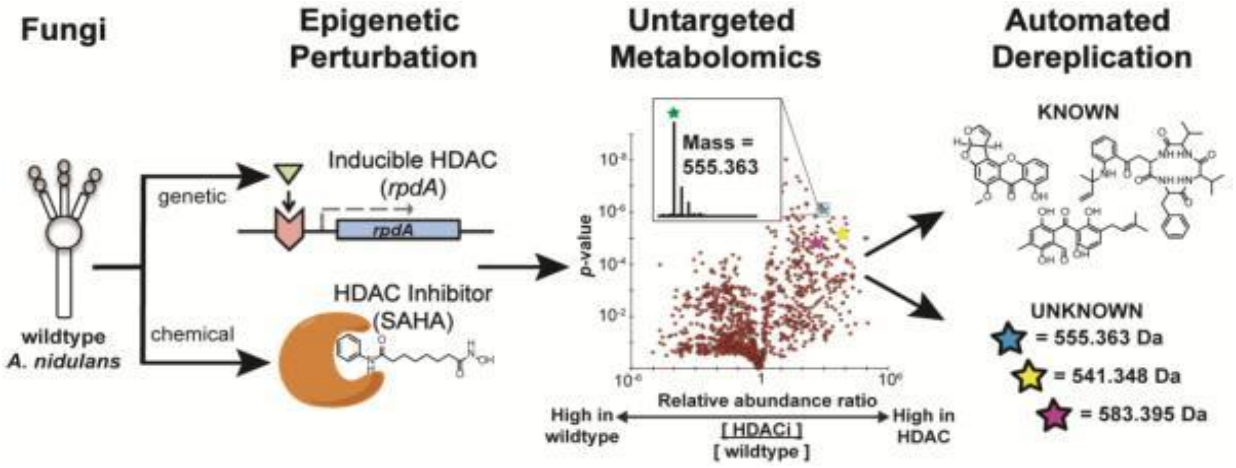

Figure 11*. Epigenetic perturbation and metabolomic analysis by Volcano plots in A. nidulans (from Albrigh et al., 2015).*

To address the challenges of discerning chemical communication between microorganisms, an emerging methodology is Imaging Mass Spectrometry (IMS); a technology applied for the detection of metabolic signals involved in microbial interactions. IMS has successfully been developed for agar cultivated microbial samples to provide a three-dimensional visualization of metabolite distribution (Yang *et al*., 2009; 2012). Metabolomic features generated via IMS consist of *m/z* ion distribution versus abundances in agar cultures. The *m/z* images obtained by these techniques provide a deep insight into microbe - microbe interactions, metabolic exchanges and identification of SMs (Figure 12). Matrix assisted laser desorption ionization (MALDI) is a commonly used technique for IMS in microbiology. MALDI requires the application of an ionization matrix to enable a laser-facilitated ionization of embedded metabolites from cells and agar.

Introduction

Several applications of MALDI-IMS have been performed to visualize spatial metabolite information and to identify the induced ones during microbial interactions (Yang *et al*., 2009; Moree *et al*., 2012; Graupner *et al*., 2012; Scherlach *et al*., 2013). Recently, miniaturized Desorption Electrospray Ionization (DESI) or nano-DESI and secondary ion mass spectrometry (SIMS), which do not require the addition of an ionizing matrix to the sample, have also been applied to obtain IMS (Hsu and Dorrestein, 2014). The information about the spatial distribution of the SMs produced during microbial interaction that can be obtained using these techniques may boost the discovery of induced NPs.

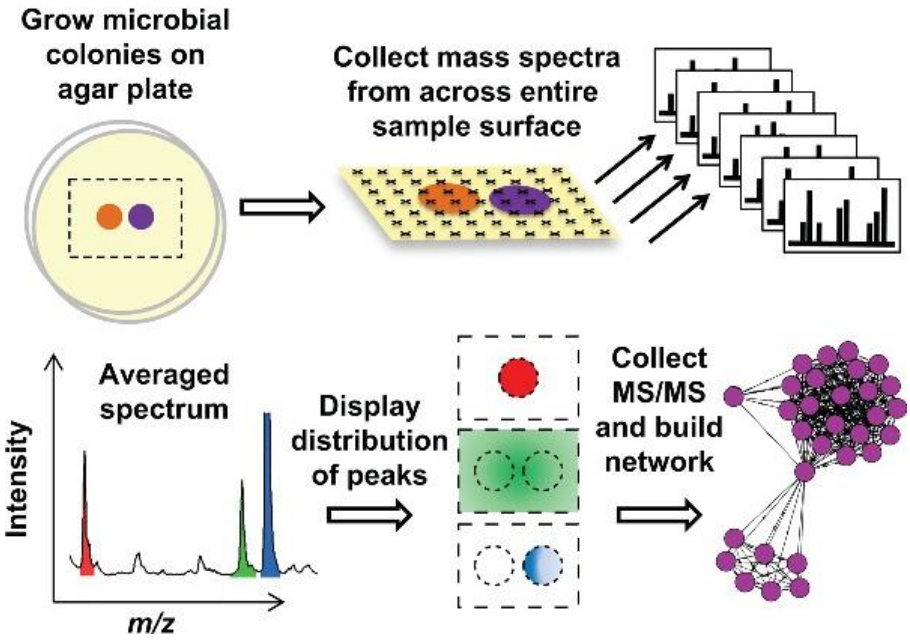

Figure 12. *Scheme of IMS to assist the identification and visualization of the distributions of SMs in microbial interactions (from Stasulli & Shank, 2016).*

Several strategies have been developed to identify and annotated SMs of microbial origin, from fingerprint matching of ultraviolet visible spectroscopy (UV-VIS), mass spectrometry (MS) and molecular formula (MF), nuclear magnetic resonance (NMR) to molecular networking maps based on MS/MS fragmentations. The process to identify already known SMs by fingerprint comparison with reference standard data is known as dereplication. A fast dereplication process is a key step during drug discovery and microbial pathway elucidation programs (Nielsen *et al*., 2011). HPLC-MS is often combined with UV spectra acquired with a diode array detector (DAD) to provide information on chromophores which can be used for database searching (de la Cruz *et al*., 2012), but normally DAD is used as a second criterion after a high resolution MS based search because many metabolites share the same chromophore and therefore UV spectra are quite less informative for the identification. In addition, available databases such as Dictionary of Natural Products (DNP) or AntiBase index more accurate mass data than UV data (Neilsen and Larsen, 2015).

Initially, the combination of LC-UV-Low resolution mass spectrometry (LC-LR-MS) collecting mass spectra in both the positive and negative modes was applied for the identification of SMs from complex microbial extracts (Bills *et al*., 2009). Databases were created based on UV-VIS spectra, retention time (RT) and positive and negative low-resolution MS fingerprints of microbial SM standards acquired or accumulated among the different research projects, and software applications compared the LC‑UV‑MS data of every components present in the samples to these fingerprints for ID matching. Nowadays advanced dereplication methodologies by LC-DAD-HRMS in combination with high resolution commercial and public databases are currently used because LR-MS fingerprints are very dependent on the detection spectrometer whereas, molecular formula inferred from high resolution spectrometers are universal. Accurate mass and predicted molecular formula, in combination with strain taxonomical information, can now be used for searching accurate identity of SMs in these databases (Martin *et al*., 2014).

Fragmentation spectra, acquired through tandem MS, is another useful tool for SM dereplication. Metabolite fragmentation patterns observed through MS/MS analysis can be matched to those in databases to identify metabolites with similar fragmentation patterns. The limitation of this approach, right now, is the low number of SMs with fragmentation spectra available in public databases (Covington *et al*. 2017). However, in recent years, the application of MS/MS networking has been used to cluster structurally related SMs, defining the concept of Molecular families (MFs) such as "structurally related molecules based on their mass spectral fragmentation patterns" (Nguyen *et al*., 2013). Following this strategy, 58 molecules, including analogs, have been dereplicated from diverse microbial samples, confirming that MS/MS networking is an alternative approach for SM dereplication (Yang *et al*. 2013). In addition, the combination of MS/MS networking and IMS has been a useful strategy for chemical identification and characterization of several microbial interactions and the discovery of new NPs (Boya *et al*., 2017).

Nevertheless, the identification of tentative new SMs will always require additional efforts for compound identification: scale up-fermentation, whole broth extraction, solvent partition, low and high-pressure fractionations, purification and structural elucidation (with MS and NMR experiments). Bioassay-guided fractionation of microbial extracts in combination with the described dereplication approaches has successfully been applied for focusing on the isolation and identification of new bioactive SMs (Perez-Victoria *et al*., 2016).

6. CURRENT STATUS OF FUNGAL NATURAL PRODUCT DISCOVERY

Innovative drug discovery from natural products requires a multidisciplinary approach, applying available and innovative technologies, starting with targeted improvements in the isolation, characterization and genome mining of microorganisms to maximize their potential machinery to produce SMs. Advances in analytical chemistry and Omics analysis have increased the capacity to process a large amount of data in NP drug discovery. Computational and chemometric studies of this data provide a comparative metabolomic tool to identify new molecules and show the untapped richness of SM diversity predicted by whole genome sequencing.

During the past decade, genome mining has added a whole new dimension to fungal NP research (Wiemann and Keller, 2014). The technological developments in genome and transcriptome sequencing technologies have resulted in an exponential number of fungal sequence data generated. Mining of these genomic resources have led to the identification of more than 160 fungal biosynthetic gene clusters that encode the enzymatic pathways for specialized metabolite production as well as global regulators involved in their expression (van der Lee and Medema, 2016). Genetic manipulations are an efficient strategy for the discovery of novel fungal NPs. One clear example of this is the disruption of the global regulator LAeB in *Aspergillus nidulans,* resulting in the discovery of four novel NPs (Lin *et al*., 2018). In addition, once a biosynthetic gene cluster is identified and characterized, the production yield of the corresponding SMs can be improved by heterologous expression. Several heterologous expression systems have been developed for fungi, providing a potent tool for the biosynthesis of bioactive SMs from diverse fungal strains. Yeast and filamentous fungi have been developed as heterologous hosts. Several biosynthetic pathways were expressed in surrogate hosts by using this approach, resulting in the successful generation of SMs such as, citrinin, aphidicolin, paxilline and pleuromutilin (Alberti e*t al.*, 2017).

Comparative genomics of multiple strains that produce structurally related compounds and knowledge-based prediction strategies have been used with fungi for SM gene cluster discovery. For instance, the comparison of the PKS genes of *Penicillium aethiopicum* and *P. chrysogenum* have been used to search for the griseofulvin and viridicatumtoxin gene clusters (Cacho *et al*. 2015). However, as variations in the sequence of the gene cluster that encodes for the same SMs may exist, the success of these procedures depend on an accurate annotation of the genomes of producing organisms.

The compilation and the analysis of the metabolome data obtained for multiple fungal culture conditions can be correlated to the expression of key genes and help to identify associated biosynthetic gene clusters through genomic and transcriptomic experiments. The application of these approaches using the right combination of strains and/or culture

conditions (so that as many biosynthetic gene clusters as possible are expressed in at least a few condition/strain combinations), has the potential to accelerate the rate of new fungal natural product discovery. A clear example is the application of the traditional OSMAC approach or the addition of several epigenetic modifiers on a fungal population where the production of some SMs was induced in certain fermentation conditions. Indeed, this strategy followed by phenotypic HTS could enable the identification of targeted active compounds (van der Lee and Medema, 2016). The combination and application of these innovative technologies on a diverse community of fungal strains offers the opportunity to increase the effectiveness in discovering and engineering fungal biosynthetic pathways that encode the production of new SMs.

REFERENCES

- Agger, S., Lopez-Gallego, F., & Schmidt-Dannert, C. (2009). Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. Molecular Microbiology. 72(5): 1181-1195. doi:10.1111/j.1365-2958.2009.06717.x.
- Aghcheh, R.K., Kubicek, C.P. (2015). Epigenetics as an emerging tool for improvement of fungal strains used in biotechnology. Appl Microbiol Biotechnol. 99(15):6167-81. doi: 10.1007/s00253-015-6763-2.
- Alberti, F., Foster, G.D. & Bailey, A.M. (2017). Natural products from filamentous fungi and production by heterologous expression. Appl Microbiol Biotechnol 101: 493. doi: 10.1007/s00253-016-8034-2.
- Albright, J.C., Henke, M.T., Soukup, A.A., McClure, R.A., Thomson, R.J., Keller, N.P., Kelleher, N.L. (2015). Large-scale metabolomics reveals a complex response of *Aspergillus nidulans* to epigenetic perturbation ACS Chem. Biol. 10:1535–1541.
- Ali, R., Mirza, Z., Ashraf, G.M., Kamal, M.A., Ansari, S.A., Damanhouri, G.A., Abuzenadah, A.M., Chaudhary, A.G., Sheikh, I.A. (2012). New anticancer agents: recent developments in tumor therapy. Anticancer Res.;32(7):2999-3005.
- Anke, T., Oberwinkler, F., Steglich, W., Schramm, G. (1977). The strobilurins-new antifungal antibiotics from the basidiomycete *Strobilurus tenacellus*. J. Antibiot. 30:806-810. doi: 10.7164/antibiotics.30.806.
- Arendrup, M. C., Perlin, D. S. (2014). Echinocandin resistance: an emerging clinical problem?. Current opinion in infectious diseases, 27(6), 484-92.
- Asai, T., Morita, S., Taniguchi, T., Monde, K., Oshima, Y. (2016). Epigenetic stimulation of polyketide production in Chaetomium cancroideum by an NAD(+)-dependent HDAC inhibitor. Org. Biomol. Chem.14:646-651.
- Avalos, J.L.; Bever, K.M.; Wolberger, C. (2005). Mechanism of sirtuin inhibition by nicotinamide: Altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. Mol. Cell. 18: 855-868.
- Bader, J., Mast-Gerlach, E., Popović, M. K., Bajpai, R., Stahl, U. (2010). Relevance of microbial coculture fermentations in biotechnology. J. Appl. Microbiol. 109: 371-387. doi: 10.1111/j.1365-2672.2009.04659.x.
- Basilio, A., Justice, M., Harris, G., Bills, G., Collado, J., de la Cruz, M., Diez, M.T., Hernandez, P., Liberator, P., Nielsen Kahn, J., Pelaez, F., Platas, G., Schmatz, D., Shastry, M., Tormo, J.R., Andersen, G.R., Vicente, F. (2006). The discovery of

moriniafungin, a novel sordarin derivative produced by *Morinia pestalozzioides*. Bioorg Med Chem. 15;14(2):560-566.

- Bayram, O., Krappmann, S., Ni, M., Bok, J., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.J., Keller, N.P., Yu, J.H., Braus, G.H. (2008). VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science. 320:1504-1506. doi: 10.1126/science.1155888.

- Beau, J., Mahid, N., Burda, W.N., Harrington, L., Shaw, L.N., Mutka, T., Kyle, D.E., Barisic, B., van Olphen, A., Baker, B.J. (2012). Epigenetic Tailoring for the Production of Anti-Infective Cytosporones from the Marine Fungus Leucostoma persoonii. Mar. Drugs. 10: 762-774.

- Bertrand, S., Bohni N., Schnee, S., Schumpp, O., Gindro, K., Wolfender, J.-L. (2014). Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. Biotechnol. Adv. 32: 1180-1204. doi: 10.1016/j.biotechadv.2014.03.001.

- Bertrand, S., Schumpp, O., Bohni, N., Monod, M., Gindro, K., Wolfender, J.-L. (2013). Denovo production of metabolites by fungal co-culture of *Trichophyton rubrum* and *Bionectria ochroleuca*. J. Nat. Prod. 76: 1157-1165. doi: 10.1021/np400258f.

- Bhatnagar, I., & Kim, S.-K. (2010). Marine Antitumor Drugs: Status, Shortfalls and Strategies. Marine Drugs, 8(10): 2702-2720. doi: 10.3390/md8102702.

- Bigelis, R., He, H., Yang, H.Y., Chang, L.P., Greenstein, M. (2006). Production of fungal antibiotics using polymeric solid supports in solid-state and liquid fermentation. J Ind Microbiol Biotechnol. 33:815-826.

- Bills, G., González-Menéndez, V., Platas, G. (2012). *Kabatiella bupleuri* sp. nov. (Dothideales), a pleomorphic epiphyte and endophyte of the Mediterranean plant *Bupleurum gibraltarium* (Apiaceae). Mycologia. 104(4):962-73. doi: 10.3852/12-003.

- Bills, G., Martin, J., Collado, J., Platas, G., Overy, D., Tormo, J.R., Vicente, F., Verkleij, G.J.M., Crous, P.W. (2009). Measuring the distribution and diversity of antibiosis and secondary metabolites in filamentous fungi. SIM News, 133-147.

- Bills, G., Platas, G., Overy, D.P., Collado, J., Fillola, A., Jiménez, M.R., Martín, J., del Val AG, Vicente F, Tormo JR, Peláez F, Calati K, Harris G, Parish C, Xu D, Roemer T. (2009). Discovery of the parnafungins, antifungal metabolites that inhibit mRNA polyadenylation, from the *Fusarium larvarum* complex and other Hypocrealean fungi. Mycologia 101(4):449-72.

Introduction

- Bills, G., Platas, G., Pelaez, F., Masurekar, P. (1999). Reclassification of a pneumocandin producing anamorph, *Glarea lozoyensis* gen. et sp. nov., previously identified as Zalerion arboricola. Mycological Res. 103; 2:179-192.

- Bode, H.B., Bethe, B., Höfs, R., and Zeeck, A. (2002). Big effects from small changes: possible ways to explore nature's chemical diversity. Chem. Biochem. 3: 619-627.

- Bok, J.W., Keller, N.P. (2004). LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryot Cell. 3(2):527-35.

- Bongomin, F., Gago, S., Oladele, R. O., Denning, D. W. (2017). Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision. Journal of fungi (Basel, Switzerland), 3(4), 57. doi:10.3390/jof3040057

- Boya, P.C.A., Fernández-Marín, H., Mejía, L.C., Spadafora, C., Dorrestein, P.C., Gutiérrez, M. (2017). Imaging mass spectrometry and MS/MS molecular networking reveals chemical interactions among cuticular bacteria and pathogenic fungi associated with fungus-growing ants. Sci Rep. 17;7(1):5604. doi: 10.1038/s41598-017-05515-6.

- Brakhage A. A., (2013). Regulation of fungal secondary metabolism. Nat. Rev. Microbiol. 11: 21-32.

- Brakhage, A. A., Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., Hertweck, C. (2008). "Activation of fungal silent gene clusters: a new avenue to drug discovery," in Natural Compounds as Drugs, eds Petersen F., Amstutz R., editors. (Basel: Birkhäuser), 1-12.

- Brizuela, M. A., García, L., Pérez, L., Mansur, M. (1998). Basidiomicetos: nueva fuente de metabolitos secundarios. Rev. Iberoam. Micol. 15: 69-74.

- Bro, R., Smilde, A.K. (2014). Principal component analysis. Anal. Methods. 6:2812-2831.

- Brosch, G., Loidl, P., & Graessle, S. (2008). Histone modifications and chromatin dynamics: a focus on filamentous fungi. Fems Microbiology Reviews, 32(3): 409-439. doi: 10.1111/j.1574-6976.2007.00100.x.

- Bungihan, M.E., Tan, M.A., Kitajima, M., Kogure, N., Franzblau, S.G., Dela Cruz, T.E., Takayama, H., Nonato, M.G. (2011). Bioactive metabolites of Diaporthe sp. P133, an endophytic fungus isolated from Pandanus amaryllifolius. J Nat Med. 65(3-4):606-9. doi: 10.1007/s11418-011-0518-x.

- Butchko, R. A., Brown, D. W., Busman, M., Tudzynski, B., Wiemann, P. (2012). Lae1 regulates expression of multiple secondary metabolite gene clusters in Fusarium verticillioides. Fungal Genet. Biol. 49, 602–612. doi: 10.1016/j.fgb.2012.06.003.

- Cacho, R.A., Tang, Y., Chooi, Y.H. (2015). Next-generation sequencing approach for connecting secondary metabolites to biosynthetic gene clusters in fungi. Front Microbiol. 14; 5:774. doi: 10.3389/fmicb.2014.00774.

- Chandra, S. (2012). Endophytic fungi: novel sources of anticancer lead molecules. Applied microbiology and biotechnology 95, 47–59. doi: 10.1007/s00253-012-4128-7.

- Chapla, V.M., Zeraik, M.L., Ximenes, V.F., Zanardi, L.M., Lopes, M.N., Cavalheiro, A.J., Silva, D.H., Young, M.C., Fonseca, L.M., Bolzani, V.S., Araújo, A.R. (2014). Bioactive secondary metabolites from *Phomopsis* sp., an endophytic fungus from *Senna spectabilis*. Molecules. 22;19(5):6597-608. doi: 10.3390/molecules19056597.

- Charlop-Powers, Z., Milshteyn, A., Brady, S. F. (2014). Metagenomic small molecule discovery methods. Current Opinion in Microbiology, 0, 70–75. doi: 10.1016/j.mib.2014.05.021.

- Chávez, R., Fierro, F., García-Rico, R. O., Vaca, I. (2015). Filamentous fungi from extreme environments as a promising source of novel bioactive secondary metabolites. Frontiers in Microbiology. 6:903. doi: 10.3389/fmicb.2015.00903.

- Chen, H.J., Awakawa, T., Sun, J.Y., Wakimoto, T., Abe, I. (2013). Epigenetic modifier-induced biosynthesis of novel fusaric acid derivatives in endophytic fungi from Datura stramonium L. Nat. Prod. Bioprospect. 3: 20-23.

- Chiang, Y-M., Chang, S-L., Oakley, B. R., Wang, C. C. C. (2011). Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. Current Opinion in Chemical Biology. 15(1): 137-143. doi: 10.1016/j.cbpa.2010.10.011.

- Christian, O. E., Compton, J., Christian, K. R., Mooberry, S. L., Valeriote, F. A., Crews, P. (2005). Using Jasplakinolide to Turn on Pathways that Enable the Isolation of New Chaetoglobosins from *Phomospis asparagi*. J Nat Prod. 68(11): 1592-1597. doi: 10.1021/np050293f.

- Cichewicz, R. (2012). Epigenetic Regulation of Secondary Metabolite Biosynthetic Genes in Fungi. In: Witzany G, editor. Biocommunication of Fungi. Netherlands: Springer 57–69.

Introduction

- Cichewicz, R.H., Henrikson, J.C., Wang, X. and Branscum, K.M. (2010). Baltz, RH, Davies, JE, Demain, AL, Bull, AT, Junker, B., Katz, L., Lynd, LR, Masurekar, P., Reeves, CD and Zhao, H. (eds.), In Manual of Industrial Microbiology and Biotechnology.

- Collado, J., Platas, G., Paulus, B., Bills, G.F. (2007). High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. FEMS Microbiol Ecol. 60(3):521-533.

- Combès, A., Ndoye, I., Bance, C., Bruzaud, J., Djediat, C., Dupont, J., Nay, B., Prado, S. (2012). Chemical communication between the endophytic fungus *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum*. PLoS ONE 7: e47313. doi: 10.1371/journal.pone.0047313.

- Covington, B. C., McLean, J. A., & Bachmann, B. O. (2017). Comparative mass spectrometry-based metabolomics strategies for the investigation of microbial secondary metabolites. Nat Prod Rep. 34(1): 6-24. doi: 10.1039/c6np00048g

- Cox, R.J. (2007). Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. Org Biomol Chem. 5:2010-2026.

- Cueto, M., Jensen, P.R., Kauffman, C., Fenical, W., Lobkovsky, E., Clardy, J. (2001). Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. J Nat Prod. 64(11):1444-1446.

- Degenkolb, T., Heinze, S., Schlegel, B., Strobel, G., Gräfe, U. (2002). Formation of new lipoaminopeptides, acremostatins A, B, and C, by co-cultivation of *Acremonium* sp. Tbp-5 and *Mycogone rosea* DSM12973. Biosci. Biotechnol. Biochem. 66 883–886.

- Delgado, S., Morán, F., Mora, A., Merelo, J.J., Briones, C. (2015). A novel representation of genomic sequences for taxonomic clustering and visualization by means of self-organizing maps. Bioinformatics. 1;31(5):736-744. doi: 10.1093/bioinformatics/btu708

- Demain, A.L. (1957). Inhibition of penicillin formation by lysine. Arch Biochem Biophys. 67:244-245.

- Demain, A.L., Fang, A. (2000). The natural functions of secondary metabolites. Adv Biochem Eng Biotechnol. 69:1-39.

- Dettmer, K., Aronov, P. A., Hammock, B. D. (2007). MASS SPECTROMETRY-BASED METABOLOMICS. Mass Spectrometry Reviews. 26(1): 51-78.

- Dettmer K, Hammock BD. (2004). Metabolomics—A new exciting field within the “omics” sciences. Environ Health Perspect. 112: 396-397.

- De la Cruz, M., Martín, J., González-Menéndez, V., Pérez-Victoria, I., Moreno, C., Tormo, J.R., El Aouad, N., Guarro, J., Vicente, F., Reyes, F., Bills, G.F. (2012). Chemical and physical modulation of antibiotic activity in *Emericella species*. Chem Biodivers. 9:1095-1113.

- Ebrahim, W., El-Neketi, M., Lewald, L., Orfali, R.S., Lin, W., Rehberg, N., Kalscheuer, R., Daletos, G., Proksch, P. (2016). Metabolites from the Fungal Endophyte *Aspergillus austroafricanus* in Axenic Culture and in Fungal-Bacterial Mixed Cultures. J Nat Prod. 22; 79(4):914-22. doi: 10.1021/acs.jnatprod.5b00975.

- El-Hawary, S. S., Sayed, A. M., Mohammed, R., Hassan, H. M., Zaki, M. A., Rateb, M. E., Mohammed, T.A., Amin, E., Abdelmohsen, U. R. (2018). Epigenetic modifiers induce bioactive phenolic metabolites in the marine-derived fungus *Penicillium brevicompactum*. Marine Drugs. 16(8):253. doi: 10.3390/md16080253.

- Fisch, K.M., Gillaspy, A.F., Gipson, M., Henrikson, J.C., Hoover, A.R., Jackson, L., Najar, F.Z., Wagele, H., Cichewicz, R.H. (2009). Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. J. Ind. Microbiol. Biotechnol. 36:1199–1213.

- Frisvad, J.C., Andersen, B., Thrane, U. (2008). The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res. 112:231-240. doi: 10.1016/j.mycres.2007.08.018.

- Frykman, S., Tsuruta, H., Galazzo, J., Licari, P. (2006). Characterization of product capture resin during microbial cultivations. Ind Microbiol Biotechnol. 33:445-453.

- Gacek, A., Strauss, J. (2012). The chromatin code of fungal secondary metabolite gene clusters. Appl Microbiol Biotechnol 95(6):1389-13404. doi: 10.1007/s00253-012-4208-8.

- Garber, G. (2001). An Overview of Fungal Infections. Drugs 61: 1-12. doi:10.2165/00003495-200161001-00001

- García, J.B., Tormo, J.R. (2003). HPLC studio: a novel software utility to perform HPLC chromatogram comparison for screening purposes. J. Biomol Screen. 8:305-315.

- Gerth, K., Washausen, P., Hofle, G., Irschik, H., Reichenbach, H. (1996). The jerangolids: a family of new antifungal compounds from Sorangium cellulosum (myxobacteria). Production, physico-chemical and biological properties of jerangolid A. J Antibiot. 49:71-75.

- Goodwin, C.R., Covington, B.C., Derewacz, D.K., McNees, C.R., Wikswo, J.P., McLean, J.A., Bachmann, B.O. (2015). Structuring Microbial Metabolic Responses to Multiplexed Stimuli via Self-Organizing Metabolomics Maps. Chem. Biol. 22:661-670.

- Graupner, K., Scherlach, K., Bretschneider, T., Lackner, G., Roth, M., Gross H., Hertweck, C. (2012). Imaging mass spectrometry and genome mining reveal highly antifungal virulence factor of mushroom soft rot pathogen. Angew. Chem. Int. Ed. 51: 13173-13177. doi: 10.1002/anie.201206658

- Grewal, S.I., Jia, S. (2007). Heterochromatin revisited. Nat Rev Genet 8(1):35-46.

- Gross, J.H. (2011). Mass Spectrometry. 2nd ed Springer-Verlag; Berlin Heidelberg.

- Haefliger W, Hauser D. (1973). Isolation and structure elucidation of 11 desacetoxywortmannin. Helv Chim Acta. 56(8):2901-2904.

- Harris, G.H., Turner, E.T., Meinz, M. S., Nallin-Omstead, M., Helms, G. L., Bills, G., Zink, D., Wilson, K.E. (1993). Isolation and structure elucidation of viridiofungins A, B and C. Tetrahedron Letters. 34(33): 5235-5238. doi: 10.1016/S0040-4039(00)73961-X.

- Hazuda, D., Blau, C.U., Felock, P., Hastings, J., Pramanik, B., Wolfe, A., Bushman, F., Farnet, C., Goetz, M., Williams, M., Silverman, K., Lingham, R., Singh, S. (1999). Isolation and characterization of novel human immunodeficiency virus integrase inhibitors from fungal metabolites. Antivir Chem Chemother. 10(2):63-70.

- Heinke, R., Schöne, P., Arnold, N., Wessjohann, L., & Schmidt, J. (2014). Metabolite Profiling and Fingerprinting of *Suillus* Species (Basidiomycetes) by Electrospray Mass Spectrometry. European Journal of Mass Spectrometry. 20(1): 85–97. doi: 10.1255/ejms.1235.

- Herath, K., Harris, G., Jayasuriya, H., Zink, D., Smith, S., Vicente, F., Bills, G., Collado, J., González, A., Jiang, B., Kahn, J.N., Galuska, S., Giacobbe, R., Abruzzo, G., Hickey, E., Liberator, P., Xu, D., Roemer, T., Singh, S.B. (2009). Isolation, structure and biological activity of phomafungin, a cyclic lipodepsipeptide from a widespread tropical *Phoma* sp. Bioorg Med Chem. 1;17(3):1361-1369. doi: 10.1016/j.bmc.2008.12.009.

- Henrikson, J.C., Hoover, A.R., Joyner, P.M., Cichewicz, R.H. (2009). A chemical epigenetics approach for engineering the *in situ* biosynthesis of a cryptic natural

product from *Aspergillus niger*. Org Biomol Chem. 7(3):435-8. doi: 10.1039/b819208a.

- Hou, Y., Braun, D.R., Michel, C.R., Klassen, J.L., Adnani, N., Wyche, T.P., Bugni, T.S. (2012). Microbial strain prioritization using metabolomics tools for the discovery of natural products. Anal Chem. 84(10):4277-4283. doi: 10.1021/ac202623g.

- Hsu, C-C., Dorrestein, P.C. (2015). Visualizing Life with Ambient Mass Spectrometry. Current opinion in biotechnology. 0:24-34. doi: 10.1016/j.copbio.2014.07.005.

- Hur, M., Campbell, A.A., Almeida-de-Macedo, M., Li, L., Ransom, N., Jose, A., Crispin, M., Nikolau, B.J., Wurtele, E.S. (2013). A global approach to analysis and interpretation of metabolic data for plant natural product discovery. Nat. Prod. Rep. 30:565-583.

- Hynes, J., Müller, C. T., Jones, T. H., Boddy, L. (2007). Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. J. Chem. Ecol. 33 43-57. doi: 10.1007/s10886-006-9209-6.

- Igarashi, Y., Iida T, Oku, N., Watanabe, H., Furihata, K., Miyanouchi, K. (2012). Nomimicin, a new spirotetronate-class polyketide from an actinomycete of the genus *Actinomadura*. J Antibiot. 65:355-359.

- Johansson, M., Sterner, O., Labischinski, H., Anke T. (2001). Coprinol, a new antibiotic cuparane from a *Coprinus* species. Z. Naturforsch. C J. Biosci. 56:31-34. doi: 10.1515/znc-2001-1-205.

- Kamerewerd J., Zadra I., Kurnsteiner H., Kuck U. (2011). PcchiB1, encoding a class V chitinase, is affected by PcVelA and PcLaeA, and is responsible for cell wall integrity in Penicillium chrysogenum. Microbiology. 157:3036-3048. doi: 10.1099/mic.0.051896-0.

- Karimi-Aghcheh R., Bok J. W., Phatale P. A., Smith K. M., Baker S. E., Lichius A., Omann, M., Zeilinger, S., Seiboth, B., Rhee, C., Keller, N.P., Freitag, M., Kubicek, C.P. (2013). Functional analyses of *Trichoderma reesei* LAE1 reveal conserved and contrasting roles of this regulator. G3 (Bethesda). 3:369-378. doi: 10.1534/g3.112.005140.

- Kim, W., Peever, T. L., Park, J.-J., Park, C.-M., Gang, D. R., Xian, Davidson, J.A., Infantino, A., Kaiser, W.J., Chen, W. (2016). Use of metabolomics for the chemotaxonomy of legume-associated *Ascochyta* and allied genera. Sci Rep. 6: 20192. doi: 10.1038/srep20192.

Introduction

- Kinghorn, A. D., Chin, Y.-W., & Swanson, S. M. (2009). Discovery of Natural Product Anticancer Agents from Biodiverse Organisms. Current Opinion in Drug Discovery & Development, 12(2): 189-196.
- Kohonen, T. (2001) Self-organizing maps, 3rd edn. Springer-Verlag, New York
- Kumar A, Patil D, Rajamohanan PR, Ahmad A (2013). Isolation, Purification and Characterization of Vinblastine and Vincristine from Endophytic Fungus Fusarium oxysporum Isolated from Catharanthus roseus. PLoS ONE 8(9): e71805.
- Kushida, H., Nakajima, S., Uchiyama, S., Nagashima, M., Kojiri, K., Kawamura, K., and Suda, H. (1999). Antifungal substances BE-49385 and process for their production. U.S. Patent 5,928,910.
- Laupacis, A., Keown, P. A., Ulan, R. A., McKenzie, N., & Stiller, C. R. (1982). Cyclosporin A: a powerful immunosuppressant. Canadian Medical Association Journal. 126(9): 1041-1046.
- Leavitt, S. D., Esslinger, T. L., Divakar, P. K., Lumbsch, H. T. (2012). Miocene and Pliocene dominated diversification of the lichen-forming fungal genus Melanohalea (Parmeliaceae, Ascomycota) and Pleistocene population expansions. BMC evolutionary biology, 12, 176. doi:10.1186/1471-2148-12-176
- Lendínez, M. L., Marchal, F.M., Salazar, C. (2011). Estudio florístico de los medios húmedos salinos de Andalucía (S. España). Catálogo y análisis de la flora vascular halófila. Lagascalia 31:77-130.
- Li, C., Wang, J., Luo, C., Ding, W., Cox, D. G. (2014). A new cyclopeptide with antifungal activity from the co-culture broth of two marine mangrove fungi. Nat. Prod. Res. 28 616–621. doi:10.1080/14786419.2014.887074.
- Li, C., Zhang, J., Shao, C., Ding, W., She, Z., Lin, Y. (2011). A new xanthone derivative from the co-culture broth of two marine fungi (strain No. E33 and K38) Chem. Nat. Compd. 47:382-384. doi: 10.1007/s10600-011-9939-8.
- Li. W. (2012). Volcano plots in analyzing differential expressions with mRNA microarrays. J Bioinform Comput Biol. 10(6):1231003. doi: 10.1142/S0219720012310038.
- Lin, H., Lyu, H., Zhou, S., Yu, J., Keller, N.P., Chen, L., Yin, W.B. (2018). Deletion of a global regulator LaeB leads to the discovery of novel polyketides in *Aspergillus nidulans*. Org Biomol Chem. 11;16(27):4973-4976. doi: 10.1039/c8ob01326h.

- Macheleidt, J., Mattern, D.J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante,V., Brakhage, A.A. (2016). Regulation and Role of Fungal Secondary Metabolites. Annu Rev Genet. 23; 50:371-392.

- Magome, E., Harimaya, K., Gomi, S., Koyama, M., Chiba, N., Ota, K., Mikawa, T. (1996). Structure of furanocandin, a new antifungal antibiotic from *Tricothecium* sp. J Antibiot (Tokyo) 49(6):599-602.

- Mandala, S.M., Thornton, R.A., Frommer, B.R., Curotto, J.E., Rozdilsky, W., Kurtz, M.B., Giacobbe, R.A., Bills, G.F., Cabello, M.A., Martín, I., Pelaez, F., Harris, G.H. (1995). The discovery of australifungin, a novel inhibitor of sphinganine N-acyltransferase from *Sporormiella australis*. Producing organism, fermentation, isolation, and biological activity. J Antibiot (Tokyo) 48(5):349-56.

- Marique, T., Allard, O., Spanoghe, M. (2012). Use of Self-Organizing Map to Analyze Images of Fungi Colonies Grown from Triticum aestivum Seeds Disinfected by Ozone Treatment. Int J Microbiol. 2012:865175. doi: 10.1155/2012/865175.

- Marshall, V.P., McWethy, J.S., Sirotti, J.M., Cialdella, J.I. (1990). The effect of neutral resins on the fermentation production of rubradirin. J Ind Microbiol. 5:283-288.

- Marshall, V.P., McWethy, J.S., Visser, J., Cialdella, J.I., Laborde, A.L. (1987). Current fermentation technology from actinomycetes: the example of paulomycin. Dev Ind Microbiol.28:105–114.

- Martín, J., Crespo, G., González-Menéndez, V., Pérez-Moreno, G., Sánchez-Carrasco, P., Pérez-Victoria, I., Ruiz-Pérez, L.M., González-Pacanowska, D., Vicente, F., Genilloud, O., Bills, G., Reyes, F. (2014). MDN-0104, an antiplasmodial betaine lipid from *Heterospora chenopodii.* J Nat Prod. 77(9):2118-2123. doi: 10.1021/np500577v.

- Massimo, N. C., Nandi Devan, M., Arendt, K. R., Wilch, M. H., Riddle, J. M., Furr, S. H., Steen, C., U'Ren, J.M., Sandberg, D.C., Arnold, A. E. (2015). Fungal endophytes in above-ground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. Microbial Ecology. 70(1):61-76. doi: 10.1007/s00248-014-0563.

- Meyer, S.E. (1986). The Ecology of Gypsophile Endemism in the Eastern Mojave Desert. Ecology. 67(5):1303-1313.Milshteyn, A., Schneider, J.S., Brady, S.F. (2014) Mining the metabiome: identifying novel natural products from microbial communities. Chem Biol. 18;21(9):1211-1223 doi: 10.1016/j.chembiol.2014.08.006.

- Moore, J.M., Bradshaw, E., Seipke, R.F., Hutchings, M.I., McArthur, M. (2012). Use and discovery of chemical elicitors that stimulate biosynthetic gene clusters in Streptomyces bacteria. Methods Enzymol. 517:367-385.

- Moree, W.J., Phelan, V.V., Wu, C-H., Bandeira, N., Cornett, D.S., Duggan, B.M., Dorrestein, P.C. (2012). Interkingdom metabolic transformations captured by microbial imaging mass spectrometry Proc. Natl. Acad. Sci. U. S. A. 109:13811-13816.

- Moskaug, J.O., Carlsen, H., Myhrstad, M., Blomhoff, R. (2004). Molecular imaging of the biological effects of quercetin and quercetin-rich foods. Mech. Ageing Dev 125, 315–324.

- Mota, J., Aguilera-Aguilera, P., Cerrillo, M.I., Cabello, J., Rodrigez Tamayo, M.L. (2004). El Parque Nacional: Antecedentes e Introducción, in Subdesiertos de Almería: naturaleza de cine, J. Mota, Mota, J., Aguilera-Aguilera, P., Cerrillo, M.I., Cabello, J., Rodrigez Tamayo, M.L, Editors. Consejeria de Medio Ambiente. Junta de Andalucia. 15-24.

- Montanini, B., Chen, P.-Y., Morselli, M., Jaroszewicz, A., Lopez, D., Martin, F., O ttonello, S., Pellegrini, M. (2014). Non-exhaustive DNA methylation-mediated transposon silencing in the black truffle genome, a complex fungal genome with massive repeat element content. Genome Biol. 15(8): 411. doi: 10.1186/s13059-014-0411-5.

- Muller, J.M., Duhner, H., Gruner, J., Voser, W. (1976). Stoffwechselprodukte von Mikroorganismen 160. Mitteilung159. Mitteilung S. Conocandin, ein fungistatisches Antibiotikum aus Hormococcus conorum. Helvetica Chimica. Acta 59:2506-2514.

- Netzker, T., Fischer, J., Weber, J., Mattern, D. J., König, C. C., Valiante, V., Schroeckh, V., Brakhage, A.A. (2015). Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. Front. Microbiol. 6:299. doi: 10.3389/fmicb.2015.00299.

- Newmand, D.J., and Cragg, G.M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. J Nat Prod. 79(3):629-661.

- Nguyen, D. D., Wu, C.-H., Moree, W. J., Lamsa, A., Medema, M. H., Zhao, X., Gavilan, R.G., Aparicio, M., Atencio, L., Jackson, C., Ballesteros, J., Sanchez, J., Watrous, J.D., Phelan, V.V., van de Wiel, C., Kersten, R.D., Mehnaz, S., De Mot, R., Shank, E.A., Charusanti, P., Nagarajan, H., Duggan, B.M., Moore, B.S., Bandeira, N., Palsson, B.Ø., Pogliano, K., Gutiérrez, M., Dorrestein, P.C. (2013). MS/MS networking guided

analysis of molecule and gene cluster families. Proc Natl Acad Sci U S A. 110(28): 2611-2620. doi:10.1073/pnas.1303471110.

- Nichols, D., Cahoon, N., Trakhtenberg, E. M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K., Epstein, S. S. (2010). Use of Ichip for High-Throughput In Situ Cultivation of "Uncultivable" Microbial Species. Applied and Environmental Microbiology, 76(8): 2445-2450. doi: 10.1128/AEM.01754-09.

- Nielsen, K. F., Månsson M., Rank C., Frisvad J. C., Larsen T. O. (2011). Dereplication of microbial natural products by LC-DAD-TOFMS. J. Nat. Prod. 74: 2338–2348. doi: 10.1021/np200254t.

- Nielsen, K.F., Larsen, T.O. (2015). The importance of mass spectrometric dereplication in fungal secondary metabolite analysis. Front Microbiol. 17; 6:71. doi: 10.3389/fmicb.2015.00071

- Nyfeler, R., Keller-Schierlein, W. (1974). Metabolites of microorganisms. 143. Echinocandin B, a novel polypeptide-antibiotic from *Aspergillus nidulans* var. *echinulatus*: isolation and structural components. Helv Chim Acta. 57(8):2459-2477.

- Nonaka K, Abe T, Iwatsuki M, Mori M, Yamamoto T, Shiomi K, Omura S, Masuma R. (2011). Enhancement of metabolites productivity of *Penicillium pinophilum* FKI-5653, by co-culture with *Trichoderma harzianum* FKI-5655. J Antibiot (Tokyo) 64(12):769-74. doi: 10.1038/ja.2011.91.

- Nützmann, H.W., Reyes-Dominguez, Y., Scherlach, K., Schroeckh, V., Horn, F., Gacek, A., Schümann, J., Hertweck, C., Strauss, J., Brakhage, A.A. (2011). Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. Proc Natl Acad Sci U S A. 108:14282–14287.

- Oh, D.C., Jensen, P.R., Kauffman, C.A., Fenical, W. (2005). Libertellenones A-D: induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. Bioorg Med Chem. 1; 13(17):5267-5273.

- Oh, D.C., Kauffman, C.A., Jensen, P.R., Fenical, W. (2007). Induced production of emericellamides A and B from the marine-derived fungus *Emericella* sp. in competing co-culture. J Nat Prod.70(4):515-520.

- Ondeyka, J., Harris, G., Zink, D., Basilio, A., Vicente, F., Bills, G., Platas, G., Collado, J., González, A., de la Cruz, M., Martin, J., Kahn, J.N., Galuska, S., Giacobbe, R., Abruzzo, G., Hickey, E., Liberator, P., Jiang, B., Xu, D., Roemer, T., Singh, S.B. (2009). Isolation, structure elucidation, and biological activity of virgineone from *Lachnum virgineum*

using the genome-wide *Candida albicans* fitness test. J Nat Prod. 72(1):136-41. doi: 10.1021/np800511r.

- Omura, S., Tomoda, H., Kumagai, H., Greenspan, M.D., Yodkovitz, J.B., Chen, J.S., Alberts, A.W., Martin, I., Mochales, S., Monaghan, R.L., Chabala, J.C., Schwartz, R.E., Patchett, A.A. (1987). Potent inhibitory effect of antibiotic 1233A on cholesterol biosynthesis which specifically blocks 3-hydroxy-3-methylglutaryl coenzyme A synthase. J. Antibiotics 40:1356-1357.

- Ortíz-López, F.J., Monteiro, M.C., González-Menéndez, V., Tormo, J.R., Genilloud, O., Bills, G., Vicente, F., Zhang, C., Roemer, T., Singh, S.B., Reyes, F. (2015). Cyclic colisporifungin and linear cavinafungins, antifungal lipopeptides isolated from *Colispora cavincola*. J Nat Prod 27; 78(3):468-75. doi: 10.1021/np500854j.

- Palacio, S., Johnson, D., Escudero, A. & Montserrat-Martí, G. (2012). Root colonization byAMfungi differs between gypsum specialist and non-specialist plants: links to the gypsophile behavior. Journal of Arid Environments 76: 128-132.

- Palenzuela, J., Ferrol, N., Boller, T., Azcón-Aguilar, C., Oehl, F. (2008). *Otospora bareai*, a new fungal species in the Glomeromycetes from a dolomitic shrub land in Sierra de Baza National Park (Granada, Spain). Mycologia. 100(2):296-305.

- Palenzuela, J., Barea, J.M., Ferrol, N., Azcón-Aguilar, C., Oehl, F. (2010). *Entrophospora nevadensis*, a new arbuscular mycorrhizal fungus from Sierra Nevada National Park (southeastern Spain). Mycologia. 102(3):624-632.

- Palmer, J.M., Keller, N.P. (2010). Secondary metabolism in fungi: does chromosomal location matter? Curr Opin Microbiol 13(4):431-436. doi: 10.1016/j.mib.2010.04.008.

- Palmer J. M., Theisen J. M., Duran R. M., Grayburn W. S., Calvo A. M., Keller N. P. (2013). Secondary metabolism and development is mediated by LlmF control of VeA subcellular localization in *Aspergillus nidulans*. PLoS Genet. 9:e1003193. doi: 10.1371/journal.pgen.1003193.

- Park, H.B., Kwon, H.C., Lee, C.H., Yang, H.O. (2009). Glionitrin A, an antibiotic-antitumor metabolite derived from competitive interaction between abandoned mine microbes. J Nat Prod. 72(2):248-52. doi: 10.1021/np800606e.

- Parsons, R.F. (1976). Gypsophily in plants-a review. The American Midland Naturalist. 96: 1-20.

- Peiris, D., Dunn, W. B., Brown, M., Kell, D. B., Roy, I., Hedger, J. N. (2008). Metabolite profiles of interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus, *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminates*. Metabolomics 4: 52–62. doi:10.1007/s11306-007-0100-4.

- Peláez, F. (2006) The historical delivery of antibiotics from microbial natural products--can history repeat? Biochem Pharmacol.71:981-990.

- Peláez, F., Cabello, A., Platas, MulG., Díez, M.T., González del Val, A., Basilio, A., Martin, J., Vicente, F., Bills, G., Giacobbe, R.A., Schwartz, R.E., Onish, J.C., Meinz, M.S., Abruzzo, G.K., Flattery, A.M., Kong, L., Kurtz, M.B. (2000). The discovery of enfumafungin, a novel antifungal compound produced by an endophytic *Hormonema* species biological activity and taxonomy of the producing organisms. Syst Appl Microbiol. 23(3):333-343.

- Pérez-Victoria, I., Martín, J., Reyes, F. (2016). Combined LC/UV/MS and NMR strategies for the dereplication of marine natural products. Planta Med. 82: 857-871. doi:10.1055/s-0042-101763.

- Porras-Alfaro, A., Herrera, J., Sinsabaugh, R.L., Natvig, D.O. (2007). Effect of long-term nitrogen fertilization on mycorrhizal fungi associated with a dominant grass in a semiarid grassland. Plant Soil 296:65-75.

- Porras-Alfaro, A., Herrera, J., Sinsabaugh, R. L., Odenbach, K. J., Lowrey, T., & Natvig, D. O. (2008). Novel Root Fungal Consortium Associated with a Dominant Desert Grass. Applied and Environmental Microbiology. *74*(9), 2805–2813. doi: 10.1128/AEM.02769-07
- Porras-Alfaro, A., Raghavan, S., Garcia, M., Sinsabaugh, R. L., Natvig, D. O., Lowrey, T. (2014). Endophytic fungal symbionts associated with gypsophilous plants. Botany 92:295-301

- Potterat, O., Hamburger, M. (2013) Concepts and technologies for tracking bioactive compounds in natural product extracts: generation of libraries, and hyphenation of analytical processes with bioassays. Nat Prod Rep. 30:546-564

- Preyat, N., Leo, O. (2013). Sirtuin deacylases: A molecular link between metabolism and immunity. J. Leukoc. Biol. 93, 669–680.

- Pu, X., Qu, X., Chen, F., Bao, J., Zhang, G., Luo, Y. (2013). Camptothecin-producing endophytic fungus Trichoderma atroviride LY357: isolation, identification, and

fermentation conditions optimization for camptothecin production. Appl Microbiol Biotechnol 97(21):9365-9375. doi: 10.1007/s00253-013-5163-8

- Raman Sharma, R. (2010). Fungal infections of the nervous system: current perspective and controversies in management. Int J Surg 8(8):591-601.

- Rampelotto, P. H. (2013). Extremophiles and Extreme Environments. Life*:* Open Access Journal, 3(3), 482–485. doi:10.3390/life3030482

- Rateb, M. E., Hallyburton, I., Houssen, W. E., Bull, A. T., Goodfellow, M., Santhanam, R., Jaspars, M., Ebel, R. (2013). Induction of diverse secondary metabolites in *Aspergillus fumigatus* by microbial co-culture. RSC Adv. 4: 14444-14450. doi: 10.1039/c3ra42378f.

- Réblová M, Untereiner WA, Réblová K. (2013). Novel Evolutionary Lineages Revealed in the Chaetothyriales (Fungi) Based on Multigene Phylogenetic Analyses and Comparison of ITS Secondary Structure. Harris S, ed. PLoS ONE. 8(5): e63547. doi: 10.1371/journal.pone.0063547.

- Riera Mora S. (2006). Cambios vegetales holocenos en la región mediterránea de la Península Ibérica: ensayo de síntesis. Ecosistemas. 2006/1 (URL: http://www.revistaecosistemas.net/articulo.asp?Id=401&Id_Categoria=2&tipo=portada)

- Rodrıguez-Estrada, A. E., Hegeman, A., Kistler, H. C., May G. (2011). In vitro interactions between *Fusarium verticillioides* and *Ustilago maydis* through real-time PCR and metabolic profiling. Fungal Genet. Biol. 48: 874-885. doi: 10.1016/j.fgb.2011.06.006.

- Roemer, T., Xu, D., Singh, S.B., Parish, C.A., Harris, G., Wang, H., Davies, J.E., Bills, G. (2011). Confronting the challenges of natural product-based antifungal discovery. Chem Biol 18(2):148-164. doi: 10.1016/j.chembiol.2011.01.009.

- Roncal, T., Cordobés, S., Sterner, O., Ugalde, U. (2002). Conidiation in *Penicillium cyclopium* is induced by conidiogenone, an endogenous diterpene. Eukaryot Cell. 1(5):823-829.

- Roy, K., Mukhopadhyay, T., Reddy, G.C., Desikan, K.R., Ganguli, B.N. (1987). Mulundocandin, a new lipopeptide antibiotic. I. Taxonomy, fermentation, isolation and characterization. J Antibiot (Tokyo) 40(3):275-280.

- Sakata, K., Kuwatsuka, T., Sakurai, A., Takahashi, N., Tamura, G. (1983). Isolation of Aspirochlorine (═Antibiotic A30641) as a True Antimicrobial Constituent of the

Antibiotic, Oryzachlorin, from *Aspergillus oryzae*, Agricultural and Biological Chemistry, 47;11: 2673-2674. doi: 10.1080/00021369.1983.10866014.

- Sarikaya-Bayram, Ö., Palmer, J. M., Keller, N., Braus, G. H., & Bayram, Ö. (2015). One Juliet and four Romeos: VeA and its methyltransferases. Frontiers in Microbiology, 6:1 doi: 10.3389/fmicb.2015.00001.

- Scherlach, K., Hertweck, C. (2009). Triggering cryptic natural product biosynthesis in microorganisms. Org Biomol Chem. 9:1753-1760.

- Scherlach, K., Lackner, G., Graupner, K., Pidot, S., Bretschneider, T., Hertweck, C. (2013). Biosynthesis and mass spectrometric imaging of tolaasin, the virulence factor of brown blotch mushroom disease. Chembiochem. 14:2439-2443. doi: 10.1002/cbic.201300553

- Sharma, V., Singamaneni, V., Sharma, N., Kumar, A., Arora, D., Kushwaha, M., Bhushan, S.N., Jaglan, S., & Gupta, P.K. (2018). Valproic acid induces three novel cytotoxic secondary metabolites in Diaporthe sp., an endophytic fungus from Datura inoxia Mill. Bioorganic & medicinal chemistry letters, 28 (12): 2217-2221.

- Shen, B. (2015). A New Golden Age of Natural Products Drug Discovery. Cell, 163(6), 1297–1300. doi: 10.1016/j.cell.2015.11.031.

- Schneider, G., Anke, H., Sterner, O. (1995). Xylarin, an Antifungal *Xylaria* Metabolite with an Unusual Tricyclic Uronic Acid Moiety, Natural Product Letters. 7;4: 309-316. doi: 10.1080/10575639508043227.

- Singh, S.B., Ondeyka, J., Harris, G., Herath, K., Zink, D., Vicente, F., Bills, G., Collado, J., Platas, G., González del Val, A., Martin, J., Reyes, F., Wang, H., Kahn, J.N., Galuska, S., Giacobbe, R., Abruzzo, G., Roemer, T., Xu, D. (2013). Isolation, structure, and biological activity of Phaeofungin, a cyclic lipodepsipeptide from a *Phaeosphaeria* sp. using the Genome-Wide *Candida albicans* Fitness Test. J Nat Prod. 22;76(3):334-45. doi: 10.1021/np300704s.

- Singh, M.P., Leighton, M.M., Barbieri, L.R., Roll, D.M., Urbance, S.E., Hoshan, L., McDonald, L.A. (2010). Fermentative production of self-toxic fungal secondary metabolites. J Ind Microbiol Biotechnol. 37:335-340.

- Sivanandhan, S., Khusro, A., Paulraj, M. G., Ignacimuthu, S., AL-Dhabi, N. A. (2017). Biocontrol Properties of Basidiomycetes: An Overview. Journal of Fungi. 3(1): 2. doi: 10.3390/jof3010002.

- Soukup, A.A., Keller, N.P. (2012) Epigenetic approaches to natural product synthesis in fungi. In Drug discovery from natural products. Eds Genilloud O, Vicente F. The Royal Society of Chemestry 10:198-217

- Songsermsakul, P., Razzazi-Fazeli, E. (2008). A Review of Recent Trends in Applications of Liquid Chromatography-Mass Spectrometry for Determination of

Mycotoxins. J Liq Chromatogr Relat Technol. 31;11-12: 1641-1686. doi: 10.1080/10826070802126395.

- Stasulli, N.M., Shank, E.A. (2016). Profiling the metabolic signals involved in chemical communication between microbes using imaging mass spectrometry. Gibbs K, ed. *FEMS* Microbiology Reviews. 40(6):807-813. doi:10.1093/femsre/fuw032.

- Stillwell, M.A., Magasi, L.P., Strunz, G.M. (1974). Production, isolation, and antimicrobial activity of hyalodendrin, a new antibiotic produced by a species of *Hyalodendron*. Can J Microbiol. 20(5):759-64

- Stierle, A., Strobel, G., Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 9; 260(5105):214-216.

- Strahl, B.D., Allis, C.D. (2000). The language of covalent histone modifications. Nature 6; 403(6765):41-45.

- Suzuki, M.M., Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet 9:465–476.

- Tormo, J.R., García, J.B., DeAntonio, M., Feliz, J., Mira, A., Díez, M.T., Hernández, P., Peláez, F. (2003). A method for the selection of production media for actinomycete strains based on their metabolite HPLC profiles. J Ind Microbiol Biotechnol. 30:582-588.

- van der Lee, T.A.J., Medema, M.H. (2016). Computational strategies for genome-based natural product discovery and engineering in fungi. Fungal Genet Biol. 2016 Apr; 89:29-36. doi: 10.1016/j.fgb.2016.01.006.

- Vervoort, H.C., Drašković, M., Crews, P. (2011). Histone Deacetylase Inhibitors as a Tool to Up-regulate New Fungal Biosynthetic Products—Isolation of EGM-556, a Cyclodepsipeptide, from Microascus sp. Org. Lett. 13, 410–413.

- Vicente, F., Cabello, A., Platas, G., Basilio, A., Díez, M.T., Dreikorn, S., Giacobbe, R.A., Onishi, J.C., Meinz, M., Kurtz, M.B., Rosenbach, M., Thompson, J., Abruzzo, G., Flattery, A., Kong, L., Tsipouras, A., Wilson, K.E., Peláez, F. (2001). Antimicrobial activity of ergokonin A from *Trichoderma longibrachiatum*. J Appl Microbiol 91(5):806-813.

- Villas-Bôas, S.G., Mas, S., Akesson, M., Smedsgaard, J., Nielsen, J. (2005). Mass spectrometry in metabolome analysis. Mass Spectrom Rev.24(5):613-646.

- Wakefield, J., Hassan, H. M., Jaspars, M., Ebel, R., & Rateb, M. E. (2017). Dual Induction of New Microbial Secondary Metabolites by Fungal Bacterial Co-cultivation. Frontiers in Microbiology, 8, 1284. doi: 10.3389/fmicb.2017.01284.

- Wang, H., Ng, T. (2006). Ganodermin, an antifungal protein from fruiting bodies of the medicinal mushroom *Ganoderma lucidum*. Peptides. 27:27–30. doi: 10.1016/j.peptides.2005.06.009.

- Wang, Z., Zang, C., Cui, K., Schones, D.E., Barsk,i A., Peng, W., Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell. 138(5):1019-1031. doi: 10.1016/j.cell.2009.06.049.

- Warr, G.A., Veitch, J.A., Walsh, A.W., Hesler, G.A., Pirnik, D.M., Leet, J.E., Lin, P.M., Medina, I.A., McBrien, K.D., Forenza, S., Clark, J.M., Lam, K.S. (1996). BMS-182123, a fungal metabolite that inhibitsthe production of TNF-a by macrophages and monocytes. J Antibiot 49:234-240.

- Watanabe, T., Izaki, K., Takahashi, H. (1982). New polyenic antibiotics active against gram-positive and-negative bacteria. I. Isolation and purification of antibiotics produced by *Gluconobacter* sp. W-315. J. Antibiot. (Tokyo) 35, 1141–1147. doi: 10.7164/antibiotics.35.1141

- Wiemann, P., Keller, N.P. (2014). Strategies for mining fungal natural products. J. Ind. Microbiol. Biotechnol., 41:301-313. doi: 10.1007/s10295-013-1366-3.

- Williams, R.B., Henrikson J.C., Hoover, A.R., Lee, A.E., Cichewicz, R.H. (2008). Epigenetic remodeling of the fungal secondary metabolome. Org Biomol Chem. 6:1895–1897.

- Woo, E.J., Starks, C.M., Carney, J.R., Arslanian, R., Cadapan, L., Zavala, S., Licari, P. (2002). Migrastatin and a new compound, isomigrastatin, from Streptomyces platensis. J Antibiot. 55:141-146.

- Xu, D., Ondeyka, J., Harris, G.H., Zink, D., Kahn, J.N., Wang, H., Bills, G., Platas, G., Wang, W., Szewczak, A.A., Liberator, P., Roemer, T., Singh, S.B. (2011). Isolation, structure, and biological activities of Fellutamides C and D from an undescribed *Metulocladosporiella* (*Chaetothyriales*) using the genome-wide *Candida albicans* fitness test. J Nat Prod. 26;74(8):1721-1730. doi: 10.1021/np2001573.

- Yang, J.Y., Sanchez, L.M., Rath, C.M., Liu, X., Boudreau, P.D., Bruns, N., Glukhov, E., Wodtke, A., de Felicio, R., Fenner, A., Wong, W.R., Linington, R.G., Zhang, L.,

Debonsi, H.M., Gerwick, W.H., Dorrestein, P.C. (2013). Molecular networking as a dereplication strategy. J Nat Prod. 27;76(9):1686-1699. doi: 10.1021/np400413s.

- Yang, X. L., Awakawa, T., Wakimoto, T., Abe I. (2013). Induced production of novel prenyldepside and coumarins in endophytic fungi *Pestalotiopsis acaciae*. Tetrahed. Lett. 54 5814–5817. doi: 10.1016/j.tetlet.2013.08.054.

- Yang, J.Y., Phelan, V.V., Simkovsky, R., Watrous, J.D., Trial, R.M., Fleming, T.C., Wenter, R., Moore, B.S0, Golden, S.S., Pogliano, K., Dorrestein, P.C. (2012). Primer on Agar-Based Microbial Imaging Mass Spectrometry. J Bacteriol. 194; 6023-6028.

- Yang, Y-L., Xu Y, Straight, P., Dorrestein, P.C. (2009). Translating metabolic exchange with imaging mass spectrometry Nat Chem Biol. 5; 885-887.

- Yocum, R.R., Rasmussen, J.R., Strominger, J.L. (1980). The mechanism of action of penicillin. Penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. J Biol Chem. 10;255(9):3977-3986.

- Zabalgogeazcoa, I. (2008). Fungal endophytes and their interaction with plant pathogens: a review. SJAR 6. doi: 10.5424/sjar/200806S1-382

- Zhu F., Lin Y. (2006). Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea. Chin. Sci. Bull.51:1426-1430. doi: 10.1007/s11434-006-1426-4.

- Zhu F., Chen G., Chen X., Huang M., Wan X. (2011). Aspergicin, a new antibacterial alkaloid produced by mixed fermentation of two marine-derived mangrove epiphytic fungi. Chem. Nat. Compd. 47:767-769. doi: 10.1007/s10600-011-0053-8.

- Zida, A., Bamba, S., Yacouba, A., Ouedraogo-Traore, R., Guiguemdé, R.T. (2017). Anti-*Candida albicans* natural products, sources of new antifungal drugs: A review. J Mycol Med. 27(1):1-19. doi: 10.1016/j.mycmed.2016.10.002.

- Zijlstra, J.D., Hof, P. van 't, Baar, J., Verkley, G.J.M., Summerbell, R.C., Paradi, I., Braakhekke, W.G., Berendse, F. (2005). Diversity of symbiotic root endophytes of the *Helotiales* in ericaceous plants and the grass, *Deschampsia flexuosa.* Stud Mycol 53:147-162

II. AIMS OF THE THESIS

AIMS OF THE THESIS

Plant associated fungi, particularly fungal endophytes, have demonstrated their ability to produce bioactive secondary metabolites with novel structures with applications in pharmaceutical, agricultural and other industries.

The **main goal** of this study was to characterize the **microbial diversity of endophytic fungi** isolated from endemic plants adapted to arid and semi-arid areas of Andalusia, and to evaluate their biosynthetic potential to generate a high chemical diversity of secondary metabolites through the implementation of the current **innovative induction methodologies** available in the field.

SPECIFIC OBJECTIVES

1) Isolate fungi associated with plants collected in arid and semi-arid regions of Andalusia, and taxonomically identify the isolates by their micro-morphologies and by their ITS_1-5.8S-ITS_2 and partial 28S rDNA sequencing.

2) Study the metabolic productivity of the fungal isolates using a set of liquid production media and characterise their chemical diversity on secondary metabolites production by using comparative HPLC-UV-MS profile analyses.

3) Assess the effects of the addition of adsorptive polymeric resins during fungal fermentations upon secondary metabolite expression.

4) Evaluate the use of small-molecule epigenetics modifiers to induce silent pathways in fungal endophytes performing multicomponent analyses on their SMs production.

5) Determine the co-culturing differential spatial expression of secondary metabolites of cohabitant fungal isolates with clear antagonistic microbial interactions.

6) Assess the biological activity of the fungal isolates from arid and semi-arid regions of Andalusia against human and plant pathogen fungi, as well as cancer cell lines.

7) Asses the chemistry of new molecules induced by the application of approaches from Aims 2-5 on the population of fungal isolates obtained in Aim 1 of this study.

III. RESULTS

Chapter 1

Fungal endophytes from arid areas of Andalusia: high potential sources of antifungal and antitumoral agents.

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Fungal endophytes from arid areas of Andalusia: high potential sources for antifungal and antitumoral agents

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Native plant communities from arid areas present distinctive characteristics to survive in extreme conditions. The large number of poorly studied endemic plants represents a unique potential source for the discovery of novel fungal symbionts as well as host-specific endophytes not yet described. The addition of adsorptive polymeric resins in fungal fermentations has been seen to promote the production of new secondary metabolites and is a tool used consistently to generate new compounds with potential biological activities. A total of 349 fungal strains isolated from 63 selected plant species from arid ecosystems located in the southeast of the Iberian Peninsula, were characterized morphologically as well as based on their ITS/28S ribosomal gene sequences. The fungal community isolated was distributed among 19 orders including Basidiomycetes and Ascomycetes, being *Pleosporales* the most abundant order. In total, 107 different genera were identified being *Neocamarosporium* the genus most frequently isolated from these plants, followed by *Preussia* and *Alternaria*. Strains were grown in four different media in presence and absence of selected resins to promote chemical diversity generation of new secondary metabolites. Fermentation extracts were evaluated, looking for new antifungal activities against plant and human fungal pathogens, as well as, cytotoxic activities against the human liver cancer cell line HepG2. From the 349 isolates tested, 126 (36%) exhibited significant bioactivities including 58 strains with exclusive antifungal properties and 33 strains with exclusive activity against the HepG2 hepatocellular carcinoma cell line. After LCMS analysis, 68 known bioactive secondary metabolites could be identified as produced by 96 strains, and 12 likely unknown compounds were found in a subset of 14 fungal endophytes. The chemical profiles of the differential expression of induced activities were compared. As proof of concept, ten active secondary metabolites only produced in the presence of resins were purified and identified. The structures of three of these compounds were new and herein are elucidated.

Several bioprospecting reports have published the ability of endophytic fungi to produce a broad range of bioactive secondary metabolites. Many of them are used as sources of anticancer lead compounds (i.e. taxol, vincristine, vinblastine, camptothecin and podophyllotoxin)[1] or as sources of antifungal lead molecules (i.e., cryptocandin A[2], enfumafungin[3], CR377[4], ambuic acid[5], jesterone[6], moriniafungin[7], parnafungins[8] or phaeofungin[9]).

Fungal endophytes represent one of the most prolific sources of novel natural products but, low production yields and the lack of expression of cryptic gene clusters in laboratory conditions are frequently key limiting factors to exploit their high potential. Several strategies have been developed to address these issues, including mutagenesis, genetic transformation, agar co-cultivation, mixed-culture fermentations and the use of additives, such as epigenetic modifiers or adsorptive polymeric resins[10]. We recently reported the promotion of new chemical entities by the addition of Diaion® and Amberlite® resins to arrays of fungal fermentation media. Results

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concluded that this approach can induce the production of new secondary metabolites and affect consistently the production yields, at least for specific groups of fungal strains[11].

The Iberian southeast is one of the most arid regions in Europe. It is characterized by a warm and dry Mediterranean climate and it is rich in gypsum and saline soils. These unique ecological conditions have allowed the development of a wide range of endemic plant species where it is frequent to find specimens of the *Aizoaceae*, *Chenopodiaceae* or *Plumbaginaceae* families[12] which are barely represented outside of these environments[13].

The diversification of Ascomycota, the highest speciated fungal phylum, is reflected in its multiple symbiotic strategies with vascular plants, and symbiotic lifestyles (mutualistic, commensalistic, parasitic and pathogenic) in response to host genotype and environmental factors[14]. Fungal endophytes are mostly known to infect plants without causing symptoms. Latent pathogens seem to represent a relatively small proportion of the endophytic community, including latent saprophytes and mutualistic species[15]. A partial characterization of Ascomycotina from arid zones of Almeria and Baetic mountains (Andalusia) indicates that fungi associated to plants of these areas remain to be discovered[16]. Limited information is still available on the biodiversity of fungal symbionts and host-specific endophytes in these areas, including the role of these microorganisms in the ecological fitness and survival of arid plants.

Previous studies on fungal endophytes from plants of arid areas[17] have not described their potential to generate chemical diversity, they only provided scattered examples of these populations within specific medicinal plant hosts[18,19] and mentioned their bioactive potential as a whole. With this in mind, we decided to perform a broader survey in Andalusia, one of the richest areas in arid plant endemisms of Europe, isolating and characterizing both, morphologically and chemically these endophytes and their potential to generate new bioactive secondary metabolites by selecting the best combinations of media and resins to promote their chemical diversity generation based on previous results[11].

Results

Biodiversity of fungal isolates. From the individual plant species collected (63) a total of 349 fungal strains were isolated. 310 were obtained from surface-disinfected leaf or stem pieces, 32 were directly isolated from fungal reproductive structures on stems, and 7 were isolated directly from cleistothecia and/or conidiophores, which developed on plant material after incubation in moist chambers. Given the major interest in low occurring fungal species colonizing these substrates, 286 strains (45.0%) from dominant species such as *Cladosporium*, *Penicillium* and *Aspergillus* were not considered in further studies as they are generally considered as ubiquitous epiphytes.

The remaining isolates were distributed among 19 orders including Basidiomycetes and Ascomycetes (Supplementary Information Fig. S2). More than half of the isolates (208; 59.6%) were included within the *Pleosporales* order, followed by *Dothideales* (22; 6.0%) and *Xylariales* (19; 5.5%). Within the *Pleosporales* order, our isolates were distributed among 42 genera belonging to 14 families, being the *Pleosporaceae*, *Didymellaceae* and *Sporormiaceae* the most abundant with 76, 43 and 19 isolates respectively. In total, 107 different genera were identified being the 10 genera with the highest number of isolates: *Neocamarosporium* (37), *Preussia* (19), *Alternaria* (18), *Ascochyta* (17), *Phoma* (14), *Comoclathris* (13), *Neomicrosphaeropsis* (10), *Aureobasidium* (7), *Pleospora* (7) and *Fusarium* (7) (see full list in Supplementary Information, Table S1).

Phylogenetic analyses. Phylogenetic analysis of the isolates belonging to the above-mentioned genera within the *Pleosporales* order were based on ITS/28S rDNA excluding *Preussia* which was the subject of a specific review study recently published by our group[20]. The different runs of the Bayesian and ML analyses yielded the same topology. The consensus phylogenetic tree of the 130 isolated strains with 95 GenBank™ sequences of representative strains (see Table S2 in Supplementary information) included the endophytic strains isolated recently from other plants of the Arizona desert[17,21]. The resulting tree also showed a very similar topology to the phylogenetic trees obtained recently in other *Pleosporales* characterization studies[22–24].

The ITS/28S rDNA tree revealed six main clades (Fig. 1): The *Coniothyriaceae* clade clustered *Coniothyrium* and *Hazslinszkyomyces* species and included six of our isolates supported statistically (posterior probability values = 91%/maximum likelihood bootstrap = 95%); The *Incertae sedis* clade grouped *Camarosporium* species including the *Camarosporium* isolate CF-285350 with high statistical support (pp = 100%/bs = 100%); The highly supported *Montagnulacea* clade grouped three different genera, *Pseudocamarosporium* (pp = 100%/bs = 98%), *Paracamarosporium* (pp = 100%/bs = 100%) and *Kalmusia* (pp = 99%/bs = 97%) including six of our isolates; The *Didymellaceae* clade, including species of *Xenodidymella*, *Neodidymelliopsis*, *Neomicrosphaeropsis*, *Didymella*, *Leptosphaerulina* and *Ascochyta* grouped 28 of our isolates with moderate statistical support (pp = 92%/bs = 89%); The highly supported *Leptosphaeriaceae* clade (pp = 100%/bs = 100%) included the *Neosetophoma* species and three *Phoma*-like isolates (CF-092164, CF-090312 and CF-091947); And the *Pleosporaceae* clade that was the largest one and clustered eight subclades including 82 of our isolates: *Libertasomyces*/*Neoplatysporoide*s, *Alternaria*, *Pleospora*, *Comoclathris*, *Tamaricicola*, *Decorospora*, *Phoma*-like and *Neocamarosporium*/*Dimorphosporicola*, all supported statistically (pp = 95–100%/bs > 70%) and where *Neocamarosporium* was the most representative genera with 35 isolates.

Activity distribution of fungal isolates. The fungal endophytes population was explored for their ability to generate antifungal and antitumoral activities. For this purpose, all 349 fungal isolates were grown in four media with and without the addition of polymeric resins. The corresponding 2792 crude extracts were tested against two fungal plant pathogens (*Magnaporthe grisea* and *Colletotrichum acutatum*), and two human fungal pathogens (*Aspergillus fumigatus* and *Candida albicans)*. Activities from these isolates were then classified into three groups according to their fermentation conditions: (i) hits induced by the use of polymeric resins, (ii) hits only produced without the resins and (iii) hits generated in both conditions (Fig. 2); we also grouped the strains

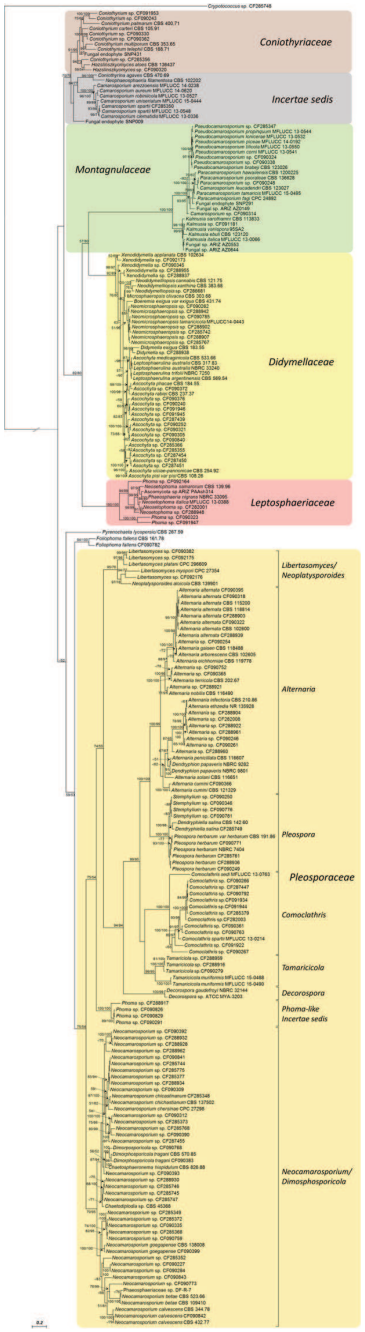

Figure 1. Consensus tree from Bayesian-phylogeny inferences based on ITS/28S sequences of almost all isolated genera within *Pleosporales* order and related genera. Clade probability values/maximum likelihood bootstrap values are indicated respectively on the branches. Values < 50 are designated by "−". *Cryptococcus* sp. CF-285748 was used as an outgroup.

according to their activity spectra in cytotoxic/non-cytotoxic or in broad/specific antifungal spectra (see Table S3 in Supplementary Information).

From the 349 isolates tested, 126 exhibited significant activity using the classical 'One Strain Many Conditions' (OSMAC) approach[25]. Among them, 35 isolates showed both cytotoxic and antifungal activities in multiple assays, and as many as three strains were found to be active in all assays tested (*Albifimbria verrucaria* CF-285778, and *Trichothecium roseum* CF-285757 and CF-277739). These strains produced several toxins in each fermentation

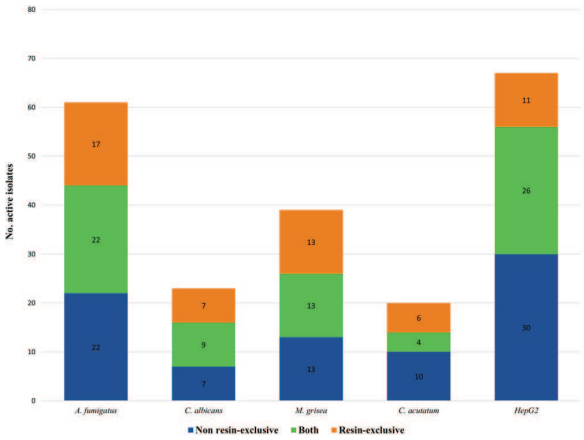

Figure 2. Activity distribution of isolates according to the fermentation conditions on each of the assays: (i) hits induced by adsorptive resins (orange), (ii) hits only produced without resins (blue) or (iii) hits produced in both conditions (green).

condition tested. Toxins were identified as verrucarin, roridin, iludin, and trichothecene when analyzed by mass spectrometry. Regarding the generation of antifungal activities against plant pathogens, 39 isolates showed inhibition zones larger than 6 mm of diameter against the rice phytopathogen *Magnaporthe grisea* in agar-based assays. For the bioassay of the fruit pathogen *Colletotrichum acutatum* a total of 20 isolates produced inhibition halos with more than 6 mm of diameter. When evaluated against human fungal pathogens, 61 fungal isolates showed more than 70% of growth inhibition against the opportunistic fungus *Aspergillus fumigatus* ATCC 46645 and 23 fungal isolates presented inhibition of the dimorphic fungus *Candida albicans* MY1055. The detailed distribution of activities induced by the addition of polymeric resins is described in Fig. 2.

In order to evaluate the production of antitumoral/cytotoxic compounds, all fungal isolates were also tested against the human hepatocellular carcinoma cell line HepG2 ATCC HB 8065. Among them, a total of 67 isolates showed HepG2 cell proliferation inhibitions higher than 70%, only 11 presented activity when grown in the presence of resins (six of them without antifungal activity), 30 when cultivated without the addition of resins (14 non-antifungal) and 26 isolates when grown in both conditions (23 with no antifungal activity). Regarding the non-cytotoxic strains, 16 presented complex antifungal spectra: *Stagonospora* sp. CF-281556 and *Camarosporium* sp. CF-090324 showed a broad spectrum against all fungal pathogens tested; *Fusarium equiseti* CF-285462, *Neocamarosporium* spp. CF090393 and CF-285768, *Phoma* sp. CF-285355 and *Pleospora* sp. CF-090792 showed a partial antifungal spectrum against human and plant pathogens; *Phaeosphaeria* sp. CF-288952 was active only against plant pathogens; *Comoclathris* sp. CF-090267, *Cryptococcus* sp. CF-285752, *Hormonema carpetanum* CF-090352, *Phaeotheca triangularis* CF-285358, *Phoma macrostoma* CF-287454, *Preussia australis* CF-288933 and *Xylaria* sp. CF-285461 were active only against the two human pathogens tested; Whereas, the remaining 75 strains showed specific activity against single fungal pathogens or the HepG2 cancer cell line (among them, 22 strains produced specific activity only when grown in the presence of resins, 31 only when grown without resins and 22 in both conditions; See Table S3 in Supplementary Information).

Chemical dereplication of active extracts. Regarding the distribution of known bioactive compounds among the active strains, active extracts from 126 strains were dereplicated by LC-MS against MEDINA's internal databases of known natural products by fingerprint matching of their HPLC retention time, ultraviolet and mass spectroscopy data. Fifty-four known bioactive molecules were identified from 75 strains both in resin- exclusive and non-exclusive production conditions which are represented in Fig. 3.

The extracts from the 51 active strains that did not present any matches in our databases were further analyzed by HR-MS (high resolution mass spectroscopy) for the tentative identification of additional molecules by matching their predicted molecular formula with the updated Dictionary of Natural Products databases for fungal natural products (DNP)[26]. Ascochlorin, coriolide, cerebroside B, solanopyrone B, brefeldin A, naematolin, ACTG toxin C, phaeosphenone, ilicicolin H, phomotone, phenopyrrozin, taurocholic acid, chrysoxanthone and sequalestatin H1were identified as produced by only 21 isolates (see Supplementary Table S3). We did not find any known molecules that could explain the activity observed in the remaining 30 active strains. In 16 cases we found predicted molecular formulas that had only been described previously in plant extracts and for the remaining 14 strains we found molecules whose molecular formulas did not show any coincidence in the DNP, with high chances of corresponding to bioactive molecules not yet described (Table 1).

Regarding the distribution of the known bioactive compounds among the fungal isolates, 35 compounds were detected in single strains whereas 19 were found in more than one strain. Among all these compounds, 12 were only detected in fermentations performed in presence of resins (Fig. 3). In general, phomasetin ($C_{25}H_{35}NO_4$) was the most frequent compound detected, being produced by 12 different strains, including four *Comoclatrhis* (CF-090792, CF-091944, CF-282003, CF-287447), two *Neomicrosphaeropsis* (CF-285741, CF-285767), one *Neo*camarosporium CF-090228, one *Pleospora* sp. CF-091933, one *Pleiochaeta* sp. CF-285364, one unidentified

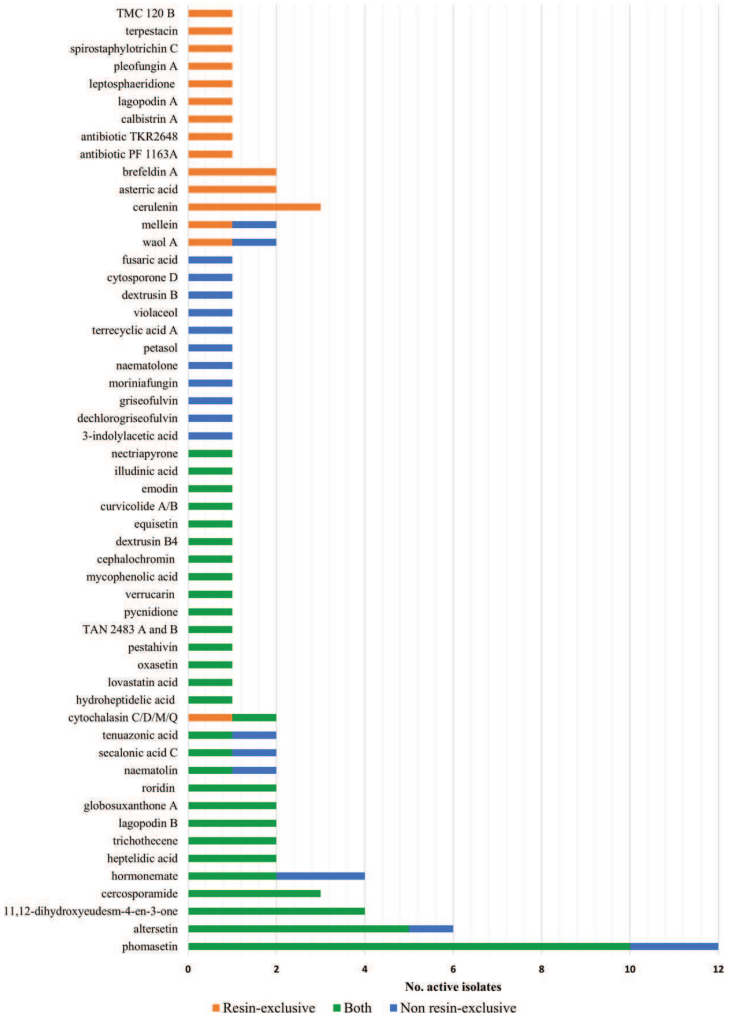

Figure 3. Distribution of dereplicated known fungal compounds in active isolates according to the fermentation condition of the producers. (i) Compounds detected in the presence of adsorptive resins (orange), (ii) compounds only produced without resins (blue) and (iii) compounds produced in both conditions (green).

Pleosporales CF-282344 and one *Leptosphaeria hispanica* CF-090357. This tetramic acid, isolated from *Phoma* sp., is closely related to equisetin, but with opposite stereochemistry[27]. The related compound, altersetin[28], was found to be produced by the four *Comoclathris* strains and the *Pleospora* sp. On the other hand, cercosporamide, a selective antifungal inhibitor of Pkc1 kinase[29], was produced by three different *Phoma*-like strains (CF-285355, CF-287454, CF-285365).

Differential expression of antifungal activities. The addition of adsorptive polymeric resins during fungal fermentations produced an increase in antifungal activities ranging from 20% (against *C. acutatum*) to 36% (against *A. fumigatus*). In order to characterize, in detail, the chemical diversity of the active isolates when antifungal activities were induced by the addition of resins (Fig. 3), secondary metabolite (SMs) profiles from fermentations generated with and without the resins were compared, and known bioactive molecules were identified by matching LC/MS databases.

In the presence of resins, 37 isolates presented antifungal activity (see Table 3 in Supplementary Information) and five of them resulted to be active against more than one fungal pathogen. Five isolates showed chemical profiles containing peaks not observed in the strains cultivated without resin, and 14 had components not described as known antifungals in the DNP database. For the remaining 12 isolates, increased production titers were observed in the presence of resins, and five produced compounds not described as known antifungals in DNP database.

Several examples of new activities obtained by the addition of resins during fermentation were due to known compounds identified by LCMS: (a) The broad-spectrum antifungal activity of the extracts of

Strain	RT (min)	$[M+H]^+$ Exp.	Proposed Ion	Main Secondary Experimental Ions	Production Media	Proposed Formula	Compound
CF-285754	3.91	497.11	$C_{22}H_{25}O_{11}S^+$	498.1138; 183.1372; 295.2259	LSFM + XAD16	$C_{22}H_{24}O_{11}S$	A
CF-285755	3.92	497.1104	$C_{22}H_{25}O_{11}S^+$	498.1138; 271.0587; 183.1217	LSFM + XAD16	$C_{22}H_{24}O_{11}S$	
CF-285753	3.91	497.1102	$C_{22}H_{25}O_{11}S^+$	498.1135; 499.1105; 519.0918	LSFM + XAD16	$C_{22}H_{24}O_{11}S$	
CF-288957	5.04	656.3848	$C_{30}H_{55}O_{14}^+$	657.3879; 658.39	YES & HP20	$C_{30}H_{54}O_{14}$	B
CF-090351	5.01	656.3852	$C_{30}H_{55}O_{14}^+$	657.3885; 658.3911	MMK2 & XAD16	$C_{30}H_{54}O_{14}$	
CF-091924	5.33	933.5659	$C_{46}H_{74}N_7O_{12}^+$	916.5391; 458.7725; 934.5688	YES & HP20	$C_{46}H_{73}N_7O_{12}$	C
CF-091924	5.49	947.5815	$C_{47}H_{76}N_7O_{12}^+$	930.5549; 948.5846; 931.5579	YES & HP20	$C_{47}H_{75}N_7O_{12}$	D
CF-091924	6.11	914.5596	$C_{47}H_{76}N_7O_{11}^+$	457.7832; 931.5863; 932.5891	YES & HP20	$C_{47}H_{75}N_7O_{11}$	E
CF-090782	5.08	328.2475	$C_{39}H_{28}NO_8S^+$	293.2104; 329.2508; 275.1997	MMK2 + XAD16	$C_{39}H_{27}NO_8S$	F
CF-288938	4.65	328.2471	$C_{39}H_{28}NO_8S^+$	275.1994; 311.2206; 279.2308	MMK2	$C_{39}H_{27}NO_8S$	
CF-287465	6.27	780.5469	$C_{40}H_{75}O_{13}^+$	781.5503; 745.5095; 785.5019	LSFM & XAD16	$C_{40}H_{74}O_{13}$	G
CF-285758	4.85	415.1392	$C_{22}H_{23}O_8^+$	416.1421; 359.1124; 301.0704	YES + HP20	$C_{22}H_{22}O_8$	H
CF-285760	2.61	521.2349	$C_{18}H_{33}N_8O_{10}^+$	522.2374; 538.2608; 523.2389	MMK2	$C_{18}H_{32}N_8O_{10}$	I
CF-285773	2.54	521.2335	$C_{18}H_{33}N_8O_{10}^+$	522.2363; 539.2626; 523.239	XPMK + HP20	$C_{18}H_{32}N_8O_{10}$	
CF-285765	5.54	551.1503	$C_{36}H_{23}O_6^+$	568.1767; 552.1532; 559.1794	YES	$C_{36}H_{22}O_6$	J
CF-090361	3.76	343.1926	$C_{18}H_{31}O_4S^+$	235.2048; 344.1957; 943.5241	LSFM& MMK2	$C_{18}H_{30}O_4S$	K
CF-090361	7.99	532.439	$C_{30}H_{59}O_4S^+$	515.4127; 532.44226; 516.416	LSFM & MMK2	$C_{30}H_{58}O_4S$	L

Table 1. Characterization of tentative new secondary metabolites by HPLC-ESI-TOF-MS.

Psudocamarosporium sp. CF-090324 when grown in LSFM medium with XAD-16 resin could be explained by the presence of calbistrin A, a compound only produced in this condition (Fig. 4A); (b) Dextrusin B_4, detected in the XPMK fermentation of *Alternaria* sp. CF-090752 explained the activity against A. *fumigatus* and HepG2. On the contrary, cultivation of the strain with HP-20 resin showed the induction of the antifungal agent terpestacin, extending the antifungal activity spectrum to *M. grisea* (Fig. 4B); (c) The production of mycotoxin secalonic acid C, identified in the extract of *Sclerostagonospora* sp. CF-281856, from a MMK2 fermentation explained the activity against *A. fumigatus*, *C. albicans* and *C. acutatum*. When this strain was cultivated in MMK2 with XAD-16, we observed an extended activity spectrum against *M. grisea*, due to the fact that the production of secalonic acid C was inhibited under these conditions and replaced by the production of a compound with molecular formula $C_{16}H_{14}O_{16}$. This compound was dereplicated by the DNP database as the phytotoxic alternethanoxin A (UV matching) (Fig. 4C); Finally, (d) the sesquiterpenoid quinone lagopodin B was detected in the extract of the basidiomycete *Coprinopsis episcopalis* CF-279244 culture grown in YES medium. However, the addition of the polymeric HP-20 resin induced the production of lagopodin A, and only trace amounts of lagopodin B. This extract only showed activity against *M. grisea* when produced in the presence of resin (Fig. 4D).

Differential expression of antitumoral activities. Similarly, eleven isolates presented inhibitory activities against the HepG2 cell line only when fermented in the presence of resins. In all cases, the presence of differential compounds in the secondary metabolite profiles was relevant when comparing fermentation conditions with and without resins. Some examples include: (a) *Preussia grandispora* CF-090835 that produced TMC-120C when grown in XPMK medium, whereas in presence of HP-20 resin, the fungus produced the related active compound TMC-120B with no trace of TMC-120C detected (Fig. 5A); (b) The semipreparative fractionation of the crude extract of *Neocamarosporium* sp. CF-285372 in LSFM with XAD-16 showed the accumulation of one mycotoxin of the spirostaphylotrichin family (C, D, G or H)[30] (Fig. 5B), only present in small quantities in the inactive LSFM condition; (c) Five induced metabolites were detected in the HepG2 active extract from the strain *Xylaria* sp. CF-285461 when grown with XAD-16 resin. Bio-assay guided semipreparative HPLC fractionation confirmed the production of five secondary metabolites not present in the condition without resin: the antibiotic TKR 2648[31], the α-pyrones 6-(1-hydroxypentyl)-4-methoxy-pyran-2-one and 6-pentyl-4-methoxy-pyran-2-one[32] and the mellein derivates (R)-(-)-5-carboxymellein[33] and (3 R)-5-cormylmellein[34] (Fig. 5C); (d) *Eutypa consobrina* CF-090213, that showed different LC/MS profiles when grown in the presence of resin, was selected for further analyses and a follow-up chemical fractionation (Fig. 5D).

New bioactive compounds induced in the presence of resins. For some of the fungal isolates, the addition of polymeric resins induced antifungal or antitumoral activities that correlated with the production of unknown chemical diversity[11]. To prove this concept, we selected the strain that produced the most potent activity against both human pathogens and that potentially showed new chemical diversity for scale-up isolation and identification of potential new bioactive compounds. As a result, a 2-liter scale-up fermentation of the *Xylariales* strain *Eutypa consobrina* CF-090213 was prepared in LSFM medium containing XAD-16 resin for the purification of its active components. After bioassay guided fractionation of the CF-090213 extract, three active compounds (**1–3**, Fig. 6) were identified from this fungal isolate, a compound with molecular formula $C_{17}H_{26}O_4$ (**1**; **MDN-0209**), produced both with and without the resin addition, and $C_{17}H_{24}O_4$ (**2**; **MDN-0210**) and $C_{10}H_{12}O_5$ (**3**; **MDN-0211**), induced only in presence of the XAD-16 resin. All three compounds were purified in amounts of

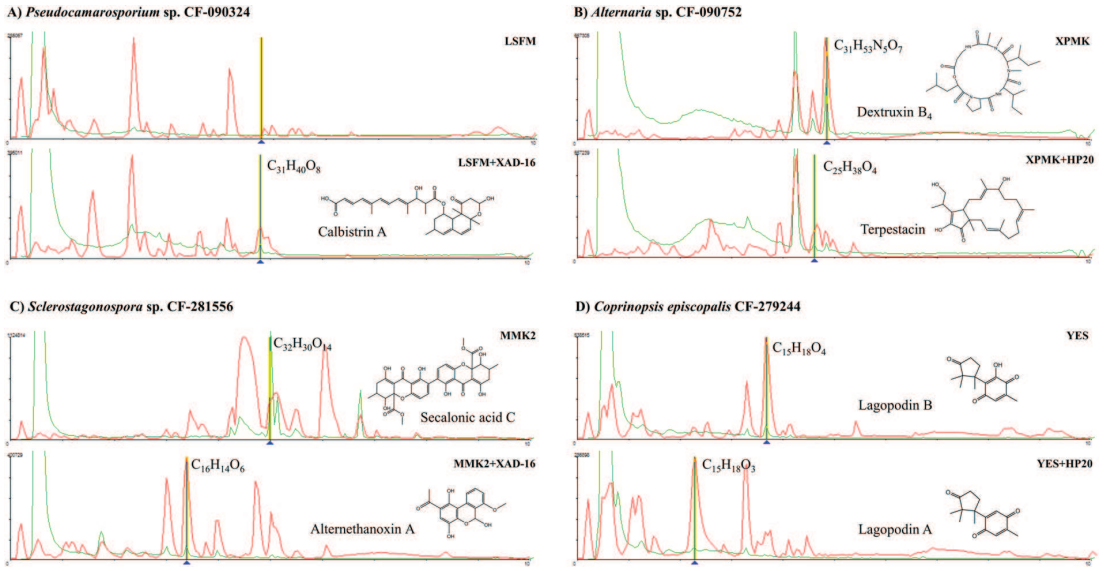

Figure 4. (**A**–**D**) Examples of the differential expression of antifungal agents induced by the presence of resins during the fermentation. Comparison of SMs profiles and identification of relevant components performed by LCMS.

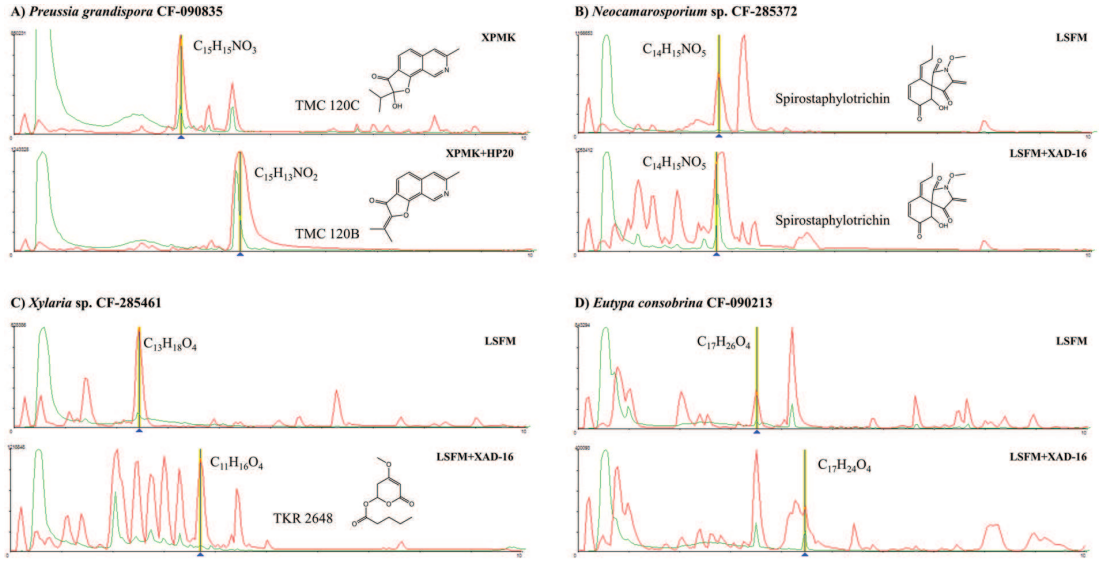

Figure 5. (**A**–**D**) Examples of the differential expression of cytotoxic agents induced by the presence of resins during fermentation. Comparison of SMs profiles and identification of relevant components performed by LCMS.

27, 2.4 and 1.4 mg, respectively. NMR analyses confirmed that these compounds were new secondary metabolites not yet described in nature (see Supplementary Information).

Further characterization of the antitumoral properties of these three compounds was carried out, testing them for cell proliferation inhibition against a panel of six human cancer cell lines, including hepatocarcinoma (HepG2), breast adenocarcinoma (MCF-7), pancreas cancer (Miapaca-2), melanoma (A-2058), lung cancer (A-549) and colon cancer (HT-29). Dose-response curves were performed at concentrations ranging from 0.01 to 100 µM while cells were treated for 72 h (see supplementary information) and ED_{50} (µM) was determined (Fig. 6). The most potent compound, **2**, presented a ED_{50} for HepG2 of 8.7 µM, and similar inhibition values for A-2058, HT-29 and Miapaca-2 cell lines, while for MCF-7 and A549 this compound was half as effective. Compound **1**, structurally related to **2**, showed a similar activity pattern, but less potent. On the contrary, compound **3** was

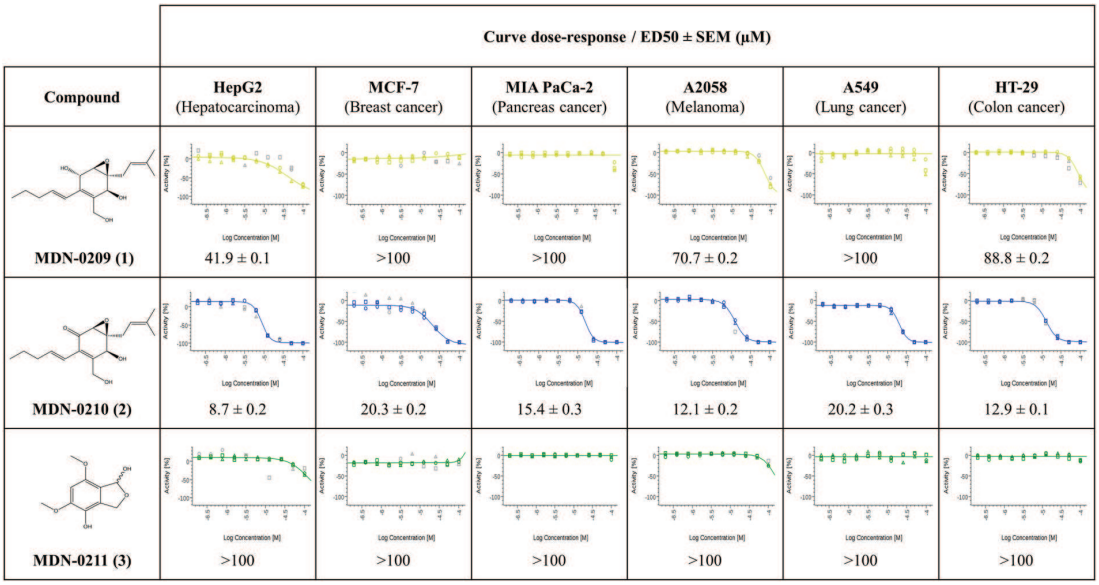

Figure 6. Dose-response curves and ED50 (μM) for new compounds **1–3** against a diverse panel of human cancer cells lines.

inactive with an effective ED_{50} above 100 μM for the HepG2 cell line. None of the three purified compounds displayed any antifungal activities.

Discussion

Few studies have reported the characterization of fungal endophytes in halophytic plants and generally, a low number of host species have been studied[35,36]. This report represents an exhaustive effort to extend the study to a large number of plant specimens from these biodiverse natural reserves, in order to isolate the culturable fungal endophyte community in halophytic and xerophytic plants. The plants included in this study are a representative example of the high diversity of species with restricted distribution due to the fact that they have adapted to these extreme environments, such as *Centaurea dracunculifolia*, *Euzomodendron bourgeanum*, *Helianthemum almeriense*, *Limonium majus* or *Moricandia foetida*. The wide community of plants studied has permitted to show that plants collected in arid zones of Andalusia are rich sources of fungal biodiversity with as many as 349 fungal isolates distributed among 19 taxonomic orders.

Pleosporales are the most frequent taxa identified among endophytes[37]. In line with this observation, in the present work *Pleosporales* was the most isolated order in the collected plants. Being the most abundant *Ascochyta*, *Phoma*, and the two recently described genera *Neomicrosphaeropsis*[23] and *Neocamarosporium*[38]. *Neocamarosporium* was the most frequently isolated genus, mostly from halophytes including the important plant endemisms *Salsola papillosa* or *Salsola genistoides*. To date, various species of this genus have been described from saline soil or halophytic plants[24,39,40]. Their presence in hypersaline environments and the ability of our isolates to grow in a medium containing more than 3% of NaCl suggest that this genus is naturally halotolerant coinciding with a recent study[39] (see Supplementary Information Fig. S2). The second most frequent genus was *Preussia*, confirming previous studies from plants collected in the Arizona desert[17]. Recently, our group has characterized and discussed the biodiversity and chemotaxonomy of these *Preussia* isolates[20]. *Alternaria* was the third genus present in the plants collected, in agreement with previous studies[41]. This genus features cosmopolitan fungi that include saprobic, endophytic and pathogenic species associated with a wide variety of substrates[42]. In addition, we have found several taxa from different plant species to those initially described, e.g. The genera *Neomicrosphaeropsis* and *Tamaricicola*, both reported as saprobic or weak pathogens of *Tamarix* species[23], were also isolated from *Frankenia*, *Arthrocnemum*, *Lycium* and *Limonium* (see Supplementary Information Table S1).

The *Pleosporales* phylogenetic analyses showed several clades containing isolates that could represent undescribed species, such as, *Tamaricicola* sp. CF-288959 isolated from the endemic *Limonium majus*, and *Phoma* sp. CF-288917, *Neocamarosporium* sp. CF-288932, *Xenodydimella* sp. CF-090345 or *Camarosporium* sp. CF-090314. Although the phylogenetic analysis in this study was focused mainly on *Pleosporales*, we also found potentially undescribed species among the isolates of *Dothideales*, namely belonging to *Aureobasidium*, *Kabatiella* or *Selenophoma* genera. A new family of hormonemate derivatives with cytotoxic activity has been recently purified from the culture of *Dothiora* sp. CF-285353[43], confirming the ability of this fungal community to produce new bioactive metabolites. Further studies to describe their morphologies and, in some cases, phylogenetic analyses based on housekeeping genes, should be carried out to confirm the taxonomic position of the new members of these orders.

The antifungal hit rates observed for our isolates were within the range of values reported for other studies with endophytic strains from Mediterranean areas[41,44]. Regarding the cytotoxic hit rates (19.2%), no previous data was available related with other antitumoral screenings performed with endophytes from arid areas. Our strains showed higher hit rates than endophytes reported from other environments[18,19,45]. Additionally, our isolates were able to produce a diversity of bioactive compounds from several chemical classes such as sesquiterpenoids (e.g. avocettin, trichothecene, lagopodins), polyketides (e.g. calbistrin A, phomasetin, altersetin), peptides (e.g. cyclo(phenyalanyl-prolyl)) and peptide polyketides (e.g. pleofungin A, naematolin).

It has been reported that the positive effects of resin additions to fermentation media are due to the adsorption of non-stable products or to the removal of a product involved in secondary metabolite pathway feedback repression[46]. The addition of polymeric resins to culture media during fungal fermentations induced and/or increased the production of bioactive molecules that were detected in all the assays tested in our screening (Fig. 2). A good example of resin induced antifungal activity was the generation of calbistrin A by the *Pseudocamarosporium* sp. CF-090324. This antifungal compound was previously isolated from various *Penicillium* and *Aspergillus* species[47,48]. Recently, the deletion of a putative polyketide synthase (PKS) in *Aspergillus aculeatus* and *Penicillium decumbens* has linked its biosynthesis to this gene cluster[49]. A homologous PKS is herein hypothesized to be cryptic in standard fermentation conditions, being this gene cluster expression activated in the presence of the resin. In addition, another five different compounds were isolated from *Xylaria* sp. CF-285461 in presence of XAD-16 resin: TKR2648, an inhibitor of metastasis of EL-4 and B16 tumor cells[31], two α-pyrones, and two mellein derivatives. Two different PKS biosynthetic pathways have been described for the biosynthesis of mellein and α-pyrones in fungi[50,51]. In our case the addition of the resin could be indirectly modulating gene expression in this fungus in more than one pathway.

Other interesting cases were those in which the resin captured the bioactive compound and protected it against post-biosynthetic degradation or biotransformation as in the case of *Coprinopsis episcopalis* CF-279244 and lagopodin A. Lagopodin A is an unstable compound in aqueous solution is transformed into lagopodin B in neutral or slightly alkaline solutions[52]. The addition of resin during the fermentation of *C. episcopalis* avoided the transformation from form A to B. Other examples are the isoquinoline alkaloids TMC-120A, B and C, described as bioactive compounds produced by *Aspergillus ustus*. Previous time-course studies with this strain showed that TMC-120B is produced in early stages of fermentation and that compounds A and C are produced sequentially, suggesting that TMC-120A and C are derived from TMC-120B by biological or chemical transformation[53]. In our producer isolate, *Preussia grandispora* CF-090385, the resin captured the bioactive TMC-120B preventing its posterior transformation. On the other hand, Spirostaphylotrichins and related compounds have been reported as metabolites produced by different *Pleosporales*[54–56]. These compounds belong to a chemical class of bioactive metabolites which includes curvupallides and phaeosphaerides, which share the same precursor and therefore the same biosynthetic pathway[57]. This type of gene clusters may be widespread among *Pleosporales*. In our case the use of resin captured spirostaphylotrichin C during the fermentation of *Neocamarosporium* sp. CF-285372, being this the first report in this genus.

Herein we hypothesized and confirmed that the addition of adsorptive resins could promote displacement of secondary metabolites in solution during fungal fermentations with the potential induction of new secondary metabolites. When applied in an extensive prospection of endophytic fungi from arid areas, resins promoted the production of three new compounds **1–3** isolated from *Eutypa consobrina* CF-090213. *Eutypa* spp are frequently found as phytopathogens of grape crops[58]. To date, 18 secondary metabolites have been reported from *Eutypa* species (DNP), including siccayne, also known to have antimicrobial activity[59]. A number of related epoxycyclohexenone-based natural products similar to **1** and **2** have been reported[60]. Some of these products are known to be phytotoxic[61,62], hence compounds **1–2** could be related to the phytopathogenic activity of *Eutypa* strains. More studies are necessary to understand the role of epoxycyclohexenones as virulence factors associated to the symptoms of *Eutypa* dieback.

This is the first study involving an extensive number of halophytic and xerophytic plant specimens surveyed from arid zones of southern Europe to culture and characterize their fungal symbiont community. We observed the dominant presence of members of *Pleosporales* in this fungal community along host plant species and sites, as well as host-specific fungal symbionts. This contributes to the understanding of ecological affiliations of fungal symbionts at regional and continental scales, where more studies are necessary to unravel the roles of these microorganisms in this plant community. Furthermore, the combination of the OSMAC approach, including the addition of adsorptive resins, with chemical diversity analyses, allowed us to highlight the high potential of these fungi as sources of bioactive secondary metabolites with biotechnological applications. As proof of concept, three new secondary metabolites were isolated and structurally elucidated from *Eutypa consobrina* when grown in the presence of resins. Further efforts on the isolation and structure elucidation of additional likely unknown bioactive secondary metabolites produced by 14 isolates of this fungal community are under way.

Methods

Plant collection. Representative arid areas of the Iberian southeast were surveyed, namely the provinces of Almeria and Granada, including Tabernas desert, Sierra de la Alhamilla, Cabo de Gata, Torre Garcia beach, Los Vados and the salt marshes of El Margen (Cullar). In these regions, the climatic conditions favor the accumulation of gypsum and other more soluble salts that benefit the development of endemic halophile or salt tolerant vegetation. A total of 63 characteristic plant species were collected from these arid areas (see Supplementary Information Table S4).

Isolation Cultures and Characterization. Fungal endophytes were isolated using standard indirect isolation techniques[16]. Stems and leaves removed from each plant were cut into pieces of approximately 5 mm. These pieces were surface-disinfected by sequential washing with 95% ethanol (30 s), 25% commercial bleach (1 min)

and 95% ethanol (30 s). Ten pieces of each plant sample were aseptically transferred to a Petri dish with corn meal agar (CMA) supplemented with streptomycin sulfate and oxytetracycline (50 mg/mL). Epiphyte fungi were also directly isolated from cleistothecia or conidiophores formed on plants by incubation in moist chambers. Isolates were cultured in YM agar (malt extract 10 g, yeast extract 2 g, agar 20 g, 1000 mL distilled H_2O), to study their macroscopic and microscopic characteristics. Strains, designated with unique IDs (e.g., CF-285353), were preserved as frozen conidia and mycelia in 10% glycerol at −80 °C and are maintained in Fundación MEDINA's fungal culture collection. DNA extraction, PCR amplification and DNA sequencing were performed as previously described[20]. Sequences of the complete ITS_1-5.8S-ITS_2-28S region or independent ITS and partial 28S rDNA sequences were compared with sequences at GenBank®, the NITE Biological Resource Center (http://www.nbrc.nite.go.jp) and CBS strain database (http://www.westerdijkinstitute.nl) by using the BLAST® application.

Phylogenetic analysis. Species and genus affinities of *Pleosporales* were inferred from a Bayesian analysis using the Markov Chain Monte Carlo (MCMC) approach with MrBayes 3.01[63]. To improve mixing of the chains, four incrementally heated simultaneous Monte Carlo Markov chains were run over 2×10^6 generations. Hierarchical likelihood ratio tests with the MrModeltest® 2.2 software[64] were used to calculate the Akaike Information Criterion (AIC) of the nucleotide substitution models. The model selected by AIC for the alignment was GTR + I + G that is based on six classes of substitution types, a portion of invariant alignment positions and mean substitution rates, varied across the remaining positions according to a gamma distribution. The MCMC processes were followed by a Dirichlet process prior (DPP) to obtain the substitution rates and nucleotide frequencies, and a unification of the rate parameter for the gamma distribution. The MCMC analysis was performed using a sampling frequency parameter of 100 and the first 1.000 trees were discarded before the majority rule consensus tree was calculated. In addition, Maximum Likelihood method (ML) and ultrafast bootstrap support values for phylogenetic trees were assessed calculating 1000 replicates with IQ-TREE software[65]. All parameters were estimated with this software (TPM2u + F + I + G4 nucleotide substitution model was selected), assuming a shape parameter of the Invar + Gamma distributed substitution rates (gamma shape alpha = 0.5355) to accommodate rate variations among sites and an estimation of nucleotide frequencies as A = 0.25, C = 0.23, G = 0.26 and T = 0.26.

Generation of fungal extracts. The 349 fungal isolates were grown in four culture media with different carbon and nitrogen sources (LSFM, MMK2, XPMK and YES media) with and without the two adsorptive polymeric resins that presented best results per media in previous studies[11]. XAD-16N (Amberlite® from Sigma-Aldrich™) was added in LSFM and MMK2 media, whereas Diaion® HP-20 (styrene-divinylbenzene Supelco™) was added in XPMK and YES media. After 14 days of incubation fermentation broths were extracted according to the procedure previously described[11].

Extract bioactivity characterization. The evaluation of antifungal activities against phytopathogenic fungi was performed using two important plant pathogens (*Colletotrichum acutatum* CF-137177 and *Magnaporthe grisea* CF-105765). The microorganisms were incubated with the extracts in an agar-based assay for 24 h at 25 °C and the activities were scored by using an image analyzer to measure the diameter of inhibition halos[66]. Fungal extracts were also evaluated against two human pathogens: *Candida albicans* MY1055 and *Aspergillus fumigatus* ATCC 46645. Target microorganisms were incubated with the extracts in a liquid-based assay for 18–30 h at 37 °C and activities were scored using resazurin, an oxidation-reduction indicator of cell viability[67]. The cytotoxicity of the different extracts against the HepG2 cell line (hepatocellular carcinoma, ATCC HB 8065) was evaluated by the MTT reduction colorimetric assay, with the same incubation times and assay concentrations as those used for their antifungal evaluation[68,69]. Furthermore, for the characterization of the antitumoral activity profiles of purified compounds **1**–**3**, MTT assays were also performed against the MCF-7 (breast adenocarcinoma ATCC HTB-22), MIA PaCa-2 (pancreas carcinoma ATCC CRL 1420), A2058 (melanoma ATCC CLR-11147), A549 (lung carcinoma ATCC CCL-185) and HT-29 (colon adenocarcinoma ATCC HTB-38) cell lines and ED50 values were determined for each cell line[69].

Dereplication of bioactive extracts. Chemical profiling of extracts was performed by LC/MS and compared with our internal proprietary databases for the identification of known secondary metabolites by low resolution LC-LRMS (UV signal, retention time, and fragmentation patterns) against 970 standards, and high resolution LC-HRMS (retention time and accurate mass) against 1073 standards[70]. In addition, compounds that were not identified in the databases of standards were enriched by semi-preparative HPLC and, once detected by LC-HRMS, their predicted molecular formulas were searched against the Chapman & Hall Dictionary of Natural Products (DNP; v25.1) and confirmed by LC-ESI-HRMS/MS fragmentation in order to determine if they matched other compounds previously described in literature.

Isolation of induced bioactive compounds. Bioactive fungal extracts were selected for 2 L scale-up fermentation in flasks containing 100 mL of LSFM medium with Amberlite XAD-16 resin (3% v/v) and extraction with acetone (2 L) under continuous shaking at 220 rpm for 1 h. The mycelium was then pelleted by centrifugation and the supernatant (4 L) was concentrated to 1.8 L under a stream of nitrogen. This solution was loaded (with continuous 1:1 water dilution, discarding the flow-through) on a column packed with SP-207SS reversed phase resin (brominated styren polymer, 65 g, 35 × 120 mm) previously equilibrated with water. The loaded column was further washed with water (2 L) and afterwards eluted at 8 mL min^{-1} on an automated flash-chromatography system (CombiFlash Rf®, Teledyne VERTEX™) using a linear gradient from 10% to 100% acetone in water for 30 min and a final 100% acetone step for 15 min. 20 mL fractions were collected. DMSO (700 µL) was added to each fraction to avoid precipitation of molecules while samples were concentrated to evaporate acetone and

water in a centrifugal vacuum evaporator. Fractions containing the bioactive compounds were characterized by LC-UV-MS for dereplication and identification of the compounds of interest. Preparative reverse phase HPLC fractionation (Agilent™ Zorbax® SB-C8, 22 × 250 mm, 7 μm; 20 mL min^{-1}, UV detection at 210 nm) was performed with a linear gradient of acetonitrile in water from 5% to 100% over 37 min, and enriched fractions with compounds **1**–**3** were obtained at retention times of 27.7, 28.8 and 26.6 min, respectively. Subsequent preparative fractionation with a linear gradient from 6% to 10% of acetonitrile in water with 0.1% of TFA yielded 1.4 mg of **3** with 90% purity after 26 minutes; semipreparative reversed phase HPLC fractionation (Waters™ XBridge® C18, 10 × 150 mm, 5 μm; 3.6 mL min^{-1}, UV detection at 210 nm) with 25.5% of acetonitrile in water was required to obtain pure compound **1** (27 mg) after 27 minutes of retention time. Finally, semipreparative reverse-phase HPLC fractionation (Agilent™ Zorbax® SB-C8, 9.4 × 250 mm, 5 μm; 3.6 mL min^{-1}, UV detection at 210 nm) was required to obtain compound **2** (2.4 mg), at a retention time of 27 minutes in a 37.5% isocratic run. HRMS, NMR spectroscopy and Mosher analysis were employed in the structure elucidation of all three of the compounds (detailed in Supplementary Information).

References

1. Chandra, S. Endophytic fungi: novel sources of anticancer lead molecules. *Applied microbiology and biotechnology* **95**, 47–59, https://doi.org/10.1007/s00253-012-4128-7 (2012).
2. Strobel, G. A. *et al*. Cryptocandin, a potent antimycotic from the endophytic fungus Cryptosporiopsis cf. quercina. *Microbiology (Reading, England)* **145**(Pt 8), 1919–1926, https://doi.org/10.1099/13500872-145-8-1919 (1999).
3. Pelaez, F. *et al*. The discovery of enfumafungin, a novel antifungal compound produced by an endophytic Hormonema species biological activity and taxonomy of the producing organisms. *Systematic and applied microbiology* **23**, 333–343 (2000).
4. Brady, S. F. & Clardy, J. CR377, a new pentaketide antifungal agent isolated from an endophytic fungus. *Journal of natural products* **63**, 1447–1448 (2000).
5. Li, J. Y. *et al*. Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from Pestalotiopsis spp. and Monochaetia sp. *Phytochemistry* **56**, 463–468 (2001).
6. Li, J. Y. & Strobel, G. A. Jesterone and hydroxy-jesterone antioomycete cyclohexenone epoxides from the endophytic fungus Pestalotiopsis jesteri. *Phytochemistry* **57**, 261–265 (2001).
7. Basilio, A. *et al*. The discovery of moriniafungin, a novel sordarin derivative produced by Morinia pestalozzioides. *Bioorganic & medicinal chemistry* **14**, 560–566, https://doi.org/10.1016/j.bmc.2005.08.046 (2006).
8. Bills, G. F. *et al*. Discovery of the parnafungins, antifungal metabolites that inhibit mRNA polyadenylation, from the Fusarium larvarum complex and other Hypocrealean fungi. *Mycologia* **101**, 449–472 (2009).
9. Singh, S. B. *et al*. Isolation, structure, and biological activity of Phaeofungin, a cyclic lipodepsipeptide from a Phaeosphaeria sp. Using the Genome-Wide Candida albicans Fitness Test. *Journal of natural products* **76**, 334–345, https://doi.org/10.1021/np300704s (2013).
10. Venugopalan, A. & Srivastava, S. Endophytes as *in vitro* production platforms of high value plant secondary metabolites. *Biotechnology advances* **33**, 873–887, https://doi.org/10.1016/j.biotechadv.2015.07.004 (2015).
11. Gonzalez-Menendez, V. *et al*. Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity. *Mycology* **5**, 179–191, https://doi.org/10.1080/21501203.2014.942406 (2014).
12. Armas, C., Miranda, J. D., Padilla, F. M. & Pugnaire, F. I. Special issue: The Iberian Southeast. *Journal of Arid Environments* **75**, 1241–1243, https://doi.org/10.1016/j.jaridenv.2011.08.002 (2011).
13. Poveda, J. F. M., Mota, J. F., Cueto, M. & Merlo, M. E. *Flora amenazada de la provincia de Almería*. (Universidad de Almería, Servicio de Publicaciones, 2003).
14. Singh, L. P., Gill, S. S. & Tuteja, N. Unraveling the role of fungal symbionts in plant abiotic stress tolerance. *Plant signaling & behavior* **6**, 175–191 (2011).
15. Zabalgogeazcoa, I. Fungal endophytes and their interaction with plant pathogens: a review. 2008 **6**, 9, https://doi.org/10.5424/sjar/200806S1-382 (2008).
16. Bills, G. F., Menendez, V. G. & Platas, G. Kabatiella bupleuri sp. nov. (Dothideales), a pleomorphic epiphyte and endophyte of the Mediterranean plant Bupleurum gibraltarium (Apiaceae). *Mycologia* **104**, 962–973, https://doi.org/10.3852/12-003 (2012).
17. Massimo, N. C. *et al*. Fungal endophytes in aboveground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. *Microbial ecology* **70**, 61–76, https://doi.org/10.1007/s00248-014-0563-6 (2015).
18. Ratnaweera, P. B., de Silva, E. D., Williams, D. E. & Andersen, R. J. Antimicrobial activities of endophytic fungi obtained from the arid zone invasive plant Opuntia dillenii and the isolation of equisetin, from endophytic Fusarium sp. *BMC complementary and alternative medicine* **15**, 220, https://doi.org/10.1186/s12906-015-0722-4 (2015).
19. Li, H., Qing, C., Zhang, Y. & Zhao, Z. Screening for Endophytic Fungi with Antitumour and Antifungal Activities from Chinese Medicinal Plants. *World Journal of Microbiology and Biotechnology* **21**, 1515–1519, https://doi.org/10.1007/s11274-005-7381-4 (2005).
20. Gonzalez-Menendez, V. *et al*. Biodiversity and chemotaxonomy of Preussia isolates from the Iberian Peninsula. *Mycological Progress* **16**, 713–728, https://doi.org/10.1007/s11557-017-1305-1 (2017).
21. U'Ren, J. M., Lutzoni, F., Miadlikowska, J. & Arnold, A. E. Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. *Microbial ecology* **60**, 340–353, https://doi.org/10.1007/s00248-010-9698-2 (2010).
22. Ariyawansa, H. A. *et al*. Fungal diversity notes 111–252—taxonomic and phylogenetic contributions to fungal taxa. *Fungal Diversity* **75**, 27–274, https://doi.org/10.1007/s13225-015-0346-5 (2015).
23. Thambugala, K. M. *et al*. Microfungi on Tamarix. *Fungal Diversity* **82**, 239–306, https://doi.org/10.1007/s13225-016-0371-z (2017).
24. Crous, P. W. & Groenewald, J. Z. The Genera of Fungi – G 4: Camarosporium and Dothiora. *IMA Fungus* **8**, 131–152, https://doi.org/10.5598/imafungus.2017.08.01.10 (2017).
25. Hewage, R. T., Aree, T., Mahidol, C., Ruchirawat, S. & Kittakoop, P. One strain-many compounds (OSMAC) method for production of polyketides, azaphilones, and an isochromanone using the endophytic fungus Dothideomycete sp. *Phytochemistry* **108**, 87–94, https://doi.org/10.1016/j.phytochem.2014.09.013 (2014).
26. Buckingham, J. *Dictionary of Natural Products*. (Taylor & Francis, 1993).
27. Singh, S. B. *et al*. Equisetin and a novel opposite stereochemical homolog phomasetin, two fungal metabolites as inhibitors of HIV-1 integrase. *Tetrahedron Letters* **39**, 2243–2246, https://doi.org/10.1016/S0040-4039(98)00269-X (1998).
28. Hellwig, V. *et al*. Altersetin, a new antibiotic from cultures of endophytic Alternaria spp. Taxonomy, fermentation, isolation, structure elucidation and biological activities. *The Journal of antibiotics* **55**, 881–892 (2002).
29. Sussman, A. *et al*. Discovery of Cercosporamide, a Known Antifungal Natural Product, as a Selective Pkc1 Kinase Inhibitor through High-Throughput Screening. *Eukaryotic Cell* **3**, 932–943, https://doi.org/10.1128/EC.3.4.932-943.2004 (2004).
30. Masi, M. *et al*. Spirostaphylotrichin W, a spirocyclic γ-lactam isolated from liquid culture of Pyrenophora semeniperda, a potential mycoherbicide for cheatgrass (Bromus tectorum) biocontrol. *Tetrahedron* **70**, 1497–1501, https://doi.org/10.1016/j.tet.2013.12.056 (2014).
31. Takesako, K., Saito, H., Ueno, M., Awazu, N. & Kato, I. (Google Patents, 1998).

32. Evidente, A. *et al*. Phytotoxic alpha-pyrones produced by Pestalotiopsis guepinii, the causal agent of hazelnut twig blight. *The Journal of antibiotics* **65**, 203–206, https://doi.org/10.1038/ja.2011.134 (2012).
33. Klaiklay, S. *et al*. Metabolites from the mangrove-derived fungus Xylaria cubensis PSU-MA34. *Archives of pharmacal research* **35**, 1127–1131, https://doi.org/10.1007/s12272-012-0701-y (2012).
34. Sumarah, M. W., Puniani, E., Blackwell, B. A. & Miller, J. D. Characterization of Polyketide Metabolites from Foliar Endophytes of Picea glauca. *Journal of natural products* **71**, 1393–1398, https://doi.org/10.1021/np800192f (2008).
35. You, Y. H. *et al*. Fungal diversity and plant growth promotion of endophytic fungi from six halophytes in Suncheon Bay. *Journal of microbiology and biotechnology* **22**, 1549–1556 (2012).
36. Khalmuratova, I. *et al*. Diversity and Plant Growth Promoting Capacity of Endophytic Fungi Associated with Halophytic Plants from the West Coast of Korea. *Mycobiology* **43**, 373–383, https://doi.org/10.5941/MYCO.2015.43.4.373 (2015).
37. Bills, G. F. In *Endophytic Fungi in Grasses and Woody Plants* (ed Redlin S. C. ans Carris L. M) 31–65 (APS Press, 1996).
38. Crous, P. W. *et al*. Fungal Planet description sheets: 281–319. *Persoonia: Molecular Phylogeny and Evolution of Fungi* **33**, 212–289, https://doi.org/10.3767/003158514X685680 (2014).
39. Papizadeh, M. *et al*. Neocamarosporium jorjanensis, N. persepolisi, and N. solicola spp. nov. (Neocamarosporiaceae, Pleosporales) isolated from saline lakes of Iran indicate the possible halotolerant nature for the genus. *Mycological Progress*. https://doi.org/10.1007/s11557-017-1341-x (2017).
40. Wanasinghe, D. N. *et al*. Phylogenetic revision of Camarosporium (Pleosporineae, Dothideomycetes) and allied genera. *Studies in Mycology* **87**, 207–256, https://doi.org/10.1016/j.simyco.2017.08.001 (2017).
41. Peláez, F. *et al*. Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycological Research* **102**, 755–761, https://doi.org/10.1017/S0953756297005662 (1998).
42. Woudenberg, J. H. C., Groenewald, J. Z., Binder, M. & Crous, P. W. Alternaria redefined. *Studies in Mycology* **75**, 171–212, https://doi.org/10.3114/sim0015 (2013).
43. Perez-Bonilla, M., Gonzalez-Menendez, V. & Perez-Victoria, I. *Hormonemate Derivatives from Dothiora sp., an Endophytic Fungus.* **80**, 845–853, https://doi.org/10.1021/acs.jnatprod.6b00680 (2017).
44. Luo, Z. P., Lin, H. Y., Ding, W. B., He, H. L. & Li, Y. Z. Phylogenetic Diversity and Antifungal Activity of Endophytic Fungi Associated with Tephrosia purpurea. *Mycobiology* **43**, 435–443, https://doi.org/10.5941/myco.2015.43.4.435 (2015).
45. Huang, Y., Wang, J., Li, G., Zheng, Z. & Su, W. Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants Taxus mairei, Cephalataxus fortunei and Torreya grandis. *FEMS immunology and medical microbiology* **31**, 163–167 (2001).
46. Fryķman, S., Tsuruta, H., Galazzo, J. & Licari, P. Characterization of product capture resin during microbial cultivations. *Journal of Industrial Microbiology and Biotechnology* **33**, 445–453, https://doi.org/10.1007/s10295-006-0088-1 (2006).
47. Jackson, M. *et al*. Calbistrins, novel antifungal agents produced by Penicillium restrictum. I. Production, taxonomy of the producing organism and biological activity. *The Journal of antibiotics* **46**, 34–38 (1993).
48. Petersen, L. M., Hoeck, C., Frisvad, J. C., Gotfredsen, C. H. & Larsen, T. O. Dereplication guided discovery of secondary metabolites of mixed biosynthetic origin from Aspergillus aculeatus. *Molecules (Basel, Switzerland)* **19**, 10898–10921, https://doi.org/10.3390/molecules190810898 (2014).
49. Grijseels, S. *et al*. in *29th Fungal genetics conference Genetics Society of America*. Vol. 29 (Genetics Society of America, Pacific Grove, CA, 2017).
50. Chooi, Y.-H. *et al*. An In Planta-Expressed Polyketide Synthase Produces (R)-Mellein in the Wheat Pathogen Parastagonospora nodorum. *Applied and Environmental Microbiology* **81**, 177–186, https://doi.org/10.1128/AEM.02745-14 (2015).
51. Schaberle, T. F. Biosynthesis of alpha-pyrones. *Beilstein journal of organic chemistry* **12**, 571–588, https://doi.org/10.3762/bjoc.12.56 (2016).
52. Bu'Lock, J. D. & Darbyshire, J. Lagopodin metabolites and artefacts in cultures of Coprinus [lagopus, Fungi] (1976).
53. Kohno, J. *et al*. Production, isolation and biological properties of TMC-120A, B and C, novel inhibitors of eosinophil survival from Aspergillus ustus TC 1118. *The Journal of antibiotics* **52**, 913–916 (1999).
54. de Almeida, T. T. *et al*. Curvulin and spirostaphylotrichins R and U from extracts produced by two endophytic Bipolaris sp. associated to aquatic macrophytes with antileishmanial activity. *Natural product research*, 1–8, https://doi.org/10.1080/14786419.2017.1380011 (2017).
55. Maloney, K. N. *et al*. Phaeosphaeride A, an inhibitor of STAT3-dependent signaling isolated from an endophytic fungus. *Organic letters* **8**, 4067–4070, https://doi.org/10.1021/ol061556f (2006).
56. Masi, M. *et al*. Pyrenophoric acids B and C, two new phytotoxic sesquiterpenoids produced by Pyrenophora semeniperda. *Journal of agricultural and food chemistry* **62**, 10304–10311, https://doi.org/10.1021/jf5035515 (2014).
57. Yokoyama, M. *et al*. Integration of Chemical, Genetic, and Bioinformatic Approaches Delineates Fungal Polyketide-Peptide Hybrid. *Biosynthesis.* **19**, 2002–2005, https://doi.org/10.1021/acs.orglett.7b00559 (2017).
58. Trouillas, F. P., Urbez-Torres, J. R. & Gubler, W. D. Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. *Mycologia* **102**, 319–336 (2010).
59. Kim, J. H., Mahoney, N., Chan, K. L., Molyneux, R. J. & Campbell, B. C. Secondary metabolites of the grapevine pathogen Eutypa lata inhibit mitochondrial respiration, based on a model bioassay using the yeast Saccharomyces cerevisiae. *Current microbiology* **49**, 282–287, https://doi.org/10.1007/s00284-004-4349-9 (2004).
60. Ciavatta, M. L. *et al*. Cytosporin-related compounds from the marine-derived fungus Eutypella scoparia. *Tetrahedron* **64**, 5365–5369, https://doi.org/10.1016/j.tet.2008.03.016 (2008).
61. Andolfi, A. *et al*. Phytotoxins produced by fungi associated with grapevine trunk diseases. *Toxins* **3**, 1569–1605, https://doi.org/10.3390/toxins3121569 (2011).
62. Shiono, Y. *et al*. Three oxygenated cyclohexenone derivatives produced by an endophytic fungus. *Bioscience, biotechnology, and biochemistry* **69**, 287–292, https://doi.org/10.1271/bbb.69.287 (2005).
63. Ronquist, F. & Huelsenbeck, J. P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics (Oxford, England)* **19**, 1572–1574 (2003).
64. Nylander, J. A. A. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University (2004).
65. Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution* **32**, 268–274, https://doi.org/10.1093/molbev/msu300 (2015).
66. Serrano, R. *et al*. Co-culturing of Fungal Strains Against Botrytis cinerea as a Model for the Induction of Chemical Diversity and Therapeutic Agents. *Frontiers in Microbiology* **8**, 649, https://doi.org/10.3389/fmicb.2017.00649 (2017).
67. Monteiro, M. C. *et al*. A new approach to drug discovery: high-throughput screening of microbial natural extracts against Aspergillus fumigatus using resazurin. *Journal of biomolecular screening* **17**, 542–549, https://doi.org/10.1177/1087057111433459 (2012).
68. de Pedro, N. *et al*. Analysis of cytotoxic activity at short incubation times reveals profound differences among Annonaceus acetogenins, inhibitors of mitochondrial Complex I. *Journal of bioenergetics and biomembranes* **45**, 145–152, https://doi.org/10.1007/s10863-012-9490-8 (2013).
69. Cautain, B. *et al*. Identification of the Lipodepsipeptide MDN-0066, a Novel Inhibitor of VHL/HIF Pathway Produced by a New Pseudomonas Species. *PloS one* **10**, e0125221, https://doi.org/10.1371/journal.pone.0125221 (2015).
70. Gonzalez-Menendez, V. *et al*. Multicomponent Analysis of the Differential Induction of Secondary Metabolite Profiles in Fungal Endophytes. *Molecules (Basel, Switzerland)* **21**, https://doi.org/10.3390/molecules21020234 (2016).

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Author Contributions

V.G.-M., J.R.T. and O.G. conceived and designed the experiments; V.G.-M., isolated, grown and identified the fungal strains, N.D., C.D., R.S., T.A.M., screened the compounds; G.C., C.J., V.G.-M., J.R.T. and F.R. isolated the compounds and determined their structures; V.G.-M., J.R.T. and O.G. analyzed the data; M.R.G.-T., M.C., J.M., F.V., F.R. contributed to reagents/materials/analysis tools; V.G.-M. wrote the main manuscript text and J.R.T. and O.G. critically revised the manuscript.

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Chapter 2

Biodiversity and chemotaxonomy of *Preussia* isolates from Iberian Peninsula

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ORIGINAL ARTICLE

Biodiversity and chemotaxonomy of *Preussia* isolates from the Iberian Peninsula

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Abstract This work documents 32 new *Preussia* isolates from the Iberian Peninsula, including endophytic and saprobic strains. The morphological study of the teleomorphs and anamorphs was combined with a molecular phylogenetic analysis based on sequences of the ribosomal rDNA gene cluster and chemotaxonomic studies based on liquid chromatography coupled to electrospray mass spectrometry. Sixteen natural compounds were identified. On the basis of combined analyses, 11 chemotypes are inferred.

Keywords *Preussia* · Chemotypes · Mass spectrometry · Secondary metabolites

Introduction

The combination of geo-climatic factors that influence the Iberian Peninsula have shaped an extraordinary variety of habitats. These privileged areas for biodiversity studies have great richness in flora and fauna, where endemic and singular plants are likely to be present. Although more than 10,000 fungal species have been described in Spain (Moreno-Arroyo 2004), most of them were mushrooms, leaving this environment open to other exhaustive fungal studies. Very few examples of fungal endophytes have been described from the Iberian Peninsula, suggesting that a large number of new fungal species will be discovered (Collado et al. 2002; Oberwinkler et al. 2006; Bills et al. 2012).

Members of the Sporormiaceae are widespread and, despite that they are most commonly found on various types of animal dung, they can also be isolated from soil, wood, and plant debris. Fungi of Sporormiaceae form dark brown, septate spores with germ slits, and include approximately 100 species divided into ten genera, including the recently described genera *Forliomyces* and *Sparticola* (Phukhamsakda et al. 2016) and *Chaetopreussia*, *Pleophragmia*, *Preussia*, *Pycnidiophora*, *Sporormia*, *Sporormiella*, *Sporormiella*, *Sporormiella*, *Sporormiella*, and *Westerdykella*. Among these, *Sporormiella* and *Preussia* are particularly species-rich (Barr 2000).

The genus *Preussia* was erected by Fuckel (1866) to include bitunicate ascomycetes with non-ostiolate, globose to subglobose ascomata, 8-spored, broadly clavate or subglobose asci, and ascospores with germ slits that are mostly surrounded by a gelatinous sheath. *Preussia* species are isolated from soil, wood, or plant debris. Later, *Sporormiella* was defined to include coprophilous bitunicate ascomycetes with ostiolate perithecioid ascomata and cylindrical to cylindric-claviform asci (Ellis and Everhart 1892). In 1961, Cain (1961) reviewed the genus *Preussia*, included new coprophilous species, and, accordingly, broadened the ecological concept of the genus. von Arx (1973) highlighted that the presence or absence of ostioles may vary with the growth conditions, indicating that this morphological character could not be considered as a valid taxonomic criterion. In 2009, a

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systematic analysis on the phylogenetic relationships based on four loci (ITS, 28S, 18S, and β-tubulin) proposed 12 new *Preussia* combinations (Kruys and Wedin 2009). Nevertheless, recent publications maintain the genera *Preussia* and *Sporormiella* (Doveri and Sarrocco 2013).

Previous studies identified 33 *Preussia* species from the Iberian Peninsula. *Preussia intermedia* was the first species cited by Urries (1932), followed by *P. dakotensis* cited in a study of ascomycetes of the Iberian Peninsula and the Balearic Islands (Unamuno 1941). Lundqvist (1960) reported four additional species of *Preussia* (*P. lageniformis*, *P. longispora*, *P. megalospora*, and *P. minima*) in a report on coprophilous ascomycetes from northern Spain. Later reported species were *P. pascua* (de la Torre 1974), *P. australis*, *P. grandispora*, *P. vexans* (Barrasa and Moreno 1980), *P. clavispora* (Guarro et al. 1981), *P. thypharum* (Guarro Artigas 1983), *P. cylindrospora*, *P. dubia*, *P. heptamera*, *P. irregularis*, *P. leporina*, *P. ovina*, *P. teretispora*, *P. pyriformis* (Barrasa 1985), *P. capybarae*, *P. cymatomera*, *P. systenospora* (Soláns 1985), *P. tenerifae* (von Arx and Van der Aa 1987), *P. splendens* (Sierra López 1987), *P. fleischhakii* (Barrasa and Checa 1989), *P. affinis* and *P. funiculata* (Valldosera and Guarro 1990), and *P. mediterranea* (Arenal et al. 2007). All previously cited species were isolated from dung except *P. mediterranea*, which was isolated from the plant *Cistus albidus*. More recently, the hairy species *Sporormiella octomegaspora* was isolated from deer dung in Andalusia (Doveri and Sarrocco 2013).

Coprophilous fungi play an important ecological role in decomposing and recycling nutrients from animal dung. They have the ability to produce a large array of bioactive secondary metabolites (Sarrocco 2016). Bioactive secondary metabolites produced by these fungi are typically involved in defense mechanisms against other competing microbes (Bills et al. 2013). Most of these bioactive compounds are antifungals, such as australifungin, an inhibitor of the sphingolipid synthesis (Hensens et al. 1995), preussomerins, inhibitors of the ras farnesyl-protein transferase (Weber et al. 1990), and zaragozic acids, potent inhibitors of squalene synthase (Bergstrom et al. 1995).

Bioactive secondary metabolites produced by *Preussia* species such as 7-chloro-6-methoxymellein, hyalopyrone, leptosin, cissetin, or microsphaeropsone A are also produced by other fungi, while auranticins, australifungin, zaragozic acid B, terezines, and sporminarins are known to be produced exclusively by *Preussia* sp. (Table 1).

The purpose of this study was to review the fungal biodiversity of *Preussia* species from environmental samples of the Iberian Peninsula and characterize occurring chemotypes. The biodiversity of *Preussia* endophytes isolated from plants in arid zones from the south of Spain and a small number of strains from soils and herbivore dung were compared with *Preussia* strains from Arizona desert plants (Massimo et al. 2015) and other Sporormiaceae obtained from public collections.

Materials and methods

Isolation, culturing, and morphology

Nine areas, including Mediterranean and Eurosiberian regions, were surveyed. Different plant species, characteristic of each geographic region, soil, and animal dung were sampled. Standard indirect techniques were performed to isolate plant endophytes: plant specimens such as stems or leaves were cut into 5-mm^2 fragments. Their surface was disinfected by serial immersion in 95% ethanol (30 s), 25% bleach (1.25% NaClO) (1 min), and 95% ethanol (30 s). Ten sterilized fragments were aseptically transferred to corn meal agar (CMA) and supplemented with streptomycin sulfate (50 mg/mL) and oxytetracycline (50 mg/mL) (Bills et al. 2012). Soil fungi were obtained following a particle filtration method (Bills et al. 2004). Coprophilous fungi were isolated directly from perithecia developed on animal dung after incubation in moist chambers.

Isolates were cultured on 2% malt agar (MEA), CMA, oat meal agar (OMA, Difco™), and synthetic nutrient agar (SNA; Nirenberg 1976) to study their macroscopic and microscopic characteristics. Microscopic features were evaluated by observing the structures in 5% KOH. Axenic strains were preserved as frozen suspensions of conidia, ascospores, or sterile mycelium in 10% glycerol at −80 °C. Strains are currently maintained in the Fundación MEDINA culture collection (http://www.medinadiscovery.com). ID coding, geographical origin, isolation substrata, and GenBank accession numbers of their rDNA gene sequences are listed in Table 2.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from aerial mycelia of strains grown on malt-yeast extract agar (Bills et al. 2012). DNA fragments containing the ITS1–5.8S–ITS2 (ITS) and the initial 600 nucleotides of the 28S rDNA gene (28S) were amplified with the 18S3 (5′-GATGCCCTTAGATGTTCTGGGG-3′) (Bills et al. 2012) and NL4 primers (O'Donnell 1993). Polymerase chain reaction (PCR) amplifications followed standard procedures (5 min at 93 °C, 40 cycles of 30 s at 93 °C, 30 s at 53 °C, and 2 min at 72 °C), using the Taq DNA polymerase (QBiogene™ Inc.), following the manufacturer-recommended procedures. Amplification products (0.1 mg/mL) were sequenced with the Big Dye Terminator Cycle Sequencing Kit® (Applied Biosystems™), also following manufacturer recommendations. Each PCR product was sequenced bidirectionally with the same primers that were used for the PCR reactions. Partial sequences obtained during sequencing reactions were assembled with the GeneStudio® software (GeneStudio™ Inc., Georgia). Sequences of the complete ITS1–5.8S–ITS2–28S region or independent ITS and partial 28S rDNA sequences were

Table 1 Bioactive secondary metabolites reported from *Preussia* species

Compounds	Species	Major nutrient sources	Fermentation state	Biological activity	References
Asterric acid	*Preussia* sp.	Rice-based media	Solid	Endothelin binding inhibitor	Talontsi et al. (2014)
Auranticins A, B	*P. aurantiaca*	Rice-based media	Solid	Antifungal and antibacterial	Poch and Gloer (1991)
7-Chloro-6-methoxymellein	*P. affinis*	Corn starch and molasses	Liquid	Antifungal	McGahren and Mitscher (1968)
Antibiotic FR 173945	*P. aemulans*	Starch and peach power	Liquid	Antifungal	Sato et al. (1998)
Antibiotic WF 15604A	*P. minima*	Starch acid and cotton flour	Liquid	Antifungal	Hatori et al. (2004)
Australifungin	*P. australis*	Corn-based media	Solid	Cytotoxic	Hensens et al. (1995)
$C_{28}H_{42}N_4O_8$	*P. minimoides*	Corn meal and sucrose	Liquid	Antifungal	Clapp-Shapiro et al. (1998)
Cissetin	*Preussia* sp.	Rice-based media	Solid	Antibacterial	Talontsi et al. (2014)
Cryptosporiopsin	*P. affinis*	Corn starch and molasses	Liquid	Antifungal	McGahren et al. (1969)
Culpin	*Preussia* sp.	Soluble starch and glucose	Liquid	Antifungal and antibacterial	Robinson et al. (1988)
Cyperin	*P. fleischhakii*	Potato dextrose broth	Liquid	Phytotoxic	Weber and Gloer (1988)
Hyalopyrone	*P. teretispora*	Soy flour medium	Liquid	Phytotoxic	Wang et al. (1995)
Leptosin A, C	*P. typharum*	Cheerios breakfast cereal	Solid	Cytotoxic	Du et al. (2014)
Preussiadin A, B	*P. typharum*	Cheerios breakfast cereal	Solid	Cytotoxic	Du et al. (2014)
Microsphaeropsone A	*P. minima*	Rice-based media	Solid	Antifungal	Xiong et al. (2014)
Preussiafuran A, B	*Preussia* sp.	Rice-based media	Solid	Cytotoxic	Talontsi et al. (2014)
Preussin	*Preussia* sp.	Soluble starch and glucose	Liquid	Antifungal	Johnson et al. (1989)
Preussochromone A, B, C, D	*P. africana*	Rice-based media	Solid	Cytotoxic	Zhang et al. (2012)
Preussomerin A, E, D, G, B	*P. isomera*	Potato dextrose broth	Liquid	ras Farnesyl-protein	Weber and Gloer (1991)
Similin A, B	*P. similis*	Soybean and dextrose	Liquid	Antifungal	Weber et al. (1992)
Spiropreussione	*Preussia* sp.	Wheat bran and glucose	Liquid	Cytotoxic	Chen et al. (2009)
Sporminarin A, B	*P. minimoides*	Rice-based media	Solid	Antifungal	Mudur et al. (2006)
Sporostatin	*Preussia* sp.	Glucose and peptone broth	Liquid	Cytotoxic	Kinoshita et al. (1997)
Sporovexin A, B, C	*P. vexans*	Potato dextrose broth	Liquid	Antifungal and antibacterial	Soman et al. (1999)
Terezine A, B, C, D	*P. teretispora*	Soy flour medium	Liquid	Antifungal and antibacterial	Wang et al. (1995)
Zaragozic acid B	*P. intermedia*	Cerelose and cottonseed	Liquid	Antifungal	Bergstrom et al. (1995)

compared with sequences deposited at GenBank® or the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/) by using the BLAST® application.

Phylogenetic analysis

Species and genus affinities were inferred in a Bayesian analysis by using the Markov chain Monte Carlo (MCMC) approach with MrBayes 3.01 (Ronquist and Huelsenbeck 2003). To improve mixing of the chains, four incrementally heated simultaneous Monte Carlo Markov chains were run over 2×10^6 generations. Hierarchical likelihood ratio tests with the MrModeltest® 2.2 software (Nylander 2004) were used to calculate the Akaike information criterion (AIC) of the nucleotide substitution models. The model selected by the AIC for the alignment was GTR + I + G, which is based on six classes of substitution types, a portion of invariant alignment positions, and mean substitution rates, varied across the remaining positions according to a gamma distribution. Priors used for the MCMC processes were followed by a Dirichlet distribution for the substitution of rates and nucleotide frequencies, and a unification of the rate parameter for the gamma distribution. The MCMC analysis used the following parameters:

Table 2 *Preussia* strains included in the phylogenetic analysis (newly isolated strains from the Iberian Peninsula are in **bold**)

Species	Strain code[a]	Substrate	Origin	GenBank accession numbers[b]		Reference
				ITS	28S	
Forliomyces uniseptata	MFLUCC 15-0765 (ex-type)	*Spartium junceum*	Spain	KU721772	KU721762	Phukhamsakda et al. (2016
Preussia aemulans	CBS 287.67	Soil	The Netherlands	DQ468017	DQ468037	Arenal et al. (2007)
Preussia aemulans	CBS 318.81	Soil	The Netherlands	KX710218		–
Preussia africana	S17 (holotype)	*Viburnum tinus* leaf	Tenerife, Spain	AY510418	AY510383	Arenal et al. (2005)
Preussia africana	**CF-279770**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710221**		–
Preussia africana	**CF-098213**	***Erica araganensis***	**Spain, Lugo**	**KX710222**		–
Preussia africana	S14	Zebra dung	South Africa	AY510417	AY510382	Arenal et al. (2005)
Preussia africana	DN136	*Ramalina calicaris*	China	JQ031265		Zhang et al. (2012)
Preussia africana	S15	Zebra dung	South Africa	AY510421	AY510385	Arenal et al. (2005)
Preussia africana	S12	Goat dung	Tanzania	AY510420	AY510384	Arenal et al. (2005)
Preussia cymatomera	CBS 396.81	*Juniperus communis*	Switzerland	KX710252		–
Preussia flanaganii	CBS 112.73 (isotype)	Sandy soil	Mexico	AY943061		–
Preussia fleischhakii	CBS 565.63	Soil	Germany	GQ203761	GQ203721	Kruys and Wedin (2009)
Preussia fleischhakii	CBS 361.49	Man's nail	The Netherlands	DQ468018	DQ468038	Arenal et al. (2007)
Preussia funiculata	UPS:Huhndorf et al. 2577 (F)	Porcupine dung	USA	GQ203762	GQ203722	Kruys and Wedin (2009)
Preussia isomera	CBS 415.82	–	Venezuela	KX710243		–
Preussia isomera	NBRC 30581	Soil	Nepal	NBRC 03058101		–
Preussia isomera	CBS 388.78	Cow dung	Venezuela	GQ203763	GQ203723	Kruys and Wedin (2009)
Preussia isomera	CBS 671.77	–	Japan	KX710241		–
Preussia mediterranea	S23 (holotype)	*Cistus albidus* leaf	Spain, Caceres	DQ468022	DQ468042	Arenal et al. (2007)
Preussia mediterranea	S30	*Alnus glutinosa* leaf	Spain, Caceres	DQ468023	DQ468043	Arenal et al. (2007)
Preussia mediterranea	S34	*Daphne gnidium* leaf	Spain, Caceres	DQ468025	DQ468045	Arenal et al. (2007)
Preussia mediterranea	S31	*Quercus suber* leaf	Spain, Caceres	DQ468024	DQ468044	Arenal et al. (2007)
Preussia mediterranea	S22	*Quercus ilex* leaf	Spain, Guadalajara	DQ468021	DQ468041	Arenal et al. (2007)
Preussia minimoides	MEXU 26355	*Hintonia latiflora*	Mexico	KF557658	KF557659	Leyte-Lugo et al. (2013)
Preussia minimoides	NRRL 37629	*Trametes hirsutum*	Hawaii	GU183123		Mudur et al. (2006)
Preussia minimoides	S10	Pig dung	Argentina	AY510423	AY510388	Arenal et al. (2005)
Preussia minimoides	S18	*Prunus lusitanica*	Canary island	AY510422	AY510387	Arenal et al. (2005)
Preussia persica	CBS 117680	Barley dead leaf	Iran	GQ292750	GQ292752	Asgari and Zare (2010)
Preussia polymorpha	CBS 117679 (holotype)	Barley dead leaf	Iran	GQ292749	GQ292751	Asgari and Zare (2010)
Preussia sp.	ELV3.2	*Eustrephus latifolius*	Australia	JN418773	KF269206	Mapperson et al. (2014)
Preussia sp.	ELV3.11	*Eustrephus latifolius*	Australia	JN418774	KF269205	Mapperson et al. (2014)
***Preussia* sp.**	**CF-095571**	***Pinus pinaster***	**Spain, Lerida**	**KX710247**		–
	CF-155367					–
Preussia sp.	WO-009	Soil	Spain	KX710239		–
Preussia sp.	CF-209171	Kudu	South Africa	KX710223		–
***Preussia* sp.**	**CF-277787**	**Soil**	**Spain, Granada**	**KX710262**		–
***Preussia* sp.**	**CF-277801**	**Soil**	**Spain, Granada**	**KX710260**		–
***Preussia* sp.**	**CF-277817**	**Soil**	**Spain, Granada**	**KX710251**		–
***Preussia* sp.**	**CF-277822**	**Soil**	**Spain, Granada**	**KX710220**		–
***Preussia* sp.**	**CF-277849**	**Soil**	**Spain, Granada**	**KX710263**		–
***Preussia* sp.**	**CF-277856**	**Soil**	**Spain, Granada**	**KX710253**		–
***Preussia* sp.**	**CF-277965**	**Soil**	**Spain, Granada**	**KX710261**		–
***Preussia* sp.**	**CF-279766**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710250**		–
***Preussia* sp.**	**CF-279773**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710256**		–
***Preussia* sp.**	**CF-282341**	***Dittrichia viscosa***	**Spain, Granada**	**KU295582**		**González-Menéndez et al.** (2016)
***Preussia* sp.**	**CF-285370**	***Anabasis articulata***	**Spain, Almeria**	**KX710254**		–
***Preussia* sp.**	**CF-285378**	***Salsola oppositifolia***	**Spain, Almeria**	**KX710249**		–
***Preussia* sp.**	**CF-285772**	***Phragmites australis***	**Spain, Granada**	**KX710255**		–
Preussia sp.	SNP008	*Larrea tridentata*	USA	KP335214		Massimo et al. (2015)
Preussia sp.	SNP034	*Simmondsia chinensis*	USA	KP335239		Massimo et al. (2015)
Preussia sp.	SNP057	*Parkinsonia microphylla*	USA	KP335262		Massimo et al. (2015)
Preussia sp.	SNP156	*Phoradendron californicum*	USA	KP335359		Massimo et al. (2015)
Preussia sp.	SNP164	*Larrea tridentata*	USA	KP335365		Massimo et al. (2015)
Preussia sp.	SNP208	*Larrea tridentata*	USA	KP335402		Massimo et al. (2015)
Preussia sp.	SNP220	*Larrea tridentata*	USA	KP335411		Massimo et al. (2015)
Preussia sp.	SNP223	*Larrea tridentata*	USA	KP335414		Massimo et al. (2015)
Preussia sp.	SNP232	*Larrea tridentata*	USA	KP335422		Massimo et al. (2015)
Preussia sp.	SNP235	*Parkinsonia microphylla*	USA	KP335425		Massimo et al. (2015)
Preussia sp.	SNP252	*Simmondsia chinensis*	USA	KP335440		Massimo et al. (2015)
Preussia sp.	SNP301	*Parkinsonia microphylla*	USA	KP335487		Massimo et al. (2015)
Preussia sp.	SNP309	*Simmondsia chinensis*	USA	KP335495		Massimo et al. (2015)
Preussia sp.	SNP334	*Larrea tridentata*	USA	KP335519		Massimo et al. (2015)
Preussia sp.	SNP392	*Larrea tridentata*	USA	KP335569		Massimo et al. (2015)
Preussia sp.	SNP408		USA	KP335584		Massimo et al. (2015)

Table 2 (continued)

Species	Strain code[a]	Substrate	Origin	GenBank accession numbers[b]		Reference
				ITS	28S	
		Phoradendron californicum				
Preussia sp.	SNP419	*Simmondsia chinensis*	USA	KP335595		Massimo et al. (2015)
Preussia sp.	SNP420	*Simmondsia chinensis*	USA	KP335596		Massimo et al. (2015)
Preussia sp.	SNP437	*Simmondsia chinensis*	USA	KP335613		Massimo et al. (2015)
Preussia sp.	SNP458	*Parkinsonia microphylla*	USA	KP335634		Massimo et al. (2015)
Preussia sp.	SNP459	*Larrea tridentata*	USA	KP335638		Massimo et al. (2015)
Preussia subticinensis	CBS 443.9	–	France	KX710258		–
Preussia subticinensis	CBS 125.66	–	Germany	KX710259		–
Preussia subticinensis	**CF-278595**	**Soil**	**Spain, Granada**	**KX710257**		**–**
Preussia terricola	CBS 317.65	*Musa sapientum*	Honduras	GQ203765	GQ203725	Kruys and Wedin (2009)
Preussia terricola	CBS 527.84	Elephant dung	Tanzania	GQ203764	GQ203724	Kruys and Wedin (2009)
Preussia typharum	CBS 107.69	Deer dung	Japan	GQ203766	GQ203726	Kruys and Wedin (2009)
Preussia typharum	718249	Degraded organic matter	USA	JX143871		
Preussia typharum	NBRC 32847	Unidentified plant	Iraq	NBRC 03284701		–
Preussia typharum	**CF-085890**	**Microbial mats**	**Spain, Tarragona**	**KX710219**		**–**
Preussia vulgaris	UPS:Strid 18884	Hare dung	Sweden	GQ203767	GQ203727	Kruys and Wedin (2009)
Sparticola junci	MFLUCC 15-0030 (ex-type)	*Spartium junceum*	Spain	KU721775	KU721765	Phukhamsakda et al. (2016)
Sparticola forlicesenica	MFLUCC 14-1097 (ex-type)	*Spartium junceum*	Spain	KU721773	KU721763	Phukhamsakda et al. (2016)
Sporormiella affinis	UPS:Lundqvist 17739-j	Rabbit dung	Denmark	GQ203770	GQ203730	Kruys and Wedin (2009)
Sporormiella alloiomera	UPS:Lundqvist 21345-p	Goat dung	Norway	GQ203771	GQ203731	Kruys and Wedin (2009)
Sporormiella antarctica	CBS 222.89	Soil	Norway	KX710224		–
Sporormiella australis	S5	Gazelle dung	South Africa	AY510411	AY510376	Arenal et al. (2005)
Sporormiella australis	**CF-285375**	***Launaea arborescens***	**Spain, Almeria**	**KU295583**		**González-Menéndez et al.** (2016)
Sporormiella australis	NBRC 101144	Soil	Israel	NBRC 11731401		–
Sporormiella australis	S7	Zebra dung	South Africa	AY510413	AY510378	Arenal et al. (2005)
Sporormiella australis	**CF-091932**	***Tamarix canariensis***	**Spain, Almeria**	**KX710240**		**–**
Sporormiella australis	UPS:Lundqvist 20884-a	Rabbit dung	France	GQ203773	GQ203732	Kruys and Wedin (2009)
Sporormiella australis	S6	Gazelle dung	Namibia	AY510412	AY510377	Arenal et al. (2005)
Sporormiella bipartis	UPS:Lundqvist 17250-a	*Lagopus muta* dung	Sweden	GQ203774	GQ203733	Kruys and Wedin (2009)
Sporormiella borealis	UPS:Lundqvist 16745-c	Horse dung	Romania	GQ203775	GQ203734	Kruys and Wedin (2009)
Sporormiella dakotensis	UPS:Thulin 2570-g	Cow dung	Ethiopia	GQ203776	GQ203735	Kruys and Wedin (2009)
Sporormiella dubia	UPS:Strid 19562-G	Horse dung	Iceland	GQ203777	GQ203736	Kruys and Wedin (2009)
Sporormiella grandispora	S37	*Phragmites communis*	Madrid, Spain	DQ468032	DQ468052	Arenal et al. (2007)
Sporormiella heptamera	UPS:Lundqvist 3090-b	Horse dung	Sweden	GQ203778	GQ203737	Kruys and Wedin (2009)
Sporormiella intermedia	UPS:Kruys 304	Cow dung	Sweden	GQ203779	GQ203738	Kruys and Wedin (2009)
Sporormiella intermedia	S1	Elk dung	USA	AY510415	AY510380	Arenal et al. (2005)
Sporormiella intermedia	CF-208569 WQ-056	Dung	Spain	KX710225		–
Sporormiella intermedia	**CF-279774**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710226**		**–**
Sporormiella intermedia	**CF-209155**	***Lithodora diffusa***	**Portugal, Algarve**	**KX710227**		**–**
Sporormiella intermedia	S4	Goat dung	Greece	AY510416	AY510381	Arenal et al. (2005)
Sporormiella intermedia	S3	Goat dung	Greece	AY510414	AY510379	Arenal et al. (2005)
Sporormiella intermedia	UAMH 7460	*Populus tremuloides*	Canada	DQ468020	DQ468040	Arenal et al. (2007)
Sporormiella irregularis	UPS:Lundqvist 16568-f	Cow dung	Hungary	GQ203780	GQ203739	Kruys and Wedin (2009)
Sporormiella isabellae	S25 (holotype)	Leaf litter	Puerto Rico	AY510424	AY510389	Arenal et al. (2005)
Sporormiella leporina	UPS:Richardson MJR93/04	Spruce grouse dung	Canada	GQ203782	GQ203741	Kruys and Wedin (2009)
Sporormiella leporina	UPS:Lundqvist 19873-a	Hare dung	Sweden	GQ203781	GQ203740	Kruys and Wedin (2009)
Sporormiella lignicola	**CF-282334**	***Dittrichia viscosa***	**Spain, Granada**	**KX710231**		**–**
Sporormiella lignicola	CBS 363.69	Rabbit dung	The Netherlands	GQ203783	DQ384098	Kruys and Wedin (2009)
Sporormiella lignicola	**CF-282002**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710232**		**–**
Sporormiella lignicola	**CF-282345**	***Dittrichia viscosa***	**Spain, Granada**	**KX710233**		**–**
Sporormiella lignicola	**CF-279765**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710234**		**–**
Sporormiella lignicola	**CF-097553**	***Viscum album***	**Spain, Guadalajara**	**KX710235**		**–**
Sporormiella lignicola	**CF-121346**	***Erica australis***	**Spain, Caceres**	**KX710236**		**–**
Sporormiella lignicola	**CF-279767**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710237**		**–**
Sporormiella lignicola	CF-214984 WQ-124	Dung	Spain	KX710238		–
Sporormiella lignicola	**CF-090241**	***Genista umbellata***	**Spain, Almeria**	**KX710230**		**–**
Sporormiella longisporopsis	UPS:Lundqvist 16551-g	Rabbit dung	Hungary	GQ203784	GQ203742	Kruys and Wedin (2009)
Sporormiella megalospora	UPS:Kruys 305	Cow dung	Sweden	GQ203785	GQ203743	Kruys and Wedin (2009)
Sporormiella minima	S13	Gazelle dung	Namibia	AY510426	AY510391	Arenal et al. (2005)
Sporormiella minima	CF-160935	Elephant dung	India	KX710243		–
Sporormiella minima	CF-066028	Vegetation	Dominican Republic	KX710244		–
Sporormiella minima	**CF-279768**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710245**		**–**
Sporormiella minima	CF-215748 WQ-140	Dung	Spain	KX710246		–
Sporormiella minima	NBRC 32842	Garden soil	India	NBRC 03284201		–
Sporormiella minima	CBS 524.50	Goat dung	Panama	DQ468026	DQ468046	Arenal et al. (2007)
Sporormiella minima	UPS:Lundqvist 17212-a	Cow dung	Sweden	GQ203786	GQ203744	Kruys and Wedin (2009)

Table 2 (continued)

Species	Strain code[a]	Substrate	Origin	GenBank accession numbers[b]		Reference
				ITS	28S	
Sporormiella minima	NBRC 8595	Soil	–	NBRC -00859501		–
Sporormiella minima	S26	Leaf litter	USA	AY510427	AY510392	Arenal et al. (2005)
Sporormiella minima	S21	Rhinoceros dung	South Africa	AY510425	AY510390	Arenal et al. (2005)
Sporormiella minima	CF-209022 WQ-064	Dung	Spain	KX710248		–
Sporormiella minipascua	UPS:Kruys 306	Cow dung	Sweden	GQ203787	GQ203745	Kruys and Wedin (2009)
Sporormiella muskokensis	NBRC 8539	Soil	–	NBRC 00853901		–
Sporormiella octomera	UPS:Huhndorf et al. 2579	Porcupine dung	USA	GQ203788	GQ203746	Kruys and Wedin (2009)
Sporormiella pilosella	S38	*Quercus ilex* twigs	Guadalajara Spain	DQ468033	DQ468053	Arenal et al. (2007)
Sporormiella pulchella	UPS:MJR67/01, #216605	Rabbit dung	USA	GQ203789	GQ203747	Kruys and Wedin (2009)
Sporormiella septenaria	UPS:Espigores 00036	Sheep dung	Argentina	GQ203790	GQ203748	Kruys and Wedin (2009)
Sporormiella similis	**CF-285357**	***Asparagus horridus***	**Spain, Almeria**	**KX710228**		–
Sporormiella similis	S19	Undetermined dung	USA	AY510419	AY510386	Arenal et al. (2005)
Sporormiella similis	CBS 804.73	Saline dessert soil	Kuwait	DQ468028	DQ468048	Kruys and Wedin (2009)
Sporormiella similis	CF-210023 WQ-023	Dung	Spain	KX710229		–
Sporormiella vexans	UPS:23.VIII.1995, Andersson	Moose dung	Sweden	GQ203793	GQ203751	Kruys and Wedin (2009)
Sporormiella tenerifae	CBS 354.86	Rabbit dung	Tenerife	GQ203794	GQ203752	Kruys and Wedin (2009)
Verruculina enalia	CF-090068	Soil	Singapore	KX710217		–
Westerdykella dispersa	CBS 297.56	*Phlox drummondii*	USA	GQ203797	GQ203753	Kruys and Wedin (2009)
Westerdykella ornata	CBS 379.55 (holotype)	Mangrove mud	Mozambique	AY943045	GU301880	Schoch et al. (2009)
Westerdykella purpurea	NBRC 9428 (ex-type)	Soil	–	LC146766		–

[a] *CBS* CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; *CF* Fundación MEDINA Private Fungal Collection, Granada, Spain; *MFLUCC* Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; *NBRC* Biological Resource Center, National Institute of Technology and Evolution, Tokyo, Japan; *NRRL* Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture, Peoria, Illinois, USA; *UAMH* University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada; *UPS* The Museum of Evolution Herbarium, Sweden

[b] Accession numbers of sequences newly generated in this study are indicated in **bold**. *28S* Large subunit of the nrDNA; *ITS* internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDNA

sampling frequency = 100; first 1000 trees were discarded before the majority rule consensus tree was calculated.

In addition, the maximum likelihood (ML) method and ultrafast bootstrap support values for the phylogenetic tree were assessed calculating 1000 replicates with IQ-TREE software (Nguyen et al. 2015). All parameters were estimated by the software [the TIM2e + I + G4 model of nucleotide substitution was selected, assuming the shape parameter of the Invar + Gamma distributed substitution rates (gamma shape alpha = 0.4917) to accommodate rate variations among sites and an estimation of nucleotide frequencies as A = 0.25, C = 0.25, G = 0.25, and T = 0.25]. Aligned sequence data and phylogenetic trees were deposited in TreeBASE (SN 20908) http://purl.org/phylo/treebase/phylows/study/TB2:S20908

Preparation of extracts and metabolomic analysis

Thirty-seven fungal strains (23 Iberian isolates plus 14 *Preussia* strains from public collections) were grown in duplicate in two culture media with different carbon and nitrogen sources (MMK2 and YES media; González-Menéndez et al. 2014). Extracts generated from submerged fungal cultures were analyzed by low-resolution mass spectrometry (LR-MS) in the range of positive m/z for each extract. Four sets of m/z data ranging from 150 to 1500 Da were generated for each culture. The differential chemotypes in the crudes were identified using a matrix that correlated the intensity of each m/z per strain and a multivariate statistical analysis using Bionumerics® (Applied Maths™). The resulting dendrogram, built on a similarity matrix based on the m/z signals according to the Pearson correlation coefficient (see the supplementary material) and unweighted pair group method with arithmetic mean (UPGMA) allowed the identification of differential secondary metabolites and chemotypes among the studied species.

Chemical profiles were performed and compared to our internal proprietary databases for the identification of known secondary metabolites by low-resolution LC-LRMS (UV signal, retention time, and fragmentation patterns) against 950 standards and high-resolution LC-HRMS (retention time and accurate mass) against 835 standards (González-Menéndez et al. 2016; Pérez-Victoria et al. 2016). In addition, the compounds that were not identified from the database of standards were isolated by semi-preparative HPLC. Their predicted molecular formulas were confirmed by LC-ESI-HRMS/MS and compared to the entries in the Chapman & Hall Dictionary of Natural Products (v25.1) in order to identify compounds already described in the literature.

Results

Phylogenetic analysis and morphological observations

DNA fragments consisting of 465–485 bp (ITS) and 584–587 bp (28S) were obtained for the sequenced *Preussia* isolates. The different runs of the Bayesian analyses that were performed and ML analyses yielded the same topology (TreeBASE SN 20908). The consensus phylogenetic tree of 32 isolated strains with 104 GenBank™ sequences of representative strains including endophytic *Preussia* strains isolated recently from plants of the Arizona desert (Massimo et al. 2015) showed a very similar topology to the phylogenetic tree obtained previously by Kruys and Wedin (2009). Overall, all the *Preussia* strains are grouped in a single cluster that accommodates numerous, monophyletic, statistically supported subclades of both algorithms (posterior probability values = 95–100%/maximum likelihood bootstrap >70%). The only exception was the clade containing the strains of *P. minima*, *P. persica*, *P. isabellae*, and *P. mediterranea* that, despite the lack of support by Bayesian analyses, was well-supported by ML bootstrap (98%).

In detail, the ITS/28S rDNA tree revealed 19 clades named according to Kruys and Wedin (2009) (Fig. 1): (a) the clades "Sparticola", "Forliomyces", and "Westerdykella" were supported as previously shown in other phylogenetic studies (pp = 100%/bs = 100%) for each clade (Phukhamsakda et al. 2016); (b) the "Megalospora" clade grouped *Preussia* sp. SNP235, *Preussia* sp. (CF-277965 and CF-277801), *Preussia* sp. (CF-277787 and CF-277849), *P. terricola*, *Sporormiella megalospora*, and *P. polymorpha* with high statistical support (pp = 100%/bs = 100%); (c) the "Sporminula" clade with *P. cymatomera*, *P. pilosella*. *P. longisporopsis*, *P. grandispora*, *P. tenerifae*, and *Sporormia subticinensis* was not supported (pp = 87%/bs = 84%); (d) the highly supported "Vexans" clade, including the species *P. affinis*, *P. heptamera*, *P. octomera*, and *P. vexans*, clustered with the monospecific "Leporina" clade (pp = 100%/bs = 100%); (e) *P. dubia*, *P. irregularis*, and *P. muskokensis* cluster in the highly supported "Irregularis" clade (pp = 100%/bs = 100%); (f) the "Preussia" clade grouped seven species, *P. fleischhakii*, *P. flanaganii*, *P. alloiomera*, *P. thypharum*, *P. funiculata*, *P. vulgaris*, and *P. aemulans*, with strong support (pp = 100%/bs = 100%), clearly distinguished from the *Preussia* sp. strains SNP459 and SNP392 (pp = 100%/bs = 100%), and *P. septenaria* and *Preussia* sp. CF-282341 (pp = 100%/bs = 100%); (g) a main branch that contained the five statistically well-supported clades "Africana", "Intermedia", "Similis", "Lignicola", and "Australis" (pp = 100%); (h) relatedness of the two monospecific clades "Isomera" and "Minimoides" was supported by (pp = 100%/bs = 100) and (pp = 97%/bs = 93%), respectively; and (i) relatedness of the "Isabellae", "Minima", and "Mediterranea" complex, including the *Preussia* sp. strains CF-285378, SNP309, SNP057, SNP220, and SNP156, was supported by a 98% of bootstrap but not by Bayesian analyses, with the posterior probability value of 66%.

Based on their phylogenetic position, 14 of our isolates could be identified as *P. grandispora*, *P. subticinensis*, *P. typharum*, *P. funiculata*, *P. africana*, *P. intermedia*, *P. similis*, *P. australis*, and *P. minima*, all of which have been previously collected in the Iberian Peninsula. Their tentative phylogenetic position was verified following the methodology described by Arenal et al. (2004, 2005). Nine strains from different plants were morphologically and phylogenetically identified as *P. lignicola* (Fig. 2), a species that has not previously been cited from the Iberian Peninsula. Isolates currently not identifiable at the species level and distributed in the new clades were selected for morphological studies in order to compare them with other phylogenetically related *Preussia* species.

The asci and ascospore morphology of CF-277856 resembled that of *P. cymatomera* (Soláns 1985). *Preussia* sp. CF-277801 showed compact asci and four-celled, biseriately arranged ascospores showing parallel and diagonal germ slits extending over the entire spore length. *Preussia* sp. (CF-285378) showed similar colony morphologies, ornamental hyphae, and peridial cells as *P. isabellae* and *P. minima*. On the other hand, strains CF-155367, CF-279766, CF-279733, and CF-277817 only developed non-sporulating, darkly pigmented, and septate mycelium. A phoma-like anamorph was seen in CF-282341 and CF-209171 and a chrysosporium-like anamorph in CF-277787. The first report of a chrysosporium-like anamorph associated with a *Preussia* species was reported by Asgari and Zare (2010), who described the anamorphic state of *P. polymorpha*. Prior to this study, only *Phoma* sp. had been reported as anamorphs of *Preussia* species (de Gruyter et al. 2013).

Dereplication of known compounds and identification of chemotypes

The LC-HRMS dereplication of fungal extracts by comparison with more than 900 microbial natural product standards (González-Menéndez et al. 2016) identified 32 known compounds. Among them, we could identify seven compounds known to be produced by *Preussia* sp., including australifungin, australifunginol, asterric acid, preussomerins A and B, and sporminarins A and B. Twenty-three compounds previously described in other distantly related taxa were also identified, including altersetin, antibiotic FR 198248, bisdechlorodihydrogeodin, brefeldin A, brevianamide F, calbistrin A, chloro-6-methoxymellein, cis-4-hydroxy-6-deoxyscytalone, citrinin, cytochalasin F or B, curvicollide A, 11-deacetoxywortmannin, equisetin, funicone, globosuxanthone A, 2-(2-hydroxy-5-

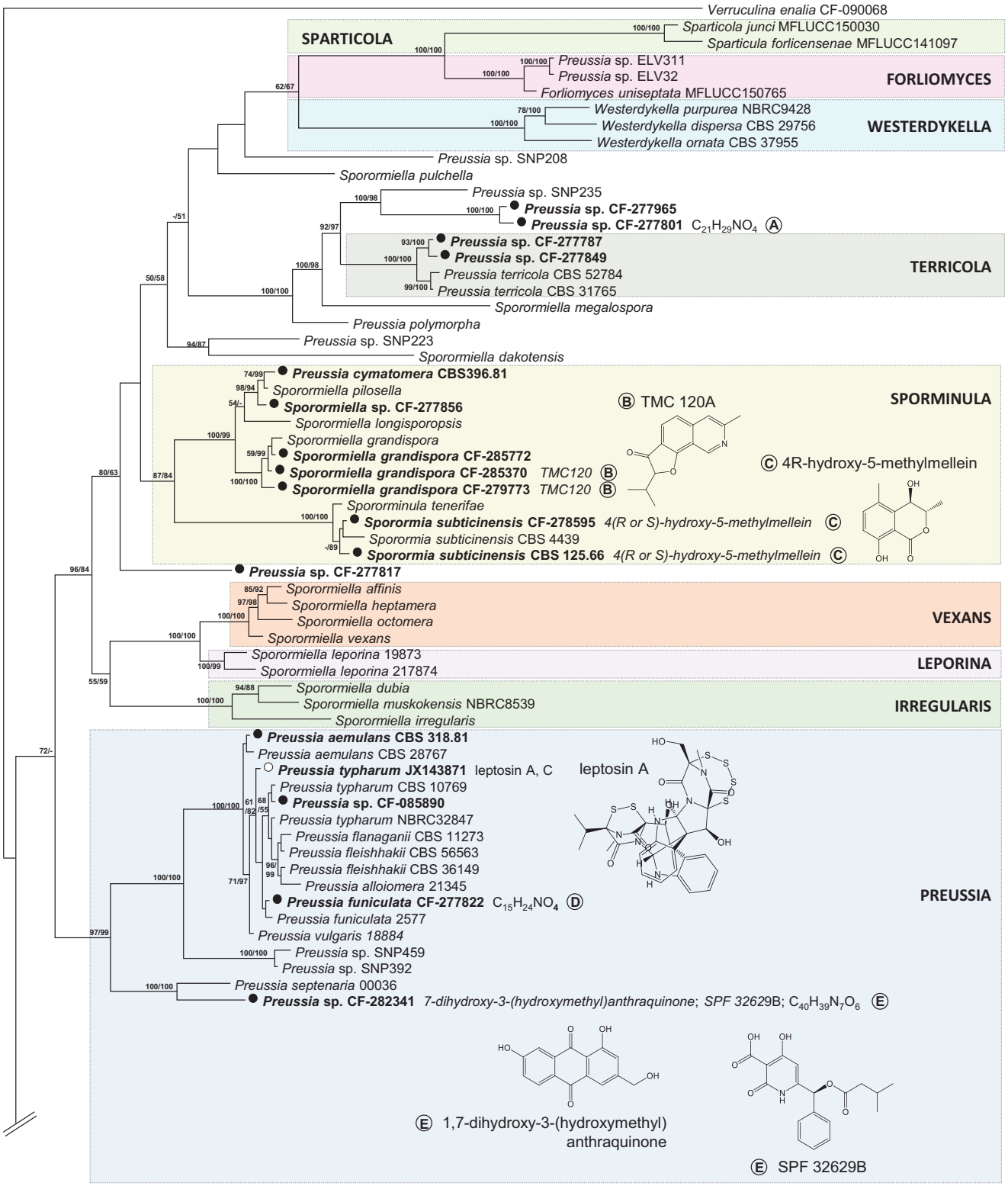

Fig. 1 Consensus tree from Bayesian phylogeny inferences based on ITS/28S sequences of selected *Preussia* species and related genera. The symbol ● identifies strains from the Iberian Peninsula isolated in this study and the symbol ○ indicates producers of compounds described in the literature. The most relevant compounds, including those newly identified in this study, are printed next to their producing taxa. Differential chemotypes are identified by **A**–**K**. Clade probability values/maximum likelihood bootstrap values are indicated respectively at the branches. Values <50 are designated by –. *Verruculina enalia* CF-090068 was used as an outgroup

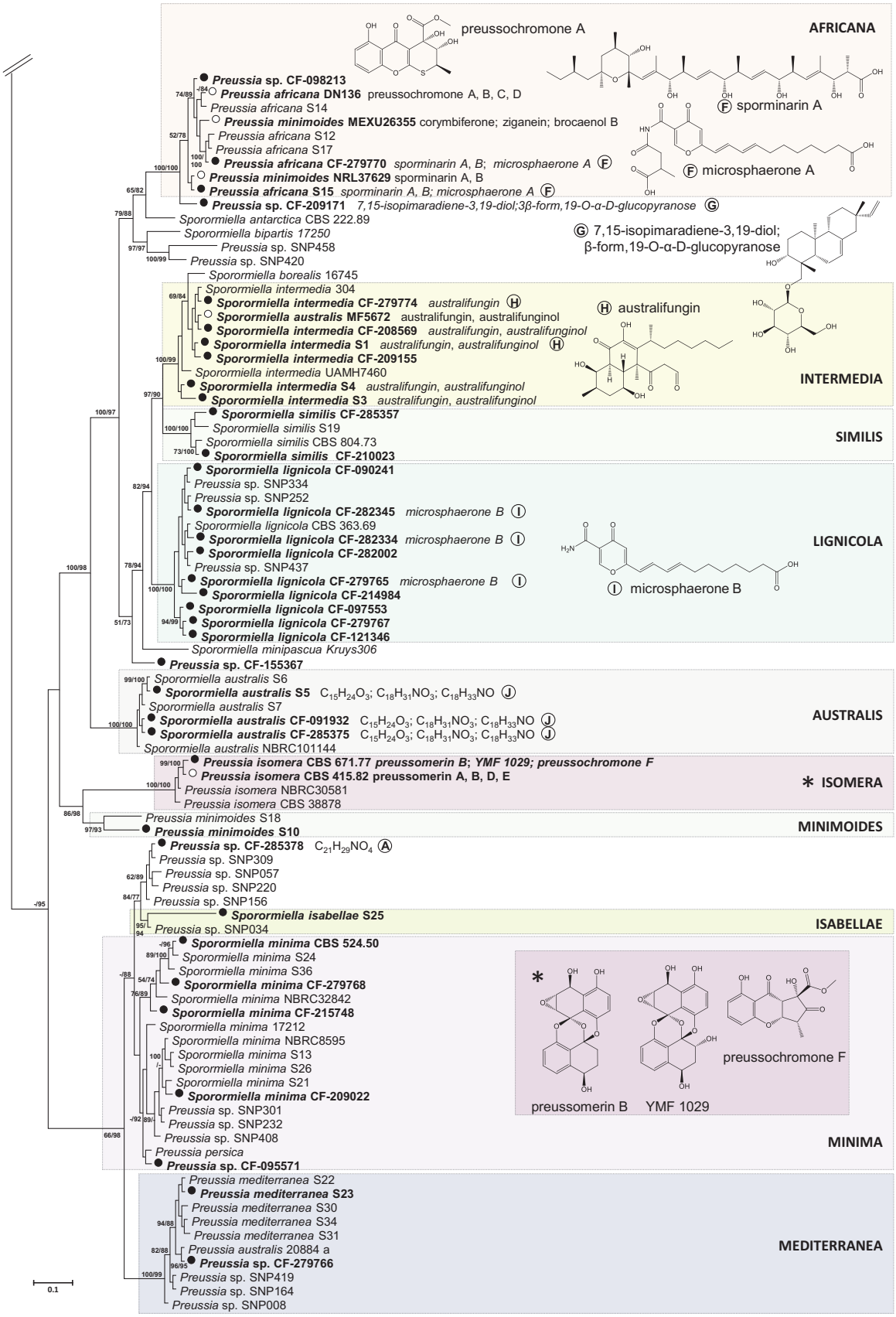

Fig. 1 (continued)

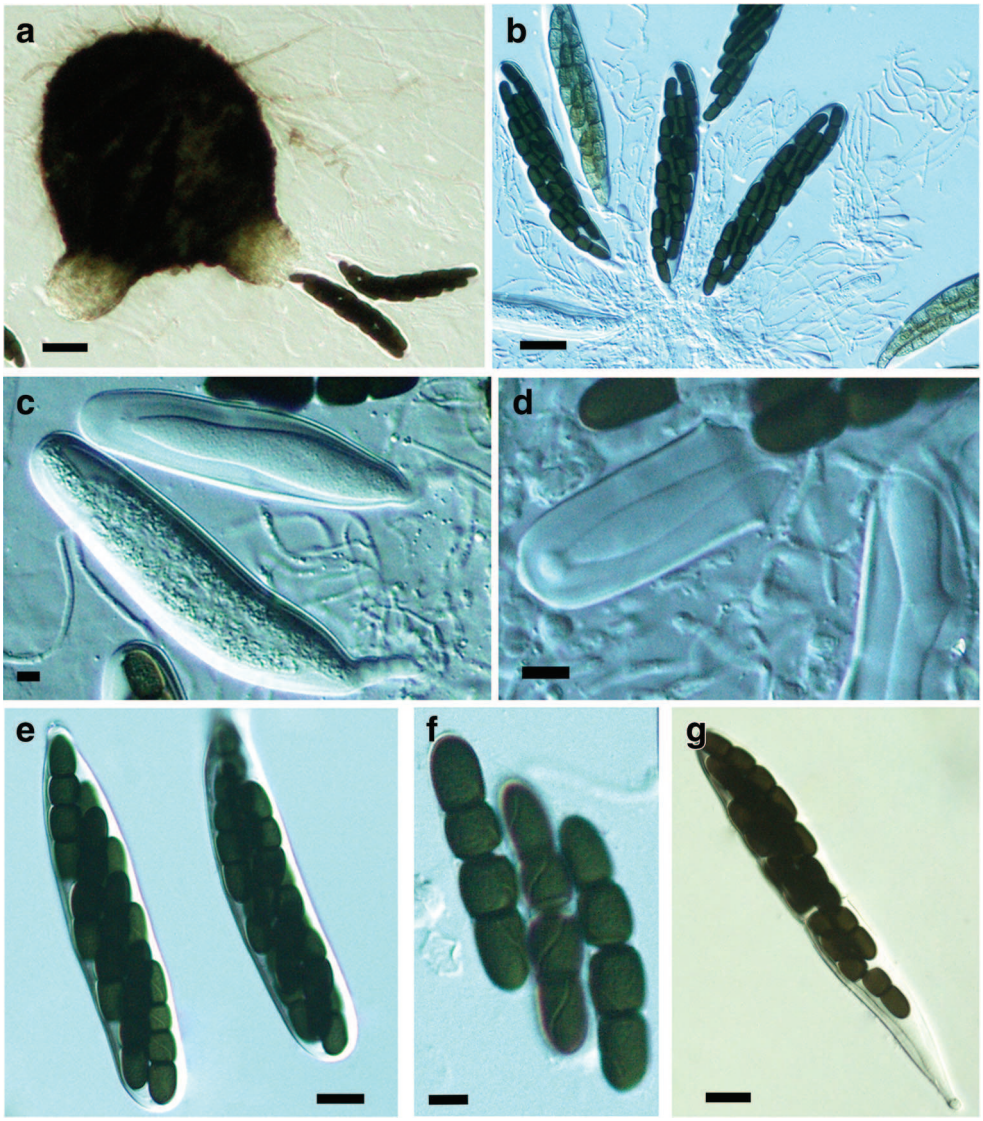

Fig. 2 *Preussia lignicola* CF-279765. **a** Ascoma formed on CMA. **b**–**e**, **g** Mature and immature asci. **f** Ascospores showing germ slits. Scale bars = 80 μm (**a**), 35 μm (**b**), 10 μm (**c**, **d**, **f**), 20 μm (**e**), 25 μm (**g**)

methoxyphenoxy)acrylic acid, 3-hydroxymellein, palmarumycin C15, penicillic acid, phomasetin, rugulosin, ulocladol, and waol A.

A metabolite profiling approach was also applied for the characterization of the 23 Iberian isolates and the 14 strains from public collections, encompassing 22 different species of *Preussia*. Mass spectrometry (MS) metabolite profiles from two different liquid media conditions were compared. The mass to charge ratio (*m*/*z*) and intensity of ionized molecules allowed the identification of known compounds and chemotypes characterizing different species of the genus.

The dendrogram obtained after multivariate statistical analysis of these profiles proved the relationships between the different strains (supplementary Fig. 1). Four strains (CBS 318.51, CF-277787, CF-279766, and CF-155367) belonging to four *Preussia* species presented profiles with a low number of metabolites and 70% similarity with the unfermented control media profile. Several sterile isolates that could not be identified produced chemical profiles closely related to other *Preussia* species. For example, strain CF-091932 showed a compound profile similar to that of one of the *P. australis* strains. This analysis grouped 12 strains in five monophyletic clades, where they clustered with *P. australis*, *P. lignicola*, *P. cymatomera*, *P. grandispora*, and *P. subticinensis*, regardless of their geographical origin or isolation source. Three of the five strains of *P. minima*, CF-095571, CF-206340, and CF-215745, clustered with *Preussia* sp. CF-277801 and *P. minimoides* S10, with 80% similarity. The two other strains of *P. minima*, CF-209022 and CF-279768, clustered with *P. mediterranea* S23, *P. isomera* CBS 671.77, *P. isabellae* S25, and two plant isolates (CF-285357 and CF-285378). In addition, both clades clustered together with *P. africana* S15 and *P. intermedia* S3, with 75% similarity. Finally, two strains with phoma-like

anamorphs (CF-209171 and CF-282341) were positioned outside the central clade, suggesting that they present different metabolic profiles (supplementary Fig. 1).

On the basis of the similarity matrix, we could determine compounds characteristic for each cluster. No specifically characterizing compounds were seen in 11 species (17 strains). Sixteen secondary metabolites were found to present good signal to noise ratios and could be used as differential compounds of the *Preussia* species analyzed. The presence or absence of a given compound, or a combination of more than one of these molecules, permitted the establishment of 11 different chemotypes (A–K) that grouped 21 of the studied strains into nine *Preussia* species (chemotypes C–K) (Fig. 1). Six species-specific secondary metabolites were identified for *P. subticinensis* (chemotype C), *Preussia* sp. CF-282341 (E), *P. africana* (F), *Preussia* sp. CF-209171 (G), *P. lignicola* (I), and *P. australis* (J). The "Grandispora" clade was characterized differentially by the compounds $C_{15}H_{18}O_3$, TMC120, and $C_{15}H_{27}NO_4$ (chemotype B). The "Subticinensis" clade presented differentially 4-hydroxy-5-methylmellein (chemotype C). The "Africana" clade showed $C_{15}H_{18}O_3$, $C_{16}H_{12}O_7$, and microsphaerone A (chemotype F). All members of the "Lignicola" clade were characterized as producers of TMC120, microsphaerone B, and $C_{21}H_{29}NO_4$ (chemotype I). The "Australis" clade (chemotype J) included $C_{15}H_{24}O_3$, $C_{18}H_{33}NO$, and $C_{18}H_{31}NO_3$ producers (see Tables 3 and 4).

Discussion

Although a comprehensive classification requires extensive efforts to recollect, culture, and phylogenetically characterize the full range of predominantly coprophilous *Preussia* species, our study has focused mainly on endophytic *Preussia* strains from Spain and Portugal, and only few were isolated from soil and dung.

Fungal endophytes form a very diverse group composed mostly of phylogenetically unrelated ascomycetes (Arnold 2007; Rodriguez et al. 2009). There have been many reports on endophytic species of *Preussia* isolated from different plant species (Mapperson et al. 2014; Zaferanloo et al. 2014; Massimo et al. 2015), but the life cycle of these fungi within their host plants is still unknown. It is possible that these fungi colonize internal plant tissues, beneath the epidermal cell layers, without causing any apparent harm or symptomatic infections to their host. They may live within the intercellular spaces of the tissues of living cells.

Many endophytic species of grasses are also known as common coprophilous fungi (Sánchez-Márquez et al. 2012). Other endophytes of non-grass hosts remain viable after passing throughout the gut of herbivores (Devarajan and Suryanarayanan 2006). These observations suggest that the coprophilous stage is an alternate phase in the life cycle of some endophytic fungi, and that certain coprophilous fungi

Table 3 Differential compounds identified by LC-LRMS and LC-HRMS analyses for *Preussia* species

No.	RT (min)	$[M + H]^+$ exp.	Proposed ion	Main secondary experimental ions	Production media	Proposed formula	Proposed compound
1	3.75	209.0810	$C_{11}H_{13}O_4^+$	210.0838; 191.0698	MMK2	$C_{11}H_{12}O_4$	4(R or S)-Hydroxy-5-methylmellein
2	3.19	247.1322	$C_{15}H_{19}O_3^+$	248.1355; 495.7545	MMK2	$C_{15}H_{18}O_3$	*34 possible matches in DBs*
3	2.15	253.1790	$C_{15}H_{25}O_3^+$	292.1167; 348.1794; 492.2807	YES	$C_{15}H_{24}O_3$	*94 possible matches in DBs*
4	3.13	258.1150	$C_{15}H_{16}NO_3^+$	259.1145	MMK2	$C_{15}H_{15}NO_3$	TMC120*
5	2.30	271.0603	$C_{15}H_{11}O_5^+$	272.0634; 293.042	MMK2	$C_{15}H_{10}O_5$	7-Dihydroxy-3-(hydroxymethyl) anthraquinone*
6	5.10	280.2637	$C_{18}H_{34}NO^+$	281.2663	YES	$C_{18}H_{33}NO$	–
7	3.59	286.2009	$C_{15}H_{28}NO_4^+$	287.2038	YES	$C_{15}H_{27}NO_4$	–
8	2.44	307.0841	$C_{15}H_{15}O_7^+$	324.1078; 308.0843; 209.0705	MMK2	$C_{15}H_{14}O_7$	Preussochromone F*
9	2.77	310.2369	$C_{18}H_{32}NO_3^+$	311.2400	YES	$C_{18}H_{31}NO_3$	–
10	2.47	317.0659	$C_{16}H_{13}O_7^+$	334.0925; 285.0394; 318,059	MMK2	$C_{16}H_{12}O_7$	*16 possible matches in DBs*
11	2.87	335.0763	$C_{16}H_{15}O_8^+$	317.0657; 318.0690; 691.1268	MMK2	$C_{16}H_{14}O_8$	Microsphaerone A*
12	3.17	320.1491	$C_{17}H_{22}NO_5^+$	302,1379; 321,1521; 368,2062	MMK2	$C_{17}H_{21}NO_5$	Microsphaerone B
13	0.93	346.1284	$C_{18}H_{20}NO_6^+$	309.1325; 347,1313; 399.1544	MMK2	$C_{18}H_{19}NO_6$	SPF 32629B
14	1.73	360.2167	$C_{21}H_{30}NO_4^+$	361.2198	MMK2	$C_{21}H_{29}NO_4$	*6 possible matches in DBs*
15	5.63	449.2907	$C_{26}H_{43}O_7^+$	450.2936; 484.3273; 950.6221	MMK2	$C_{26}H_{42}O_7$	7,15-Isopimaradiene-3,19-diol; 3β-form, 19-O-α-D-ose*
16	2.73	714.3028	$C_{40}H_{10}N_7O_6^+$	715,3061; 716,3085	MMK2	$C_{40}H_{39}N_7O_6$	–

*Additional semi-preparative HPLC fractionation and LC-HRMS/MS were performed for accurate identifications of the compounds proposed

Table 4 Differential chemotypes identified for the analyzed *Preussia* species, sorted according their position in the phylogenetic tree. Species-specific compounds are highlighted in **bold**

Strain	Taxonomy	Compounds (Table 3)																Chemotype
		1	2	**3**	4	**5**	**6**	7	8	**9**	**10**	**11**	**12**	**13**	14	**15**	**16**	
CF-277801	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	**A**
CBS 395.81	*Preussia cymatomera*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-277586	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-258370	*Preussia grandispora*	−	+	−	+	−	−	+	−	−	−	−	−	−		−	−	**B**
CF-279773	*Preussia grandispora*	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	**B**
CBS 125.66	*Preussia subticinensis*	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**C**
CF-278595	*Preussia subticinensis*	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**C**
CF-277817	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CBS 318.51	*Preussia aemulans*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-277822	*Preussia funiculata*	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	**D**
CF-282341	*Preussia* sp.	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	**E**
CF-160923 (S15)	*Preussia africana*	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	**F**
CF-279770	*Preussia africana*	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	**F**
CF-209171	*Preussia* sp.	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	**G**
CF-160907 (S1)	*Preussia intermedia*	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	**H**
CF-160910 (S3)	*Preussia intermedia*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279774	*Preussia intermedia*	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	**H**
CF-210023	*Preussia similis*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-285357	*Preussia similis*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279765	*Preussia lignicola*	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	**I**
CF-282334	*Preussia lignicola*	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	**I**
CF-282345	*Preussia lignicola*	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	**I**
CF-155365	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-091932	*Preussia australis*	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	**J**
CF-160911	*Preussia australis*	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	**J**
CF-285375	*Preussia australis*	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	**J**
CBS 671.77	*Preussia isomera*	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	**K**
CF-160916	*Preussia minimoides*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-285378	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	**A**
CF-160936 (S25)	*Preussia isabellae*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-095571	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CBS 524.50	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-209022	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-215748	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279768	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-160934 (S23)	*Preussia mediterranea*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279766	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None

might have coevolved with grazing animals and plants (Porras-Alfaro et al. 2008).

The spores of coprophilous species are often surrounded by mucilage or have gelatinous appendices that attach easily to plant surfaces. When a plant is foraged by a herbivore, the spores travel through their digestive tract and, finally, when ending up in a new dung pile, the spores germinate and produce new fruit bodies (Kruys and Wedin 2009). An alternative hypothesis is that some of these coprophilous fungi were erroneously reported as endophytes, as their surface-sterilant resistant propagules could also occur passively on plant surfaces (Newcombe et al. 2016).

From the total number of 32 *Preussia* strains that were isolated in our study, the most frequent species was *P. lignicola*,

with eight isolates obtained from five different plant species (*Dittrichia viscosa*, *Retama sphaerocarpa*, *Viscum album*, *Erica australis*, and *Genista umbellata*) collected from all habitats sampled (Table 2). This confirmed previous results that indicated a wide distribution of this species in desert plants and its broad host range (Massimo et al. 2015). This is the first report of *P. lignicola* from the Iberian Peninsula. Another strain of *P. lignicola* was isolated from dung, as was *P. lignicola* strain CBS 264.69 from the Netherlands. Our second most frequently isolated species is *P. minima*, with four isolates. It was obtained from animal dung and plants, which may highlight its ability to alternate between endophytic and coprophilous lifestyles and explain why this species was isolated from different substrates worldwide.

The general topology of the ITS/28S phylogenetic tree was in agreement with previous studies (Kruys and Wedin 2009; Massimo et al. 2015). Eleven of the 21 *Preussia* sp. isolates from Arizona desert plants (Massimo et al. 2015) are included within the "Minima" complex.

Many known and frequent taxa represent heterogeneous species complexes, which remain to be resolved by a combination of genotype- and phenotype-derived data (Stadler 2011). Several polyphasic studies using chemotaxonomic, morphological, and molecular data have clarified the similarities between the different genera among Xylariales; for example, between *Daldinia*, *Entonaema*, and *Rhopalostroma* (Stadler et al. 2014). Although a relevant number of chemotaxonomic studies have been carried out, secondary metabolites have only been examined extensively in species of *Aspergillus*, *Penicillium*, and *Fusarium*.

Although few other studies exist that compare the secondary metabolite profiles and phylogeny (Frisvad et al. 2008), chemotaxonomic affinities only in *Alternaria* and *Ascochyta* but not other Dothideomycetes have been examined (Andersen et al. 2008; Kim et al. 2016). These recent studies highlight that this approach has the potential to provide valuable information related to ecology, and that its use in fungal biology needs to be further explored (Kim et al. 2016).

The evaluation of the different chemotypes present in the studied *Preussia* isolates revealed 16 compounds that can be used to distinguish *Preussia* species. We proposed component identities for eight of the 16 compounds. Four presented several possible compounds for each molecular formula identified and the other four could not be identified in the databases, suggesting that they may correspond to undescribed compounds (Table 3). Eleven of them were uniquely formed by certain species and they could be used to resolve groups of closely related species. This is the case for microsphaerone A formed in *P. africana* and microsphaerone B formed in *P. lignicola* (CF-279765) while no such compounds were encountered in other closely related *Preussia* sp.

The first fungal strain described to produce microsphaerone A and B was the mitosporic fungus *Microsphaeropsis* sp. (Wang et al. 2002). *Preussia subticinensis* also produced a specific ochratoxin derivative (Cole et al. 2003), previously described in a strain of *Microsphaeropsis* sp. as 4(R/S)-hydroxy-5-methyl-mellein (Höller et al. 1999). Young *Microsphaeropsis* pycnidia may be easily mistaken for a *Phoma* species (Boerema et al. 2004), with still colorless conidia when immature. This raises the question whether the strain was misidentified as a *Preussia* anamorph. A recent publication from *P. minima* reported the isolation of three novel linear pyran–furan fused furochromones, sporormielleins A–C, and three biogenetically related compounds, sporormiellones A and B, and microsphaeropsone A (Xiong et al. 2014). Microsphaeropsone A is a secondary metabolite intermediate generated by the sporormielleins AC production pathway, confirming our hypothesis that these metabolites are present in another species of *Preussia* (Xiong et al. 2014). On the other hand, the comparative analysis of the presence or absence of several specific *m*/*z* ions (chemotypes) for each *Preussia* strain proved to be also useful for discriminating species that did not present species-specific compounds, as in *P. grandispora* (chemotype B) or *P. funiculata* (chemotype D) (Fig. 1, Tables 3 and 4).

Regarding the bioactive secondary metabolites dereplicated in the extracts, several mellein (ochracein) derivatives were also found in three *Preussia* strains (CF-282341, CF-277856, and CBS 125.66). These precursors of ochratoxins (Harris and Mantle 2001) were originally discovered in *Aspergillus ochraceus* and then in different taxa of the Botryosphaeriales, Pleosporales, and Xylariales (Rukachaisirikul et al. 2013; Stadler 2011). Preusserin (Johnson et al. 1989) is produced by *A. ochraceus* (Schwartz et al. 1988) and several species of *Preussia*. The analyzed strains of *P. africana* produced sporminarin A and B and strains of *P. similis* contained brefeldin A and 11-deacetoxywortmannin. The compounds cytochalasin, globosuxanthone A, or brevianamide F were produced by some strains included in the clades "Australis", "Intermedia", and "Minima".

Limitations to the detection of already known active compounds in these species can be explained by a differential production under the specific fermentation conditions used in this study (MMK2 and YES). Most of the discussed molecules had been previously reported from rice- or corn-based solid media cultures (Hensens et al. 1995; Mudur et al. 2006; Zhang et al. 2012; Xiong et al. 2014) (Table 1). It is well known that culture media compositions affect the production of fungal secondary metabolites. Microorganisms growing on a solid medium are in various physiological conditions, which may stimulate the expression of different biosynthetic gene clusters (de la Cruz et al. 2012). To confirm this hypothesis, we studied the production of australifungin and

australifunginol by adopting the same solid media and conditions used by Mandala et al. (1995) and using the original australifungin producer strain (MF5672). Australifungin was detected in five and australifunginol in four species of the "Intermedia" clade (Fig. 1). This experiment confirms that specific conditions and taxon-specific optimizations are required for triggering the production of certain compounds.

Conclusions

Preussia lignicola, a species reported for the first time from the Iberian Peninsula, was encountered in five of the 14 different plant species analyzed. Another 19 *Preussia* species were identified from the phylogenetic and morphological analyses, of which three either formed phoma- or chrysosporium-like anamorphs, while four did not sporulate in culture.

Eleven of the 16 identified secondary metabolites produced by the *Preussia* isolates can be chemotaxonomically used to distinguish six species. In addition, phylogenetic analysis identified 11 different chemotypes among 22 of the species studied, supporting that secondary metabolites characterization is a useful tool for taxonomic descriptions. More culturing conditions should be added to further identify other chemotypes to distinguish the rest of the *Preussia* species.

This analysis also identified four putative new secondary metabolites with no matches in the natural products databases of known compounds, suggesting that the potential of *Preussia* species for the discovery of new natural products is untapped.

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References

Andersen B, Dongo A, Pryor BM (2008) Secondary metabolite profiling of *Alternaria dauci*, *A. porri*, *A. solani*, and *A. tomatophila*. Mycol Res 112:241–250

Arenal F, Platas G, Peláez F (2004) Variability of spore length in some species of the genus *Preussia* (*Sporormiella*). Mycotaxon 89:137–151

Arenal F, Platas G, Peláez F (2005) Two new *Preussia* species defined based on morphological and molecular evidence. Fungal Divers 20: 1–15

Arenal F, Platas G, Peláez F (2007) A new endophytic species of *Preussia* (Sporormiaceae) inferred from morphological observations and molecular phylogenetic analysis. Fungal Divers 25:1–17

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 21:51–66

Asgari B, Zare R (2010) Two new species of *Preussia* from Iran. Nova Hedwigia 90:533–548

Barr ME (2000) Notes on coprophilous bitunicate ascomycetes. Mycotaxon 76:105–112

Barrasa JM (1985) Estudio de los Ascomycetes coprófilas en España. Thesis, University of Alcalá de Henares

Barrasa JM, Checa J (1989) Dothidelaes coprófilas del Parque Natural de Monfragüe (Cáceres). VIII Simposios Ciencias Criptográmicas

Barrasa JM, Moreno G (1980) Contribución al estudio de los hongos que viven sobre materias fecales (2a aportación). Acta Bot Malacitana Málaga 6:111–148

Bergstrom JD, Dufresne C, Bills GF, Nallin-Omstead M, Byrne K (1995) Discovery, biosynthesis, and mechanism of action of the zaragozic acids: potent inhibitors of squalene synthase. Ann Rev Microbiol 49: 607–639

Bills GF, Christensen M, Powell M, Thorn G (2004) Saprobic soil fungi. In: Mueller GM, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic Press, Oxford, pp 271–302

Bills GF, González-Menéndez V, Platas G (2012) *Kabatiella bupleuri* sp. nov. (*Dothideales*), a pleomorphic epiphyte and endophyte of the Mediterranean plant *Bupleurum gibraltarium* (Apiaceae). Mycologia 104:962–973

Bills GF, Gloer JB, An Z (2013) Coprophilous fungi: antibiotic discovery and functions in an underexplored arena of microbial defensive mutualism. Curr Opin Microbiol 16(5):549–565

Boerema GH, de Gruyter J, Noordeloos ME, Hamers MEC (2004) A *Phoma* sect. *Phoma*. In: Boerema GH, de Gruyter J, Noordeloos ME, Hamers MEC (eds) *Phoma* identification manual. Differentiation of specific and infra-specific taxa in culture. CAB International, Wallingford, pp 32–118

Cain RF (1961) Studies of coprophilous ascomycetes VII. *Preussia*. Can J Bot 39:1633–1666

Chen X, Shi Q, Lin G, Guo S, Yang J (2009) Spirobisnaphthalene analogues from the endophytic fungus *Preussia* sp. J Nat Prod 72: 1712–1725

Clapp-Shapiro WH, Burgess BW, Giacobbe RA, Harris GH, Mandala S, Polishook J, Rattray M, Thornton RA, Zink DL, Cabello A, Diez MT, Martin I, Pelaez F (1998) Antifungal agent from *Sporomiella minimoides*. US patent US5801172A

Cole RJ, Jarvis BB, Schweikert MA (2003) Handbook of secondary fungal metabolites. Academic Press, New York

Collado J, González A, Platas G, Stchigel AM, Guarro J, Peláez F (2002) *Monosporascus ibericus* sp. nov., an endophytic ascomycete from plants on saline soils, with observations on the position of the genus based on sequence analysis of the 18S rDNA. Mycol Res 106:118–127

de Gruyter J, Woudenberg JH, Aveskamp MM, Verkley GJ, Groenewald JZ, Crous PW (2013) Redisposition of phoma-like anamorphs in Pleosporales. Stud Mycol 75:1–36

de la Cruz M, Martín J, González-Menéndez V, Pérez-Victoria I, Moreno C, Tormo JR, El Aouad N, Guarro J, Vicente F, Reyes F, Bills GF (2012) Chemical and physical modulation of antibiotic activity in *Emericella* species. Chem Biodivers 9:1095–1113

de la Torre M (1974) Estudio sistemático, ecológico y corológico de Ascomycetes españoles. Thesis doctoral (ined), Facultad de Farmacia, Universidad Complutense de Madrid, 264 pp

Devarajan PT, Suryanarayanan TS (2006) Evidence for the role of phytophagous insects in dispersal of non-grass fungal endophytes. Fungal Divers 23:111–119

Doveri F, Sarrocco S (2013) *Sporormiella octomegaspora*, a new hairy species with eight-celled ascospores from Spain. Mycotaxon 123: 129–140

Du L, Robles AJ, King JB, Mooberry SL, Cichewicz RH (2014) Cytotoxic dimeric epipolythiodiketopiperazines from the ascomycetous fungus *Preussia typharum*. J Nat Prod 77:1459–1466

Ellis JB, Everhart BM (eds) (1892) The North American Pyrenomycetes. A contribution to mycologic biology. Ellis & Everhart, Newfield, NJ, 793 pp

Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res 112:231–240

Fuckel L (1866) Fungi Rhenani Exsiccati Cent. XVI–XVIII 17–18:1601–1800

González-Menéndez V, Asensio F, Moreno C, de Pedro N, Monteiro MC, de la Cruz M, Vicente F, Bills GF, Reyes F, Genilloud O, Tormo JR (2014) Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity. Mycology 5: 179–191

González-Menéndez V, Pérez-Bonilla M, Pérez-Victoria I, Martín J, Muñoz F, Reyes F, Tormo JR, Genilloud O (2016) Multicomponent analysis of the differential induction of secondary metabolite profiles in fungal endophytes. Molecules 21:234–250

Guarro Artigas J (1983) Hongos coprófilos aislados en Cataluña. Ascomycetes. Anales Jard Bot Madrid 39:229–245

Guarro J, Calvo MA, Ramirez C (1981) Soil ascomycetes from Catalunya (Spain). Nova Hedw 34:285–299

Harris JP, Mantle PG (2001) Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry 58:709–716

Hatori H, Shibata T, Nishikawa M, Ueda H, Hino M, Fujii T (2004) FR171456, a novel cholesterol synthesis inhibitor produced by *Sporormiella minima* no. 15604: II. Biological activities. J Antibiot 57:260–263

Hensens OD, Helms GL, Jones ETT, Harris GH (1995) Structure elucidation of australifungin, a potent inhibitor of sphinganine N-acyltransferase in sphingolipid biosynthesis from *Sporormiella australis*. J Org Chem 60:1772–1776

Höller U, König GM, Wright AD (1999) Three new metabolites from marine-derived fungi of the genera *Coniothyrium* and *Microsphaeropsis*. J Nat Prod 62:114–118

Johnson JH, Phillipson DW, Kahle AD (1989) The relative and absolute stereochemistry of the antifungal agent preussin. J Antibiot 42: 1184–1185

Kim W, Peever TL, Park JJ, Park CM, Gang DR, Xian M, Davidson JA, Infantino A, Kaiser WJ, Chen W (2016) Use of metabolomics for the chemotaxonomy of legume-associated *Ascochyta* and allied genera. Sci Rep 6:20192

Kinoshita K, Sasaki T, Awata M, Takada M, Yaginuma S (1997) Structure of sporostatin (M5032), an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. J Antibiot 50:961–964

Kruys Å, Wedin M (2009) Phylogenetic relationships and an assessment of traditionally used taxonomic characters in the Sporormiaceae (Pleosporales, Dothideomycetes, Ascomycota), utilising multigene phylogenies. Syst Biodivers 7:465–478

Leyte-Lugo M, Figueroa M, del Carmen González M, Glenn AE, González-Andrade M, Mata R (2013) Metabolites from the entophytic fungus *Sporormiella minimoides* isolated from *Hintonia latiflora*. Phytochemistry 96:273–278

Lundqvist NI (1960) Coprophilous ascomycetes from northern Spain. Svensk Bot Tidskr 54:523–529

Mandala SM, Thornton RA, Frommer BR, Curotto JE, Rozdilsky W, Kurtz MB, Giacobbe RA, Bills GF, Cabello MA, Martín I, Peláez F, Harris GH (1995) The discovery of australifungin, a novel inhibitor of sphinganine N-acyltransferase from *Sporormiella australis*. Producing organism, fermentation, isolation, and biological activity. J Antibiot 48:349–356

Mapperson RR, Kotiw M, Davis RA, Dearnaley JD (2014) The diversity and antimicrobial activity of *Preussia* sp. endophytes isolated from Australian dry rainforests. Curr Microbiol 68:30–37

Massimo NC, Nandi Devan MM, Arendt KR, Wilch MH, Riddle JM, Furr SH, Steen C, U'Ren JM, Sandberg DC, Arnold AE (2015) Fungal endophytes in aboveground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. Microb Ecol 70:61–76

McGahren WJ and Mitscher LA (1968) Dihydroisocoumarins from a *Sporormia* fungus. J Org Chem 33:1577–1580

McGahren WJ, van den Hende JH, Mitscher LA (1969) Chlorinated cyclopentenone fungitoxic metabolites from the fungus, *Sporormia affinis*. J Am Chem Soc 91:157–162

Moreno-Arroyo B (2004) Inventario Micológico Básico de Andalucía. Consejería de Medio Ambiente, Junta de Andalucía, Córdoba

Mudur SV, Gloer JB, Wicklow DT (2006) Sporminarins A and B: antifungal metabolites from a fungicolous isolate of *Sporormiella minimoides*. J Antibiot 59:500–506

Newcombe G, Campbell J, Griffith D, Baynes M, Launchbaugh K, Pendleton R (2016) Revisiting the life cycle of dung fungi, including *Sordaria fimicola*. PLoS One 11:e0147425

Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32:268–274. doi:10.1093/molbev/msu300

Nirenberg HI (1976) Untersuchungen uber die morphologische und biologische Differenzierung in der Fusarium-Sektion Liseola. Mitt Biol Bundesanst Land-u Forstwirtsch (Berlin-Dahlem) 169:1–117

Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233

Oberwinkler F, Kirschner R, Arenal F, Villarreal M, Rubio V, Begerow D, Bauer R (2006) Two new pycnidial members of the Atractiellales: *Basidiopycnis hyalina* and *Proceropycnis pinicola*. Mycologia 98: 637–649

Pérez-Victoria I, Martín J, Reyes F (2016) Combined LC/UV/MS and NMR strategies for the dereplication of marine natural products. Planta Med 82:857–871

Phukhamsakda C, Ariyawansa HA, Phillips AJL, Wanasinghe DN, Bhat DJ, McKenzie EHC, Singtripop C, Camporesi E, Hyde KD (2016) Additions to Sporormiaceae: introducing two novel genera, *Sparticola* and *Forliomyces*, from *Spartium*. Cryptogamie Mycol 37:75–97

Poch GK, Gloer JB (1991) Auranticins A and B: two new depsidones from a mangrove isolate of the fungus *Preussia aurantiaca*. J Nat Prod 54:213–217

Porras-Alfaro A, Herrera J, Sinsabaugh RL, Odenbach KJ, Lowrey T, Natvig DO (2008) Novel root fungal consortium associated with a dominant desert grass. Appl Environ Microbiol 74:2805–2813

Robinson GW, O'Sullivan J, Meyers E, Wells JS, Del Mar JH (1988) Culpin. US patent US4914245A

Rodriguez RJ, White JF Jr, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Rukachaisirikul V, Buadam S, Sukpondma Y, Phongpaichit S, Sakayaroj J, Hutadilok-Towatana N (2013) Indanone and mellein derivatives from the Garcinia-derived fungus *Xylaria* sp. PSU-G12. Phytochem Lett 6:135–138

Sánchez-Márquez S, Bills GF, Herrero N, Zabalgogeazcoa Í (2012) Non-systemic fungal endophytes of grasses. Fungal Ecol 5:289–297

Sarrocco S (2016) Dung-inhabiting fungi: a potential reservoir of novel secondary metabolites for the control of plant pathogens. Pest Manag Sci 72:643–652

Sato T, Hanada T, Arioka M, Ando K, Sugiyama J, Uramoto M, Yamasaki M, Kitamoto K (1998) S19159, a modulator of neurite outgrowth produced by the ascomycete *Preussia aemulans*. I. Producing strain, fermentation, isolation and biological activity. J Antibiot (Tokyo) 51:897–901

Schoch CL, Crous PW, Groenewald JZ, Boehm EW, Burgess TI, de Gruyter J (2009) A class-wide phylogenetic assessment of Dothideomycetes. Stud Mycol 64:1–15

Schwartz RE, Liesch J, Hensens O, Zitano L, Honeycutt S, Garrity G, Fromtling RA, Onishi J, Monaghan R (1988) L-657,398, a novel antifungal agent: fermentation, isolation, structural elucidation and biological properties. J Antibiot 41:1774–1779

Sierra López D (1987) Aportación al conocimiento de los ascomicetes (*Ascomycotina*) de Cataluña. Societat Catalana de Micologia vol I, 481 pp

Soláns MJ (1985) Tres especies del genero *Preussia* Fuckel (*Sporormiella* Ell. & Ev.). Novedades para el catalogo micológico español. Bol Soc Micol Castellana 9:29–36

Soman AG, Gloer JB, Koster B, Malloch D (1999) Sporovexins A–C and a new preussomerin analog: antibacterial and antifungal metabolites from the coprophilous fungus *Sporormiella vexans*. J Nat Prod 62: 659–661

Stadler M (2011) Importance of secondary metabolites in the Xylariaceae as parameters for assessment of their taxonomy, phylogeny, and functional biodiversity. Curr Res Environ Appl Mycol 1:75–133

Stadler M, Læssøe T, Fournier J, Decock C, Schmieschek B, Tichy H-V, Peršoh D (2014) A polyphasic taxonomy of *Daldinia* (Xylariaceae). Stud Mycol 77:1–143

Talontsi FM, Lamshöft M, Douanla-Meli C, Kouam SF, Spiteller M (2014) Antiplasmodial and cytotoxic dibenzofurans from *Preussia* sp. harboured in *Enantia chlorantha* Oliv. Fitoterapia 93:233–238

Unamuno PLM (1941) Enumeración y distribución geográfica de los ascomicetos de la Península Ibérica y de las Islas Baleares. Men R Acad Madr, Ser Cienc Nat 8:1–403

Urries MJ (1932) Datos sobre macromicetos de la provincia de Huesca. Bol Soc Esp His Nat 32:213–229

Valldosera M, Guarro J (1990) Estudios sobre hongos coprófilos aislados en España. XV. El género *Preussia* (*Sporormiella*). Boletín de la Sociedad Micológica de Madrid 14:81–94

von Arx JA (1973) Ostiolate and nonostiolate pyrenomycetes. Proc Kon Ned Akad Wet Ser C 76:289–296

von Arx JA, Van der Aa HA (1987) *Sporormula tenerifae* gen. et sp. nov. Trans Br Mycol Soc 89:117–120

Wang Y, Gloer JB, Scott JA, Malloch D (1995) Terezines A–D: new amino acid-derived bioactive metabolites from the coprophilous fungus *Sporormiella teretispora*. J Nat Prod 58:93–99

Wang CY, Wang BG, Brauers G, Guan HS, Proksch P, Ebel R (2002) Microsphaerones A and B, two novel gamma-pyrone derivatives from the sponge-derived fungus *Microsphaeropsis* sp. J Nat Prod 65:772–775

Weber HA, Gloer JB (1988) Interference competition among natural fungal competitors: an antifungal metabolite from the coprophilous fungus *Preussia fleischhakii*. J Nat Prod 51:879–883

Weber HA, Gloer JB (1991) The preussomerins: novel antifungal metabolites from the coprophilous fungus *Preussia isomera* Cain. J Org Chem 56:4355–4360

Weber HA, Baenziger NC, Gloer JB (1990) Structure of preussomerin a: an unusual new antifungal metabolite from the coprophilous fungus *Preussia isomera*. J Am Chem Soc 112:6718–6719

Weber HA, Swenson DC, Gloer JB, Malloch D (1992) Similins A and B: new antifungal metabolites from the coprophilous fungus *Sporormiella similis*. Tetrahedron Lett 33:1157–1160

Xiong H, Xiao GK, Chen GD, Chen HR, Hu D, Li XX, Zhong SW, Guo LD, Yao XS, Gao H (2014) Sporormiellin A, the first tetrahydrofuran-fused furochromone with an unprecedented tetracyclic skeleton from *Sporormiella minima*. RSC Adv 46: 24295–24299

Zaferanloo B, Bhattacharjee S, Ghorbani MM, Mahon PJ, Palombo EA (2014) Amylase production by *Preussia minima*, a fungus of endophytic origin: optimization of fermentation conditions and analysis of fungal secretome by LC-MS. BMC Microbiol 14:55

Zhang F, Li L, Niu S, Si Y, Guo L, Jiang X, Che Y (2012) A thiopyranchromenone and other chromone derivatives from an endolichenic fungus, *Preussia africana*. J Nat Prod 75:230–237

Chapter 3

Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity

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INVITED ARTICLE

Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity

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Adsorptive polymeric resins have been occasionally described to enhance the production of specific secondary metabolites (SMs) of interest. Methods that induce the expression of new chemical entities in fungal fermentations may lead to the discovery of new bioactive molecules and should be addressed as possible tools for the creation of new microbial chemical libraries for drug lead discovery. Herein, we apply both biological activity and chemical evaluations to assess the use of adsorptive resins as tools for the differential expression of SMs in fungal strain sets. Data automation approaches were applied to ultra high performance liquid chromatography analysis of extracts to evaluate the general influence in generating new chemical entities or in changing the production of specific SMs by fungi grown in the presence of resins and different base media.

Keywords: HPLC-Studio; fungal fermentations; Diaion® resin; Amberlite® resin; secondary metabolites; chemical diversity

1. Introduction

Fungal metabolites have historically provided a rich source of lead compounds for drug discovery. However, a general perception is emerging that rates of discovery of new molecules, especially new antibiotics, are decreasing after half a century of continued screening of fungal diversity by fungal fermentation processes (Bills et al. 2009). Recent advances in genome sequencing, facilitated by lowered costs and fast service, have revealed untapped reservoirs of microbial natural products in unexpressed biosynthetic pathways. The number of presumable secondary metabolite (SM) gene clusters in fungal genome analysed so far widely exceeds the number of detected metabolites for these fungal strains (Bergmann et al. 2007; Gross 2007; Hornung et al. 2007). This highlights the opportunity of finding possibly unexpressed genes that potentially could encode the biosynthesis of novel SMs, yet unknown because of our inability to simulate *in vitro* their environmental conditions required for expression (Gross 2007).

The value of empirical strategies for manipulating nutritional and environmental parameters to stimulate the metabolic diversity of a microorganism has long been recognized. In many industrial microbial screening laboratories, empirical comparisons of an organism metabolic response to a large number of medium formulations, over a range of temperatures and aerations, were often the first-line approach for improving product formation and associated bioactivity from a newly discovered fungus or actinomycete. The impact of varied media formulations was also evident during the initial fermentation of large numbers of new strains and testing extracts across multiple biological assays (Yarbrough et al. 1993). Review of screening data often recognized that interesting active metabolites were formed or produced in larger quantities in only one of several media. Recently, several approaches have been applied to foster the expression of these unexpressed pathways and to promote SM biosynthesis. These strategies include the manipulation of medium and growth conditions in miniaturized nutritional arrays (Bills et al. 2008), the application of transformation techniques for the generation of gene knockouts, the exchange of native gene promoters with constitutive or inducible promoters or the overexpression of transcription factors (Brakhage and Schroeckh 2011), the co-cultivation of one or more microorganisms in constant interaction (Combès et al. 2012), the addition of small-molecule elicitors such as epigenetic modifiers, inhibitors of histone deacetylase or DNA methyltransferase and the fermentation in the presence of non-ionic adsorption resins (de la Cruz et al. 2012).

Adsorptive polymeric resins have been incorporated since 1970s into fermentation processes for myxobacteria,

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fungi and actinomycetes, basically in order to increase titres by sequestering metabolites, preventing degradation or decreasing the cytotoxic effects of the final metabolites produced (Phillips et al. 2013). Several examples of microbial SMs demonstrate that the addition of resins cause some of these effects, including: (a) the production of rubradirin, an antibiotic produced by *Streptomyces achromogenes* subsp. *rubradiris* (Marshall et al. 1990) in which the addition of non-ionic polymeric adsorbents XAD-2, XAD-7, XAD-16, HP-20 and HP-21 led to a two- to fourfold increase in rubradirin titres after 3–4 days of fermentation; (b) paulomycin production, wherein the resin prevented the transformation of paulomycin to paulomenol (Marshall et al. 1987); (c) the production of BMS-182123, a metabolite produced by *Penicillium chrysogenum* which acts as an inhibitor of tumour necrosis factor alpha (TNF-α), whose production was increased by 5.5-fold by adding XAD-8 resin in the fermentation medium (Warr et al. 1996); (d) the production of migrastatin and isomigrastatin, potential anticancer agents produced by *Streptomyces platensis* (Woo et al. 2002), where XAD-16 added to the fermentation medium enhanced its production; (e) the production of ambruticins and jerangolids, new antifungal compounds produced by the myxobacterium *Sorangium cellulosum* (Gerth et al. 1996) and (f) the production of nomimicin, a new spirotetronate class of polyketide obtained from an actinomycete of the *Actinomadura* genus fermented in the presence of the polymeric resin HP-20 (Igarashi et al. 2012).

Our research group also described recently that the production of some metabolites is increased in the presence of non-ionic adsorption resins such as Amberlite® XAD-7 (de la Cruz et al. 2012), sometimes resulting in a modulation of whole-cell antibiotic production. So far, no clear evidence indicated that the addition of non-ionic adsorptive resins to fermentation processes is capable of activating unexpressed biosynthetic pathways, or increasing the chemical diversity produced in fungal fermentations (Phillips et al. 2013). To examine this hypothesis, we have evaluated the effect of different resin additions on fungal SM diversity production using ultra high performance liquid chromatography (uHPLC)-UV profiles and a new data-automated analysis method. Our aim was to generate collections of natural product extracts with high chemical diversity, with the final goal of finding new potential non-cytotoxic antifungal and antibacterial agents in our drug discovery programs.

2. Material and methods

2.1. Inocula preparation

Cryotubes containing fungal inocula or mycelia discs in 10% (v/v) glycerol stored at −80°C were thawed, and strains were revived and transferred to 60-mm Petri dishes containing 10 ml of YM agar (malt extract 10 g, yeast extract 2 g, agar 20 g, 1000 ml distilled H_2O) at 22°C for 14–20 days. Five mycelial discs were cut from each 60-mm plate with a sterile Transfer Tube (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Mycelia discs were extruded from the Transfer Tube and crushed in the bottom of tubes (25 × 150 mm) containing 12 ml of SMYA medium (Difco™ neopeptone 10 g, maltose 40 g, Difco™ yeast extract 10 g, agar 4 g, distilled H_2O 1000 ml) and two cover glasses (22 × 22 mm). Tubes were incubated on an orbital shaker (200 rpm; 1.5 cm throw), where rotation of the cover glasses continually sheared hyphae and mycelial disc fragments to produce nearly homogenous hyphal suspensions consisting of minute hyphal aggregates and fine mycelia pellets. Tubes were incubated for 7 days at 22°C (Bills et al. 2008).

2.2. Media formulation

MMK2 (mannitol 40 g, Difco™ yeast extract 1 g, Sigma-Aldrich (St. Louis, MO, USA) Murashige & Skoog salt 4.3 g, distilled H_2O 1000 ml), SCAS (Panreac (Barcelona, Spain) soluble starch from potato 40 g, Sigma-Aldrich casein hydrolysate 5 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g and distilled H_2O 1000 ml), MV8 (maltose 75 g, V8 juice 200 ml, Sigma-Aldrich soy flour 1 g, L-proline 3 g, 2-(N-morpholino)ethanesulfonic acid 16.2 g and distilled H_2O 800 ml), YES (Difco™ yeast extract 20 g, sucrose 150 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, trace elements 1 ml ($ZnSO_4 \cdot 7H_2O$ 1 g/100 ml and $CuSO_4 \cdot 5H_2O$ 0.5 g/100 ml) and distilled H_2O 1000 ml), LSFM (glycerol 18.7 g, glucose 40 g, NH_4SO_4 2 g, Sigma-Aldrich yeast autolysate 5 g, soybean flour 5 g, tomato paste 5 g, sodium citrate 2 g and distilled H_2O 1000 ml) and XPMK (D-Xylose 60 g, Difco™ peptone 8 g, KH_2PO_4 0.5 g, Murashige & Skoog salt 4.3 g, distilled H_2O 1000 ml). Fermentation vials (42 ml EPA vials) containing 10 ml of medium were autoclaved for 21 min at 121°C.

2.3. Addition of adsorptive resins

Four adsorptive polymeric resins were added to fermentations: Amberlite® XAD-2, XAD-7, XAD-16N (hydrophobic polyaromatic resins, Sigma-Aldrich) and Diaion® HP-20 (styrene-divinylbenzene, Supelco (Bellefonte, PA, USA)) polyaromatic adsorbent resin for hydrophobic retention of compounds as antibiotics and other molecules. The resins were washed in twice their volume of methanol and stirred for 1 h, followed by six washes with distilled water, and were finally stored at 4°C in distilled water for 48 h (Singh et al. 2010). Afterwards, they were vacuum filtered through a 125 mm diameter and 6 μm pore size filter. Once filtered, the resins were oven dried at 75°C, and 0.3 g of each resin was added to the fermentation vials before autoclaving for 21 min at 121°C.

2.4. Growth

Cultures of each fungus were inoculated by adding 0.3 ml of inoculum suspension into five 42-ml EPA scintillation vials containing 10 ml of culture media with and without each of the resins. The vials were incubated for 14 days in a shaking incubator (Kühner AG, Birsfelden, Suiza) at 22°C, 220 rpm and 70% relative humidity.

2.5. Extraction

After 14 days of incubation, fermentation broths with cells were extracted by adding 9 ml of acetone using a Multiprobe II robotic liquid handler and shaking at 220 rpm for 1 h. After centrifugation, 12 ml of supernatant from each vial was transferred to glass tubes containing 0.6 ml of dimethyl sulfoxide (DMSO). Solvent was evaporated under a hot nitrogen stream to a final volume of 3 ml (80/20 water/DMSO solution) and a final concentration of 2× whole broth equivalents. Each fermentation batch included extracts from control culture media to discriminate their components. This extraction procedure was validated using antibiotics covering a wide range of polarities, molecular weights (MWs) and chemical structures, measuring in each case if compounds were detected after the extraction and preparation of the samples. For this purpose, water solutions containing 1 mg/ml of Terramycin®, cephalothin, amphotericin B, novobiocin, fusidic acid and actinomycin and unsaturated resins at 3% weight/volume were extracted applying the same methodology.

2.6. Bioactivity characterization

The antimicrobial activities of the extracts were evaluated against methicillin-resistant *Staphylococcus aureus* (MRSA) MB5393 (de la Cruz et al. 2012), *Candida albicans* MY1055 (de la Cruz et al. 2012) and *Aspergillus fumigatus* ATCC 46645 (Monteiro et al. 2012). Briefly, the microorganisms were incubated with the extracts for 18–30 h at 37°C. Sample 1:10 or 2:25 dilutions were used depending on the target strain. The activities were measured by monitoring the absorbance differences at 600 nm between the final and the initial incubation times, except for *A. fumigatus* where the activity was scored by using resazurin, an oxidation–reduction indicator of the cell viability (Monteiro et al. 2012). Additionally, the cytotoxicity of the different extracts against the HepG2 cell line (hepatocellular carcinoma, ATCC HB 8065) was evaluated by a classical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction colorimetric assay, with the same incubation times and assay concentrations as used for the antibiotic evaluation (de Pedro et al. 2013).

2.7. Chemical analysis

Chemical profiles of fermentation extracts were analysed using an Agilent 1290 Infinity uHPLC-diode array detector (DAD). A Kinetex C-18 (1.7 μm, 2.1 × 150 mm) from Phenomenex (Torrance, CA, USA), a 10-min gradient from 1% to 99% (v/v) of acetonitrile in water, with 1.3 mM ammonium formate and 1.3 mM trifluoroacetic acid as chromatographic modifiers, a flow rate of 0.315 ml/min, a controlled temperature of 40°C and UV detection at 210, 280 and 340 nm were used for each analysis. An inert internal plastifier control (IC) was present in each sample to individually validate and normalize if necessary each chromatographic run. Additional methanol blanks were injected every 10 samples for joint monitoring of each analytical batch. Agilent integration uHPLC parameters included tangent skip mode (standard), tail peak skim height ration (0), front peal skim height ration (0), skim valley ratio (0), baseline correction (advanced), peak to valley ratio (1), slope sensitivity (15), peak width (0), area reject (0), height reject (0) and shoulders (TAN).

2.8. Data automation

The samples and positions in the storage plates were recorded in a computer database Oracle system (Nautilus LIMS by Thermo) where the producing microorganism, the fermentation medium, the additive, the extraction procedure and the location of each sample in the storage plates were recorded. An improved version of HPLC-Studio (Tormo et al. 2003; Tormo & García 2005) correlated all injected samples and extracts to their corresponding unfermented fermentation broths and allowed the chemical comparisons.

This recent version of HPLC-Studio (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005), with improvements for its application in uHPLC with Oracle databases (without limitations on the number of traces that could be processed at the same time), was used for data processing. No intensity peak thresholds were used. All the peaks eluting between 1.5 and 9.0 min were processed.

Variations found in the uHPLC retention times in the analysis of complex extracts by HPLC-DAD and uHPLC-DAD were due to several factors, such as the composition of the mixtures, the concentration of the compounds, the existence of molecules with very close retention times and the degree of resolution of the DAD detection and were corrected by applying a window of 0.065 min (double of the DAD scan detecting resolution), previously established as an adequate resolution time to distinguish between two distinct SMs among a large batch of sequential chromatograms (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005).

2.9. Characterization of chemical diversity by profile clustering

Only data related to the presence or absence of newly produced metabolites were used. Peak areas were not taken into account for the analysis to avoid biases due to very large

areas and to a possible deficient extraction of all quantities of the produced metabolites. Therefore, interpretations were strictly based on the presence or absence of peaks, without the quantitative data. To validate this approach, extracts of a water solution of several antibiotics (see Section 2.5) were also subjected to uHPLC analysis. Although in some cases the recovery titres were low (especially the one with the lowest polarity, actinomycin; see Supplementary information), the presence of all the antibiotics in the acetone extract confirmed that, in terms of presence or absence of the compounds, the extraction method could be used for chemical comparisons of the microbial SM profiles obtained under different fermentation conditions.

Components of the control fermentation media were subtracted from any SM counting. Chemical characterization of the diversity of the extracts started with the determination of the number of metabolites that were detected in the fermentations versus the control media. Next, the number of different peaks in a single condition versus the number of peaks of the metabolic footprint obtained from all the peaks present among all conditions for a single strain indicated the chemical diversity generated by that strain in each condition (a ratio expressed as a percentage). All fermentation conditions were classified based on their contribution to the chemical diversity following this procedure. The matrix of percentages of overlapping diversity between each condition was also determined.

BioNumerics software version 6.0 (Applied Maths) was used for the comparison of the metabolic footprints of each strain in the different fermentation conditions. The BioNumerics software was loaded with the resulting data matrix generated for the analysis of the common and uncommon peaks generated by the software (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005). The statistical analysis in BioNumerics was based on the determination of the Dice similarity coefficient (Dice 1945). From the estimated similarity coefficients, similarity dendrograms were generated by the unweighted pair group with arithmetic averages (UPGMA) method. This method uses the arithmetic average to generate clusters, showing the similarities between the different fermentation conditions based on the presence or absence of common SMs in each case. Principal component analysis (PCA) was also calculated with the BioNumerics software to facilitate visualization of relationships among the different fermentation conditions per strain.

2.10. *Chemical profiling validation for adsorptive resins comparisons*

Strategies for automated evaluation on SM profiles with automated HPLC peak database comparison have been outlined previously by our group to select the most chemically diverse condition before scale-up (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005). The same methods could be used statistically to assess the real influence on the metabolic profiles caused by the adsorptive polymeric resin additions. However, the degree of sensitivity and accuracy of the method had to be validated to confirm that the degree of variation observed within triplicates was always smaller than the one observed among the different fermentation conditions evaluated for the resins.

For this purpose, three different species of fungi were selected and fermented in triplicate, in two different base media each, with three different fermentation conditions (control media, control media plus XAD-16 and control media plus HP-20 additives). Chemical profile results proved that the previous method developed for the comparison of different metabolic profiles (presence or absence of detected metabolites by uHPLC at 210 nm and further statistical analysis by Dice + UPGMA from uHPLC-Studio + BioNumerics; Tormo & García 2005) was sufficiently sensitive that the triplicates had narrower distance coefficients than the statistical distances observed among the varied fermentation conditions.

3. Results and discussion

3.1. *Bioactivity evaluation of sets of strains*

Ninety-six different species of filamentous fungi from the Fundación MEDINA strains collection, belonging to orders known to be typical sources of biologically active molecules, were selected: *Chaetothyriales, Diaporthales, Dothideales, Eurotiales, Helotiales, Hypocreales* and *Pleosporales*. After 14 days of fermentation in MMK2 medium in the presence or absence of resins, differences in texture, colour, morphology and biomass were observed among the different resins when harvested (Figure 1).

Figure 1. Fungal strain CF-212941 grown on MMK2, MMK2 +HP-20, MMK2+XAD-2, MMK2+XAD-7 and MMK2+XAD-16 at 14 days, showing differences in texture, colour, morphology and quantity of biomass.

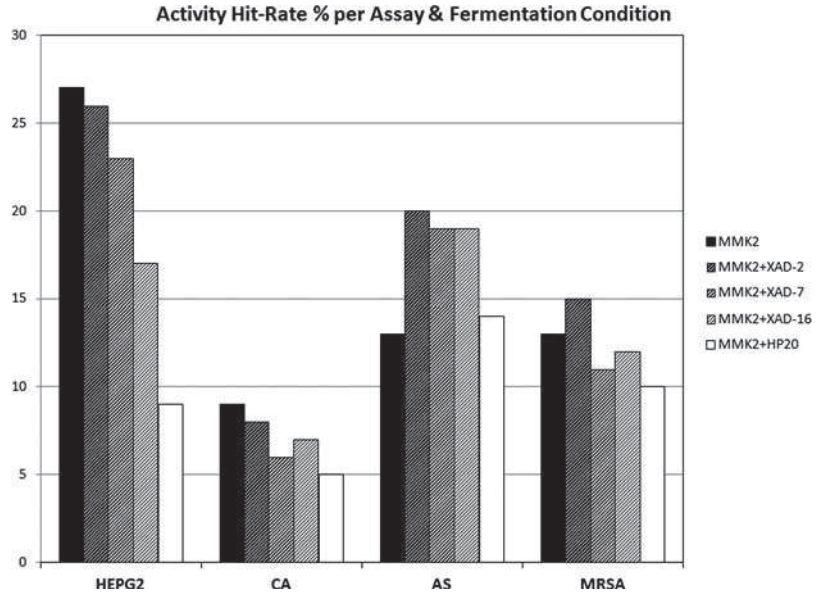

Figure 2. Activity hit-rate (%) of extracts, per assay and fermentation condition for the 96 fungal strains tested. HEPG2, cytotoxicity assay in HepG2 cell line. CA (*Candida albicans*), AS (*Aspergillus fumigatus*) and MRSA (methicillin-resistant *Staphylococcus aureus*) anti-infective tests.

Assuming that the phenotypic difference could indicate different SM production and therefore different potential new chemical entities, we proceeded to their extraction and evaluation for anti-infective and cytotoxic properties against three relevant human pathogens: one Gram-positive bacterium, MRSA and two fungi (*Candida albicans* and *Aspergillus fumigatus*). Since many antibacterial and antifungal metabolites also exhibit human cell cytotoxicity, this biological activity was also added to the assay panel (Figure 2).

Results indicated that, in general, for the MMK2 base fermentation medium, the addition of Diaion® HP-20 resin resulted in a decrease in cytotoxic and antimicrobial active hit-rates, whereas the addition of Amberlite® XAD-2 showed generally higher antimicrobial and cytotoxicity activity hit-rates than XAD-16 or XAD-7, which presented intermediate activity hit-rates.

Because most of the strains did not differ significantly in their activity profiles whether fermented in the presence or absence of the resins, we therefore focused further detailed studies on the subset of strains that exhibited consistent changes of their activity profiles when fermented in the presence of the resins, as a clear indicator of the existence of real changes in their metabolite profiles, including changes in metabolite concentrations. For this purpose, 14 strains from the original 96 were selected for chemical analysis because they presented consistent clear variation patterns on their activity profiles among the different target strains (Table 1). This subset of strains covered a wide taxonomic space for assessing the effect of the addition of the resins on a wide variety of fungi, including orders: Eurotiales (*Penicillium*), Hypocreales (*Cordyceps, Bionectria, Fusarium, Cosmospora*), Pleosporales (*Preussia, Phoma, Sydowiella*), Chaetothyriales and Diaporthales (*Pilidiella, Diaporthe*).

Among all the fermentation conditions tested, XAD-16 and HP-20 resins were also initially selected for further detailed studies because they were associated with larger differences in activity hit-rates versus base medium conditions (Figure 2). According to the manufacturers, both resins have similar chemical properties (styrene-divinylbenzene): XAD-16 is mostly used for adsorption of organic compounds of medium MWs from aqueous systems and polar solvents and HP-20 is used for refining of pharmaceuticals and natural extracts because it adsorbs larger molecules due to its relatively large pore sizes. XAD-16 characteristics included 560–710 µm of particle size, 200 Å of pore size and 800 m^2/g of surface area, whereas HP-20 characteristics included 250–850 µm of particle size, 260 Å of pore size and 500 m^2/g of surface area. The larger particle surface and slightly smaller pore size, but higher number of pores, make XAD-16 more suitable for hydrophobic compounds up to MW of 40,000, especially proteins. On the contrary, HP-20, with slightly larger pore sizes, can also be used for larger MW compounds.

3.2. *Effect of resins on the fungal growth*

Furthermore, in order to determine whether the changes in SM obtained by the fermentation in presence of the resin additives were due to changes in the initial composition of

Table 1. Antibiotic activities of the fungal strains selected.

Strain ID	TAXINIT	Country	HEPG2	CA	AS	MRSA
CF-194989	*Phoma* sp.	Puerto Rico	+++++	−+−−−	+++++	−+−−−
CF-195017	*Preussia* sp.	Puerto Rico	−+−−−	−−−−−	−+−−−	−+−−−
CF-209155	*Preussia intermedia*	Portugal	+−−−−	−−−−−	−−−−−	−−−++
CF-209171	*Preussia* sp.	South Africa	−+−+−	−+−+−	−+−+−	−+−+−
CF-209591	*Cordyceps* sp.	New Zealand	−++−−	−++++	−+++−	+++−−
CF-210345	*Sydowiella* sp.	Puerto Rico	−−−−−	−−−+−	−−−+−	−++++
CF-210367	*Cosmospora vilior*	Spain	+−−−−	−−−−−	+−−−−	+−−−−
CF-210370	*Bionectria* sp.	Spain	+−−−−	−−−−−	−−−−−	+−−−−
CF-210988	*Penicillium* sp.	C. African Republic	−−−+−	−−−−−	−++++	−−−+−
CF-210989	*Fusarium* sp.	C. African Republic	−−−+−	−−−−−	−−−−−	−+−−−
CF-214546	*Diaporthe* sp.	C. African Republic	−+−−−	−−−−−	−−−−−	−+−−+
CF-214552	*Pilidiella castaneicola*	New Zealand	+−−−	+−−−−	−−−−−	+−−−−
CF-214558	*Penicillium* sp.	C. African Republic	−−−+−	−−−−−	−−−−−	−+−−−−
CF-214575	*Chaetothyriales* sp.	Spain	−−−−	−++++	−++++	−−−−−

Notes: HEPG2: hepatocellular carcinoma ATCC HB 8065; CA: *Candida albicans* MY1055; AS: *Aspergillus fumigatus* ATCC 46645; MRSA: methicillin-resistant *Staphylococcus aureus* MB5393. Sequentially: MMK2 (±), MMK2+XAD2 (±), MMK2+XAD7 (±), MMK2+XAD16 (±) and MMK2+HP-20 (±).

the medium (i.e. by having the resin capturing essential components prior to the inoculation), we pre-treated the MMK2 control media with the resins prior to their filtration and autoclaving. The sucrose-rich medium (YES) was also tested to evaluate if possible differences could be medium dependent. YES medium was selected because of its historical success for enhancing the production of active SMs (Bills et al. 2008).

In general, the addition of the resins to the fermentations caused variations in the morphology, colour and amount of biomass in many of the broths, with fermentation triplicates behaving homogeneously. For example, in *Penicillium* sp. strain CF-210988 grown in YES medium (Figure 3), the treated and pre-treated conditions with XAD-16 or HP-20 produced more biomass than the control medium, with no substantial chemical differences between pre-treated and treated media. Interestingly, colour from either pre-treated or treated conditions was equally different when compared to the control condition, indicating that the pre-treatment with the resins had affected the growth to a certain extent. These effects might be explained by a potential sequestration by the resin of trace elements or some components of the medium during the pre-treatment.

However, in the case of the MMK2 medium, the XAD-16 pre-treated condition was very similar to the control medium, whereas the HP-20 pre-treated condition was more similar to both XAD-16- and HP-20-treated media. When the same strain was grown in MMK2 medium, only the XAD-16-treated medium exhibited increased biomass production and different chemical profile compared to the control. Interestingly, for both media, the HP-20 resin surface remained white after harvesting, whereas XAD-16 resin became strongly pigmented in both media (Figure 3).

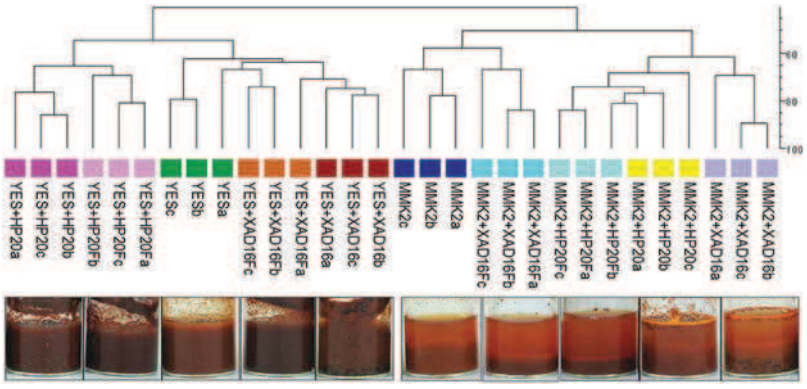

Figure 3. UPGMA dendrogram showing overall similarities between SMs produced by fungal strain (CF-210988) when grown on 10 different fermentation conditions per triplicate, comparing the different fermentation triplicates (a, b, c), the different resins (HP-20 or XAD-16) and two base media autoclaved in the presence of the resins or just pre-treated before inoculating the fungal strain (Fa, Fb, Fc). Similarities were determined by Dice's coefficient values.

In general, differential production profile and more biomass quantities were always observed when treatments with resins were performed along the whole fermentation processes. This observation and the literature references to the production of certain metabolites related to product displacement mechanisms along the whole fermentation process determined us to focus our further chemical profiling studies only on full-treated fermentation conditions.

3.3. Chemical evaluation of the resin addition to fungal fermentations

To characterize in detail the differences observed within the set of 14 fungal strains with or without adding the resins during the whole fermentation process, we decided to compare their SM profiles by uHPLC. Analysis of their SM chemical profiles (Figure 4) proved that for each of these strains, there could be: (i) conditions with clear improvement in the quantity of production of given compounds in the presence of the resins, (ii) compounds that only were produced in the control medium (without resins) and (iii) compounds that were only present when specific resins were added. The large number of cases found with variations in the presence or absence of specific SMs, determined by counting the variations in the number of compounds detected by uHPLC, led us to a practical representation on the real effect of the resins for this set of fungal strains.

Initially, the difference in the number of SMs produced by each strain with added resins was not significant for any of the four additives tested in the MMK2 medium (Figure 5). However, to determine the best condition for enhanced chemical diversity production, we scored the frequencies of the conditions that generated the maximum non-overlapping chemical diversity for each strain versus the total number of conditions used per strain. An averaged scoring was obtained for the representative subset of 14 strains (Table1, Figure 6). According to this methodology (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005), displacements of a condition from the 1:1 diagonal linear correlation clearly highlight different chemical diversity influences: (i) the greater the displacements towards the upper part of the graph, the better the medium results for exclusive new SM generation; whereas (ii) displacements towards the lower half of the graph indicate lesser differential metabolite generation (Figure 6).

This analysis clearly indicated that the order of influence of the resins in inducing more to less chemical diversity was sequentially XAD-16 followed by XAD-2, XAD-7 and HP-20. This result could be correlated, to a

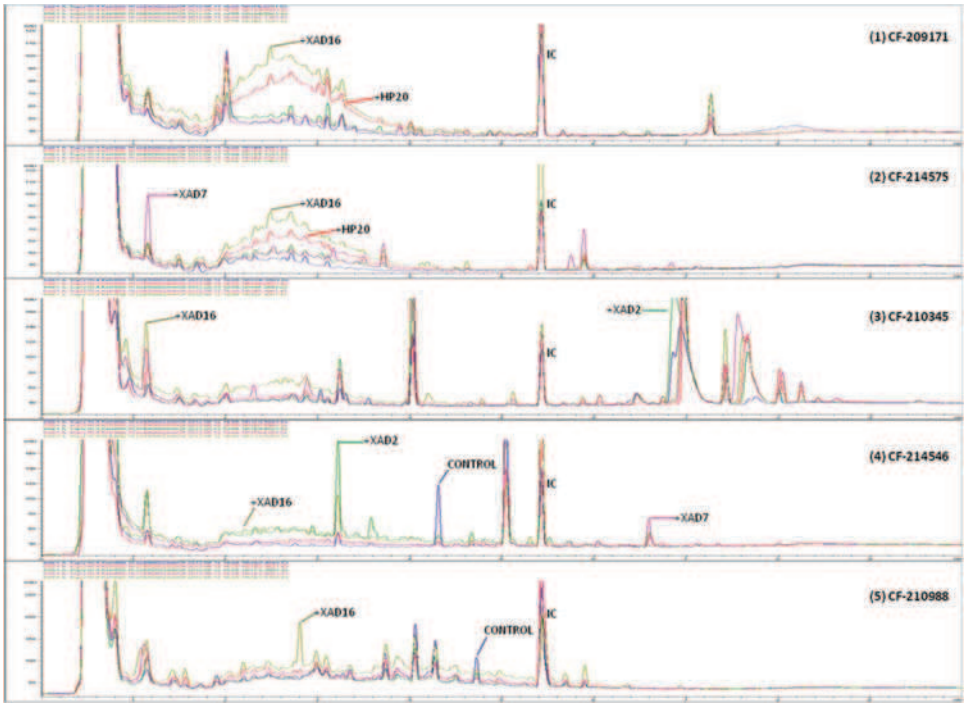

Figure 4. Comparative analysis of different uHPLC-UV 210 nm secondary metabolite profiles produced by five different fungal strains: (1) *Preussia* sp. (CF-209171); (2) *Chaetothyriales* sp. (CF-214575); (3) *Sydowiella* sp. (CF-210345); (4) *Diaporthe* sp. (CF-214546); (5) *Penicillium* sp. (CF-210988) when these fungus were grown on MMK2 fermentation medium with and without XAD-2, XAD-7, XAD-16 and HP-20 resins. Relevant uHPLC traces are indicated in the figure. Internal control (IC) was added homogeneously to each sample to allow accurate comparisons of the chromatographic runs.

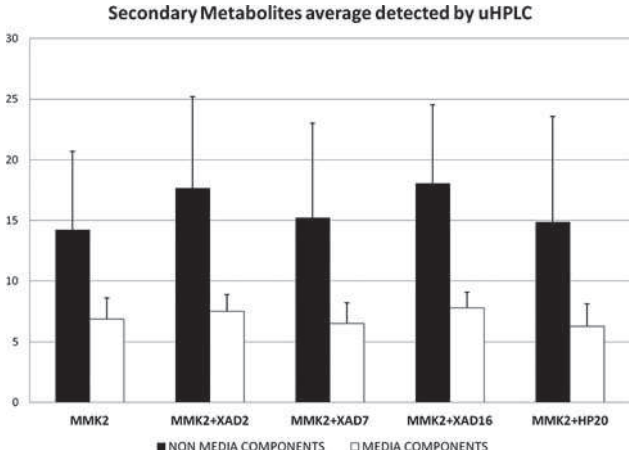

Figure 5. Average number of secondary metabolites detected by uHPLC-DAD for each additive to the MMK2 control medium fermentation for the 14 fungal strains that responded positively to the resin addition. The *T*-test method was used to compare pairs; pair differences were not statistically significant at $\alpha = 0.05$.

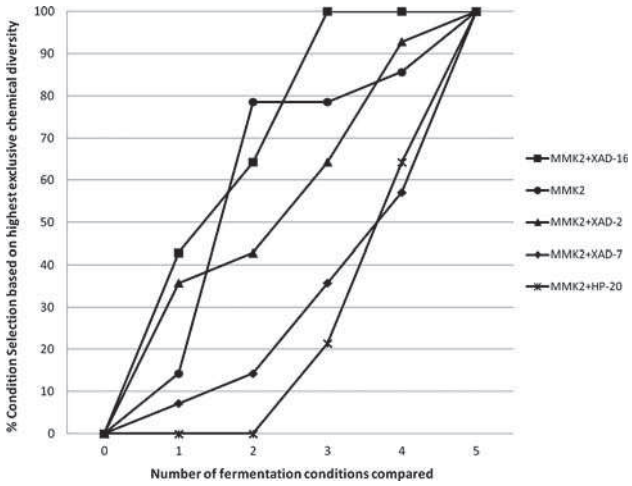

Figure 6. Percentage of fermentation conditions that presented highest exclusive chemical diversity as a function of number of fermentation conditions compared to evaluate the contribution to greater chemical diversity detected by uHPLC for five different fermentation conditions tested in 14 fungal strains that responded positively to the resin addition.

certain extent, with the low bioactivity hit-rates observed in the anti-infective and cytotoxic assays for the HP-20 resin (Figure 2), which in turn, could be related to the reduction in the chemical diversity also observed of these fermentations.

To determine whether these effects were dependent or not on the composition of the basal medium and, whether changes in carbon, nitrogen or trace elements sources could have greater influences on the production of SM than simply the presence of the resins, the same 14 fungi were also fermented in other five media with completely different compositions in carbon, nitrogen and trace elements sources (LSFM, MV8, SCAS, XPMK and YES) (Bills et al. 2008), with and without XAD-16 and HP-20 resins.

In general, and in agreement to the MMK2 results (Figure 5), the number of SMs produced did not differ significantly in any of the conditions (results not shown). Dice + UPGMA comparison of the chemical profiles for all 14 strains confirmed that the adsorptive polymeric resins generated slightly lower modifications in the SM profiles than changes in the composition of the base media (Figure 7A). This could also be observed by other statistical methods when principal components were calculated for this set of strains (Figure 7B). Clear grouping of all conditions belonging to each separate medium indicated that more chemical diversity was achieved by changing the medium composition than by adding the Diaion® or Amberlite® resins.

Specifically, the medium that generated a higher range of chemical diversity was LSFM followed by MV8, XPMK, YES and SCAS (Figure 8). This analysis also confirmed that changes in the composition of the fermentation medium determined higher variations in the chemical profiles than the addition of the resins. However, the detailed scoring of each condition (LSFM > LSFM+XAD-16 > LSFM+HP-20; MV8+XAD-16 > MV8 > MV8+HP-20, XPMK+HP-20 > XPMK > XPMK+XAD-16, YES +XAD-16 > YES+HP-20 > YES and SCAS+XAD-16 > SCAS+HP-20 > SCAS) indicated that, for fermentation media where a large chemical diversity production is initially observed, such as in LSFM, MV8 and MMK2, the fermentation media without resins generated similar or higher diversity than the one obtained by the addition of XAD-16 or HP-20. However, for fermentation media that induced lower metabolite diversity production, such as SCAS and YES, the addition of XAD-16 or HP-20 resulted in a clear improvement of the chemical diversity profiles generated (Figure 8).

3.4. *Unique secondary metabolites and changes in production obtained by resin addition*

During the statistical characterization of the chemical profiles generated with the addition of the resins, it became apparent that most of the fermentation conditions presented unique SMs and differential production titres of specific compounds. Figure 9 depicts an example (*Preussia* sp. CF-209171) where only MV8, SCAS and XMPK+PH-20 extracts resulted in inhibition zones against *Candia albicans*. In this strain, unique uHPLC peaks could be detected in 66% of the conditions: MV8, MV8+XAD-16, SCAS, SCAS+HP-20, SCAS+XAD-16, LSFM, LSFM+XAD-16, XPMK, XPMK+XAD-16 and XPMK+HP-20, and increments in the quantity of certain metabolites with respect to the control media could be

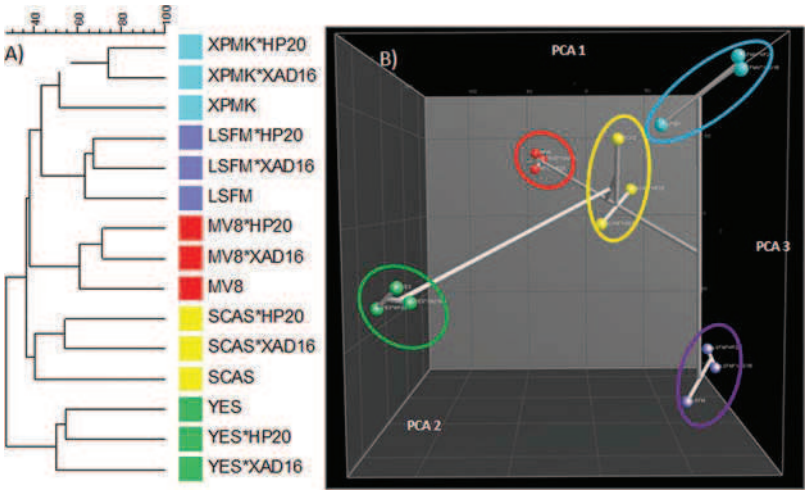

Figure 7. (A) DICE-UPGMA dendrogram. (B) Principal component analysis (PCA) of the secondary metabolites produced by the 14 selected strains from the study that responded positively to the resin addition grown on 15 fermentation conditions. PCA three-dimensional representation only depicts the three principal statistical components.

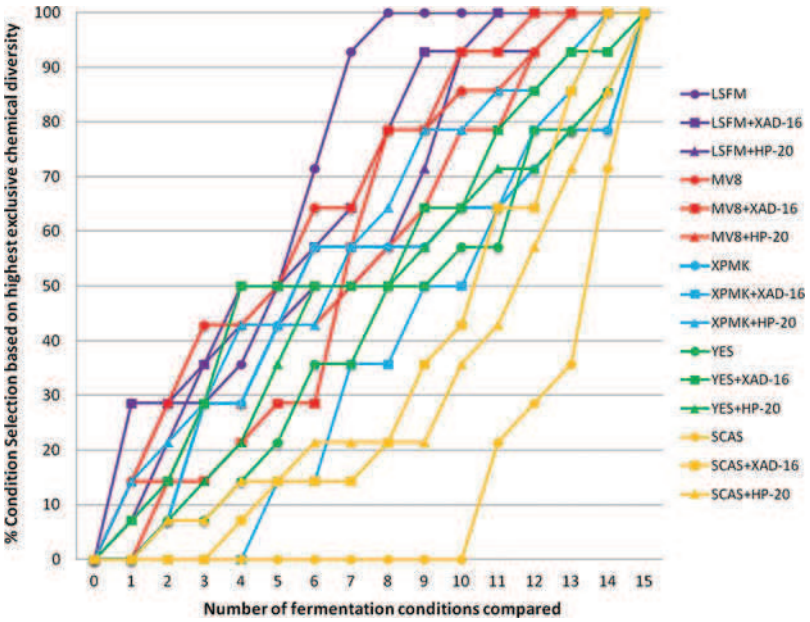

Figure 8. Ranking in percentage of the fermentation conditions that presented the highest exclusive chemical diversity as a function of number of all fermentation conditions compared per strain. To evaluate the contribution to greater chemical diversity detected by uHPLC, 15 different fermentation conditions were tested for the 14 selected fungal strains that responded positively to the resin addition.

detected in 50% of the resin conditions: MV8+XAD-16, SCAS+XAD-16, LSFM+XAD-16, XMPK+XAD-16 and YES+XAD-16.

Scoring on the number of unique metabolites per condition in the set of 14 strains supported this hypothesis (Table 2) where 102 unique metabolites were only obtained with the addition of the resins. These numbers clearly indicated that the real value of the addition of the resins to fungal fermentation on large sets of strains really consists in a general increment of the chemical diversity as an overall feature. However, the cause of these improvements remains unknown; they could be due to increased production beyond the detection threshold due to relief from feedback inhibition, relief from auto-toxicity, prevention of end product degradation, change on media composition by adsorption of certain components on the resins or other mechanisms. Moreover, and for some specific cases, the addition of the adsorptive polymeric resins could result even in larger changes in the SM profiles than the ones obtained

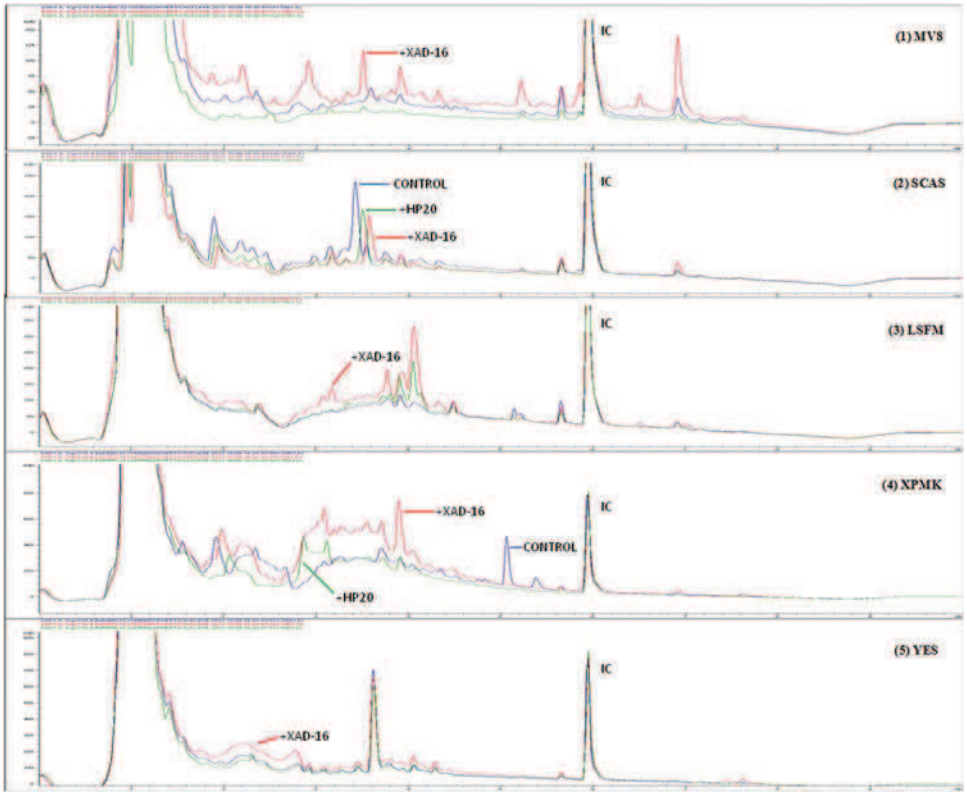

Figure 9. Comparative analysis of different uHPLC-UV 210 nm secondary metabolite profiles produced by strain *Preussia* sp. (CF-209171) when this fungus was grown on five different fermentation media (MV8, SCAS, LSFM, XPMK and YES) with and without XAD-16 and HP-20 resins. Relevant uHPLC traces are indicated in the figure. Internal control (IC) was added to each sample to allow accurate comparisons of the chromatographic runs.

by changing the composition of the base medium (Figure 10). These results clearly support the application of the adsorptive polymeric Amberlite® or Diaion® resins for specific strains where an increase in the production of specific metabolites or the generation of new bioactive metabolite cannot be achieved solely by introducing major changes in the medium components, e.g. addition of complex plant-based nutrients, or varying the carbon, nitrogen sources and the trace elements.

4. Conclusions

Several studies, on bacteria and fungi, have described the ability of adsorptive polymeric resins to increase the production titres of certain compounds of microbial origin (Frykman et al. 2006), and the possible mechanisms of action for these have been reviewed (Phillips et al. 2013). However, the question of whether one resin is consistently better than others for increasing metabolite chemical diversity has not been examined for any sets of strains. In order to evaluate this possibility, we selected the four most common resins used as microbial fermentation additives – Amberlite® XAD-2, XAD-7 and XAD-16, and Diaion® HP-20 – and we tested them in a pilot study for a set of 96 diverse fungal strains, grown in MMK2 fermentation medium, looking for increasing hit-rates of anti-infective activity and enhancements on metabolite production.

We were unable to correlate the additions of Amberlite® or Diaion® resins to the fermentation media of any fungal species with a direct increase in the production of non-cytotoxic antimicrobial metabolites for the widely diverse set of 96 fungal strains tested. Our bioactivity evaluation tests indicated that the addition of Diaion® HP-20 resin to the base fermentations medium MMK2 resulted in a decrease in the generation of cytotoxic and antimicrobial activities for these strains, whereas their fermentation in MMK2 medium, in the presence of Amberlite® XAD-2, slightly increased the frequency of detection of antibiotic and cytotoxic activity.

Analyses of their SM profiles indicated that variations in the composition of the base media increased the chemical diversity generated by the fungal strains to a larger extent than the addition of Amberlite® or Diaion® resins. It was also observed that variations on the end products of fungal fermentations in the presence of these polymeric resins could be related to an initial capture of essential nutrients by the resins or to a continuous action of the

Table 2. Number of unique secondary metabolites detected per microorganism and fermentation condition.

Strain ID	LSFM	LSFM +HP-20	LSFM +XAD-16	MV8	MV8 +HP-20	MV8 +XAD-16	SCAS	SCAS +HP-20	SCAS +XAD-16	XPMK	XPMK +HP-20	XPMK +XAD-16	YES	YES +HP-20	YES +XAD-16
CF-194989				*5*	*4*				*1*		*1*		*1*		
CF-195017	*1*	*1*	*2*						*3*		*1*			*1*	
CF-209155	*1*		*1*	*2*	*1*			*1*	*2*	*1*				*1*	*2*
CF-209171	*3*		*1*	*2*		*5*	*1*	*1*	*1*	*1*	*1*	*1*			
CF-209591	*2*		*2*	*3*		*6*		*1*	*4*	*1*	*1*	*1*	*1*		
CF-210345	*1*	*2*	*2*	*2*	*1*	*1*			*1*		*1*		*1*		*1*
CF-210367	*2*		*1*		*2*	*1*							*1*	*2*	
CF-210370						*1*	*1*			*1*					
CF-210988		*1*		*3*						*2*		*1*		*1*	*1*
CF-210989	*1*		*3*					*2*	*1*		*1*		*1*	*1*	*1*
CF-214546		*1*			*1*	*1*								*3*	*2*
CF-214552			*1*	*3*		*1*	*1*		*3*	*2*			*2*	*1*	
CF-214558	*1*		*1*			*1*	*1*		*3*		*1*		*1*		
CF-214575	*1*	*1*	*2*	*1*					*1*	*1*			*3*		*2*
Accumulated	*13*	*6*	*16*	*21*	*9*	*17*	*4*	*5*	*20*	*9*	*7*	*3*	*11*	*10*	*9*

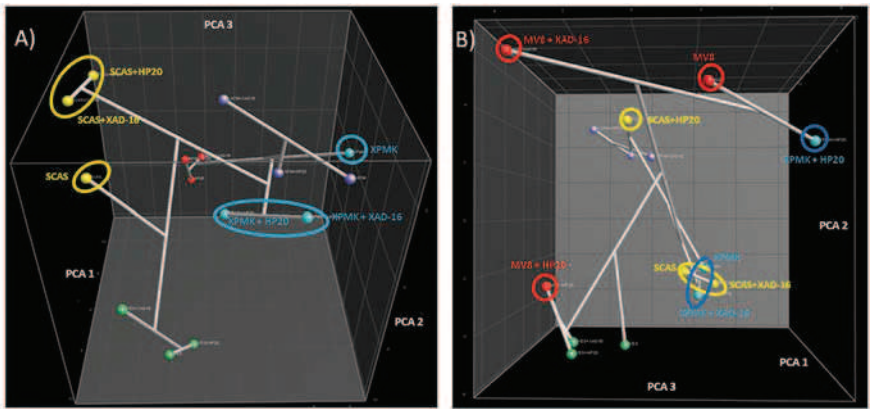

Figure 10. Principal component analysis (PCA) of the total of secondary metabolites produced by (A) *Pilidiella castaneicola* (CF-214558) and (B) *Preussia* sp. (CF-209171) grown in 15 different fermentation conditions. Relevant fermentation conditions are indicated in the figure.

resin throughout the whole fermentation process depending on the base medium composition.

Most of the fermentation conditions tested, either base medium changes, or additions of Amberlite® or Diaion® resins, produced unique compounds not found in any of the other conditions or induced changes in the production of specific compounds in most of the strains. Moreover, for certain strains, the addition of the adsorptive polymeric resins can result in greater changes in the SM profiles than changes obtained solely by varying the carbon, nitrogen and trace element composition. These findings might justify testing the addition of these resins when the production of a specific metabolite encoded by the fungal genome is desired.

In summary, this study is the first data-automated analysis on the effect of the addition of Diaion® and Amberlite® resins to fungal fermentations and their chemical diversity. Although changes in the medium composition more strongly influenced the SMs produced than the addition of adsorptive resins, the results clearly supported the hypothesis that the application of resins in fungal fermentations can determine the production of new SMs and provide a tool to consistently increase the production of low titre compounds. We believe these methods can be generally applied for the generation of natural product extract collections to get access to new bioactive chemical entities in drug discovery programs.

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Supplemental data

Supplemental data for this article can be accessed here.

References

Bergmann S, Schümann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. Nat Chem Biol. 3:213–217. doi:10.1038/nchembio869

Bills G, Martin J, Collado J, Platas G, Overy D, Tormo JR, Vicente F, Verkleij GJM, Crous PW. 2009. Measuring the distribution and diversity of antibiosis and secondary metabolites in filamentous fungi. SIM News. 59:133–147.

Bills G, Platas G, Fillola A, Jiménez MR, Collado J, Vicente F, Martín J, González A, Bur-Zimmermann J, Tormo JR, Peláez F. 2008. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. J Appl Microbiol. 104:1644–1658. doi:10.1111/j.1365-2672.2008.03735.x

Brakhage AA, Schroeckh V. 2011. Fungal secondary metabolites – strategies to activate silent gene clusters. Fungal Genet Biol. 48:15–22. doi:10.1016/j.fgb.2010.04.004

Combès A, Ndoye I, Bance C, Bruzaud J, Djediat C, Dupont J, Nay B, Prado S, Chaturvedi V. 2012. Chemical communication between the endophytic fungus *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum*. PLoS One. 7:e47313. doi:10.1371/journal.pone.0047313

de la Cruz M, Martín J, González-Menéndez V, Pérez-Victoria I, Moreno C, Tormo JR, El Aouad N, Guarro J, Vicente F, Reyes F, Bills GF. 2012. Chemical and physical modulation of antibiotic activity in *Emericella* species. Chem Biodivers. 9:1095–1113. doi:10.1002/cbdv.201100362

de Pedro N, Cautain B, Melguizo A, Vicente F, Genilloud O, Peláez F, Tormo JR. 2013. Mitochondrial Complex I inhibitors, acetogenins, induce HepG2 cell death through the induction of the complete VI apoptotic mitochondrial pathway. J Bioenerg Biomembr. 45:153–164. doi:10.1007/s10863-012-9489-1

Dice LR. 1945. Measures of the amount of ecologic association between species. Ecology. 26:297–302. doi:10.2307/1932409.

Frykman S, Tsuruta H, Galazzo J, Licari P. 2006. Characterization of product capture resin during microbial cultivations. Ind Microbiol Biotechnol. 33:445–453. doi:10.1007/s10295-006-0088-1

García JB, Tormo JR. 2003. HPLC-studio: a novel software utility to perform HPLC chromatogram comparison for screening purposes. J Biomol Screen. 8:305–315. doi:10.1177/1087057103008003008

Gerth K, Washausen P, Höfle G, Irschik H, Reichenbach H. 1996. The jerangolids: a family of new antifungal compounds from *Sorangium cellulosum* (myxobacteria). Production, physico-chemical and biological properties of jerangolid A. J Antibiot. 49:71–75. doi:10.7164/antibiotics.49.71

Gross H. 2007. Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects. Appl Microbiol Biotechnol. 75:267–277. doi:10.1007/s00253-007-0900-5

Hornung A, Bertazzo M, Dziarnowski A, Schneider K, Welzel K, Wohlert SE, Holzenkämpfer M, Nicholson GJ, Bechthold A, Süssmuth RD, et al. 2007. A genomic screening approach to the structure-guided identification of drug candidates from natural sources. ChemBioChem. 8:757–766. doi:10.1002/cbic.200600375

Igarashi Y, Iida T, Oku N, Watanabe H, Furihata K, Miyanouchi K. 2012. Nomimicin, a new spirotetronate-class polyketide from an actinomycete of the genus *Actinomadura*. J Antibiot. 65:355–359. doi:10.1038/ja.2012.30

Marshall VP, McWethy JS, Sirotti JM, Cialdella JI. 1990. The effect of neutral resins on the fermentation production of rubradirin. J Ind Microbiol. 5:283–287. doi:10.1007/BF01578202

Marshall VP, McWethy JS, Visser J, Cialdella JI, Laborde AL. 1987. Current fermentation technology from actinomycetes: the example of paulomycin. Dev Ind Microbiol. 28:105–114.

Monteiro MC, Dela Cruz M, Cantizani J, Moreno C, Tormo JR, Mellado E, De Lucas JR, Asensio F, Valiante V, Brakhage AA, et al. 2012. A new approach to drug discovery: high-throughput screening of microbial natural extracts against *Aspergillus fumigatus* using resazurin. J Biomol Screen. 17:542–549. doi:10.1177/1087057111433459

Phillips T, Chase M, Wagner S, Renzi C, Powell M, DeAngelo J, Michels P. 2013. Use of in situ solid-phase adsorption in microbial natural product fermentation development. J Ind Microbiol Biotechnol. 40:411–425. doi:10.1007/s10295-013-1247-9

Singh MP, Leighton MM, Barbieri LR, Roll DM, Urbance SE, Hoshan L, McDonald LA. 2010. Fermentative production of self-toxic fungal secondary metabolites. J Ind Microbiol Biotechnol. 37:335–340. doi:10.1007/s10295-009-0678-9

Tormo JR, García JB. 2005. Automated analyses of HPLC profiles of microbial extracts: a new tool for drug discovery screening. In: Zhang L, Demain AL, editors. Natural products: drug discovery and therapeutic medicine. Vol. 3. Totowa (NJ): Humana Press; p. 57–75.

Tormo JR, García JB, De Antonio M, Feliz J, Mira A, Díez MT, Hernández P, Peláez F. 2003. A method for the selection of production media for actinomycete strains based on their metabolite HPLC profiles. J Ind Microbiol Biotechnol. 30:582–588. doi:10.1007/s10295-003-0084-7

Warr GA, Veitch JA, Walsh AW, Hesler GA, Pirnik DM, Leet JE, Lin PM, Medina IA, McBrien KD, Forenza S, et al. 1996. BMS-182123, a fungal metabolite that inhibits the production of TNF-α. by macrophages and monocytes. J Antibiot. 49:234–240. doi:10.7164/antibiotics.49.234

Woo EJ, Starks CM, Carney JR, Arslanian R, Cadapan L, Zavala S, Licari P. 2002. Migrastatin and a new compound, isomigrastatin, from *Streptomyces platensis*. J Antibiot. 55:141–146. doi:10.7164/antibiotics.55.141

Yarbrough GG, Taylor DP, Rowlands RT, Crawford MS, Lasure LL. 1993. Screening microbial metabolites for new drugs – theoretical and practical issues. J Antibiot. 46:535–544. doi:10.7164/antibiotics.46.535

Chapter 4

Multicomponent analysis of the differential induction of secondary metabolites profiles in fungal endophytes

molecules

Chapter 4 MDPI

Article

Multicomponent Analysis of the Differential Induction of Secondary Metabolite Profiles in Fungal Endophytes

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Abstract: Small molecule histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors are commonly used to perturb the production of fungal metabolites leading to the induction of the expression of silent biosynthetic pathways. Several reports have described the variable effects observed in natural product profiles in fungi treated with HDAC and DNMT inhibitors, such as enhanced chemical diversity and/or the induction of new molecules previously unknown to be produced by the strain. Fungal endophytes are known to produce a wide variety of secondary metabolites (SMs) involved in their adaptation and survival within higher plants. The plant-microbe interaction may influence the expression of some biosynthetic pathways, otherwise cryptic in these fungi when grown *in vitro*. The aim of this study was to setup a systematic approach to evaluate and identify the possible effects of HDAC and DNMT inhibitors on the metabolic profiles of wild type fungal endophytes, including the chemical identification and characterization of the most significant SMs induced by these epigenetic modifiers.

Keywords: fungal endophytes; epigenetic modifiers; HDAC; DNMT; volcano plots; secondary metabolites; metabolomics

1. Introduction

Arid zones in Andalucía have special edaphological and climatic conditions such as low rainfall, high sunshine levels and specific lithology where loamy materials and evaporites abound. Native plant communities from these arid zones possess distinctive survival characteristics in these special conditions, which have led to the existence of a high degree of endemic plants with highly adapted endophyte ecosystems poorly studied. It is precisely this singularity which turns them into a valuable potential source for the isolation of new unique host-specific endophytes.

Fungal endophytes consist mainly of members of the *Ascomycota* class of mitosporic fungi, but can also include members of the *Basidiomycota*, *Zygomycota* and *Oomycota* classes [1]. Some of these groups are widespread and found on different plant species; others are highly specific to single hosts and single environments. Fungal endophytes are organisms inhabiting plants which are characterized by being neutral or beneficial to the host plant. These fungi can promote plant growth and confer abiotic and biotic stress tolerances throughout one or more interactions with their host, for example, by producing a wide variety of other secondary metabolites (SMs). These interactions may influence

the expression of some biosynthetic pathways, otherwise cryptic when these fungi are grown *in vitro* in laboratory conditions.

Epigenetic small-molecule modifiers (Figure 1) of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) activities are being used to perturb the fungal secondary biosynthetic mechanisms, which can lead to the induction of the expression of silent metabolite pathways. DNMT inhibitors such as 5-azacitidine (**1**) and hydralazine hydrochloride (**2**) have demonstrated their ability to reduce DNA-methylation-mediated silencing of different resistance genes and cellular processes in a wide variety of fungal species [2].

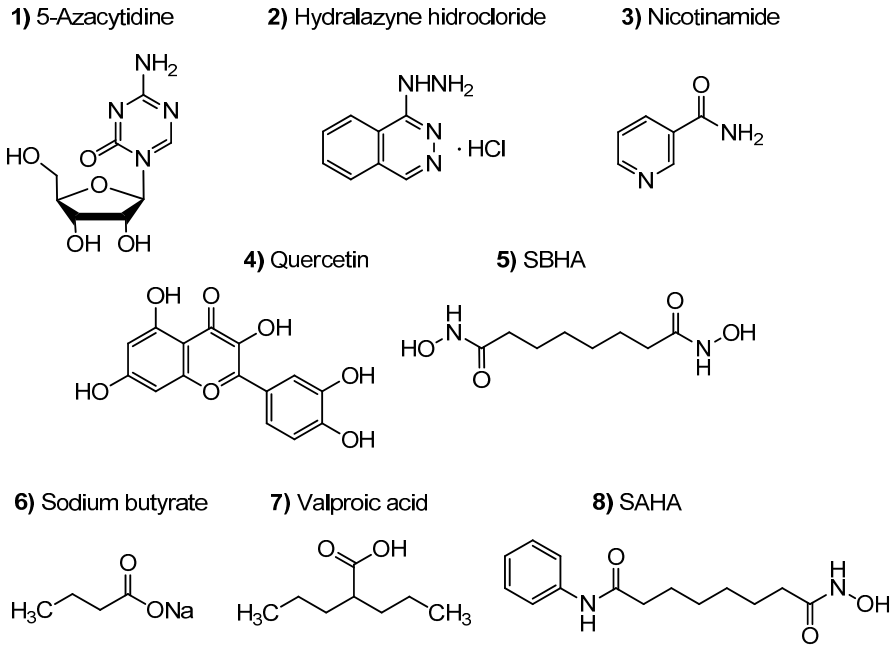

Figure 1. Epigenetic small-molecule modifiers **1**–**2** of DNA methyltransferase and **3**–**8** of histone-deacetylase activities described in the study.

Three classes of histone deacetylases (HDAC) (I, II and III) have been identified in fungi [3]. Sirtuins are the most described ones (HDAC of class III). They are NAD-dependent protein deacetylases involved in a wide variety of biological processes, including transcriptional silencing, regulation of apoptosis, fat mobilization, and stress resistance [4]. Sirtuins' activities are regulated by nicotinamide (**3**), a noncompetitive inhibitor that promotes a base-exchange reaction at the expense of deacetylation [5], and quercetin (**4**), a natural product belonging to the flavonoids family, that acts as an activator of sirtuins activities [6]. Quercetin has antioxidant properties, modulates the expression of specific enzymes attributed to an inhibitory action on protein kinases, inhibits DNA topoisomerases and regulates gene expression [7]. Recent examples on the use of nicotinamide (HDAC of class III inhibitor) can be the induction in *Chaetomium cancroideum* of the secondary metabolites chaetophenol G and cancrolides A and B [8].

Suberohydroxamic acid (**5**), sodium butyrate (**6**) and valproic acid (**7**) are potent inhibitors of HDAC of classes I and II. Several reports have described the variable effects of HDAC inhibitors on fungi. The majority of these epigenetic studies report the effect of using hydroxamic-acid compounds, such as SAHA (**8**), with similar HDAC inhibitory effects; e.g.,: the production of a new fungal biosynthetic cyclodepsipeptide (EMG-556) by *Microascus* sp. when cultured with SAHA [9], the production of new anti-infective cytosporones by the marine fungus *Leucostoma persoonii* [10] or, the biosynthesis of novel fusaric acid derivatives by the *Datura stramonium* endophyte when SBHA was added to its production medium [11].

Recently Albright *et al.* [12] proved global changes in *Aspergillus nidulans* metabolome upon treatment with epigenetic modifiers in response to an upregulated reduced expression of rpdA by using volcano-plots statistical analyses [13]. This study suggested that, although HDAC inhibition generally leads to an up-regulation of the *Aspergillus nidulans* biosynthetic machinery as evidenced by transcriptomics, the response at the level of the secondary metabolome is more complex than just a global increase in the abundance of the secondary metabolites already produced by the strain.

The principal aim of this study was to evaluate the differential expression of biosynthetic pathways of wild fungal endophytes upon systematic treatment with different small molecule elicitors. The multicomponent analysis of the differential induction of secondary metabolite profiles by applying metabolomic techniques may result in a robust methodology for assessing the potential effects of HDAC and DNMT epigenetic modifiers on the secondary metabolites profiles of fungal endophytes of interest, and also, promoting systematically the expression of their cryptic pathways typically silent when fermented *in vitro*.

2. Results and Discussion

2.1. Fungal Isolation and Identification

The collection areas were localized in arid regions of the provinces of Granada and Almeria (Andalucía, Spain). Twelve different species of representative plants characteristic of each geographic region were collected. The geographical origins and isolation substrata of the strains are listed in Table 1. For the taxonomic determination of their genus and species, sequencing of the complete ITS1-5.8S-ITS2-28S region or independent ITS and 28S rDNA were performed and compared with GenBank and the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/) databases by using the BLAST application [14].

Table 1. Host plant and geographical origin of the fungal strains isolated.

Family	Strain Code	Species	Host Plant	Origin	Genbank
Dothideaceae	CF-277039	*Kabatiella bupleuri*	*Bupleurum gibraltarium*	Presa de Quentar, (Granada)	JN886788
Dothideaceae	CF-277101	*Selenophoma* sp.	*Spartium junceum*	Fuente del Hervidero, (Granada)	JN886791
Dothideaceae	CF-280549	*Kabatiella* sp.	*Bupleurum spinosum*	Camino de los neveros (Granada)	KU295574
Dothideaceae	CF-285353	*Dothiora* sp.	*Launaea arborescens*	Tabernas (Almeria)	KU295575
Dothideaceae	CF-285359	*Kabatiella* sp.	*Asparagus horridus*	Tabernas (Almeria)	KU295576
Dothideaceae	CF-285463	*Selenophoma juncea*	*Salsola oppositifolia*	Tabernas (Almeria)	KU295578
Dothideaceae	CF-285762	*Aureobasidium pullulans*	*Inula chrihtmoides*	Punta Entinas (Almeria)	KU295579
Chaetothyriaceae	CF-285360	*Knufia* sp.	*Asparagus horridus*	Tabernas (Almeria)	KU295577
Phaeosphaeriaceae	CF-285372	*Chaetosphaeronema* sp.	*Anabasis articulata*	Tabernas (Almeria)	KU295581
Planistromellaceae	CF-282001	*Loratospora* sp.	*Retama sphaerocarpa*	Albuñuelas (Granada)	KU295580
Sporormiaceae	CF-282341	*Preussia* sp.	*Dittrichia viscosa*	Alhendin (Granada)	KU295582
Sporormiaceae	CF-285375	*Preussia australis*	*Launaea arborescens*	Tabernas (Almeria)	KU295583
Xylariaceae	CF-285461	*Xylaria* sp.	*Thymelaea hirsuta*	Tabernas (Almeria)	KU295584

Thirteen different fungal strains were finally isolated and identified from these plants, that can be grouped into two taxonomic categories: (i) a taxonomically homogeneous group consisting of seven different species of the *Dothideaceae* family, and (ii) an heterogeneous group made up of six endophytes belonging to different fungal families (*Chaetothyriaceae*, *Phaeosphaeriaceae*, *Planistromellaceae*, *Sporormiaceae* and *Xylariaceae*, Table 1).

2.2. Screening of Epigenetic Modifiers Effects on Fungal Endophytes

All strains were grown in the same production medium (YES) in the absence (control) and presence (100 μM) of seven small-molecule epigenetic modifiers that were added: (i) only during the production stage (− +), or (ii) during both, seed and production, fermentation stages (+ +). According

to previous reports [10,15], all epigenetic modifiers were tested at 100 μM as the standard concentration in the study. After 14 days of fermentation, ensuring the generation of enough biomass in the control conditions, submerged culture differences in morphology and SMs profiles were analyzed visually and by UHPLC-UV at 210 nm. Changes in production titers (as changes in 210 nm UV areas) and presence of new peaks were observed among the different treatments with respect to their corresponding controls. Increasing changes in production profiles were classified into three categories as ***p-pp-ppp*** (as increasing changes: ×2, ×4 and ×8 respectively, in the area of the peaks under the uHPLC UV-210 nm trace compared to its corresponding control areas). Changes in the chemical diversity induced were also categorized based on the number of new UHPLC-UV 210 nm peaks detected as ***d***: 1–3 peaks; ***dd***: 3–5 peaks and ***ddd*** > 5 peaks (Table 2). In addition, any changes in the fermentation morphology (pigmentation, final biomass or conidia/hyphae conversion rate), were also referred as ***m*** in the table.

Three of the 13 fungal strains screened by using the 14 epigenetic modifiers fermentation conditions showed: clear significative changes in their final growth morphologies, changes in their production titers and/or generation of new peaks when cultured in the presence of the epigenetic modifiers. Among them, the strain that showed more changes in these three components was the strain *Dothiora* sp. CF-285353. This strain was therefore selected for a deeper and more detailed metabolomic study on the effects of the three elicitors that induced most morphological and SMs profile changes when compared to its corresponding control fermentation condition (Figure 2).

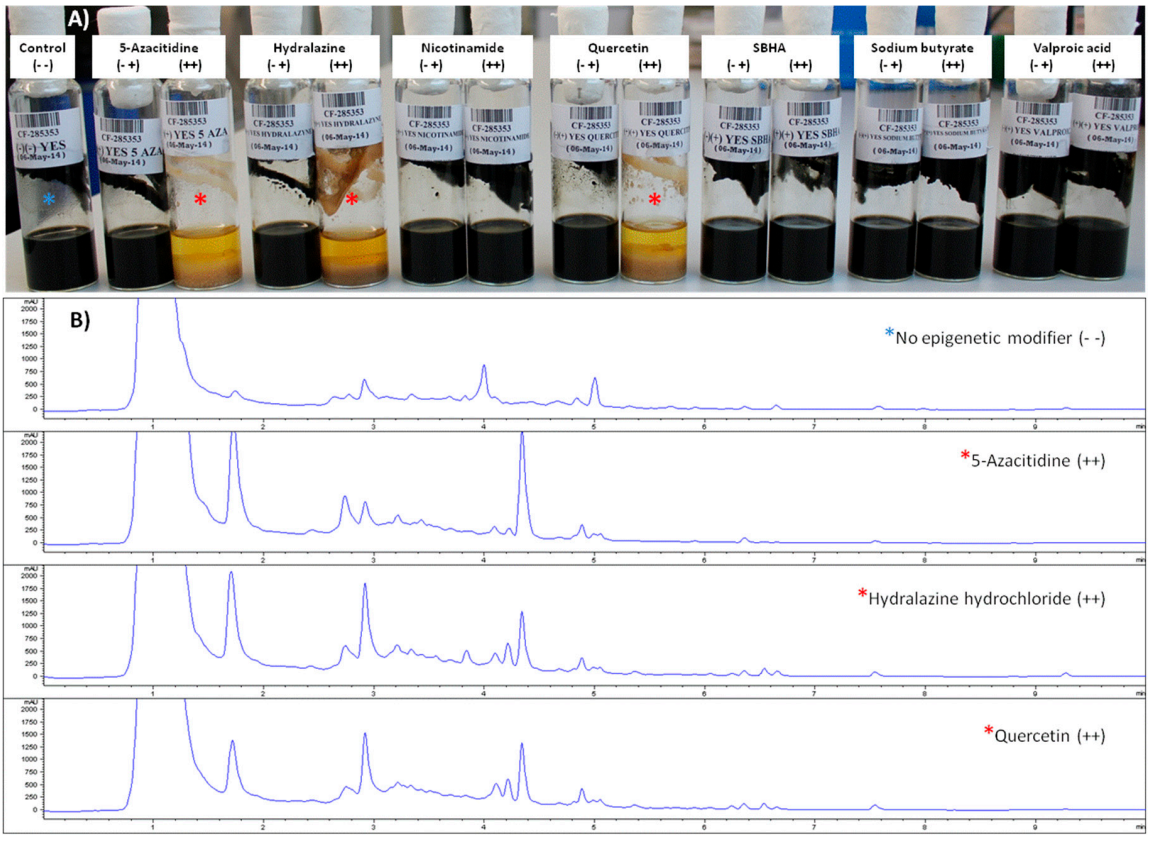

Figure 2. (**A**) Fungal strain CF-285353 grown, for 14 days in YES medium with and without all the epigenetic modifiers studied, showing differences in texture, colour, morphology and biomass amount; (**B**) Comparative analysis of the differential UHPLC-UV 210 nm secondary metabolite profiles produced by the strain with and without the addition, during seed and the production steps (+ +), of the three epigenetic modifiers of interest (red asterisks) *vs*. control (blue asterisk).

Table 2. Effect of the different epigenetic treatments on the UHPLC-UV 210 nm chemical profiles of the fungal endophytes of the study.

Strain ID	5-Azacitidine (1)		Hydralazine (2)		Nicotinamide (3)		Quercetin (4)		SBHA (5)		Sodium Butyrate (6)		Valproic (7)	
	(− +)	(+ +)	(− +)	(+ +)	(− +)	(+ +)	(− +)	(+ +)	(− +)	(+ +)	(− +)	(+ +)	(− +)	(+ +)
CF-277039	-	-	-	-	-	-	-	-	*p*	*m*	*pp*	*mp*	*m*	m
CF-277101	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***p***	***dmp***	***p***	***p***	***p***	**p**
CF-280549	*m*	*m*	*m*	-	*m*	-	*m*	*m*	*m*	*mp*	*m*	-	*p*	-
CF-285353	**-**	***ddmpp***	**-**	***ddmpp***	**-**	**-**	**-**	***ddmpp***	***p***	***m***	***p***	**-**	**-**	**m**
CF-285359	-	*m*	-	*m*	-	*m*	*m*	-	-	*m*	-	-	-	m
CF-285463	-	*mp*	-	-	-	*mp*	-	*mp*	-	*mp*	-	*mp*	-	mp
CF-285762	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF-285360	-	-	-	-	-	*p*	-	-	-	*p*	-	*p*	-	-
CF-285372	*p*	*p*	*p*	*p*	-	-	-	*p*	*p*	*p*	*p*	*p*	-	-
CF-282001	***pp***	***p***	***ppp***	***pp***	**-**	**-**	***p***	***dppp***	***p***	***pp***	***pd***	***ddpp***	***m***	**m**
CF-282341	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF-285375	*p*	*p*	-	-	-	-	-	-	-	-	*p*	-	-	-
CF-285461	-	-	-	-	-	-	-	-	-	-	-	-	-	-

***m**: changes in morphology; **p-pp-ppp**: increasing changes in production titers; **d-dd**: increasing changes in chemical diversity.*

2.3. Standardization of Cultivation Conditions of *Dothiora sp.*

The study on the effect of the epigenetic modifiers has shown that the two DNMT inhibitors, 5-azacitydine (**1**) and hydralazine hydrochloride (**2**), and the sirtuin activator quercetin (**4**), induced more changes in the SMs profiles of the strain CF-285353 when added both, to the seed and production stages (+ +), than when added only during the production stage (− +). To verify that these changes were significative and reproducible, extensive studies were designed and carried out with submerged cultures per triplicate, in the presence and absence of these three elicitors, both in the seed and/or the production stages.

The cultivated strain CF-285353 presented different morphologies after addition of some of the epigenetic modifiers (Figure 2). To ensure the reproducibility of the experimental conditions, we characterized the morphology of the strain both in the inoculum and in the different fermentation steps. We considered, as well, the risk of introducing mutations and strain degeneration after each transfer and cell division by the liquid-liquid sub-culturing required to obtain enough inoculum for the study [16]. This effect has been previously observed in studies comparing jasmonic acid production by a fresh and a sub-cultured producing strain, which concluded that liquid-liquid sub-culturing led to a significative downfall of jasmonic acid production by *Lasiodiplodia theobromae* [17].

Therefore, three experimental designs where tested for minimizing these inoculum sub-culturing effects: (i) a method involving the use of one single stage seeds from agar plugs, similar to the original process performed during the previous screening of the epigenetic modifier additives (Figure 3A); (ii) a second method where a pre-inoculum enrichment step was added for generating a large amount of inoculum from a unique production batch (Figure 3B); and (iii) a third one involving a pre-inoculum, preserved as frozen stocks, from which agar plates of the endophyte with a controlled differentiation morphology were prepared, and were later used to inoculate seeds for each production batch (Figure 3C).

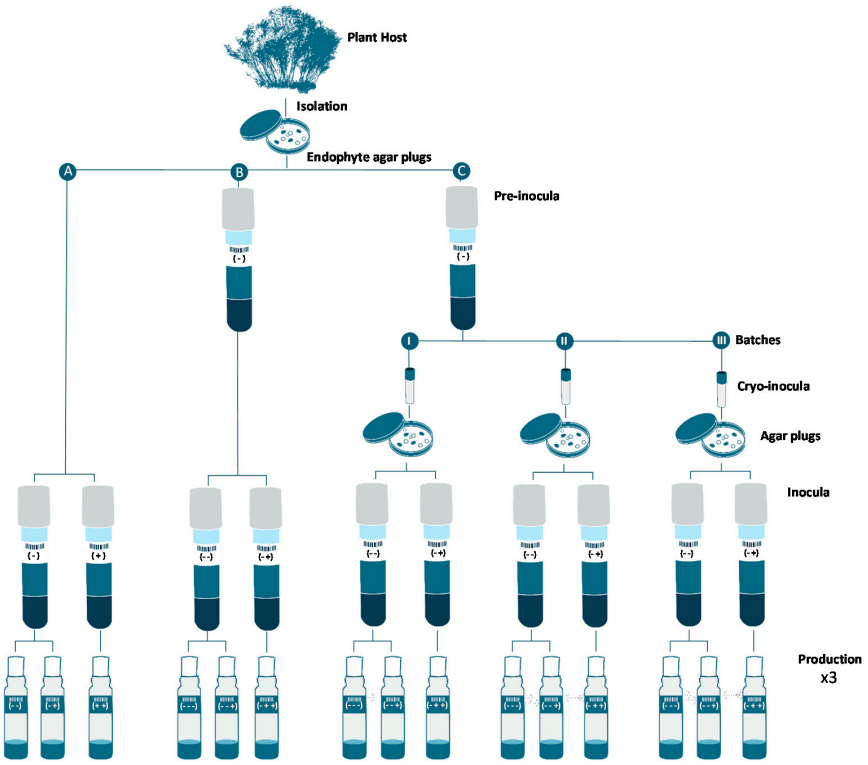

Figure 3. Experimental design for evaluating the effect of sub-culturing and epigenetic modifiers in *Dothiora* sp. (**A**) Initial fungal fermentation process; (**B**) Pre-inocula step added; (**C**) Pre-inocula and agar plugs steps added.

Finally, and similarly to the screening procedure, each inoculum generated was then seeded in the production medium (YES), in absence (control) and presence (100 μM) of the three best small-molecule epigenetic modifiers selected: (i) added only during production or; (ii) added during all fermentation steps, seed and production.

2.3.1. Effects of Epigenetic Modifiers on *Dothiora* sp. Submerged Culturing

To assess the effect of the epigenetic modifiers, all fungal cultures were harvested after 14 days of incubation, and were characterized according to their morphology (color, biomass and mycelial-to-yeast conversion phase; Figure 4), extracted with organic solvent and analyzed by UHPLC-MS.

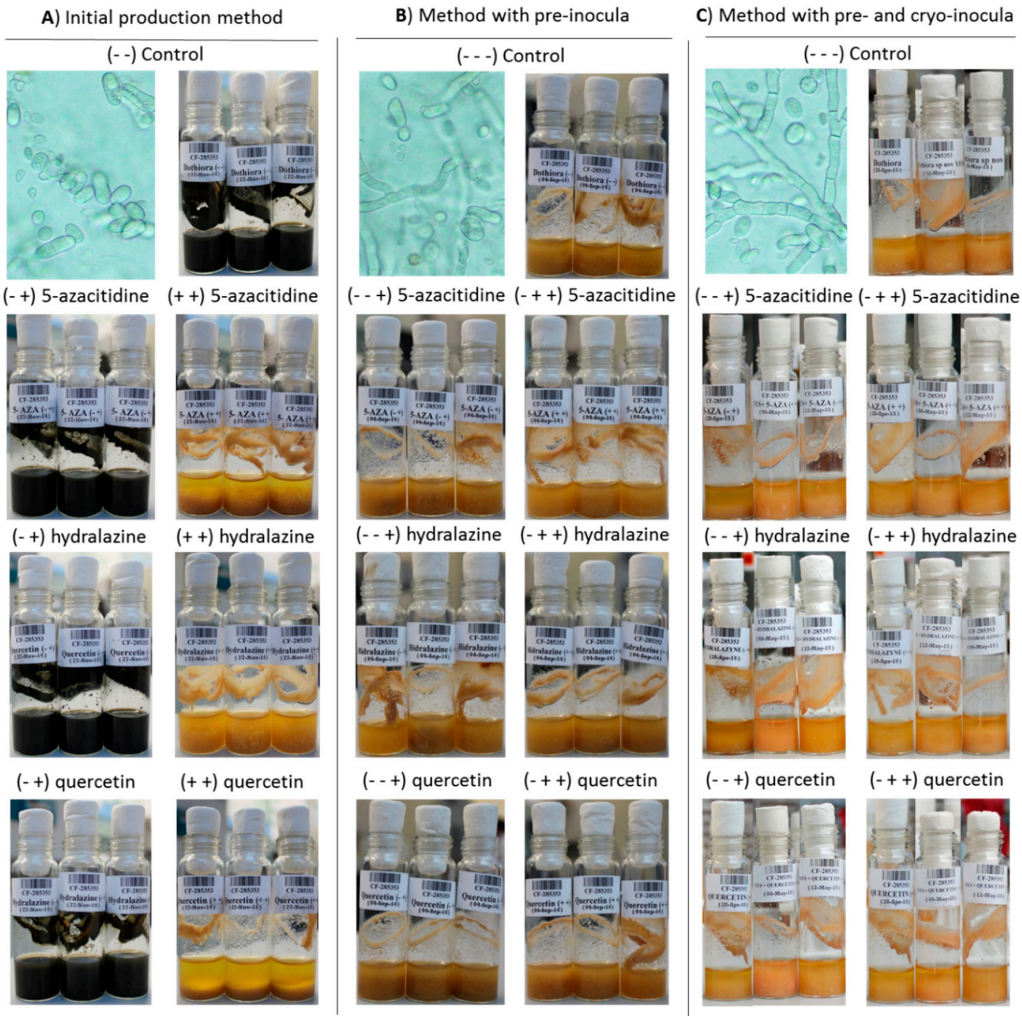

Figure 4. Fermentation batches obtained for: the initial production method (**A**); when a pre-inocula step was added (**B**); and when a pre-inocula and agar-plug steps were added (**C**).

Regarding the morphology, clear differences were observed between the three control fermentation batches for each sub-culturing methods tested even without any epigenetic modifier. The fermentation triplicates performed using the initial screening approach (Figure 3A) presented, consistently, black thick broth with a high density of conidia (Figure 4A), whereas fermentation controls for the other two inoculating methods (Figure 3B,C) resulted in a yellowish broth with plenty of hyphae and few conidia (Figure 4B,C). A slightly higher amount of hyphae was present when using the frozen inocula (Figure 3C), even when harvested after at 7 days, half of the time used for the other two

methods (14 days). No other morphology differences could be related to the presence or absence of any of the epigenetic modifiers, thus all three methods and the two growth morphologies were decided to be included in further metabolomic differential analyses.

2.3.2. Metabolomic Evaluation of the Changes in the SM Profile of *Dothiora* sp. by Epigenetic Modifiers

HPLC-UV traces can be used for evaluating the effect of epigenetic modifiers on fungal strains and have proved to be suitable for a fast detection of the conditions that induce strong changes in their SM profiles. In our case, extended studies required deeper analytical methods to quantify and identify the SMs that could be induced by the addition of the modifiers. We decided to use mass spectrometry analyses, as metabolomics is currently getting stronger foundation on LC/MS data. In recent studies, Volcano-plots have been applied to these data with success in describing and obtaining conclusions on the biosynthesis of fellutamides by *A. nidulans*, hence supporting the use of HDAC inhibitors and said techniques for the discovery of cryptic secondary metabolites [12,13].

Volcano-plots constitute a scatter-plots representation that can describe very visually how two different experimental conditions may affect a large set of components. Statistically, these plots determine if significative differences exist between averages of two populations of the same component treated with the two conditions of interest, depicting these results for, in our case, the secondary metabolites of a given extract (or the mass ions that can be detected by LCMS and can be inferred as components).

In *Dothiora* sp. fermentations (Figures 3 and 4), we observed molecular species whose production increased or decreased significantly with respect to the corresponding controls for each condition (see Figure 5 for a detailed number of the ions statistically different in production for each modifier and fermentation method). In general, the addition of small-molecule elicitors, both during the inocula and the production fermentations, resulted in a significant increase of the diversity and amount of secondary metabolites generated for the three epigenetic modifiers of the study. For the two seeding methods that have included a pre-inoculum step, 5-azacytidine (**1**) was the epigenetic modifier that induced more changes when added both during the seed and production fermentations, and the number of significant unique molecular species detected by HPLC-MS at least doubled when compared to its addition only during the production fermentation.

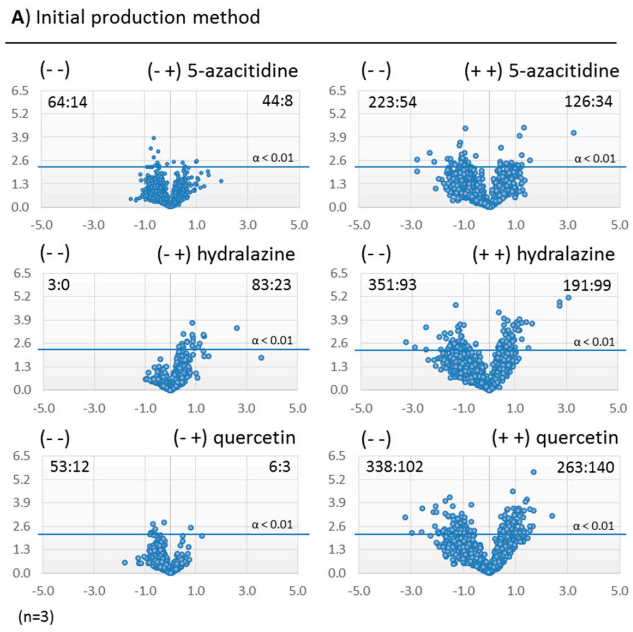

Figure 5. *Cont.*

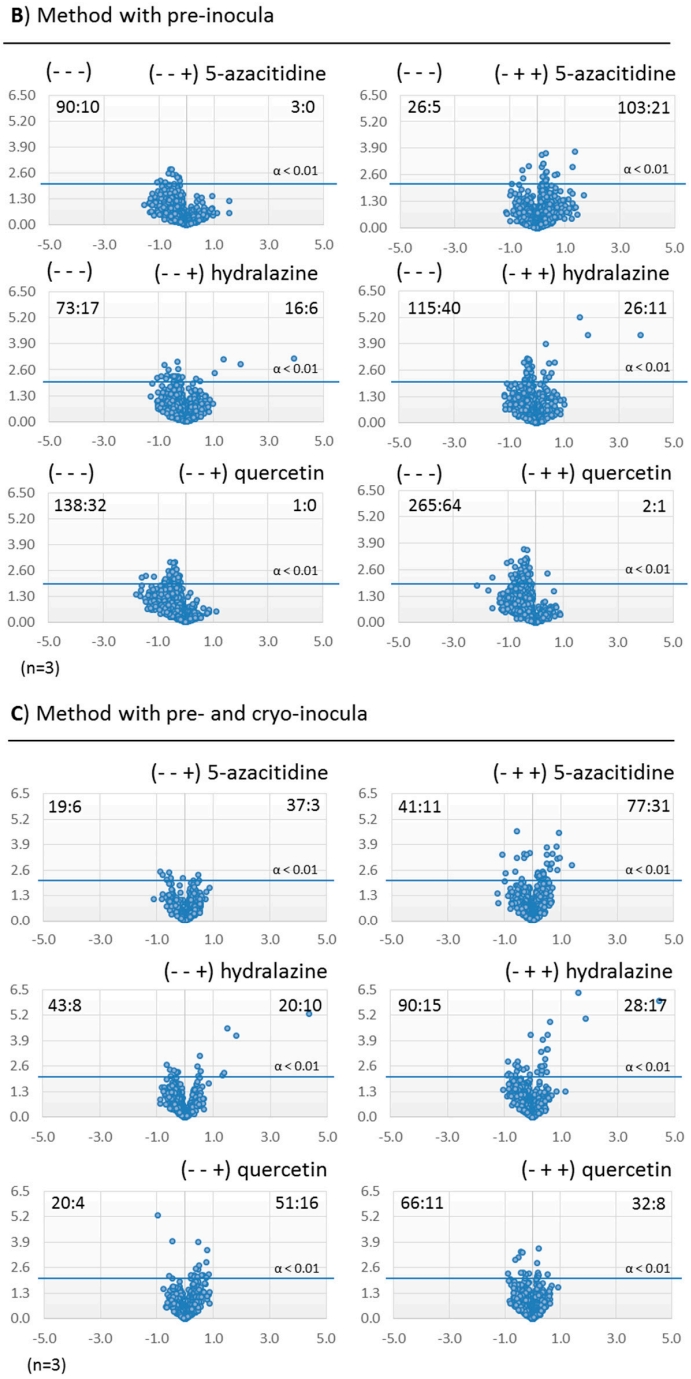

Figure 5. Volcano-plots for the three production methods tested, comparing the different growth conditions tested with their corresponding controls (−log 10 of *t*-test statistical *p*-value in *y*-axis *vs.* −log 2 of ion masses areas ratio in *x*-axes): (**A**) Initial production method; (**B**) pre-inoculum step added; (**C**) pre-inoculum and agar-plug steps added. The number of statistically different mass ions due to higher production for each growth condition is indicated as n_1:n_2, where n_1 and n_2 are respectively the number of differential mass ions with statistical confidences of 95% and 99% ($n = 3$; $\alpha < 0.05$ and $\alpha < 0.01$ respectively; 99% of significance ($\alpha < 0.01$ limit) depicted).

In most of the cases we observed a continuous dispersion of the *p*-values for the significance of the differential components for confidences above 95% and 99%. This indicated a general and continuous epigenetic modifiers effect on the SMs profiles, without highlighting any outlier group of ions that

could indicate a unique strongly induced secondary metabolite pathway. Thus, results confirmed the general non-specificity of the effects induced by the modifiers on the culture broths SMs profiles. In fact, only hydralazine was observed to induce clear groups of outlier mass ions in the scatter-plots.

The conditions showing less production changes on the SMs abundances compared to their controls were the fermentations that were prepared with the pre- and cryo-inocula method (Figure 5C). In contrast, the higher dispersions were obtained with the initial production method (Figure 5A). Although fermentations were harvested with similar biomasses, the method that included pre- and cryo-inocula steps presented the lowest dispersion of their volcano-plots (Figure 5C), suggesting an important influence of the methodology used.

2.3.3. Identification of Molecules Produced in the Fermentations with Modifiers

As previously commented, hydralazine (**2**) was the epigenetic modifier that induced most of the differential mass ion populations for CF-285353. In fact a total number of 23 and 99, 6 and 11, and 10 and 17 ions were identified respectively as statistically differential, with a 99% of significance, for treatments with hydralazine in (− +) and (+ +) for methods A, B and C (see Figure 5 for details). Among them, we could identify some molecules with 8 to 32 fold higher production rates in the presence of this modifier, and some others that were only produced in its presence.

In an initial attempt to identify several of the natural products induced by this modifier, we compared every ion that presented a statistical significance above 99% to our internal de-replication UV-HPLC-HRMS databases. Database matching was performed by using an in house developed application where the UV signal, retention time, mass signal and moleculat formula of the selected ions are compared to the UV-HPLC-HRMS data of known metabolites stored in our proprietary database of 845 microbial natural products, including commercial compounds and molecules obtained from internal purification campaigns. Among the metabolites with increased production, we could identify the presence of curvicollide A/B (m/z 432; $C_{26}H_{40}O_5$) and fusidic acid (m/z 516; $C_{31}H_{48}O_6$), and more tentativelly (matching of their molecular formulas with the commercial database Dictionary of Natural Products, DNP): pyrophen (m/z 309; $C_{18}H_{17}NO_5$), cyclo-isoleucyl-leucyl-isoleucyl-leucyl (m/z 452; $C_{24}H_{44}N_4O_4$), melledonal C (m/z 497; $C_{24}H_{29}ClO_8$) and rhizoxin S (m/z 613; $C_{34}H_{47}NO_9$), previously described to be produced by a endosymbiotic bacteria in *Rhizopus* spp. On the contrary, when compared to the control condition, the production of several secondary metabolites was repressed in the presence of hydralazine (**2**). These melecules were identified tentatively, by comparison to our databases and DNP, as monascuspyrone (m/z 340; $C_{19}H_{32}O_5$), pleurotin (m/z 354; $C_{21}H_{22}O_5$), roseotoxin B (m/z 591; $C_{30}H_{49}N_5O_7$) and 12-hydroxy-8,10-octadecadienoic acid (m/z 653; $C_{18}H_{32}O_3$). It is important to mention that all these molecules were only identified tentatively and future chemically directed purifications and HRMS/NMR confirmations are needed for a definitive confirmation.

Most differential ions detected with the volcano-plots methodology were not found in our chemical de-replication databases and could not be identified. These ions, according to their highest to lowest statistical significance p-value, included, for the production method A) (− +): m/z 243–244, 185, 374–375, (+ +): m/z 266–267–268, 243–244–245, 185–186–187; Production method B) (− − +): m/z 185–186, 157, 243, 247 and 225, (− + +): m/z 185–186, 171, 243, 247 and 227; and production method C) (− − +): m/z 185–186, 243 and 431, (− + +): 185–186, 243, 225, 171 (continuous ions, that are listed here together with hyphens, may belong to high intensity low resolution LC/MS signals detected in the raw data as contiguous m/z ions). In general, some of these most outlier ion masses were induced independently of the production method applied, being four of them the most statistically significative: m/z 171, 185–186, 225–226–227 and 243–244–245. These four sets of ions were studied then by UHPLC/HRMS-MS and their molecular formulae identified as $C_9H_6N_4$ (**9**), $C_{10}H_8N_4$ (**10**), $C_{13}H_{14}N_4$ (**11**) and $C_{14}H_{16}N_2O_2$ (**12**). Interestingly the first three outlier molecules presented a molecular formula closely related to that of hydralazine ($C_8H_8N_4$, **2**), and could not be found also in public nor commercial natural products databases. Further scale up and purification studies were setup for their identification.

Medium volume (600 mL) fermentations of the strain in the presence and absence of hydralazine (**2**) were extracted and fractionated for the isolation and identification of these differential molecules. HRMS/NMR results indicated that m/z 171, m/z 185–186, and m/z 227 ions corresponded to biotransformation products (**9**–**11**) of hydralazine (**2**) ocurring in the fermentation broth. On the other hand, the molecular formula $C_{14}H_{16}N_2O_2$, deduced from the ions m/z 243–244–245, that could be associated to 19 possible molecules in the natural products databases, was finally purified and identified as the diketopiperazine natural product cyclo(phenylalanyl-prolyl) (**12**) by HRMS and NMR data (Figure 6 and Supplementary Materials).

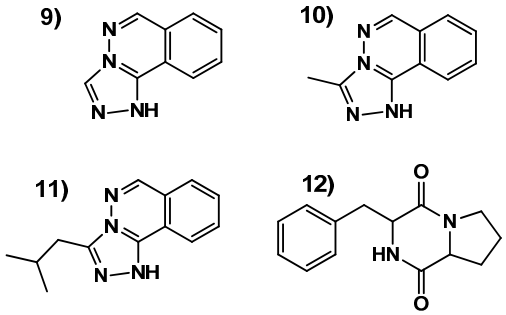

Figure 6. Most differential metabolites detected by the Volcano-plots methodology described, from the biotransformation (**9**–**11**), or the elicitor induction (**12**), of the epigenetic modifier hydralazine (**2**) on the fermentation broth of the endophyte fungus CF-285353.

2.3.4. *Dothiora* sp. Biomarkers Related to Growth Morphology

The differential growth morphologies observed in the strain CF-285353 and the availability of the metabolomic tools implemented for the evaluation of the epigenetic modifiers effects, also prompted us to perform the comparison of the profiles generated by the strain in the different morphologies, with the aim of identifing possible morphology biomarkers that could help us to monitor and fine tune future *Dothiora* sp. fermentations.

A representative population of eight CF-285353 extracts that presented the yellow hyphal morphology were compared with a similar population of extracts that presented the black conidial morphology, by using the volcano-plots approach (Figure 7). Statistically, and with a 99% of confidence, 713 mass ions were present in the conidial growths with significative higher titers than in the corresponding hyphal growths. On the other hand, 237 mass ions were present significatively in higher amounts in the hypha than in the conidia growth. Additionally, clear outlier populations of ions were observed for each condition, highlighting several SM pathways differentially expressed according to the fermentation morphology presented by the fungus.

The outlier ion masses with more significative abundance in the hyphal morphology were m/z 268, 269 and 226, whereas significative abundant ion masses in the conidial morphology were m/z 222, 465, 486 and 503. Both sets of components were studied, identified and their structures confirmed by UHPLC/HRMS-MS to correspond respectively to the primary metabolite adenosine (**13**) and the natural product mycosporin: glutamicol-5′-*O*-β-D-glucopyranoside (**14**) (Figure 7A,B).

Previous studies on the morphology, growth kinetics, and main chemical components of solid or liquid cultures of *Tolypocladium* fungi isolated from wild *Cordyceps sinensis*, showed that the *in vitro* hyphae mycelium of *Tolypocladium* presented much higher contents of adenosine (1116.8 μg *vs.* 264.6 μg) than when the fungus was directly isolated from *C. sinensis* [18]. Similarly to what was observed for *Tolypocladium*, adenosine could represent a potential biomarker of the hyphae growth morphology state as observed in our strain. Previous work by Pirttila *et al.* [19] suggests that the adenosine secretion of endophytes microorganisms may play an important role in the morphological development of the host plant and therefore, it could be playing a role in the secondary metabolism of the endophytes microorganism.

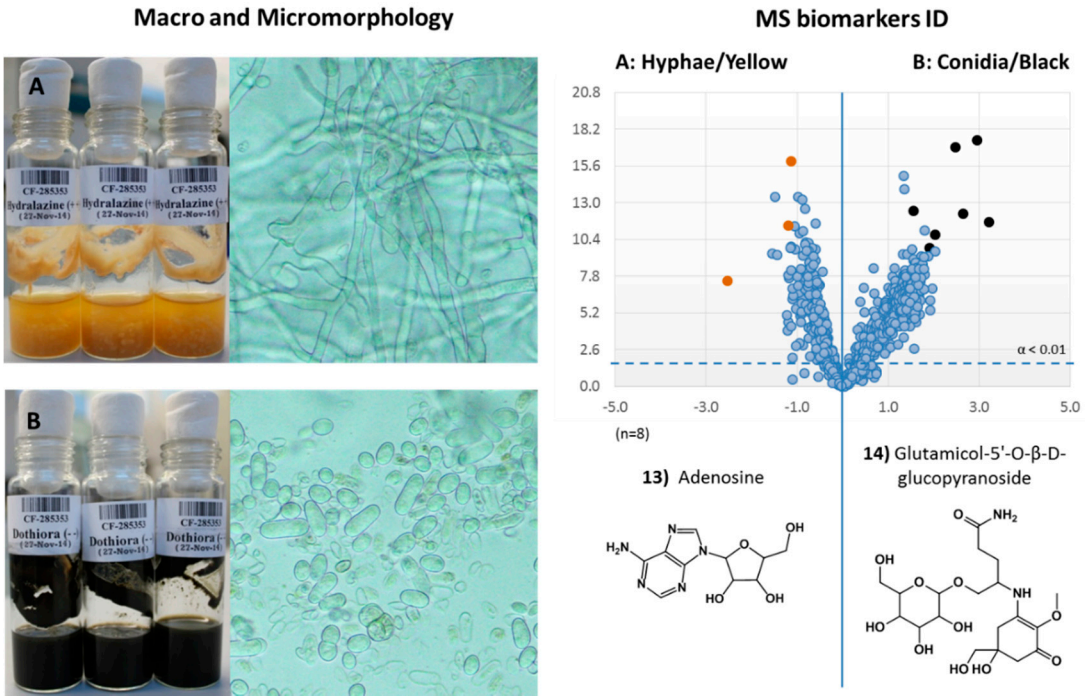

Figure 7. Volcano-plots representation for hyphae/yellow (**A**) *vs.* conidia/black (**B**) *Dothiora* sp. growth morphologies (−log 10 of *t*-test statistical *p*-value in *y*-axis *vs.* −log 2 of ion masses areas ratio in *x*-axes; $n = 8$), and the most differential SMs produced for each morphology determined by UHPLC-HRMS.

In the case of mycosporins, extremophilic microcolonial fungi have been described to constitutively synthesize considerable amounts of mycosporins, also known to be involved in morphogenesis and sporulation [20–22]. Many reports regarding different yeast species indicate the ability of these fungi to synthesize mycosporin-like amino acids, which have been also proposed to reflect phylogenetic relationships among species, suggesting their utility in yeast systematics. In addition, mycosporins have also been described in the extracellular matrix and in the outer cell wall layers of microcolonial fungi, in which they mediate a wide range of intracellular reactions. They are present in the mucilage that surrounds conidia of some fungi, confirming its direct presence in cultures with this morphology, with no evidences of their presence inside the conidia [23]. In some fungi, mycosporins are also described to protect conidia from solar radiation during atmospheric dispersal and prevent untimely germination [24] prolonging their survival. Therefore, the modulated production of these molecules by yeasts represents an interesting subject for further research due to its ecological, taxonomical and biotechnological implications [25]. For example, cosmetics applications as UV protectors and activators of cell proliferation, with potential therapeutic properties, could be interesting for other commercial developments [26].

Both detected molecules, adenosine (**13**) and mycosporin glutamicol-5′-*O*-β-D-glucopyranoside (**14**), confirmed in the literature as related to these morphology growth stages of some fungi, also corroborate the volcano-plots methology as a suitable approach for a succesful identification of metabolites produced differentially between two fermentation conditions, allowing a fast and robust approach for the identification of fermentation biomarkers.

3. Experimental Section

3.1. Isolation Cultures and Characterization

Standard indirect isolation techniques were followed to isolate the endophytes. Stems or leaves removed from each plant were cut into pieces of approximately 5 mm. These pieces were

surface-disinfected sequentially through washing with 95% ethanol (30 s), 25% household bleach (1 min) and 95% ethanol (30 s). 10 pieces of each vegetal sample were aseptically transferred to a Petri dish with CMA supplemented with streptomycin sulfate and oxytetracycline (50 mg/mL) [14]. Epiphyte fungi were also directly isolated from cleistothecia or conidiophores formed on plants by incubation in moist chambers. Isolates were cultured in YM agar (malt extract 10 g, yeast extract 2 g, agar 20 g, 1000 mL distilled H_2O), to study their macro- and microscopic characteristics. Strains designated with an ID (e.g., CF-282341) were preserved as frozen conidia and mycelia in 10% glycerol at −80 °C and are maintained in the culture collection of Fundación MEDINA (www.medinadiscovery.com). DNA extraction, PCR amplification and DNA sequencing were performed as previously described by Bills *et al.* in 2012 [14].

3.2. Epigenetic Modifiers Stock Solutions

Seven epigenetic modifiers were selected for its addition into inocula and fungal fermentations (Figure 1): two potent inhibitors of DNA methylation [(5-azacytidine (A2385, Sigma-Aldrich, St. Louis, MO, USA) and hydralazine hydrochloride (H1753, Sigma-Aldrich)], an inhibitor of sirtuins [nicotinamide (72340, Sigma-Aldrich)], a sirtuins activator [quercetin (Q4951, Sigma-Aldrich)] and three histone deacetylase (HDAC) inhibitors [suberohydroxamic acid (SBHA) (390585, Sigma-Aldrich), sodium butyrate (303410, Sigma-Aldrich) and valproic acid (P4543, Sigma-Aldrich)].

3.3. Inocula Preparation

The seed medium for all inocula was SMYA (DIFCO neopeptone 10 g, maltose 40 g, DIFCO yeast extract 10 g, agar 4 g, distilled H_2O 1000 mL). For the control inocula five mycelial discs of fungal strains grown on YM agar at 22 °C for 7 days were cut from each 60 mm plate with a sterile "Transfer Tube" (Spectrum Laboratories, Rancho Dominguez, CA, USA). Mycelia discs were extruded from the "Transfer Tube" and crushed in the bottom of inocula tubes (25 × 150 mm) containing 12 mL of SMYA and two cover glasses (22 × 22 mm). Tubes were incubated on an orbital shaker (200 rpm; 1.5 cm throw), where rotation of the cover glasses continually sheared hyphae and mycelial disc fragments to produce nearly homogenous hyphal suspensions consisting of minute hyphal aggregates and fine mycelia pellets [27].

For each epigenetic modifier treatment during inocula, SMYA medium was prepared and each epigenetic modifier dissolved in DMSO or distilled water was aseptically added to each tube to attain for a final concentration of 100 µM. Tubes were then incubated for 7 days at 22 °C (Figure 3A). When pre-inocula was prepared from agar plugs (Figure 3B), the incubation time was also 7 days. When cryo-tubes were prepared, aliquots of 0.8 mL from this biomass were added to cryo-tubes containing 0.8 mL of glycerol and frozen at −80 °C. Along three consecutive batches (weeks), a cryo-tube per week was then thawed and added to Petri dishes containing YME agar medium to be incubated for 7 days. Agar plugs mycelia were then used to generate a new inocula for to be fermented for 7 days in SMYA before inoculating the 40 mL scintillation vials containing the production medium with or without the epigenetic modifier (Figure 3C).

3.4. Production Fermentations

The base production medium for all fermentation conditions was YES (DIFCO yeast extract 20 g, sucrose 150 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, trace elements 1 mL ($ZnSO_4 \cdot 7H_2O$ 1 g/100 mL and $CuSO_4 \cdot 5H_2O$ 0.5 g/100 mL) and distilled H_2O 1000 mL), a rich and clear medium that allows easy identification of differences in color, morphology and biomass [28]. The control condition was YES dispensed at 10 mL in 40 mL EPA vials, inoculated with 0.3 mL of mycelia inoculum, and shaked at 220 rpm [14]. Epigenetic modifiers dissolved in DMSO or distilled water were aseptically added to each vial to attain a final concentration of 100 µM [15]. All fermentations were then incubated for 7–14 days at 22 °C to equivalent biomass generation (Figure 3A–C).

3.5. Chemical Extraction

After harvesting, 9 mL whole broths were extracted by adding 9 mL of acetone using a Multiprobe II robotic liquid handler (PerkinElmer, Waltham, MA, USA) and shaking at 220 rpm for 1 h. After centrifugation, 12 mL of supernatant from each vial were transferred to glass tubes containing 0.6 mL of DMSO and mixed. Solvent was evaporated under a heated nitrogen stream to a final volume of 3 mL (80/20 water/DMSO solution) and a final concentration of 2 × WBE (whole broth equivalents). Each fermentation batch included extracts from control culture media to discriminate their components [28].

3.6. UHPLC-UV Profile Analysis

Chemical profiles of fermentation extracts (4 µL) were analyzed using an Agilent 1290 Infinity UHPLC-UV (Santa Clara, CA, USA). A Kinetex C-18 (1.7 µm, 2.1 × 150 mm) column from Phenomenex (Torrance, CA, USA), a 10 min gradient from 1% to 99% (*v*/*v*) of acetonitrile in water, with 1.3 mM ammonium formate and 1.3 mM trifluoroacetic acid as chromatographic modifiers, a flow rate of 315 µL/min, a controlled temperature of 40 °C, and UV detection at 210, 280 and 340 nm were used for each analysis. An inert internal control was present in each sample to individually validate and normalize if necessary retention times of each chromatographic run. Additional methanol blanks were injected every ten samples for joint monitoring of each analytical batch [28].

3.7. LC/MS Data and Metabolomics Evaluation

Samples selected (2 µL) were analyzed by HPLC-MS. LC analysis was performed on an Agilent 1200, using a Zorbax SB-C8 column (2.1 × 30 mm), maintained at 40 °C and with a flow rate of 300 µL/min. Solvent A consisted of 10% acetronitrile and 90% water with 0.01% trifluoroacetic acid and 1.3 mM ammonium formate, while solvent B was 90% acetronitrile and 10% water with 0.01% trifluoroacetic acid and 1.3 mM ammonium formate. The gradient started at 10% B and went to 100% B in 6 minutes, kept at 100% B for 2 min and returned to 10% B for 2 min to initialize the system. Full diode array UV scans from 100 to 900 nm were collected in 4 nm steps at 0.25 sec/scan. Mass spectrometry acquisition was performed on an Agilent MSD 1100 mass spectrometer for generating the metabolomics raw data.

Volcano-plots were calculated according to Hur *et al.* [13] but using each low resolution m/z as a component, and without introducing at this step a deconvolution for simplifying all m/z observed for a single metabolite into a single component. This simplified strongly the IT investment required for data management and processing, highlighting equally the differential components observed for each treatment comparison. UHPLC/HRMS identification and characterization of the components of interest included all those features in a following step, once the ion masses of interest were identified.

In the x-axis, volcano-plots depict the $-\log 10$ of the t-test statistical p-value from comparing two populations of the same component (m/z in our study) treated with the two conditions of interest, whereas in the y-axis, a $-\log 2$ representation on the ratios of the areas of one condition among the other is plotted for the same component [29]. In these volcano-plots, scatter plots (m/z) 1.3 units above in the y-axis presented significant differences among both conditions with a 95% of statistical confidence ($\alpha = 0.05$). Plots above 2 units presented a 99% ($\alpha = 0.01$) of significant differences between both treatments. For an easy visual interpretation, 1.3 was used as the main cross mark in the scatter-plots depicted in our study. The number of mass ions statistically different by higher production for each growth condition is indicated for 95%: 99% of statiscical confidences ($\alpha < 0.05$ and $\alpha < 0.01$ respectively). On the other hand, units in the y-axes are correlated to relative sequential abundances of 200%–400%–800% of one condition compared to the other. The ratio also helps in minimizing/normalizing the diferential ionization signals than can be observed for each metabolite [13].

3.8. UHPLC-HRMS Database Matching of Known Metabolites and Antibiotics

A Bruker maXis HR-QTOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) coupled to the previously described UHPLC system was used for characterization and identification of specific metabolites. Ionization of the eluting solvent were obtained using the standard ESI sources adjusted to a drying gas flow of 11 L/min at 200 °C and a nebulizer pressure of 40 psig. The capillary voltage was set to 4000 V. Mass spectra were collected from 150 m/z to 2000 m/z in positive mode. Database matching was performed by comparing retention time and exact mass generated with the Bruker maXis, of extracted components from the samples under study, with the retention times and exact masses from known metabolites stored in a database acquired under the same exact LC-HRMS conditions.

4. Conclusions

The systematic addition of small-molecule epigenetic elicitors during the inocula stages in several fungi of the study, determined the activation of biosynthetic pathways otherwise silent when grown under standard conditions. The increase in production titers of global SM profiles that was observed in some of the strains was slightly higher when the elicitors were present since the inoculum stage. A UHPLC-UV screening of the use of epigenetic elicitors in multiple conditions highlighted the most interesting combinations of strain/elicitor/fermentation methodology. The Volcano-plots methodology proved to be a robust way to identify and quantify in detail these effects in fungal microbial fermentations.

Among the seven epigenetic modifiers studied, hydralazine resulted the most effective one for a differential generation of SMs profiles in the CF-285353 *Dothiora* sp. strain. An extensive study on the differential profiles generated for this strain by hydralazine indicated 4 molecules as the most significative ones in differential production. Scale-up fermentations of CF-285353 identified three of these molecules as biotransformation products of hydralazine, and determined cyclo(phenylalanyl-prolyl) as a natural product whose production was clearly induced by the addition of the epigenetic modifier. Differences on the growth morphology presented by the fungus CF-285353 were also identified and quantified by the metabolomic approach described, allowing the identification of adenosine and the mycosporin glutamicol-5′-*O*-β-D-glucopyranoside as conidia/hyphae morphology biomarkers respectively. Evaluation throughout metabolomics on the other two strains highlighted in the modifiers screening (CF-277101 and CF-282001) is currently underway for a better understanding of the effect of epigenetic modifiers in the fermentation of this population of fungal endophytes.

Results also indicated the possibility that, in some cases, the epigenetic modifier added may not only be inducing the expresion or activation of criptic pathways, but can also suffer biotransformations. In both situations, the fermentation methodologies described, the systematic screening of epigenetic elicitors presented, the Volcano-plots metabolomics methodology applied for large sets of MS data, and the subsequent dereplication and identification of the differential components by HRMS, can result in a robust aproach for highlighting and quantifying both biotransformations and possible silent patways induced by small mollecule elicitors in fungal endophytes.

Supplementary Materials: Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/21/2/234/s1.

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Author Contributions: By authors order; Conceived and designed the experiments: V.G.M. J.R.T. O.G. Performed the experiments: V.G.M. M.P.B. I.P.V. J.M. F.M. Reagents/materials/analyses: V.G.M. J.M. I.P.V. F.M. J.R.T. Data process: V.G.M. M.P.B. I.P.V. F.R. J.R.T. Contributed/Wrote the paper: V.G.M. J.R.T. O.G.

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

References

1. Xiang, S.; Liang-Dong, G. Endophytic fungal diversity: Review of traditional and molecular techniques. *Mycology* **2002**, *3*, 65–76.
2. Cichewicz, R.H. Epigenome manipulation as a pathway to new natural product scaffolds and their congeners. *Nat. Prod. Rep.* **2010**, *27*, 11–22. [CrossRef] [PubMed]
3. Brosch, G.; Loidl, P.; Graessle, S. Histone modifications and chromatin dynamics: A focus on filamentous fungi. *Fems Microbiol. Rev.* **2008**, *32*, 409–439. [CrossRef] [PubMed]
4. Preyat, N.; Leo, O. Sirtuin deacylases: A molecular link between metabolism and immunity. *J. Leukoc. Biol.* **2013**, *93*, 669–680. [CrossRef] [PubMed]
5. Avalos, J.L.; Bever, K.M.; Wolberger, C. Mechanism of sirtuin inhibition by nicotinamide: Altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. *Mol. Cell* **2005**, *18*, 855–868. [CrossRef] [PubMed]
6. Moore, J.M.; Bradshaw, E.; Seipke, R.F.; Hutchings, M.I.; McArthur, M. Use and discovery of chemical elicitors that stimulate biosynthetic gene clusters in *Streptomyces* bacteria. *Methods Enzymol.* **2012**, *517*, 367–385. [PubMed]
7. Moskaug, J.O.; Carlsen, H.; Myhrstad, M.; Blomhoff, R. Molecular imaging of the biological effects of quercetin and quercetin-rich foods. *Mech. Ageing Dev.* **2004**, *125*, 315–324. [CrossRef] [PubMed]
8. Asai, T.; Morita, S.; Taniguchi, T.; Monde, K.; Oshima, Y. Epigenetic stimulation of polyketide production in Chaetomium cancroideum by an NAD(+)-dependent HDAC inhibitor. *Org. Biomol. Chem.* **2015**, *14*, 646–651. [CrossRef] [PubMed]
9. Vervoort, H.C.; Drašković, M.; Crews, P. Histone Deacetylase Inhibitors as a Tool to Up-regulate New Fungal Biosynthetic Products—Isolation of EGM-556, a Cyclodepsipeptide, from *Microascus* sp. *Org. Lett.* **2011**, *13*, 410–413. [CrossRef] [PubMed]
10. Beau, J.; Mahid, N.; Burda, W.N.; Harrington, L.; Shaw, L.N.; Mutka, T.; Kyle, D.E.; Barisic, B.; van Olphen, A.; Baker, B.J. Epigenetic Tailoring for the Production of Anti-Infective Cytosporones from the Marine Fungus *Leucostoma persoonii*. *Mar. Drugs* **2012**, *10*, 762–774. [CrossRef] [PubMed]
11. Chen, H.J.; Awakawa, T.; Sun, J.Y.; Wakimoto, T.; Abe, I. Epigenetic modifier-induced biosynthesis of novel fusaric acid derivatives in endophytic fungi from *Datura stramonium* L. *Nat. Prod. Bioprospect.* **2013**, *3*, 20–23. [CrossRef]
12. Albright, J.C.; Henke, M.T.; Soukup, A.A.; McClure, R.A.; Thomson, R.J.; Keller, N.P.; Kelleher, N.L. Large-scale metabolomics reveals a complex response of Aspergillus nidulans to epigenetic perturbation. *ACS Chem. Biol.* **2015**, *10*, 1535–1541. [CrossRef] [PubMed]
13. Hur, M.; Campbell, A.A.; Almeida-de-Macedo, M.; Li, L.; Ransom, N.; Jose, A.; Wurtele, E.S. A global approach to analysis and interpretation of metabolic data for plant natural product discovery. *Nat. Prod. Rep.* **2013**, *30*, 565–583. [CrossRef] [PubMed]
14. Bills, G.F.; González-Menéndez, V.; Platas, G. *Kabatiella bupleuri* sp. nov. (Dothideales), a pleomorphic epiphyte and endophyte of the Mediterranean plant *Bupleurum gibraltarcum* (Apiaceae). *Mycologia.* **2012**, *104*, 962–973. [CrossRef] [PubMed]
15. DelaCruz, M.; Martín, J.; González-Menéndez, V.; Pérez-Victoria, I.; Moreno, C.; Tormo, J.R.; El Aouad, N.; Guarro, J.; Vicente, F.; Reyes, F.; *et al*. Chemical and physical modulation of antibiotic activity in emericella species. *Chem. Biodivers.* **2012**, *9*, 1095–1113. [CrossRef] [PubMed]
16. Stanbury, P.F.; Whitakerm, A.; Hall, S.J. The isolation, preservation and improvement of industrially important microorganisms. In *Principles of Fermentation Technology*, 2nd ed.; Elsevier Science: Kidlington, UK, 1997; pp. 35–85.
17. Dhandhukia, P.C.; Thakkar, V.R. Standardization of growth and fermentation criteria of Lasiodiplodia theobromae for production of jasmonic acid. *J. Biotechnol.* **2007**, *6*, 707–712.

18. Leung, P.H.; Zhang, Q.X.; Wu, J.Y. Mycelium cultivation, chemical composition and antitumour activity of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *J. Appl. Microbiol.* **2006**, *101*, 275–283. [CrossRef] [PubMed]
19. Pirttilä, A.M.; Joensuu, P.; Pospiech, H.; Jalonen, J.; Hohtola, A. Bud endophytes of Scots pine produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures. *Physiol. Plant.* **2004**, *121*, 305–312. [CrossRef] [PubMed]
20. Gorbushina, A.A.; Whitehead, K.; Dornieden, T.; Niesse, A.; Schulte, A.; Hedges, J.I. Black fungal colonies as units of survival: Hyphal mycosporines synthesized by rock-dwelling microcolonial fungi. *Can. J. Bot.* **2003**, *81*, 131–138. [CrossRef]
21. Leach, C.M. Ultraviolet-absorbing substances associated with light-induced sporulation in fungi. *Can. J. Bot.* **1965**, *43*, 185–200. [CrossRef]
22. Trione, E.J.; Leach, C.M.; Mutch, J.T. Sporogenic substances isolated from fungi. *Nature* **1966**, *212*, 163–164. [CrossRef] [PubMed]
23. Young, V.J.; Patterson, A. UV protective compound from *Glomerella cingulate* a mycosporine. *Phytochemistry* **1982**, *21*, 1075–1077. [CrossRef]
24. Leite, B.; Nicholson, R.L. Mycosporine-alanine: A self-inhibitor of germination from the conidial mucilage of *Colletotrichum graminicola*. *Exp. Mycol.* **1992**, *16*, 76–86. [CrossRef]
25. Sommaruga, R.; Libkind, D.; van Broock, M.; Whitehead, K. Mycosporine-glutaminol-glucoside, a UV-absorbing compound of two Rhodotorula yeast species. *Yeast* **2004**, *21*, 1077–1081. [CrossRef] [PubMed]
26. Carreto, J.I.; Carignan, M.O. Mycosporine-like amino acids: Relevant secondary metabolites. Chemical and ecological aspects. *Mar Drugs.* **2011**, *9*, 387–446. [CrossRef] [PubMed]
27. Bills, G.F.; Platas, G.; Fillola, A.; Jiménez, M.R.; Collado, J.; Vicente, F.; Martín, J.; González, A.; Bur-Zimmermann, J.; Tormo, J.R.; *et al*. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *J. Appl. Microbiol.* **2008**, *104*, 1644–1658. [CrossRef] [PubMed]
28. González-Menéndez, V.; Asensio, F.; Moreno, C.; de Pedro, N.; Monteiro, M.C.; de la Cruz, M.; Vicente, F.; Bills, G.F.; Reyes, F.; Genilloud, O.; *et al*. Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity. *Mycology* **2014**, *5*, 179–191. [CrossRef] [PubMed]
29. Cui, X.; Churchill, G.A. Statistical tests for differential expression in cDNA microarray experiments. *Genome Biol.* **2003**, *4*, 210. [CrossRef] [PubMed]

Sample Availability: Samples of the compounds **9**–**12** are available from the authors.

Chapter 5

Ultraviolet (IUV) and Mass Spectrometry (IMS) imaging for the deconvolution of microbial interactions

Chapter 5
BMC Systems Biology

RESEARCH Open Access

Ultraviolet (IUV) and mass spectrometry (IMS) imaging for the deconvolution of microbial interactions

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Abstract

Background: Spatial localization of natural products or proteins during microbial interactions can help to identify new antimicrobials both as offensive or defensive agents. Visible spatial interactions have been used for decades to enhance Drug Discovery processes both in industry and academia.

Results: Herein we describe an automated micro-extraction methodology, that coupled with the previously described HPLC-Studio 2.0 software and the new development, the MASS-Studio 1.0 software, can combine multiple chemical analyses to generate ultraviolet (UV) and mass spectrometry (MS) images from traditional affordable analytical equipment. As a proof of concept, we applied this methodology on two microbial antagonisms observed among co-habitant endophytes isolated from endemic plants of arid areas of the south of Europe.

Conclusions: The use of UV and MS images highlighted interacting naturals products and allowed clear identification of induced molecules of interest not produced by the strains when cultured individually.

Keywords: IUV, IMS, Metabolomics, Co-culturing, Microbial interactions

Background

Drug Discovery for new chemical entities and innovative compound design relies on natural products for more than half of the drugs in development [1]. So far more than 42% of known bioactive compounds have been described as produced by filamenting fungi and many of these molecules with pharmacological applications were developed for clinical uses, especially as antibiotics and antifungals among other applications [2, 3]. However, a general perception is that the emerging rates of discovery of new molecules, especially new antibiotics, are decreasing after half a century of continued research on fungal diversity and axenic fermentation-based processes [4].

Fungal genome mining has revealed the high number of gene clusters involved in the biosynthesis of fungal secondary metabolites (SMs) that are not produced in strains cultivated axenically in laboratory conditions [5, 6]. Recently, several approaches have been applied to foster the expression of these unexpressed pathways and to promote their biosynthesis. These strategies have included: the manipulation of medium and growth conditions in miniaturized nutritional arrays [7], the application of transformation techniques for the generation of gene knockouts, the exchange of native gene promoters with constitutive or inducible promoters or the overexpression of transcription factors [8], or the co-cultivation of more than one microorganism in constant interaction [9].

Traditional screening processes to discover new bioactive molecules, involve culturing a single microbial strain, however the use of co-cultures presents new opportunities for the activation of cryptic biosynthetic pathways. Microorganisms can present an antagonistic reaction in presence of other microorganisms that promotes changes

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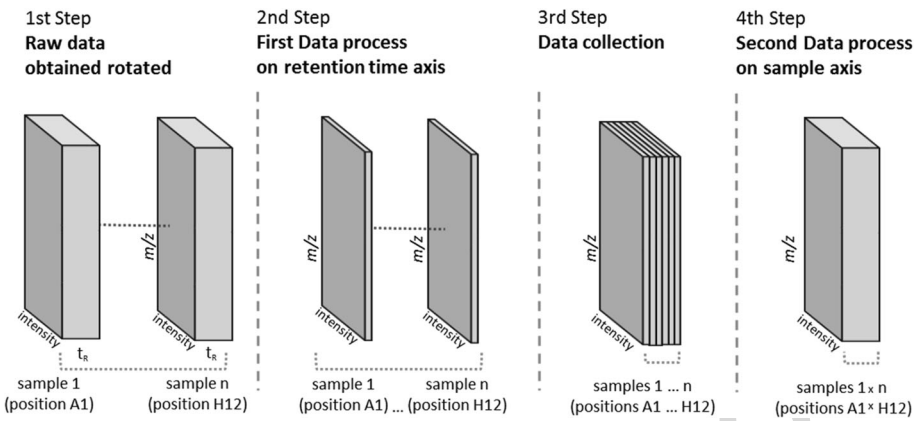


Fig. 1 Data management in MASS-Studio 1.0 where components are processed twice

in their morphology and the production of SMs, enzymes and other compounds in the interaction zone [10].

Co-culturing has proved to be an effective tool to simulate the physiological conditions that occur during microbial interaction in their natural environment and may have an enormous potential for the discovery of new molecules with therapeutic approaches [11–13].

Recent detection techniques try to evaluate natural products by Image Mass Spectrometry (IMS) [14, 15] by remarking its high level of suitability for analyzing microbial interactions and detecting the activation of cryptic pathways [16, 17]. Unfortunately, most of these techniques rely on the use of very expensive or innovative ionization heads in the Mass Spectrometer (MS) (ie MALDI, or nano-DESI respectively). Most of the common MS for natural products are electrospray units that cover mass ranges from 150 to 1500 Da, more suitable for natural products extracts. Recently, we have developed in our lab the MASS-Studio 1.0 software tool for high-throughput analysis of batches of samples analyzed by LC-ESI-MS equipment. Herein we decided to combine miniaturized chemical extractions with HPLC-Studio 2.0 [18, 19] and MASS-Studio 1.0 [20] utilities to generate ultraviolet and mass images from LC-ESI-MS analyses.

Methods

Endophytic strains were isolated as described previously by Gonzalez-Menendez et al. (2016) [19]. Strains interaction were performed by co-culturing on malt agar (malt extract Difco™ 20 g, agar 20 g and 1000 mL deionized H_2O) for 14 days at 22 °C and 70% of relative humidity, and said agar of the positive antagonist was separated into 80 portions corresponding to 80 microplate wells. All co-culture portions were extracted with acetone, shaking at 220 rpm for 1 h and the samples were dried in a Genevac HT-8. Finally, the dried samples were suspended in 500 μl of 20% DMSO. The samples were analyzed by UPLC-UV and by low resolution mass spectrometry (LR-MS) in the range of positive m/z for each extract. Mass ion detection was performed in a ramp from 150 *m/z* to 1500 *m/z* in positive and negative modes. MASS-Studio 1.0 software was used for generating the mass spectrometry imaging for each co-culture. Comparison with proprietary database of more than 950 known microbial standards was performed by low resolution (LC-LRMS) using the same raw data that generated the images.

Once chemical evaluation had been performed raw data corresponding to each individual analysis was recorded in 'cvs' files by the equipment software and HPLC-Studio 2.0 [18, 19] and MASS-Studio 1.0 [20] were used to combine all individual analyses from ultraviolet or mass spectrometry

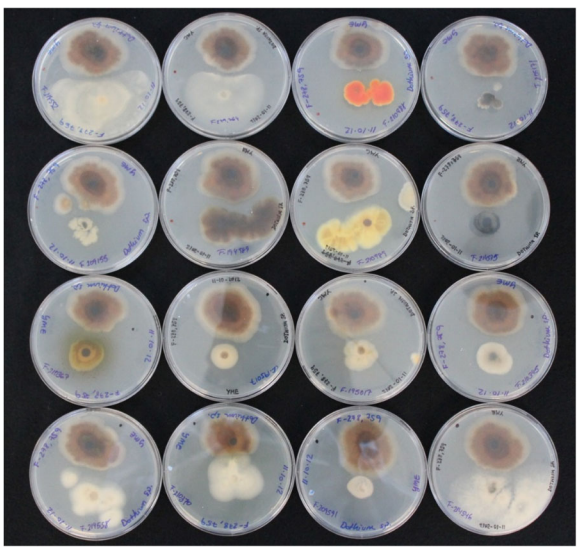


Fig. 2 Examples of antagonisms of several endophytes co-cultures on malt agar plates

detection respectively. Typical runs in these studies compare components detected in the samples and identify if they correspond to the same metabolite or not, by bucketing in the time dimension in the case of the HPLC-Studio 2.0 or in the mass direction in the MASS-Studio 1.0 software.

To obtain the image data management, a second reprocess was used where the spatial dimension was included and components were bucketed for all samples from the same microbial interaction (Fig. 1). In this sense all samples from each batch, including every sample for every spatial position for axenic cultures and co-cultures were compared within the same data matrix for each set of strains. As a result, the combined matrix could be pivoted and represented not as quantity for each component for each sample, but as quantity of each component for each position within each batch of strains (with and without the interaction). Spatial distribution of each component was then plotted by using the Excel® commercial software application (Microsoft Corp.) [20].

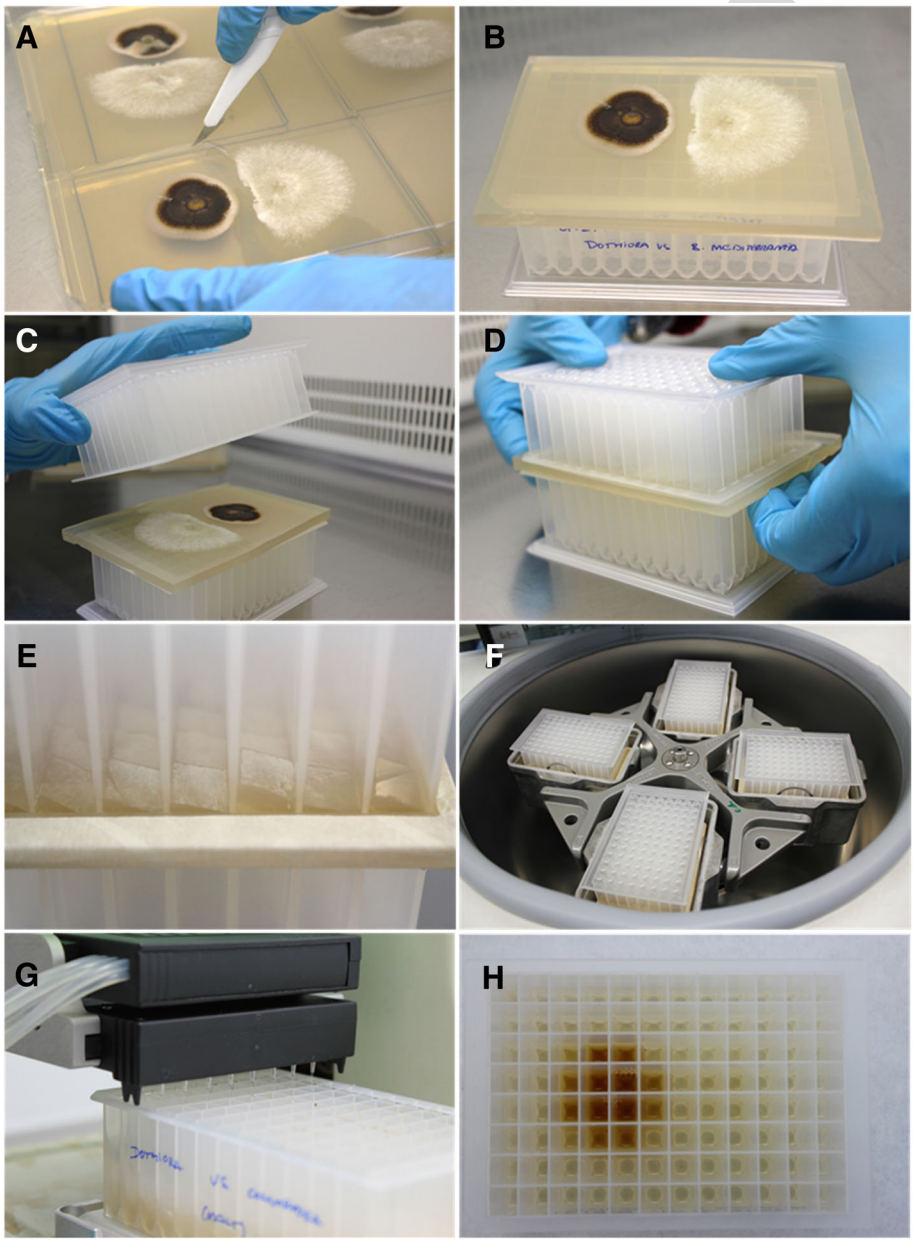


Fig. 3 Sample management methodology performed for creating analytical extracts form microbial interactions chemical analyses. Specific steps (**a-h**) described in the text

Traditional analytical methods have allowed the detection of changes in the metabolite profiles that vary depending on the interacting fungi [21, 22]. Different co-culturing techniques have been developed for this purpose including liquid and solid media, but all approaches consist on culturing two or more microorganisms in a single confined environment to facilitate interactions and induce further chemical diversity [23, 24].

An automated method based on image mass spectrometry (IMS) has been used for evaluating the presence of different secondary metabolites when a clear antagonistic effect was observed in a fungal co-culture. This approach aims to analyze the microbial interactions in terms of the natural products generated (UV and MS) to evaluate the effect of antagonism and detect molecules that could be produced as a consequence, among others, of communication, attack or defense between both microorganisms. As, some of these molecules may not be produced when these microorganisms are grown axenically, there is a high probability that some of them may show biological activities with possible therapeutic purposes.

The antagonistic activity of several endophytes isolated from arid plants of Andalucía was evaluated (Fig. 2). A clear antagonistic effect was observed for two endophyte isolates from the plant *Retama sphaerocarpa*, the host-specific fungi *Dothiora* sp. CF-279759 and *Bacillus* sp. CB-293420, against the semi-parasite *Hypoxylon mediterraneum* CF-279764, isolated also from this plant.

Agar co-cultures of these two strains interacting were selected for proof of concept (POC) for image UPLC-UV and UPLC-MS spatial metabolomics analyses. After co-culturing them per triplicate on malt agar plates, sections of the same size as the destination Thermo™ ABgene® 2.2 mL AB-0661 storage plate were cut (Fig. 3a-b). By superimposing, another plate was pressed out until the co-culture was punched out into 96 square portions (Fig. 3c-e). The next step was a centrifugation at 3000 rpm for 10 min so that the portions were deposited on the bottom of the extraction plate (Fig. 3f) for later addition of acetone (Fig. 3g). After evaporation of organic solvent, the samples were resuspended in 500 μl of DMSO 20% (Fig. 3h) and 3000 rpm 10 min supernatants were analyzed by UPLC-UV and MS.

Results and discussion

Initial data management was performed with HPLC-Studio 2.0 software from UV traces of axenic and co-culture of *Dothiora* sp. vs *H. mediterraneum*. Images highlighted four components only produced when both strains interacted, apart from a large list of other compounds produced constitutively by each axenic growth (Fig. 4a-d). UV-visible detection, although sensitive enough when components present chromophores, gives very few information on the complexity of a microbial fermentation or a natural products extract. Most of natural products present 210 nm absorption relying their identification on its retention time in an HPLC chromatography and the UV-visible absorption spectrum comparing with standards. Mass spectrometry is a detection technique with the ability to discriminate molecular mases of the different components of a mixture with more sensitivity and accuracy. MASS-Studio 1.0 was

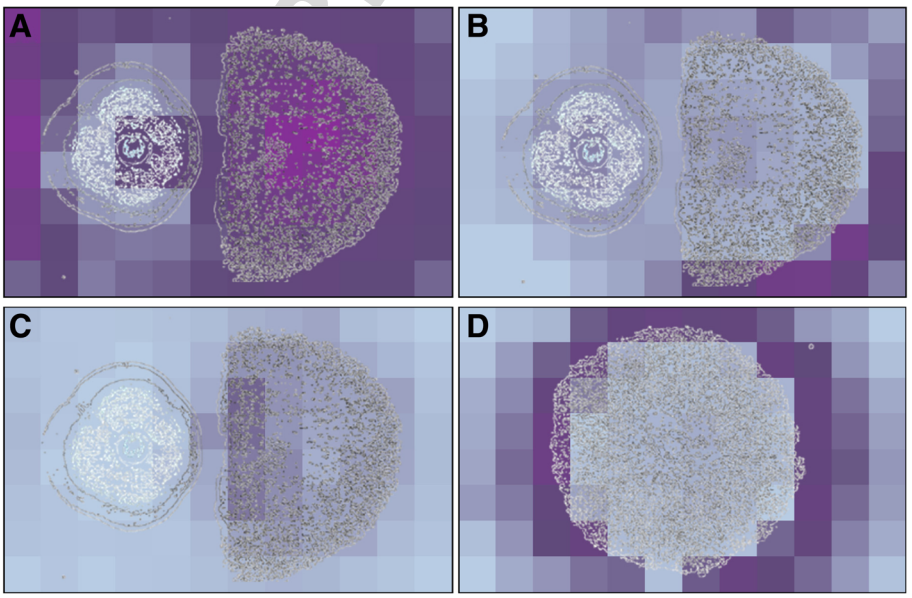


Fig. 4 Image of microbial interactions by processing LC-UV raw data with HPLC-Studio 2.0 tool for four (**a**-**d**) UV components at 3.57, 4.90, 4.16 and 4.50 min of UHPLC retention times, respectively, that presented differential spatial distributions

adapted to generate these high detailed images of both the coculture and the individual strains (Fig. 5a-c). Results highlighted many other components produced constitutively by the strains and, more interestingly, many others that were produced only during their interaction. Moreover, spatial correlation of the antimicrobial area could be correlated with the ion with a mass to charge ratio (m/z) of 419.2421. Not present in the axenic cultures (Fig. 5B, e).

The co-localization of this ion in the inhibition zone suggested that this molecule could be involved in the antagonistic activity observed. The LC-HRMS dereplication by comparison with more than 900 microbial natural product standards (60% with anti-infective activity) did not give a positive match [19]. A tentative molecular formula could be determined by BrukerTM Smart formula 3D® Software, according to its high-resolution mass isotopic pattern, as C22H33N3O5 (Fig. 6).

In the case of co-culturing *Hypoxylon mediterraneum* with *Bacillus* sp. (Fig. 5d) twelve known compounds with antimicrobial activities were dereplicated as possible responsible of part of the interactions observed. These compounds belonged to three main families produced by *Bacillus* sp.: surfactins (Fig. 5D, o and D, q), iturins (Fig. 5D, p and D, r) and mycosubtilins (Fig. 5D, s and D, t) [21, 22]. Other accumulated ions were found to be produced by *H. mediterraneum* in response to this antimicrobial attack, as (m/z) 263, 337, 429, 443, 520 or 709, but were not observed when the strain was grown axenically. This agreed with the selective production of several secondary metabolites as a signaling response or a defense mechanism.

Conclusions

A combination of compound management techniques, automated micro-extractions and the HPLC-Studio 2.0 and MASS-Studio 1.0 software tools was used for the

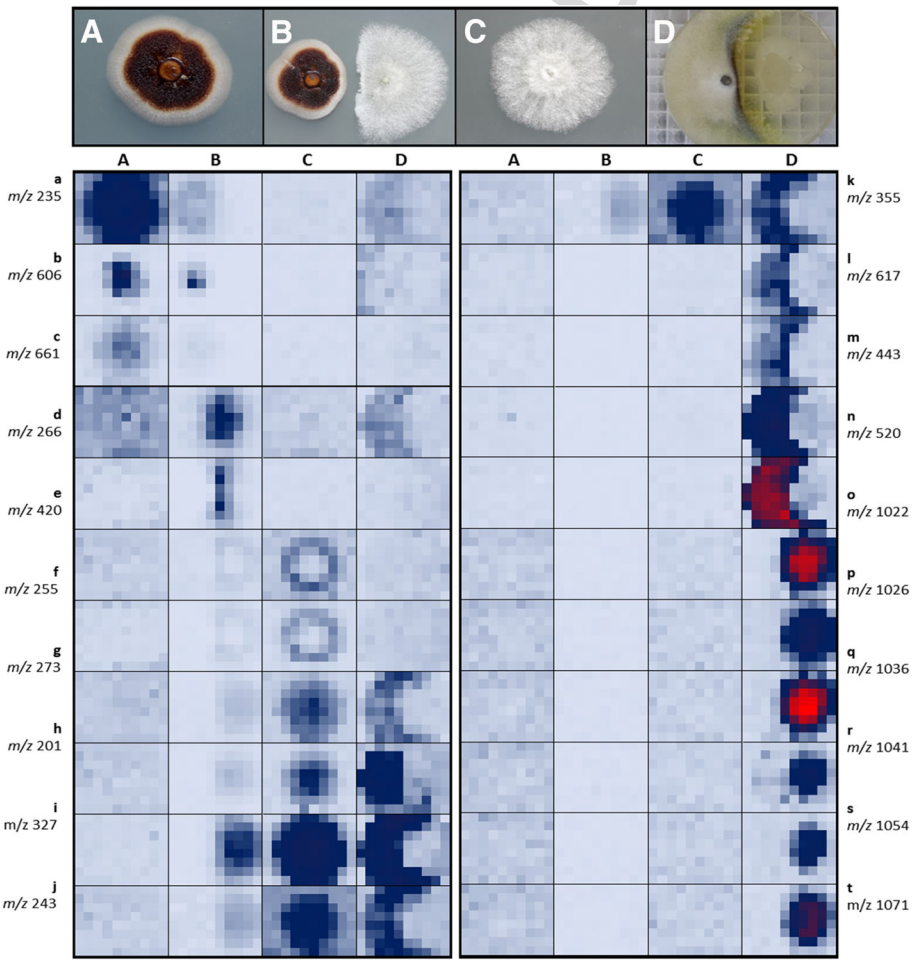
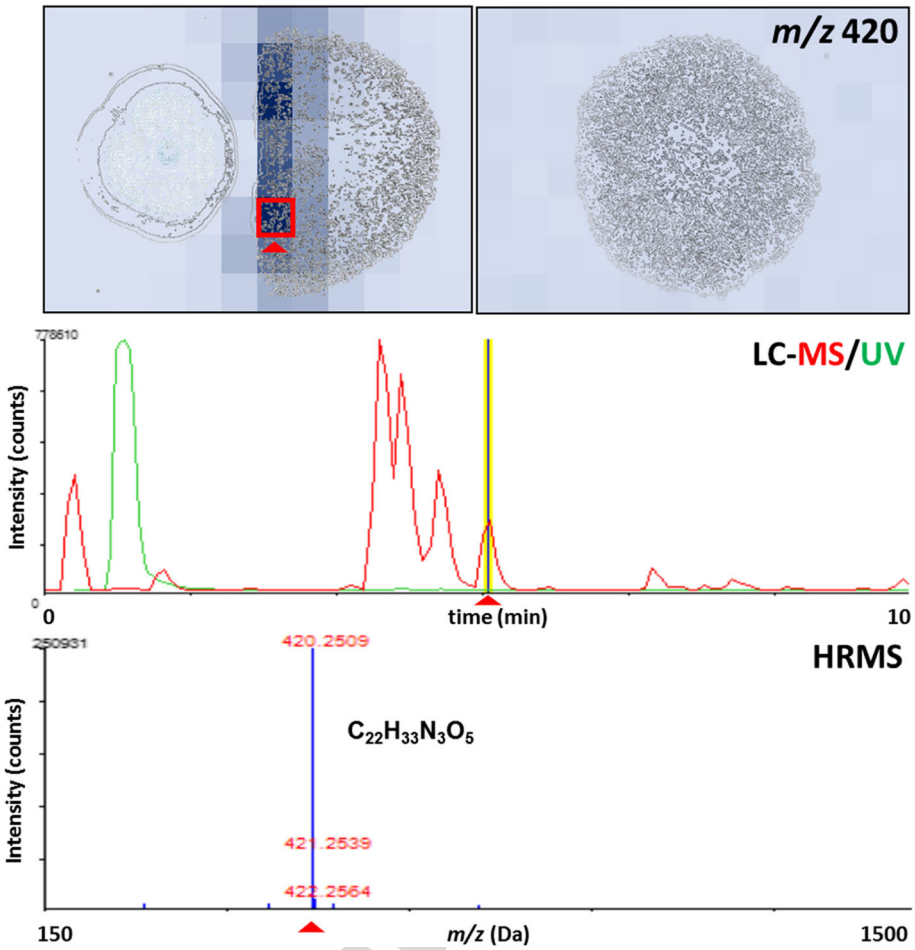


Fig. 5 Image Mass Spectrometry (IMS) for *Dothiora* sp. **a** vs *H. mediterraneum* **b** or vs *Bacillus* sp. **d**, obtained by processing LC-MS raw data with MASS-Studio 1.0 tool. Twenty (a-t) MS components that presented differential spatial distributions depicted



f6.1 **Fig. 6** Imaging Mass Spectrometry (IMS) distribution and LC/HRMS dereplication of most induced component in the interaction of *Dothiora* sp. vs
f6.2 *H. mediterraneum*
f6.3

development of Ultra Violet and Mass Spectrometry Imaging from microbial interactions. Spatial localization of secondary metabolites resulted in an advanced tool for the evaluation of the antagonistic effects among strains in ecological communities. Mass Spectrometry Imaging (IMS) resulted in a more informative analytical technique than Ultraviolet Imaging (IUV) for the evaluation of these microbial interactions. Moreover, this methodology, when combined with evaluation of antimicrobial properties, can speed up the discovery of bioactive natural products and signaling molecules.

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Authors' contributions
JRT and VGM designed the study. VGM and RS performed the microbiology including images. VGM and FM performed the sample preparation including images. JRT and GM developed the software algorithm, software application and schemas. JM performed the low-resolution MS analyses and high-resolution MS dereplications. JRM processed the data and generated the MS images. JRT wrote the manuscript. VGM and OG reviewed critically the manuscript. All authors contributed and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

References

1. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. Nat Rev Drug Disc. 2005;4:206–20.
2. Schueffler A, Anke T. Fungal natural products in research and development. Nat Prod Rep. 2014;31:1425–48.
3. Demain LA. Importance of microbial natural products and the need to revitalize their discovery. J Ind Microbiol Biot. 2014;41:185–01.
4. Bills G, Martin J, Collado J, Platas G, Overy D, Tormo JR, Vicente F, Verkleij GJM. Crous PW measuring the distribution and diversity of antibiosis and secondary metabolites in filamentous fungi. SIM News. 2009;59:133–47.
5. Brakhage AA, Schuemann J, Bergmann S, Scherlach K, Schroeckh V. Hertweck C. In: Petersen F, Amstutz R, editors. Activation of fungal silent gene clusters: a new avenue to drug discovery natural compounds as drugs. Basel: Birkhäuser; 2008. p. 1–12.
6. Rank C, Larsen T, Frisvad J. Functional systems biology of aspergillus. In: Machida M, Gomi K, editors. Aspergillus: molecular biology and genomics. Wymondham Norfolk: Caister Academic Press; 2010. p. 173–98.
7. Bills G, Platas G, Fillola A, Jiménez MR, Collado J, Vicente F, Martín J, González A, Bur-Zimmermann J, Tormo JR, Peláez F. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. J Appl Microbiol. 2008;104:1644–58.
8. Brakhage AA, Schroeckh V. Fungal secondary metabolites - strategies to activate silent gene clusters. Fungal Genet Biol. 2011;48:15–22.
9. Combès A, Ndoye I, Bance C, Bruzaud J, Djediat C, Dupont J, Nay B, Prado S. Chemical communication between the endophytic fungus *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum*. PLoS One. 2012;7(10): e47313.
10. Hynes J, Müller CT, Jones TH, Boddy L. Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare and Resinicium bicolor*. J Chem Ecol. 2007;33:43–57.
11. Bader J, Mast-Gerlach E, Popović MK, Bajpai R, Stahl U. Relevance of microbial coculture fermentations in biotechnology. J Appl Microbiol. 2010; 109:371–87.
12. Brakhage AA. Regulation of fungal secondary metabolism. Nat Rev Microbiol. 2013;11:21–32.
13. Netzker T, Fischer J, Weber J, Mattern DJ, König CC, Valiante V, Schroeckh V, Brakhage AA. Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. Front Microbiol. 2015. https://doi.org/10.3389/fmicb.2015.00299.
14. Sanchez LM, chemistry DPCA. Virulence caught green-handed. Nat Chem. 2013;5(3):155–7.
15. Hopwood DA. Imaging Mass Spectrometry reveals highly specific interactions between Actinomycetes to activate specialized metabolic gene clusters. mBio. 2013;4(5):e00612–3.
16. Traxler MF, Watrous JD, Alexandrov T, Dorrestein PC, Koltera R. Interspecies Interactions Stimulate Diversification of the *Streptomyces coelicolor* Secreted Metabolome. mBio. 2013;4(4):e00459–13.
17. Bertrand S, Schumpp O, Bohni N, Monod M, Gindro K, Wolfender J-L. Denovo production of metabolites by fungal co-culture of Trichophyton rubrum and Bionectria ochroleuca. J Nat Prod. 2013;76:1157–65.
18. Garcia JB, Tormo JR. HPLC-studio: a novel software utility to perform HPLC chromatogram comparison for screening purposes. J Biomol Scr. 2003;8: 305–15.
19. González-Menéndez V, Pérez-Bonilla M, Pérez-Victoria I, Martín J, Muñoz F, Reyes F, Tormo JR, Genilloud O. Multicomponent analysis of the differential induction of secondary metabolite profiles in fungal endophytes. Molecules. 2016;21:234–50.
20. Martínez G, González-Menéndez V, Martín J, Reyes F, Genilloud O, Tormo JR. MASS-studio: a novel software utility to simplify LC-MS analyses of large sets of samples for metabolomics. In: Rojas I, Ortuño F, editors. Proceedings bioinformatics and biomedical engineering. Basel: Springer International Publishing; 2017. p. 230–44.
21. Peiris D, Dunn WB, Brown M, Kell DB, Roy I, Hedger JN. Metabolite profiles of interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminates*. Metabolomics. 2008;4:52–62.
22. Rodriguez-Estrada AE, Hegeman A, Kistler HC, May G. In vitro interactions between fusarium verticillioides and Ustilago maydis through real-time PCR and metabolic profiling. Fungal Genet Biol. 2011;48:874–85.
23. Latoud C, Peypoux F, Michel G. Action of iturin a an antifungal antibiotic from Bacillus subtilis on the yeast *Saccharomyces cerevisiae*: modifications of membrane permeability and lipid composition. J Antibiot. 1987;40(11):1588–95.
24. Besson F, Michel G. Action of mycosubtilin an antifungal antibiotic of *Bacillus subtilis* on the cell membrane of *Saccharomyces cerevisiae*. Microbios. 1989;59(239):113–21.

IV. GENERAL DISCUSSION

GENERAL DISCUSSION

Arid areas of Andalusia have special climatic conditions and edaphological such as low rainfall, high UV levels and specific lithology where loamy materials and evaporite abound. These unique ecological conditions have influenced the development of a wide range of endemic plant species with distinctive characteristics (Lazaro *et al*., 2000), ensuring their survival under selective environmental pressures. Limited information is available on the biodiversity of fungal symbionts and host-specific endophytes in these areas. Previous biodiversity studies from arid zones of Almeria and the Baetic Mountains (Andalusia) suggest that many fungi associated to plants of these areas remain to be discovered (Bills *et al*., 2012). The lack of information encouraged the current study of plant species with limited distribution to these regions, including the most representative endemisms *Centaurea dracunculifolia*, *Euzomodendron bourgeanum*, *Helianthemum almeriense*, *Limonium majus* and *Moricandia foetida*, in order to isolate and characterize their culturable fungal communities (fulfilling the first objective of this thesis).

From the survey performed, a total of 349 fungal strains were obtained from 63 plant specimens, representing 19 taxonomic orders (from both Basidiomycetes and Ascomycetes; see Chapter 1 and Supplementary Figure 1). Taxa from *Pleosporales* were the most frequent endophytic taxa isolated and identified from these plants, in agreement with previous studies on plants collected from other related environments (Bills, 1996). Within the order *Pleosporales*, the different isolates were distributed among 42 genera belonging to 14 families, with *Pleosporaceae*, *Didymellaceae* and *Sporormiaceae* being the most abundant, with 76, 43 and 19 isolates respectively. The wide diversity of strains isolated proves that the plants in arid areas of Andalusia are rich sources of endophytic fungal biodiversity.

Within *Pleosporaceae*, the most frequently isolated genus was the recently described *Neocamarosporium* (Crous *et al.,* 2014); a genus characterized as naturally halotolerant due to its occurrence in hypersaline environments and its ability to grow in presence of salts (Papizadeh *et al*., 2018). All of the *Neocamarosporium* isolates, obtained mainly from halophyte plant endemisms such as *Salsola papillosa* and *Salsola genistoides,* were able to grow in media containing more than 3% of NaCl, thus reaffirming the halotolerance of members of this genus. In addition, this is the first time that the presence of this genus is reported in plants from the Iberian Peninsula, confirming that microorganisms from these regions have adapted to existing extreme environmental conditions.

All the isolates belonging to *Sporormiaceae* were identified as *Preussia* spp, the second most frequent genus isolated, in agreement with previous results from plants collected in the Arizona desert (Massimo *et al*., 2015). Many reports exist on endophytic species of *Preussia* from different plant specimens (Mapperson *et al*., 2014; Zaferanloo *et al*., 2014; Massimo *et al*., 2015), but their life cycle within their host plant remains unknown. One

hypothesis is that these fungi colonize internal plant tissues, beneath the epidermal cell layers, without causing any apparent harm or symptomatic infections on their host and remain viable after passing throughout the gut of herbivores (Devarajan and Suryanarayanan 2006). Observations suggest that the coprophilous stage is an alternate phase in the life cycle of some endophytic *Preussia*, and that certain coprophilous fungi may have co-evolved with grazing animals and plants (Porras-Alfaro *et al*., 2008). Another alternative hypothesis is that some of the *Preussia* spp were erroneously reported as endophytes due to the fact that their propagules are able to resist the common sterilization methodologies used for endophyte isolation (Newcombe *et al*., 2016). The spores of *Preussia* species are often surrounded by mucilage or have gelatinous appendices that attach easily to plant surfaces. When a plant is foraged by an herbivore, the spores travel through their digestive tract, and finally, when ending up in a dung pile, germinate and produce new fruit bodies (Kruys and Wedin, 2009). Nevertheless, both hypotheses explain the repeated isolation of this genus as an endophyte.

In order to determine the phylogenetic position of the isolates within the order *Pleosporales* and to identify potential new linages, two different phylogenetic analyses were performed on the basis of their ITS and 28S ribosomal DNA regions (excluding *Preussia* species which were subject of a different specific study). These analyses showed several clades containing isolates that could represent undescribed species, such as, *Tamaricicola* sp. CF-288959 isolated from the endemic *Limonium majus*, *Phoma* sp. CF-288917, *Neocamarosporium* sp. CF-288932, *Xenodydimella* sp. CF-090345 and *Camarosporium* sp. CF-090314 (Chapter 1; Figure 1), supporting the initial hypothesis that several fungal endophytes from these environments still remain to be discovered and that **Andalusia is a rich source of fungal biodiversity**.

In the case of the *Preussia* isolates, a specific study was carried out to review their taxonomy and to characterize occurring chemotypes of *Preussia* species from arid and other environmental samples of the Iberian Peninsula. For this purpose, the population of 32 *Preussia* isolates including 19 endophytes and a small number of strains from soils and herbivore dung were compared with previously reported *Preussia* strains from Arizona desert plants (Massimo *et al*., 2015) and other *Sporormiaceae* obtained from public collections. The general topology of their ITS/28S phylogenetic tree is in agreement with previous studies (Kruys and Wedin 2009; Massimo *et al.* 2015) where eleven of the 21 *Preussia* spp. isolates from Arizona desert plants (Massimo *et al*., 2015) were included within the 'Minima' species complex. However, in the current work the most frequently isolated species was *Preussia lignicola* obtained from five different plant species (*Dittrichia viscosa*, *Erica australis*, *Genista umbellata*, *Retama sphaerocarpa* and *Viscum album*) and the second most frequent isolated species was *P. minima* from a variety of substrates. This is the first report of *P. lignicola* in the Iberian Peninsula (Chapter 2; Figure 2). In addition, 19 different *Preussia* species were able to be identified from the

phylogenetic and morphological analyses (Chapter 2; Figure 1), also confirming that **Andalusia is a rich source of fungal species.**

Many known and common taxa represent heterogeneous species complexes, which remain to be resolved by a combination of genotypic and phenotypic data (Stadler, 2011). Consequently, polyphasic studies using chemotaxonomic, morphological and molecular data can be used as effective tools to clarify the similarities between different genera within a given family (Stadler *et al*., 2014). However, few reports exist comparing the SM profiles and phylogeny in *Pleosporales*. Only *Alternaria* and *Ascochyta* had been the subject of similar studies (Andersen *et al*., 2008; Kim *et al*., 2016). Therefore, a chemotaxonomic analysis of *Preussia* species was included in this thesis to present the first comprehensive evaluation of the genus in terms of chemotaxonomic, morphological and phylogenetic characteristics. SM profiles of 37 fungal strains (including 23 isolates obtained from this study and 14 *Preussia* strains from public collections) were compared to characterize their different chemotypes. The evaluation revealed sixteen compounds that could be used as biomarkers to distinguish *Preussia* species: molecular structures were proposed for eight of the metabolites (Chapter 2; Table 3), four compounds presented more than one possible identity in database matching, and the remaining four could not be identified in the databases suggesting that they could correspond to potentially new NPs. Eleven of the biomarkers were uniquely produced by certain species and could be used to resolve groups of closely related species. This is the case of microsphaerone A, generated by *P. africana* and microsphaerone B by *P. lignicola*, as these compounds were not detected in other closely related *Preussia* spp. In addition, patterns in pseudomolecular ions within the chemotypes for each *Preussia* strain discriminated between species even though unique species-specific biomarkers were absent, as demonstrated for *P. grandispora* (chemotype B) and *P. funiculata* (chemotype D) (Chapter 2; Table 4).

The lack of detection of compounds previously described in some *Preussia* species (e.g. preussomerin, australufingin and sporminarin) can be explained by differences in medium composition and fermentation conditions used in this work (MMK2 and YES liquid media) compared to rice or corn based solid media cultures, where most of these molecules have been reported (Hensens *et al*., 1995; Mudur *et al*., 2006; Zhang *et al*., 2012; Xiong *et al*., 2014). It is well known that culture media compositions drastically affect the production of fungal SMs (Bills et al.,2008). Fungi growing on a solid medium compared to a liquid may morphologically develop differently as a response, which can translate into a differential expression of SM biosynthetic gene clusters (de la Cruz *et al*., 2012). To confirm this hypothesis, the production of australifungin and australifunginol was compared by reproducing the same solid media and fermentation conditions used by Mandala *et al*. (1995). The experiment also included the original australifungin producer strain (MF 5672) as control. Australifungin production was detected in five, and

australifunginol in four, of the six *Preussia intermedia* isolates, confirming the hypothesis that specific fermentation conditions and taxon-specific optimizations are required for triggering the production of certain compounds.

It has been widely described that fungi represent one of the most prolific sources of novel NPs but low production yields and the lack of expression of cryptic gene clusters in laboratory conditions are frequently key limiting factors for exploiting the full SM potential of fungi *in vitro*. Several strategies have been developed to address this issue, including mutagenesis, genetic transformation, agar co-cultivation and the use of epigenetic modifiers or adsorptive polymeric resins in fermentation conditions (Venugopalan *et al*., 2016). Several studies have reported that the addition of adsorptive polymeric resins is an effective tool to increase the production titers of certain SMs (Marshall *et al*., 1990; Woo *et al*., 2002; Igarashi *et al*., 2012), while the use of epigenetic modifiers increases the expression of secondary metabolic genes during fungal production (Vervoort *et al.* 2011; Chen *et al*., 2013; Asai *et al*., 2016). Although both approaches have been used on specific fungal species, confirming their capacity for increasing and/or inducing the production of undescribed SMs (Phillips *et al*., 2013), no in-depth study on the effects of the addition of a set of epigenetic modifiers or adsorptive polymeric resins on the production of SMs of a taxonomically diverse group of fungi have been published. Similarly, the application of co-cultivation has also been shown to induce SMs that do not occur when these microorganisms are cultured axenically (Hynes *et al*., 2007; Combés *et al*., 2012; Serrano *et al*., 2017). Thus, the main objective of this work, once the populations of isolates had been characterized, was to stimulate and characterize their chemical diversity by applying these three methodologies.

Initially, the effect of the **addition of a set of adsorptive polymeric resins to multiple media** on the SMs production was evaluated to determine if resins had a more positive effect on the number of different metabolites produced than changes in the medium composition. For this purpose, the four most common resins previously reported as used for specific microbial fermentations (Amberlite® XAD-2, XAD-7 and XAD-16 and Diaion® HP-20) were combined with six media with different carbon, nitrogen and trace element compositions (LSFM, MMK2, MV8, SCAS, XPMK and YES; Bills *et al*., 2008). The study, starting from a selection of 98 taxonomically diverse fungal strains, and then focusing on the 14 strains that presented most relevant changes in SMs profiles, showed that there were no significant differences in the number of SMs generated with resins, compared to the conditions tested without them. Hierarchical Cluster Analysis (HCA; Dice + UPGMA) confirmed that the addition of adsorptive polymeric resins had a lower impact on the SM profiles compared to the effect of changes in the medium composition. This could also be clearly observed by principal component analysis (PCAs), indicating that there is more chemical diversity generated by modifying the media than by the addition of Diaion® or Amberlite® resins (Chapter 3; Figure 7). A detailed analysis of each fermentation condition

indicated that, for those fermentation media in which a large chemical diversity is initially observed without the addition of resins, such as LSFM, MV8 and MMK2, resins do not improve the diversity obtained. However, the addition of XAD-16 or HP-20 resulted in a clear increase in the overall number of different SMs produced in the media that without the resins had low metabolite diversity, such as SCAS and YES (Chapter 3; Figure 8). Most of the fermentation conditions tested presented condition-specific SMs and different production titers for many of the SMs detected (Chapter 3; Figure 9). These results support the use of the adsorptive polymeric Amberlite® or Diaion® resins with specific strains where an increase in the production or the generation of bioactive metabolites cannot be obtained by introducing changes in media compositions.

Through PCA and HCA metabolomic analyses, the most effective combination of media and resins was selected and applied on the fungal isolates to promote the greatest SM diversity as well as the generation of biological activities. The hit rates obtained after the screening of the isolates against human pathogens (*Aspergillus fumigatus* and *Candida albicans*), phytopathogens (*Magnaporthe grisea* and *Colletotrichum acutatum*) and the human hepatocellular carcinoma cell line HepG2 are in line with previously reported studies for other fungal communities (Pelaez *et al*., 1998; Li *et al*., 2005; Luo *et al*., 2015; Ratnaweera *et al*., 2015). In order to determine if the addition of resins increased the number of strains with activity, the distribution of the actives generated with or without resins, was compared (Chapter 1; Figure 2). Exclusive activities were produced when isolates were grown both in presence and absence of resins, for each of the assays performed. Regarding the chemical diversity detected, 75 of the 126 total active strains were able to produce 54 known bioactive compounds from several biosynthetic classes such as sesquiterpenoids (e.g. avocettin, trichothecene, lagopodins), polyketides (e.g. calbistrin A, phomasetin, altersetin), peptides (e.g. cyclo(phenyalanyl-prolyl)) and peptide polyketides (e.g. pleofungin A, naematolin) (Chapter 1; Figure 3). Twelve of these compounds, from multiple biosynthetic classes, were produced only in the presence of resin, supporting the hypothesis that resins can help to modulate the expression of different cryptic metabolic pathways by removing feedback repressors during the fermentation process (Phillips *et al*., 2013).

The addition of adsorptive polymeric resins during fungal fermentations resulted in an increase of antifungal activities per isolate ranging from 20% (against *C. acutatum*) to 36% (against *A. fumigatus*), most probably because of the increased chemical diversity. Eleven of the isolates produced cytotoxic activities against the HepG2 cell line only when fermented in the presence of resins. The comparison of the chemical profiles by HPLC-UV-MS plus database dereplication of known compounds, confirmed that these induced activities were mainly due to an increase of the production titers of specific compounds, as well as, the induction of SMs.

General Discussion

As indicated above, several previous studies have reported the capacity of resins to capture specific compounds, increasing their production titers and protecting them against post-biosynthetic degradations or biotransformations (Marshall *et al*., 1987; de la Cruz e*t al*., 2012). These effects were observed in various active isolates, such as in *Coprinopsis episcopalis* CF-279244 and *Preussia grandispora* CF-090385, where the resin XAD-16 captured the bioactive compounds lagopodin A (Chapter 1; Figure 4-D) and TMC-120B (Chapter 1; Figure 5-A), preventing their later transformation into lagopodin B (Bu´Lock and Darvyrise, 1976) and TMC-120C (Kohno *et al*., 1996) respectively. Another reported positive effect of the addition of resins was the removal of a product involved in SM pathway feedback repression (Frykman *et al*., 2006). This effect could also explain the detection of certain bioactive compounds only in presence of resins, such was the case of the generation of calbistrin A by the *Pseudocamariosporium* sp. CF-090324 (Chapter 1; Figure 4-A). Recently, the putative polyketide synthase PKS involved in the biosynthesis of calbistrin A has been described in other fungal species (Grijseels *et al.,* 2017). Probably, in this strain, a homologous PKS could remain cryptic in standard fermentation conditions and the resin may capture a repressor of this biosynthetic pathway. Something similar could explain the results obtained for the *Xylaria* sp. CF-285461 strain that produced five different compounds only in presence of the XAD-16 resin, the inhibitor of metastasis in EL-4 and B16 tumor cells, TKR2648 (Takesako *et al*., 1998), two α-pyrones, and two mellein derivatives (Chapter 1; Figure 5-C).

A specific objective of this work was to evaluate if the increase in chemical diversity by the addition of adsorptive resins could result in the discovery of new bioactive SMs. To prove this hypothesis, a subset of 6 fungal endophytes only active in the presence of resins, with 9 potentially new compounds, were studied (Chapter 1; Table 1). Their chemical profiles showed a high potential for scalability when comparing small to large scale, with components not present in MEDINA internal databases of standards, and with HRMS estimated molecular formulas not present in commercial or public databases. Among them, extracts of *Eutypa consobrina* CF-090213 produced the most potent antitumoral activities and therefore were selected for bioactivity-guided isolation of its bioactive compounds. Three active compounds (**1**-**3**) were identified from this fungal isolate: a compound with the molecular formula of $C_{17}H_{26}O_4$ (**1**; MDN-0209), produced both with and without the resin addition; and two other compounds, $C_{17}H_{24}O_4$ (**2**; MDN-0210) and $C_{10}H_{12}O_5$ (**3**; MDN-0211) produced only in presence of the XAD-16 resin. All three compounds were purified in amounts of 27.0, 2.4 and 1.4 mg, respectively. NMR analyses confirmed that these compounds were new SMs not yet described in nature (Chapter 1; Supplementary Structure elucidation). These results confirm the hypothesis that the addition of resins can promote the activation of cryptic biosynthetic pathways during fungal fermentation processes that could lead to the discovery of new active NPs.

The antitumoral activity profile of these three new compounds was carried out by testing them against a set of six human cancer cell lines, including hepatocarcinoma (HepG2), breast adenocarcinoma (MCF-7), pancreatic cancer (Miapaca-2), melanoma (A-2058), lung cancer (A-549) and colon cancer (HT-29), to characterize selective *in vitro* activities of the compounds against these cancer cell lines. The most potent compound (**2**) yielded an ED50 for HepG2 cell line of 8.7 µM, with similar values for A-2058, HT-29 and Miapaca-2 cell lines, whereas for MCF-7 and A549 cells this compound was half as effective. Compound (**1**), that was structurally related to (**2**), showed similar activity patterns, but with less potency. On the contrary, compound (**3**), not related structurally to (**1**) and (**2**), was inactive with an effective ED50 above 100µM for the HepG2 cell line (Chapter 1; Figure 6). Other structurally related, partially reduced epoxyquinones have previously been isolated from marine-derived fungi and have showed selective activity *in vitro* towards a range of 36 tumour cell lines (Sunassee and Davies-Coleman, 2012). *Eutypa* species, frequently found as phytopathogens of grape crops (Trouillas *et al*., 2010), have previously been described as producers of phytotoxic epoxyquinones, similar to compounds (**1**) and (**2**) (Andolfi *et al*., 2011; Shiono *et al*., 2005). More studies are necessary to understand the role of these compounds as possible virulence factors associated to the symptoms of *Eutypa* dieback.

In line with a specific objective of this study, the second methodology that was decided to be applied on the fungal endophyte population was the **addition of small-molecule epigenetic modifiers** to the fermentation processes (Chapter 4; Figure 1). Many studies have reported the effects of histone deacetylases (HDAC) and DNA methyltransferases (DNMT) in fungal SM profiles by the induction of the expression of silent metabolite pathways (Deepika *et al.,* 2016; Magotra *et al*., 2017). To date, no previously reported studies have evaluated the efficacy that a systematic treatment with different epigenetic modifiers added to the culture media, would have on promoting the expression of SMs for a set of diverse fungal strains. The different chemical structures and mechanisms of action of the five different HDACs (SBHA, sodium butyrate, valproic acid, nicotinamide and quercetin) and two DNMT inhibitors (5-azacytidine and hydralazine hydrochloride) selected for the study, has enabled to evaluate the differences in their efficacy to promote differential expression of SMs in wild fungal endophytes. To evaluate the effect of these epigenetic modifiers two populations of isolates were selected: a heterogenous group of six filamentous fungi from different families (*Chaetothyriaceae, Neocamarosporiaceae, Planistromellaceae, Sporormiaceae and Xylariaceae*) and a homogenous group of seven dimorphic yeasts of the *Saccotheciaceaeae* family. Chemical epigenetic modifications are traditionally based on the addition of different small-molecule elicitors, in the production media, during fungal fermentations but the effect of also adding these elicitors during the seed stage had not been evaluated before. Furthermore, the study has included the analysis of seven small-molecule epigenetic modifiers added in different culture stages, during the production stage and during both the seed and production fermentation

stages, to assess changes in morphology, production titers and chemical diversity for each strain and fermentation condition.

In general, none of the epigenetic modifiers showed a clear pattern or stimulated the production of new peaks in all the isolates tested, rather the response was found to be specific for each strain and type of molecule produced. These results suggest that the outcome cannot be predicted a priori, therefore it is necessary to test more than one chemical elicitor to improve the metabolic expression of a diverse fungal population. However, it is important to emphasize that SBHA promoted changes in the morphology and/or the production titers in a high number of strains, affecting nine of the 13 isolates when it was added in both fermentation stages (Chapter 4; Table 2). Three of the 13 fungal strains showed significant changes in their growth morphologies, in their production titers and in the generation of new SMs when cultured in the presence of the epigenetic modifiers.

A metabolomic study was performed with the strain *Dothiora* sp. CF-285353, where the effects of three elicitors induced most significative morphological and SM profile changes when compared with its corresponding control fermentation conditions. The liquid-liquid sub-culturing, required for obtaining enough inoculum for the scale-up processes, demonstrated the introduction of mutations and strain degradation after each transfer and cell division (Stanbury *et al*., 1997). Therefore, the evaluation of the sub-culturing effects in *Dothiora* sp., in combination with epigenetic elicitors, using three experimental seed stage designs, was applied in a scaled-up production (combining the direct use of agar plugs in seed and/or the addition of a pre-inoculum) (Chapter 4; Figure 3). Clear morphological differences were observed even without any epigenetics modifiers. Thus, all three methods and the two growth morphologies were selected to be included in the metabolomic analyses.

Metabolomic analyses based on LC/MS data can be used to identify induced compounds from stimuli exposure, prioritize features through molecular class, and even dereplicate prioritized SMs (Covington *et al*., 2017). Volcano-plot representations based on LC/MS have previously been used to determine significant differences between two sets of samples from two different conditions of interest (Albright *et al*., 2015). Thus, volcano-plot representations were applied to identify induced SMs in comparative analyses between epigenetic treatments and control conditions. In the case of *Dothiora* sp. the addition of small-molecule elicitors, during both the inocula and the production stages, resulted in a significant increase in the diversity and amount of SMs generated. Regarding the different epigenetic modifiers used, 5-azacytidine was the epigenetic modifier that induced more changes in SM expression when added during the seed and production fermentations compared to when added only during the production stage alone. Plots were translated into a significant number of unique molecular species that increased by two-fold when

compared to the addition of the epigenetic inhibitor only during the production stage. Although fermentations were harvested with similar biomasses, the method that included sub-culturing steps presented the lowest volcano-plot dispersion, suggesting an important influence of this fermentation methodology (Chapter 4; Figure 5).

Interestingly, hydralazine was the epigenetic modifier that induced most statistically differential pseudomolecular ions (m/z) for this strain (Chapter 4; Figure 5). After the dereplication by UV-HPLC-HRMS databases, different metabolites with increased production were identified such as curvicollide A/B and fusidic acid, and more tentativelly pyrophen, cyclo-isoleucyl-leucyl-isoleucyl-leucyl melledonal C and rhizoxin S previously described to be produced by an endosymbiotic bacteria in *Rhizopus* spp (Scherlach *et al*., 2006). The effect of production enhancement of certain SMs after the addition of DNMT and HDACs inhibitors had been described in several fungal strains, e.g, in *Aspergillus fumigatus* where the production of fumiquinazoline C was enhanced by valproic acid addition (Magotra *et al*., 2017), or the enhancement of camptothecin production when an attenuated endophytic fungus was grown in presence of 5-azacytidine (Vasanthakumari *et al*., 2015). On the contrary, when compared to the control condition, the production of several SMs, identified tentatively as monascuspyrone, pleurotin, roseotoxin B and 12-hydroxy-8,10-octadecadienoic acid, was lower in the presence of hydralazine. This effect has been also described in *A. fumigatus* where several compounds were produced in lower yields after the addition of valproic acid to the culture medium (Magotra *et al*., 2017), confirming that the epigenetic treatments with small-molecule elicitors can modulate the production of constitutive SMs, increasing or deceasing their yields. However, the increase in production titers of global SM profiles that was observed in some of the isolates was slightly higher when the elicitors were present since the inoculum stage, suggesting that the addition of elicitors in this step can improve the epigenetic modifier effects even further.

Most differential ions detected with the volcano-plot methodology were not found in the chemical dereplication databases and could not be identified. Some of these outlier ion masses were induced by the addition of hydralazine in both culture stages and regardless of the sub-culturing effects, being the four *m/z 171, 185-186, 225-226-227* and *243-244-245* the most statistically significant, later identified by UHPLC/HRMS-MS as $C_9H_6N_4$, $C_{10}H_8N_4$, $C_{13}H_{14}N_4$ and $C_{14}H_{16}N_2O_2$ (Chapter 4; Figure 6). Interestingly, the first three outlier molecules presented a molecular formula closely related to that of hydralazine ($C_8H_8N_4$) and could not be found in either public nor commercial NP databases. After scaling up and purification studies, HRMS/NMR results indicated that *m/z 171, m/z 185-186* and *m/z 227* ions corresponded to hydralazine biotransformation products occurring in the fermentation broth. However, once the induced compound with molecular formula $C_{14}H_{16}N_2O_2$ was purified, it was indeed identified as the diketopiperazine cyclo(phenylalanyl-prolyl) (Chapter 4; Figure 6-(12)); confirming the hypothesis that the

systematic addition of small-molecule epigenetic elicitors since the inocula stages of several fungi of the study, determined the activation of biosynthetic pathways otherwise silent when grown under standard conditions.

The different growth morphologies observed for the model strain of the study, *Dothiora* sp. CF-285353, ranging from the yellow hyphal growth to the black conidia growth, and the availability of the metabolomic tools implemented for the evaluation of the epigenetic modifiers, also permitted to perform the comparison of the SM profiles generated by the strain in its different morphologies. The purpose of these studies was to identify potential morphology biomarkers that could help to monitor and fine-tune future scale-ups of *Dothiora* sp. when specific morphologic conditions may be required to produce certain SMs. As a proof of concept, a representative population of extracts (n=8) from CF-285353, that presented a yellow hyphal morphology, was compared with a similar population of extracts (n=8), that presented a black conidial morphology. Clear outlier populations of ions were observed for each condition, highlighting several SM pathways differentially expressed according to the fermentation morphology presented by the fungus. The significant outlier ion masses most abundant in the hyphal morphology corresponded to the primary metabolite adenosine. On the contrary, in the case of the conidial morphology, the significant differential ion masses corresponded to the mycosporin glutamicol-5'-O-β-D-glucopyranoside. Both detected molecules have been reported in literature as related to these morphology growth stages of other fungi (Leung *et al*., 2006; Young and Patterson, 1982). These results also corroborate that the volcano-plot methodology is a suitable approach for a successful identification of metabolites produced differentially among pairs of fermentation conditions and therefore represents a fast and robust procedure for the identification of fermentation biomarkers (Chapter 4; Figure 7).

Microorganisms are rarely encountered as single species populations in the environment, where they establish a large variety of interactions that can facilitate their cohabitation, such as mutualistic, endosymbiotic, antagonistic, pathogenic and parasitic relationships (Braga *et al*., 2016). Microbial interactions with different combinations of microorganisms have been extensively reported and have shown to modulate fungal and bacterial metabolism expression, sometimes inducing the production of new bioactive molecules (Brakhage *et al*., 2008; Brizuela *et al*., 1998, Combès *et al*., 2012; Arora *et al*., 2018). Microorganisms capable of inducing an antagonistic interaction in presence of other microorganisms can promote changes in their morphology and in their production of metabolites and/or enzymes (Hynes *et al*., 2007). This was the main reason why the **co-culture approach** was herein tested as a third way of inducing cryptic metabolic pathways when interacting between the isolates from plants of arid zones of Andalusia. For this purpose, a screening was performed to look for antagonistic reactions among the population of isolates by co-culturing the strains on agar plates in pairs.

Interestingly, during the co-culturing screening, a clear antagonistic effect was observed between two endophytic isolates (*Dothiora* sp. CF-279759 and *Bacillus* sp. CB-293420) obtained from a *Retama sphaerocarpa* Mediterranean shrub, with the semi-parasite *Hypoxylon mediterraneum* CF-279764, also isolated from the same plant. This phytopathogen frequently attacks cork oaks, and it has been transmitted for many years by the tools needed for cork extraction. Its mycelium can live within the plant without causing decline symptoms for several years, increasing the probability of isolating this phytopathogen as an endophyte (Oliva and Molinas, 1984) as it was present among the isolates of this study. The endophytic strain *Dothiora* sp. is a dimorphic fungus that has recently been described as producer of new cytotoxic hormonemate compounds in monoculture (Perez-Bonilla *et al*., 2017). In addition, several *Bacillus* species have been described as producers of antifungal and cytotoxic compounds in monocultures and cocultures (Si *et al*., 2016). Microbial communities have a major influence on their immediate environment, microbiome or host (Beltrand *et al*., 2014), hence this kind of endophytes could produce interesting SMs when co-cultured with their antagonistic cohabitants.

Previous studies have reported that co-culture induced compounds (endophyte *vs* phytopathogen) were dependent on the presence of the phytopathogen and were spatially localized only in the interaction zone (Tata *et al*., 2015). In this sense, axenic monocultures and co-cultures of the three strains were performed in order to determine if these antagonistic effects observed between the isolates could be induced by co-culturing and if cryptic antifungal compounds were accumulating spatially in the interaction zone. For this purpose, an automated extraction method was designed and developed to deconvolute the co-cultures in 80 agar portions corresponding to 80 microplate wells (Chapter 5; Figure 3), which allowed the co-culture extracts to be directly analysed by HPLC-UV-MS equipment, maintaining the co-culture spatial references.

Image Mass Spectrometry (IMS) is a tool to visualize the spatial distribution of metabolites and can be used to identify induced SMs upon microbial interactions. This tool requires the use of very expensive or innovative ionization front-ends in the Mass Spectrometer (MS) (i.e. MALDI or nano-DESI). Most of the common mass spectrometers for NPs are electrospray units that cover mass ranges from 150-1500 Daltons, also suitable for NP extracts. Recently, the MASS-Studio 1.0 software tool for high-throughput analysis of batches of samples analysed by LC-ESI-MS equipment had just been developed (Martinez *et al*., 2017). For the study of these selected antagonistic strains the previously described for UV data HPLC-Studio 2.0 (Garcia and Tormo, 2003) and the new MASS-Studio 1.0 (Martinez *et al*., 2017) for MS data, utilities were combined to generate ultraviolet and mass images respectively from the UV-LC-ESI-MS analyses of the co-cultures.

General Discussion

In the case of the co-culture of *H. mediterraneum* with *Bacillus* sp., twelve known compounds with antimicrobial activities were identified and dereplicated as possibly responsible for the observed antagonistic interaction (Chapter 5; Figure 5d). These compounds belong to three main families of antimicrobials produced by *Bacillus* sp.: surfactins, iturins and mycosubtilins (Peiris *et al*., 2008; Rodriguez-Estrada *et al*., 2011). Other accumulated compounds were also found to be produced by *H. mediterraneum* in response to this antimicrobial attack as they were not observed when the strain was grown axenically, in agreement with a selective production of SMs as a signalling response or as a defence mechanism.

The first spatial mapping obtained by the different UV traces produced by axenic cultures and co-cultures of *Dothiora* sp. *vs H. mediterraneum,* showed that many SMs were produced constitutively by the strains and, more interestingly, highlighted a few that were produced only during their interaction (Chapter 5; Figure 4). After IMS analysis, where a more detailed picture of the interaction could be obtained, several compounds, not produced in the axenic cultures, showed a spatial correlation with the antimicrobial interaction area generated in their co-cultures (Chapter 5; Figure 5b). In detail, the localization of $C_{22}H_{33}N_3O_5$ in the inhibition zone, a compound with no match in the Dictionary of NPs (DNP) databases, suggested that this could be a new molecule involved in the interaction between both fungi (Chapter 5; Figure 6). This fact supports the hypothesis that some SMs are only produced by the fungal isolates when interacting with other microbes, and, in fact, that the application of this approach can speed up the discovery of new induced NPs.

Overall, this work represents an in-depth study on the importance of applying different methodologies to exploit the hidden richness on the production of SMs in microbial isolates. An exhaustive effort has been made to survey a large number of halophytic and xerophytic plant specimens from arid areas of Andalusia, **isolating and characterizing** their **culturable fungal endophyte community**. **Different microbial culture approaches** have been applied on this fungal collection to explore and **promote** its capacity to activate **silent pathways** and **generate** potentially **new SMs** as well as **their antifungal and antitumoral properties. Metabolomic studies** have also been developed to **characterize this potential chemical diversity**. Finally, to reach the aims of this thesis, some antifungal or antitumoral potentially **new natural products**, have been **identified**, **purified** and **elucidated**. Further efforts will continue to prove the high value of biodiversity in these arid areas of Andalusia (Spain) with the isolation of additional new bioactive SMs produced by this chemically rich and biodiverse fungal community.

REFERENCES

- Albright, J.C., Henke, M.T., Soukup, A.A., McClure, R.A., Thomson, R.J., Keller, N.P., Kelleher, N.L. (2015). Large-scale metabolomics reveals a complex response of *Aspergillus nidulans* to epigenetic perturbation ACS Chem. Biol.10:1535-1541.

- Andersen, B., Dongo, A., Pryor, B.M. (2008). Secondary metabolite profiling of *Alternaria dauci, A. porri, A. solani, and A. tomatophila*. Mycol Res 112:241-250.

- Andolfi, A., Mugnai, L., Luque, J., Surico, G., Cimmino, A., Evidente, A. (2011). Phytotoxins produced by fungi associated with grapevine trunk diseases. Toxins. 3:1569–1605. doi: 10.3390/toxins3121569

- Arora, D., Chashoo, G., Singamaneni, V., Sharma, N., Gupta, P., Jaglan, S. (2018). *Bacillus amyloliquefaciens* induces production of a novel blennolide K in coculture of Setophoma terrestris. J Appl Microbiol. 124(3):730-739. doi: 10.1111/jam.13683

- Asai, T., Morita, S., Taniguchi, T., Monde, K., Oshima, Y. (2016). Epigenetic stimulation of polyketide production in *Chaetomium cancroideum* by an NAD(+)-dependent HDAC inhibitor. Org. Biomol. Chem. 14: 646–651.

- Bertrand, S., Bohni N., Schnee, S., Schumpp, O., Gindro, K., Wolfender, J.-L. (2014). Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. Biotechnol. Adv. 32 1180–1204. doi: 10.1016/j.biotechadv.2014.03.001.

- Bills, G. (1996). In Endophytic Fungi in Grasses and Woody Plants (ed Redlin S. C. ans Carris L. M) 31–65 (APS Press).

- Bills, G., González-Menéndez, V., Platas, G. (2012). *Kabatiella bupleuri* sp. nov. (Dothideales), a pleomorphic epiphyte and endophyte of the Mediterranean plant *Bupleurum gibraltarium* (Apiaceae). Mycologia. 104(4):962-973. doi: 10.3852/12-003.

- Bills, G., Platas, G., Fillola, A., Jiménez, M.R., Collado, J., Vicente, F., Martín, J., González, A.,Bur-Zimmermann, J., Tormo, J.R., and Peláez, F. (2008). Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. J Appl Microbiol. 104:1644-1658.

- Braga, R. M., Dourado, M. N., & Araújo, W. L. (2016). Microbial interactions: ecology in a molecular perspective. Braz J Microbiol. 47:86-98. doi: 10.1016/j.bjm.2016.10.005.

- Brakhage, A. A., Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., Hertweck, C. (2008). “Activation of fungal silent gene clusters: a new avenue to drug discovery,”

in Natural Compounds as Drugs, eds Petersen F., Amstutz R., editors. (Basel: Birkhäuser). 1–12.

- Brizuela, M. A., García, L., Pérez, L., Mansur, M. (1998). Basidiomicetos: nueva fuente de metabolitos secundarios. Rev. Iberoam. Micol. 15: 69–74.

- Bu'Lock, J.D., Darbyshire, J. (1976). Lagopodin metabolites and artifacts in cultures of *Coprinus*. Phytochemistry 15:2004.

- Chen, H.J., Awakawa, T., Sun, J.Y., Wakimoto, T., Abe, I. (2013). Epigenetic modifier-induced biosynthesis of novel fusaric acid derivatives in endophytic fungi from Datura stramonium L. Nat. Prod. Bioprospect. 3:20-23.

- Combès, A., Ndoye, I., Bance, C., Bruzaud, J., Djediat, C., Dupont, J., Nay, B., Prado, S. (2012). Chemical communication between the endophytic fungus *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum*. PLoS ONE 7: e47313. doi: 10.1371/journal.pone.0047313.

- Covington, B. C., McLean, J. A., & Bachmann, B. O. (2017). Comparative mass spectrometry-based metabolomics strategies for the investigation of microbial secondary metabolites. Nat Prod Rep. 34(1), 6–24. doi: 10.1039/c6np00048g

- Crous, P.W., Wingfield, M.J., Schumacher, R.K., Summerell, B.A., Giraldo, A., Gené, J., Guarro, J., Wanasinghe, D.N., Hyde, K.D., Camporesi, E., Gareth Jones, E.B., Thambugala, K.M., Malysheva, E.F., Malysheva, V.F., Acharya, K., Álvarez, J., Alvarado, P., Assefa, A., Barnes, C.W., Bartlett, J.S., Blanchette, R.A., Burgess, T.I., Carlavilla, J.R., Coetzee, M.P., Damm, U., Decock, C.A., den Breeÿen, A., de Vries, B., Dutta, A.K., Holdom, D.G., Rooney-Latham, S., Manjón, J.L., Marincowitz, S., Mirabolfathy, M., Moreno, G., Nakashima, C., Papizadeh, M., Shahzadeh Fazeli, S.A., Amoozegar, M.A., Romberg, M.K., Shivas, R.G., Stalpers, J.A., Stielow, B., Stukely, M.J., Swart, W.J., Tan, Y.P., van der Bank, M., Wood, A.R., Zhang, Y., Groenewald, J.Z. (2014). Fungal Planet description sheets: 281–319. Persoonia. 33:212–289. doi: 10.3767/003158514X685680.

- De la Cruz, M., Martín, J., González-Menéndez, V., Pérez-Victoria, I., Moreno, C., Tormo, J.R., El Aouad, N., Guarro, J., Vicente, F., Reyes, F., Bills, G. (2012). Chemical and physical modulation of antibiotic activity in *Emericella species*. Chem Biodivers. 9:1095-1113.

- Deepika, V.B., Murali, T.S., Satyamoorthy, K. (2016). Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review. Microbiol Res. 182:125-140. doi: 10.1016/j.micres.2015.10.009

- Devarajan, P.T., Suryanarayanan, T.S. (2006). Evidence for the role of phytophagous insects in dispersal of non-grass fungal endophytes. Fungal Diversity 23: 111-119.
- Frykman, S., Tsuruta, H., Galazzo, J., Licari, P. (2006). Characterization of product capture resin during microbial cultivations. J Ind Microbiol Biotechnol. 33:445-453. doi: 10.1007/s10295-006-0088-1.
- García, J.B., Tormo, J.R. (2003). HPLC studio: a novel software utility to perform HPLC chromatogram comparison for screening purposes. J. Biomol Screen. 8:305-315.
- Grijseels, S. *et al*. (2017). in 29th Fungal genetics conference Genetics Society of America. Vol. 29 (Genetics Society of America, Pacific Grove, CA)
- Hensens, O.D., Helms, G.L., Jones, E.T.T., Harris, G.H. (1995). Structure elucidation of australifungin, a potent inhibitor of sphinganine N-acyltransferase in sphingolipid biosynthesis from Sporormiella australis. J Org Chem. 60:1772-1776.
- Hynes, J., Müller, C. T., Jones, T. H., Boddy, L. (2007). Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. J. Chem. Ecol. 33 43–57. doi: 10.1007/s10886-006-9209-6.
- Igarashi, Y., Iida T, Oku, N., Watanabe, H., Furihata, K., Miyanouchi, K. (2012). Nomimicin, a new spirotetronate-class polyketide from an actinomycete of the genus *Actinomadura*. J Antibiot. 65:355-359.
- Kim, W., Peever, T. L., Park, J.-J., Park, C.-M., Gang, D. R., Xian, Davidson, J.A., Infantino, A., Kaiser, W.J., Chen, W. (2016). Use of metabolomics for the chemotaxonomy of legume-associated *Ascochyta* and allied genera. Sci Rep. 6: 20192. doi: 10.1038/srep20192.
- Kohno, J., Sakurai, M., Kameda, N., Nishio, M., Kawano, K., Kishi, N., Okuda, T., Komatsubara, S. (1999). Production, isolation and biological properties of TMC-120A, B and C, novel inhibitors of eosinophil survival from *Aspergillus ustus* TC 1118. J Antibiot (Tokyo). 52:913-916. doi: 10.7164/antibiotics.52.913.
- Kruys, Å., Wedin, M. (2009). Phylogenetic relationships and an assessment of traditionally used taxonomic characters in the *Sporormiaceae* (*Pleosporales*, Dothideomycetes, Ascomycota), utilising multi-gene phylogenies. Syst Biodivers 7:465-478.
- Lazaro, R., Alexander, R, W., Puigdefabregas, J. (2000). Cover distributtion patterns of lichens, annuals and shrubs in the Tabernas Desert, Almeria, Spain. In R.W. Alexander & A.C. Millington (Eds.), Vegetation mapping: from patch to plantet. 2: 19-40

- Leung, P.H.; Zhang, Q.X.; Wu, J.Y. (2006). Mycelium cultivation, chemical composition and antitumour activity of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. J. Appl. Microbiol. 101: 275–283.

- Li, H., Qing, C., Zhang, Y., Zhao, Z. (2005). Screening for endophytic fungi with antitumour and antifungal activities from Chinese medicinal plants. World J Microbiol Biotechnol. 21: 1515-1519.

- Luo, Z.P., Lin, H.Y., Ding, W.B., He, H.L., Li, Y.Z. (2015). Phylogenetic Diversity and Antifungal Activity of Endophytic Fungi Associated with *Tephrosia purpurea*. Mycobiology. 43:435-443. doi: 10.5941/MYCO.2015.43.4.435.

- Magotra, A., Kumar, M., Kushwaha, M., Awasthi, P., Raina, C., Gupta, A. P., Shah, B.A., Gandhi, S.G., Chaubey, A. (2017). Epigenetic modifier induced enhancement of fumiquinazoline C production in *Aspergillus fumigatus* (GA-L7): an endophytic fungus from *Grewia asiatica* L. AMB Express. 7(1):43. doi:10.1186/s13568-017-0343-z

- Mandala, S.M., Thornton, R.A., Frommer, B.R., Curotto, J.E., Rozdilsky, W., Kurtz, M.B., Giacobbe, R.A., Bills, G.F., Cabello, M.A., Martín, I., Pelaez, F., Harris, G.H. (1995). The discovery of australifungin, a novel inhibitor of sphinganine N-acyltransferase from Sporormiella australis. Producing organism, fermentation, isolation, and biological activity. J Antibiot (Tokyo) 48(5):349-56.

- Mapperson, R.R., Kotiw, M., Davis, R.A., Dearnaley, J.D. (2014). The diversity and antimicrobial activity of *Preussia* sp. endophytes isolated from Australian dry rainforests. Curr Microbiol. 68(1):30-37. doi: 10.1007/s00284-013-0415-5.

- Marshall, V.P., McWethy, J.S., Sirotti, J.M., Cialdella, J.I. (1990). The effect of neutral resins on the fermentation production of rubradirin. J Ind Microbiol. 5:283-288.

- Marshall, V.P., McWethy, J.S., Visser, J., Cialdella, J.I., Laborde, A.L. (1987). Current fermentation technology from actinomycetes: the example of paulomycin. Dev Ind Microbiol.28:105–114.

- Martínez, G., González-Menéndez, V., Martín, J., Reyes, F., Genilloud, O., Tormo, J.R. (2017). MASS-Studio: A Novel Software Utility to Simplify LC-MS Analyses of Large Sets of Samples for Metabolomics. In: Rojas I, Ortuño F, editors. Proceedings Bioinformatics and Biomedical Engineering. Basel: Springer International Publishing .230-244.

- Massimo, N. C., Nandi Devan, M., Arendt, K. R., Wilch, M. H., Riddle, J. M., Furr, S. H., Steen, C., U'Ren, J.M., Sandberg, D.C., Arnold, A. E. (2015). Fungal endophytes in

above-ground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. Microbial Ecology. 70(1):61-76. doi: 10.1007/s00248-014-0563.

- Mudur, S.V., Gloer, J.B., Wicklow, D.T. (2006). Sporminarins A and B: antifungal metabolites from a fungicolous isolate of *Sporormiella minimoides*. J Antibiot. 59:500-506.

- Newcombe, G., Campbell, J., Griffith, D., Baynes, M., Launchbaugh, K., Pendleton, R. (2016). Revisiting the life cycle of dung fungi, including *Sordaria fimicola*. PLoS ONE, 11: e0147425.

- Oliva Estanyol, M., Molinas de Ferrer, M.L. (1984) Incidencia de *Hypoxylon mediterraneum* en los alcornocales gerundenses. Bol. Sts. C. de Ecol. 13 (25): 9-16

- Papizadeh, M., Wijayawardene, N.N., Amoozegar, M. A., Saba, F., Abolhassan, S., Fazeli, S. (2018). *Neocamarosporium jorjanensis*, *N. persepolisi*, and *N. solicola* spp. nov. (*Neocamarosporiaceae*, *Pleosporales*) isolated from saline lakes of Iran indicate the possible halotolerant nature for the genus. Mycological Progress.17;5:661-679

- Pelaez, F., Collado, J., Arenal, F., Basilio, A., Cabello, M.T., Diez Matas, J.B., Garcia, A., González Del Val, A., González, V., Gorrochategui, J., Hernández, P. Martín, I., Platas, G., Vicente, F. (1998). Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycol Res. 102; 6:755-761.

- Pérez-Bonilla, M., González-Menéndez, V., Pérez-Victoria, I., de Pedro, N., Martín, J., Molero-Mesa, J., Casares-Porcel, M., González-Tejero, M.R., Vicente, F., Genilloud, O., Tormo, J.R., Reyes, F. (2017). Hormonemate Derivatives from *Dothiora* sp., an Endophytic Fungus. J Nat Prod. 28;80(4):845-853. doi: 10.1021/acs.jnatprod.6b00680

- Peiris, D., Dunn, W.B., Brown, M., Kell, D.B., Roy, I., Hedger, J.N. (2008). Metabolite profiles of interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminates*. Metabolomics. 4:52-62.

- Phillips, T., Chase, M., Wagner, S., Renzi, C., Powell, M., DeAngelo, J., Michels, P. (2013). Use of in situ solid-phase adsorption in microbial natural product fermentation development. J Ind Microbiol Biotechnol. 40:411-425. doi:10.1007/s10295-013-1247-9.

- Porras-Alfaro, A., Herrera, J., Sinsabaugh, R.L., Odenbach, K.J., Lowrey, T., Natvig, D.O. (2008). Novel root fungal consortium associated with a dominant desert grass. Appl Environ Microbiol 74:2805-2813.

General Discussion

- Ratnaweera, P.B., de Silva, E.D., Williams, D.E., Andersen, R.J. (2015). Antimicrobial activities of endophytic fungi obtained from the arid zone invasive plant *Opuntia dillenii* and the isolation of equisetin, from endophytic *Fusarium* sp. BMC Complement Altern Med. 15:220. doi: 10.1186/s12906-015-0722-4.

- Rodriguez-Estrada, A.E., Hegeman, A., Kistler, H.C., May, G. (2011). In vitro interactions between *Fusarium verticillioides* and *Ustilago maydis* through real-time PCR and metabolic profiling. Fungal Genet Biol. 48:874-85.

- Scherlach, K., Partida-Martinez, L.P., Dahse, H.M., Hertweck, C. (2006). Antimitotic rhizoxin derivatives from a cultured bacterial endosymbiont of the rice pathogenic fungus *Rhizopus microsporus*. J Am Chem Soc.128:11529-11536.

- Serrano, R., González-Menéndez, V., Rodríguez, L., Martín, J., Tormo, J. R., & Genilloud, O. (2017). Co-culturing of Fungal Strains Against *Botrytis cinerea* as a Model for the Induction of Chemical Diversity and Therapeutic Agents. Front Microbiol. 8: 649. doi: 10.3389/fmicb.2017.00649

- Shiono, Y., Murayama, T., Takahashi, K., Okada, K., Katohda, S., Ikeda, M. (2005). Three oxygenated cyclohexenone derivatives produced by an endophytic fungus. Biosci Biotechnol Biochem. 69:287–292. doi: 10.1271/bbb.69.287.

- Si, T., Li, B., Zhang, K., Xu, Y., Zhao, H., Sweedler, J. V. (2016). Characterization of *Bacillus subtilis* colony biofilms via mass spectrometry and fluorescence imaging. J. Proteome Res. 15:1955-1962. doi:10.1021/acs.jproteome.6b00127.

- Stadler, M. (2011). Importance of secondary metabolites in the Xylariaceae as parameters for assessment of their taxonomy, phylogeny, and functional biodiversity. Curr Res Environ Appl Mycol 1:75-133.

- Stadler, M., Læssøe, T., Fournier, J., Decock, C., Schmieschek, B., Tichy, H-V., Peršoh, D. (2014). A polyphasic taxonomy of *Daldinia* (*Xylariaceae*). Stud Mycol 77:1-143.

- Stanbury, P.F., Whitakerm, A., Hall, S.J. (1997). The isolation, preservation and improvement of industrially important microorganisms. In Principles of Fermentation Technology, 2nd ed.; Elsevier Science: Kidlington, UK, 35-85.

- Sunassee, S.N., Davies-Coleman, M.T. (2012). Cytotoxic and antioxidant marine prenylated quinones and hydroquinones. Nat Prod Rep. 1;29(5):513-35. doi: 10.1039/c2np00086e.

- Takesako, K., Saito, H., Ueno, M., Awazu, N., Kato, I. (1998). Antibiotic TKR2648 and process for producing the same. (Google Patents).

- Tata, A., Perez, C., Campos, M.L., Bayfield, M.A., Eberlin, M.N., Ifa, D.R. (2015). Imprint Desorption Electrospray Ionization Mass Spectrometry Imaging for Monitoring Secondary Metabolites Production during Antagonistic Interaction of Fungi. Anal Chem. 15;87(24):12298-12305. doi: 10.1021/acs.analchem.5b03614

- Trouillas, F.P., Urbez-Torres, J.R., Gubler, W.D. (2010). Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. Mycologia. 102:319-336. doi: 10.3852/08-185

- Vasanthakumari, M.M., Jadhav, S.S., Sachin, N., Vinod, G., Shweta, S., Manjunatha, B.L., Kumara, P.M., Ravikanth, G., Nataraja, K.N., Uma Shaanker, R. (2015). Restoration of camptothecine production in attenuated endophytic fungus on re-inoculation into host plant and treatment with DNA methyltransferase inhibitor. World J Microbiol Biotechnol.31(10):1629-1639. doi: 10.1007/s11274-015-1916-0.

- Venugopalan, A., Potunuru, U.R., Dixit, M., Srivastava, S. (2016). Reprint of: Effect of fermentation parameters, elicitors and precursors on camptothecin production from the endophyte *Fusarium solani*. Bioresour Technol. 213:311-318. doi: 10.1016/j.biortech.2016.05.023.

- Vervoort, H.C., Drašković, M., Crews, P. (2011). Histone Deacetylase Inhibitors as a Tool to Up-regulate New Fungal Biosynthetic Products—Isolation of EGM-556, a Cyclodepsipeptide, from *Microascus* sp. Org. Lett. 13, 410–413.

- Woo, E.J., Starks, C.M., Carney, J.R., Arslanian, R., Cadapan, L., Zavala, S., Licari, P. (2002). Migrastatin and a new compound, isomigrastatin, from Streptomyces platensis. J Antibiot. 55:141-146.

- Xiong, H., Xiao, G.K., Chen, G.D., Chen, H.R., Hu, D., Li, X.X., Zhong, S.W., Guo, L.D., Yao, X.S., Gao, H. (2014). Sporormiellin A, the first tetrahydrofuran-fused furochromone with an unprecedented tetracyclic skeleton from *Sporormiella minima*. RSC Adv. 46:24295-24299.

- Young, V.J., Patterson, A. (1982). UV protective compound from *Glomerella cingulate* a mycosporine. Phytochemistry. 21: 1075–1077.

- Zaferanloo, B., Bhattacharjee, S., Ghorbani, M.M., Mahon, P.J., Palombo, E.A. (2014). Amylase production by Preussia minima, a fungus of endophytic origin: optimization of fermentation conditions and analysis of fungal secretome by LC-MS.BMC Microbiol. 7; 14:55. doi: 10.1186/1471-2180-14-55.

- Zhang, F., Li, L., Niu, S., Si, Y., Guo, L., Jiang, X., Che, Y. (2012). A thiopyranchromenone and other chromone derivatives from an endolichenic fungus, *Preussia africana*. J Nat Prod. 75:230-237.

V. CONCLUSIONS

CONCLUSIONS

I. This is the first study culturing and characterizing the fungal symbiont community of an extensive number of halophytic and xerophytic plant specimens from arid zones of southern Europe (Andalusia). A total of 349 fungal endophytes were isolated and characterized from 63 plants surveyed.

II. The phylogenetic analyses by ITS_1-5.8S-ITS_2 and partial 28S rDNA revealed 19 taxonomic orders including Basidiomycetes and Ascomycetes from the 63 plant specimens, where *Pleosporales* resulted the dominant ones.

III. A detailed study of the *Pleosporales* isolates identified *Neocamarosporium* and *Preussia* as the most frequently encountered genera. Seven clades containing *Neocamaroporium* isolates could represent previously undescribed species. In addition, 19 different *Preussia* species were identified from the phylogenetic and morphological analyses, where *Preussia lignicola* was reported for the first time in the Iberian Peninsula.

IV. Among the eleven different chemotypes found for the 22 *Preussia* species studied, eleven secondary metabolites, four of them tentatively new molecules, were identified as taxonomic biomarkers of six of the species, supporting the fact that secondary metabolite characterization is a useful tool for taxonomic descriptions.

V. The addition of adsorptive polymeric resins in the fermentations of the fungal endophytes from arid areas of Andalusia, promoted more unique secondary metabolites (12 known and 12 unknown) than fermentations without resins (11 Known and 1 unknown), with exclusive biological activities when the isolates were grown both in presence and in absence of the resins.

VI. The addition of resins improved the chemical diversity when added to fermentation media where fungi produced low chemical diversity without resins, such as SCAS and YES. Whereas, they did not produce such an effect when added to fermentation media where fungi generated a higher chemical diversity in absence of resins, such as LSFM, MV8 and MMK2.

VII. New compounds MDN-0209, MDN-0210 and MDN-0211, induced by the addition of resins in the fermentation of *Eutypa consobrina* CF-090213, were identified, purified and characterized chemically and biologically, confirming they can promote cryptic biosynthetic pathways during fungal fermentation processes, that could lead to the discovery of new active natural products.

Conclusions

VIII. The systematic addition of small-molecule epigenetic elicitors since the inocula stages of several fungi of the study, determined the activation of biosynthetic pathways otherwise silent when grown under standard conditions. The increase in production titers of global SM profiles that was observed for some of the strains, was slightly higher when the elicitors were present since the inoculum stage.

IX. The use of multiple chemical elicitors was necessary to improve the metabolic expression of the diverse fungal population of the study. However, SBHA was the elicitor that promoted changes in morphology and/or production titers for a higher number of the isolates.

X. Three new biotransformation products of hydralazine and the diketopiperazine cyclo(phenylalanyl-prolyl), induced by the addition of hydralazine in the fermentation of *Dothiora* sp. CF-285353, were identified, purified and characterized chemically, confirming they can promote cryptic biosynthetic pathways during fungal fermentation processes. This fact could lead to the discovery of new active natural products.

XI. The volcano-plot methodology proved to be a precise approach to identify and quantify metabolites produced differentially among two fermentation conditions and therefore represents a fast and robust procedure for the identification of condition-dependent fermentation biomarkers.

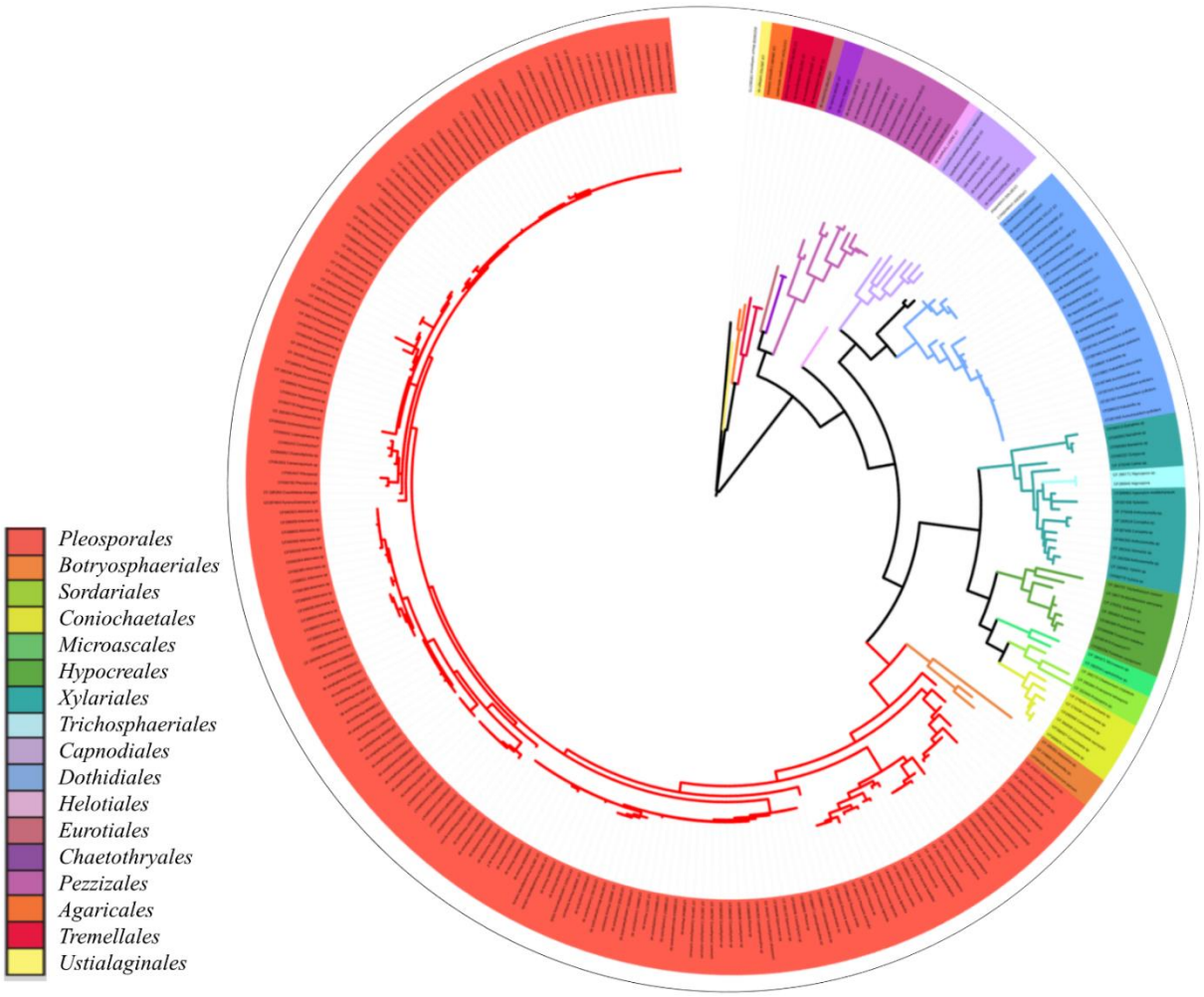
XII. Following an extensive co-culturing screening to simulate microbial interactions within the endophytes of the study, two clear microbial antagonisms for *Dothiora* sp. and *Bacillus* sp. were found against the cohabitant phytopathogen *Hypoxylon mediterraneum*.

XIII. Spatial location of secondary metabolites by Ultra Violet (IUV) and Mass Spectrometry Imaging (IMS) resulted to be an effective tool for the identification of the secondary metabolites involved in these antagonistic interactions. The new natural product $C_{22}H_{33}N_3O_5$ (*m/z* 420.2509), induced by the co-culture of *Dothiora* sp. CF-279723 and *Hypoxylon mediterraneum* CF-279764, was identified spatially by IMS, confirming that microbial interactions can promote cryptic biosynthetic pathways.

XIV. The evaluation of the antifungal and antitumoral activities of the fungal isolates showed hit-rates reaching 17.5% against a human pathogen, 11% against a plant pathogen and 20% *versus* a cancer cell line, confirming the microbial richness of arid areas of Andalusia as a valuable source for the discovery of new potentially bioactive natural products.

VI. APPENDIX

APENDIX CHAPTER 1: Fungal endophytes from arid areas of Andalusia: high potential sources for antifungal and antitumoral agents



Supplementary Figure 1. Neighbor-joining tree based on nucleotide sequences of 28S rDNA gene of the 349 fungal isolates. Major taxonomic orders are labelled using differential color coding.

Apendix

Supplementary Table 1. Total list of fungal isolates sort by genus.

Isolate	Genus	Species	Order	Substrate
CF-288903	*Alternaria*	*alternata*	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090318	*Alternaria*	*alternata*	*Pleosporales*	*Artemisia barrelieri*
CF-090322	*Alternaria*	*alternata*	*Pleosporales*	*Artemisia barrelieri*
CF-090366	*Alternaria*	*cumini*	*Pleosporales*	*Sedum sediforme*
CF-090246	*Alternaria*	*infectoria*	*Pleosporales*	*Genista umbellata*
CF-090261	*Alternaria*	*infectoria*	*Pleosporales*	*Asparagus horridus*
CF-282008	*Alternaria*	sp.	*Pleosporales*	*Retama sphaerocarpa*
CF-288921	*Alternaria*	sp.	*Pleosporales*	*Ononis tridentata*
CF-288922	*Alternaria*	sp.	*Pleosporales*	*Ononis tridentata*
CF-288909	*Alternaria*	sp.	*Pleosporales*	*Frankenia pulvirulenta*
CF-288960	*Alternaria*	sp.	*Pleosporales*	*Frankenia pulvirulenta*
CF-288961	*Alternaria*	sp.	*Pleosporales*	*Sonchus crassifolius*
CF-288939	*Alternaria*	sp.	*Pleosporales*	*Suaeda vera*
CF-288904	*Alternaria*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090752	*Alternaria*	sp.	*Pleosporales*	*Thymelaea hirsuta*
CF-090365	*Alternaria*	sp.	*Pleosporales*	*Sedum sediforme*
CF-090395	*Alternaria*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090254	*Alternaria*	sp.	*Pleosporales*	*Asparagus horridus*
CF-282338	*Anthostomella*	sp.	*Xylariales*	*Dittrichia viscosa*
CF-090350	*Anthostomella*	sp.	*Xylariales*	*Pinus halepenssis*
CF-279248	*Anthostomella*	sp.	*Xylariales*	*Retama sphaerocarpa*
CF-287454	*Ascochyta*	sp	*Pleosporales*	*Rosmarinus eriocalyx*
CF-287439	*Ascochyta*	sp.	*Pleosporales*	*Ononis fruticosa*
CF-282337	*Ascochyta*	sp.	*Pleosporales*	*Dittrichia viscosa*
CF-090376	*Ascochyta*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-090372	*Ascochyta*	sp.	*Pleosporales*	*Sedum sediforme*
CF-285758	*Ascochyta*	sp.	*Pleosporales*	*Frankenia corymbosa*
CF-285355	*Ascochyta*	sp.	*Pleosporales*	*Asparagus horridus*
CF-285366	*Ascochyta*	sp.	*Pleosporales*	*Rosmarinus eriocalyx*
CF-287450	*Ascochyta*	sp.	*Pleosporales*	*Rosmarinus eriocalyx*
CF-287451	*Ascochyta*	sp.	*Pleosporales*	*Rosmarinus eriocalyx*
CF-090321	*Ascochyta*	sp.	*Pleosporales*	*Artemisia barrelieri*
CF-090240	*Ascochyta*	sp.	*Pleosporales*	*Genista umbellata*
CF-090840	*Ascochyta*	sp.	*Pleosporales*	*Launaea arborescens*
CF-091945	*Ascochyta*	sp.	*Pleosporales*	*Launaea arborescens*
CF-091946	*Ascochyta*	sp.	*Pleosporales*	*Lycium intricatum*
CF-090305	*Ascochyta*	sp.	*Pleosporales*	*Launaea arborescens*
CF-090252	*Ascochyta*	sp.	*Pleosporales*	*Genista umbellata*
CF-282009	*Ascorhizoctonia*	sp.	*Pezizales*	*Dittrichia viscosa*
CF-287465	*Aureobasidium*	*pullulans*	*Dothideales*	*Genista umbellata*
CF-287442	*Aureobasidium*	*pullulans*	*Dothideales*	*Ononis fruticosa*
CF-288910	*Aureobasidium*	*pullulans*	*Dothideales*	*Gypsophila tomentosa*
CF-090316	*Aureobasidium*	*pullulans*	*Dothideales*	*Artemisia barrelieri*
CF-285762	*Aureobasidium*	*pullulans subglaciale*	*Dothideales*	*Limbarda crithmoides*
CF-287440	*Aureobasidium*	sp.	*Dothideales*	*Ononis fruticosa*
CF-090333	*Aureobasidium*	sp.	*Dothideales*	*Anthyllis cystisoides*
CF-090336	*Bactrodesmium*	sp.	*Incertae sedis*	*Anthyllis cystisoides*
CF-090812	*Bartalinia*	sp.	*Xylariales*	*Limonium insigne*
CF-285376	*Basifimbria*	sp.	*Xylariales*	*Launaea arborescens*
CF-288963	*Biscogniauxia*	*mediterranea*	*Xylariales*	*Tamarix canariensis*
CF-285367	*Boubovia*	sp.	*Pezizales*	*Anthyllis terniflora*
CF-279249	*Cainia*	sp.	*Xylariales*	*Retama sphaerocarpa*
CF-090379	*Camarosporium*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-090387	*Camarosporium*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-285350	*Camarosporium*	sp.	*Pleosporales*	*Euzomodendron bourgeanum*
CF-090314	*Camarosporium*	sp.	*Pleosporales*	*Artemisia barrelieri*

CF-090242	*Camarosporium*	sp.	*Pleosporales*	*Genista umbellata*
CF-285770	*Chaetomium*	*crispatum*	*Sordariales*	*Macrosyringion longiflorum*
CF-287476	*Chaetomium*	sp.	*Sordariales*	*Centaurea dracunculifolia*
CF-286679	*Chaetomium*	sp.	*Sordariales*	*Zygophylum fabago*
CF-286682	*Chaetothyriales*	sp.	*Chaetothryriales*	*Thymelaea hirsuta*
CF-091931	*Chaetothyriales*	sp.	*Chaetothryriales*	*Tamarix canariensis*
CF-090214	*Coleophoma*	*cylindrospora*	*Pleosporales*	*Ziziphus lotus*
CF-282003	*Comoclathris*	sp.	*Pleosporales*	*Retama sphaerocarpa*
CF-091922	*Comoclathris*	sp.	*Pleosporales*	*Ziziphus lotus*
CF-090361	*Comoclathris*	sp.	*Pleosporales*	*Sedum sediforme*
CF-285379	*Comoclathris*	sp.	*Pleosporales*	*Fagonia cretica*
CF-287447	*Comoclathris*	sp.	*Pleosporales*	*Rosmarinus eriocalyx*
CF-090301	*Comoclathris*	sp.	*Pleosporales*	*Launaea arborescens*
CF-091944	*Comoclathris*	sp.	*Pleosporales*	*Launaea arborescens*
CF-090266	*Comoclathris*	sp.	*Pleosporales*	*Lygeum spartum*
CF-090267	*Comoclathris*	sp.	*Pleosporales*	*Lygeum spartum*
CF-090792	*Comoclathris*	sp.	*Pleosporales*	*Lygeum spartum*
CF-091934	*Comoclathris*	sp.	*Pleosporales*	*Lygeum spartum*
CF-090763	*Comoclathris*	sp.	*Pleosporales*	*Nerium oleander*
CF-090766	*Comoclathris*	sp.	*Pleosporales*	*Nerium oleander*
CF-282006	*Coniochaeta*	*saccardoi*	*Coniochaetales*	*Retama sphaerocarpa*
CF-288927	*Coniochaeta*	sp.	*Coniochaetales*	*Ononis tridentata*
CF-288956	*Coniochaeta*	sp.	*Coniochaetales*	*Thymus zygis subs gracilis*
CF-279239	*Coniochaeta*	sp.	*Coniochaetales*	*Retama sphaerocarpa*
CF-091953	*Coniothyrium*	sp.	*Pleosporales*	*Anthyllis cystisoides*
CF-090330	*Coniothyrium*	sp.	*Pleosporales*	*Cistus albidus*
CF-090362	*Coniothyrium*	sp.	*Pleosporales*	*Sedum sediforme*
CF-285356	*Coniothyrium*	sp.	*Pleosporales*	*Asparagus horridus*
CF-090243	*Coniothyrium*	sp.	*Pleosporales*	*Genista umbellata*
CF-090236	*Constantinomyces*	sp.	*Capnodiales*	*Suaeda vera*
CF-279244	*Coprinopsis*	*episcopalis*	*Agaricales*	*Retama sphaerocarpa*
CF-285465	*Coprinus*	*cinereus*	*Agaricales*	*Salsola genistoides*
CF-285748	*Filobasidium*	*chernovii*	*Tremellales*	*Beta macrocarpa*
CF-285760	*Cryptococcus*	sp.	*Tremellales*	*Genista umbellata*
CF-285750	*Cryptococcus*	sp.	*Tremellales*	*Centaurea dracunculifolia*
CF-285752	*Cryptococcus*	sp.	*Tremellales*	*Centaurea dracunculifolia*
CF-285749	*Paradendryphiella*	*salina*	*Pleosporales*	*Centaurea dracunculifolia*
CF-091936	*Alternaria*	*penicillata*	*Pleosporales*	*Limonium insigne*
CF-285362	*Dendryphion*	sp.	*Pleosporales*	*Asparagus horridus*
CF-288958	*Devriesia*	sp.	*Capnodiales*	*Whitania frutescens*
CF-091918	*Devriesia*	sp.	*Capnodiales*	*Thymelaea hirsuta*
CF-091923	*Devriesia*	sp.	*Capnodiales*	*Nerium oleander*
CF-090344	*Diaporthe*	sp	*Diaporthales*	*Onoris ramosissina*
CF-279247	*Dictyosporium*	sp.	*Pleosporales*	*Retama sphaerocarpa*
CF-288938	*Didymella*	sp.	*Pleosporales*	*Sonchus crassifolius*
CF-090355	*Didymella*	sp.	*Pleosporales*	*Sedum sediforme*
CF-288950	*Didymocytris*	sp.	*Pleosporales*	*Teucrium capitatum sbsp. gracillium*
CF-090768	*Dimorphosporicola*	sp.	*Pleosporales*	*Suaeda vera*
CF-091943	*Dimorphosporicola*	sp.	*Pleosporales*	*Suaeda vera*
CF-090383	*Dimorphosporicola*	*traganii*	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090749	*Diplodia*	sp.	*Botryosphaeriales*	*Thymelaea hirsuta*
CF-285354	*Dothiora*	sp.	*Dothideales*	*Moricandia foetida*
CF-285353	*Dothiora*	sp.	*Dothideales*	*Launaea arborescens*
CF-285777	*Dothiorella*	sp.	*Botryosphaeriales*	*Teucrium capitatum sbsp. gracillium*
CF-278800	*Dothiorella*	sp.	*Botryosphaeriales*	*Dittrichia viscosa*
CF-285759	*Entodesmium*	sp.	*Pleosporales*	*Genista umbellata*
CF-288946	*Epicoccum*	*nigrum*	*Pleosporales*	*Teucrium capitatum sbsp. gracillium*
CF-285743	*Epicoccum*	sp.	*Pleosporales*	*Atriplex glauca*
CF-287460	*Eurotium*	sp.	*Eurotiales*	*Launaea arborescens*

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CF-090213	*Eutypa*	*consobrina*	*Xylariales*	*Ziziphus lotus*
CF-090257	*Eutypa*	sp.	*Xylariales*	*Asparagus horridus*
CF-090782	*Foliophoma*	sp.	*Pleosporales*	*Asparagus horridus*
CF-090384	*Fusarium*	*equiseti*	*Hypocreales*	*Arthrocnemum macrostachyum*
CF-285462	*Fusarium*	*equiseti*	*Hypocreales*	*Salsola genistoides*
CF-288908	*Fusarium*	*redolens*	*Hypocreales*	*Frankenia pulvirulenta*
CF-285764	*Fusarium*	sp.	*Hypocreales*	*Limonium insigne*
CF-287474	*Fusarium*	sp.	*Hypocreales*	*Asparagus horridus*
CF-090256	*Fusarium*	sp.	*Hypocreales*	*Asparagus horridus*
CF-090320	*Hazslinszkyomyces*	sp.	*Pleosporales*	*Artemisia barrelieri*
CF-282335	*Homortomyces*	*tamaricis*	*Incertae sedis*	*Dittrichia viscosa*
CF-090351	*Hormonema*	*carpetanum*	*Dothideales*	*Sedum sediforme*
CF-091927	*Hormonema*	sp.	*Dothideales*	*Genista umbellata*
CF-091949	*Hormonema*	sp.	*Dothideales*	*Rhamnus lycioides*
CF-092177	*Hortaea*	*werneckii*	*Capnodiales*	*Arthrocnemum macrostachyum*
CF-090396	*Hortaea*	*werneckii*	*Capnodiales*	*Chamaerops humilis*
CF-288947	*Kabatiella*	sp.	*Dothideales*	*Teucrium capitatum sbsp. gracillium*
CF-285359	*Kabatiella*	sp.	*Dothideales*	*Asparagus horridus*
CF-091935	*Kabatiella*	sp.	*Dothideales*	*Limonium insigne*
CF-090299	*Kabatiella*	sp.	*Dothideales*	*Launaea arborescens*
CF-090814	*Kabatiella*	sp.	*Dothideales*	*Limonium insigne*
CF-091181	*Kalmusia*	sp.	*Pleosporales*	*Asparagus horridus*
CF-287452	*Keissieriella*	sp.	*Pleosporales*	*Rosmarinus eriocalyx*
CF-285360	*Knufia*	sp.	*Incertae sedis*	*Asparagus horridus*
CF-285361	*Knufia*	sp.	*Incertae sedis*	*Asparagus horridus*
CF-285464	*Lapidomyces*	sp.	*Capnodiales*	*Fagonia cretica*
CF-090797	*Lapidomyces*	sp.	*Capnodiales*	*Lygeum spartum*
CF-287444	*Lasiobolidium*	*orbiculoides*	*Pezizales*	*Otanthus maritimus*
CF-279246	*Lecythophora*	sp.	*Coniochaetales*	*Retama sphaerocarpa*
CF-090357	*Leptosphaeria*	*hispanica*	*Pleosporales*	*Sedum sediforme*
CF-279243	*Leptospora*	sp.	*Incertae sedis*	*Retama sphaerocarpa*
CF-090382	*Libertasomyces*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-092175	*Libertasomyces*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-092176	*Libertasomyces*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-090297	*Lophiostoma*	sp	*Pleosporales*	*Thymus hyemalis*
CF-090828	*Lophiostoma*	sp.	*Pleosporales*	*Thymus hyemalis*
CF-282004	*Lophotrichus*	sp.	*Microascales*	*Retama sphaerocarpa*
CF-285351	*Masarina*	sp.	*Botryosphaeriales*	*Ziziphus lotus*
CF-285371	*Microascus*	*trigonosporus*	*Microascales*	*Anabasis articulata*
CF-279241	*Coniothyrium*	*olivaceum*	*Pleosporales*	*Retama sphaerocarpa*
CF-090371	*Morinia*	*pestalozzioides*	*Amphisphaeriales*	*Sedum sediforme*
CF-090313	*Morinia*	sp.	*Xylariales*	*Artemisia barrelieri*
CF-285778	*Albifimbria*	*verrucaria*	*Hypocreales*	*Thymus hyemalis*
CF-282342	*Nemania*	sp.	*Xylariales*	*Dittrichia viscosa*
CF-285348	*Neocamarosporium*	*chicastinanum*	*Pleosporales*	*Salsola papollosa*
CF-285352	*Neocamarosporium*	sp.	*Pleosporales*	*Atriplex glauca*
CF-285744	*Neocamarosporium*	sp.	*Pleosporales*	*Atriplex glauca*
CF-285745	*Neocamarosporium*	sp.	*Pleosporales*	*Atriplex glauca*
CF-285746	*Neocamarosporium*	sp.	*Pleosporales*	*Atriplex glauca*
CF-285747	*Neocamarosporium*	sp.	*Pleosporales*	*Beta macrocarpa*
CF-288928	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola vermiculata*
CF-288930	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola vermiculata*
CF-288932	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola vermiculata*
CF-288934	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola vermiculata*
CF-285775	*Neocamarosporium*	sp.	*Pleosporales*	*Suaeda vera*
CF-288962	*Neocamarosporium*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090335	*Neocamarosporium*	sp.	*Pleosporales*	*Anthyllis cystisoides*
CF-090390	*Neocamarosporium*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090392	*Neocamarosporium*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*

CF-090393	*Neocamarosporium*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090394	*Neocamarosporium*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090399	*Neocamarosporium*	sp.	*Pleosporales*	*Chamaerops humilis*
CF-285349	*Neocamarosporium*	sp.	*Pleosporales*	*Anabasis articulata*
CF-285368	*Neocamarosporium*	sp.	*Pleosporales*	*Anabasis articulata*
CF-285372	*Neocamarosporium*	sp.	*Pleosporales*	*Anabasis articulata*
CF-285373	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola papillosa*
CF-285374	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola papillosa*
CF-285377	*Neocamarosporium*	sp.	*Pleosporales*	*Suaeda vera*
CF-090284	*Neocamarosporium*	sp.	*Pleosporales*	*Salicornia ramossissima*
CF-090228	*Neocamarosporium*	sp.	*Pleosporales*	*Suaeda vera*
CF-090759	*Neocamarosporium*	sp.	*Pleosporales*	*Nerium oleander*
CF-090841	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola genistoides*
CF-090842	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola genistoides*
CF-090843	*Neocamarosporium*	sp.	*Pleosporales*	*Salsola genistoides*
CF-090227	*Neocamarosporium*	sp.	*Pleosporales*	*Suaeda vera*
CF-090309	*Neocamarosporium*	sp.	*Pleosporales*	*Suaeda vera*
CF-090312	*Neocamarosporium*	sp.	*Pleosporales*	*Suaeda vera*
CF-090773	*Neocamarosporium*	sp.	*Pleosporales*	*Genista umbellata*
CF-090824	*Neocamarosporium*	sp.	*Pleosporales*	*Thymus hyemalis*
CF-285768	*Neocamarosporium*	sp.	*Pleosporales*	*Lycium intricatum*
CF-287455	*Neocamarosporium*	sp.	*Pleosporales*	*Lycium intricatum*
CF-286681	*Neodidymelliopsis*	sp.	*Pleosporales*	*Cynanchum acutum*
CF-090206	*Neofusicoccum*	*parvum*	*Botryosphaeriales*	*Ziziphus lotus*
CF-090380	*Neofusicoccum*	sp.	*Botryosphaeriales*	*Ballota hirsuta*
CF-285742	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-288941	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Tamarix canariensis*
CF-288942	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Tamarix canariensis*
CF-288943	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Tamarix canariensis*
CF-288907	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Frankenia pulvirulenta*
CF-285741	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-288902	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-090262	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Tamarix canariensis*
CF-090785	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Tamarix canariensis*
CF-285767	*Neomicrosphaeropsis*	sp.	*Pleosporales*	*Lycium intricatum*
CF-285780	*Neosetophoma*	sp.	*Pleosporales*	*Thymelaea hirsuta*
CF-288948	*Neosetophoma*	sp.	*Pleosporales*	*Teucrium capitatum sbsp. gracillium*
CF-282001	*Neosetophoma*	sp.	*Pleosporales*	*Retama sphaerocarpa*
CF-285771	*Nigrospora*	sp.	*Incertae sedis*	*Phragmites australis*
CF-288945	*Nigrospora*	sp.	*Incertae sedis*	*Tamarix canariensis*
CF-287449	*Orbicula*	sp.	*Pezizales*	*Rosmarinus eriocalyx*
CF-090248	*Paracamarosporium*	sp.	*Pleosporales*	*Genista umbellata*
CF-285765	*Paraconiothyrium*	sp.	*Pleosporales*	*Lygeum spartum*
CF-282005	*Pezizales*	sp.	*Pezizales*	*Retama sphaerocarpa*
CF-282011	*Pezizales*	sp.	*Pezizales*	*Dittrichia viscosa*
CF-282336	*Pezizales*	sp.	*Pezizales*	*Dittrichia viscosa*
CF-285776	*Phaeosphaeria*	sp.	*Pleosporales*	*Teucrium capitatum sbsp. gracillium*
CF-285779	*Phaeosphaeria*	sp.	*Pleosporales*	*Thymus zygis subs gracilis*
CF-285363	*Phaeosphaeria*	sp.	*Pleosporales*	*Moricandia foetida*
CF-288952	*Phaeosphaeria*	sp.	*Pleosporales*	*Teucrium capitatum sbsp. gracillium*
CF-282010	*Phaeosphaeria*	sp.	*Pleosporales*	*Dittrichia viscosa*
CF-285358	*Phaeotheca*	*triangularis*	*Capnodiales*	*Asparagus horridus*
CF-278520	*Phoma*	sp.	*Pleosporales*	*Opuntia ficus-indica*
CF-288912	*Phoma*	sp.	*Pleosporales*	*Gypsophila tomentosa*
CF-288915	*Phoma*	sp.	*Pleosporales*	*Lepidium subulatum*
CF-288914	*Phoma*	sp.	*Pleosporales*	*Lepidium subulatum*
CF-288919	*Phoma*	sp.	*Pleosporales*	*Ononis tridentata*
CF-288917	*Phoma*	sp.	*Pleosporales*	*Lygeum spartum*
CF-288953	*Phoma*	sp.	*Pleosporales*	*Thymus zygis subs gracilis*

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CF-092178	*Phoma*	sp.	*Pleosporales*	*Chamaerops humilis*
CF-285365	*Phoma*	sp.	*Pleosporales*	*Rosmarinus eriocalyx*
CF-091947	*Phoma*	sp.	*Pleosporales*	*Suaeda vera*
CF-090323	*Phoma*	sp.	*Pleosporales*	*Artemisia barrelieri*
CF-092164	*Phoma*	sp.	*Pleosporales*	*Lygeum spartum*
CF-090291	*Phoma*	sp.	*Pleosporales*	*Thymus hyemalis*
CF-090829	*Phoma*	sp.	*Pleosporales*	*Thymus hyemalis*
CF-090223	*Phomopsis*	sp.	*Diaporthales*	*Ziziphus lotus*
CF-285364	*Pleiochaeta*	sp.	*Incertae sedis*	*Moricandia foetida*
CF-288936	*Pleospora*	*herbarum*	*Pleosporales*	*Sonchus crassifolius*
CF-285761	*Pleospora*	*herbarum*	*Pleosporales*	*Limbarda crithmoides*
CF-090249	*Pleospora*	*herbarum*	*Pleosporales*	*Genista umbellata*
CF-090771	*Pleospora*	*herbarum*	*Pleosporales*	*Genista umbellata*
CF-090753	*Pleospora*	sp.	*Pleosporales*	*Ziziphus lotus*
CF-090826	*Pleospora*	sp.	*Pleosporales*	*Thymus hyemalis*
CF-091933	*Pleospora*	sp.	*Pleosporales*	*Lygeum spartum*
CF-282343	*Podospora*	*pleiospora*	*Sordariales*	*Dittrichia viscosa*
CF-285369	*Schizothecium*	*tetrasporum*	*Sordariales*	*Anabasis articulata*
CF-288933	*Preussia*	*australis*	*Pleosporales*	*Salsola vermiculata*
CF-285375	*Preussia*	*australis*	*Pleosporales*	*Launaea arborescens*
CF-091940	*Preussia*	*australis*	*Pleosporales*	*Thymus hyemalis*
CF-285772	*Preussia*	*grandispora*	*Pleosporales*	*Phragmites australis*
CF-285773	*Preussia*	*grandispora*	*Pleosporales*	*Phragmites australis*
CF-090838	*Preussia*	*grandispora*	*Pleosporales*	*Launaea arborescens*
CF-091941	*Preussia*	*grandispora*	*Pleosporales*	*Thymus hyemalis*
CF-282339	*Preussia*	*lignicola*	*Pleosporales*	*Dittrichia viscosa*
CF-090241	*Preussia*	*lignicola*	*Pleosporales*	*Genista umbellata*
CF-090293	*Preussia*	*lignicola*	*Pleosporales*	*Thymus hyemalis*
CF-285357	*Preussia*	*similis*	*Pleosporales*	*Asparagus horridus*
CF-288918	*Preussia*	sp.	*Pleosporales*	*Lygeum spartum*
CF-288931	*Preussia*	sp.	*Pleosporales*	*Salsola vermiculata*
CF-288906	*Preussia*	sp.	*Pleosporales*	*Arthrocnemum macrostachyum*
CF-279766	*Preussia*	sp.	*Pleosporales*	*Retama sphaerocarpa*
CF-287459	*Preussia*	sp.	*Pleosporales*	*Launaea arborescens*
CF-285370	*Preussia*	sp.	*Pleosporales*	*Anabasis articulata*
CF-285378	*Preussia*	sp.	*Pleosporales*	*Salsola oppositifolia*
CF-090835	*Preussia*	sp.	*Pleosporales*	*Thymus hyemalis*
CF-090338	*Pseudocamarosporium*	sp.	*Pleosporales*	*Anthyllis cystisoides*
CF-285347	*Pseudocamarosporium*	sp.	*Pleosporales*	*Helianthemum almeriense*
CF-090324	*Pseudocamarosporium*	sp.	*Pleosporales*	*Artemisia barrelieri*
CF-285753	*Pseudodiplodia*	*ruticola*	*Botryosphaeriales*	*Cynanchum acutum*
CF-285754	*Pseudodiplodia*	*ruticola*	*Botryosphaeriales*	*Cynanchum acutum*
CF-285755	*Pseudodiplodia*	*ruticola*	*Botryosphaeriales*	*Cynanchum acutum*
CF-285756	*Pseudodiplodia*	sp.	*Botryosphaeriales*	*Cynanchum acutum*
CF-279245	*Pseudopithomyces*	*chartarum*	*Pleosporales*	*Retama sphaerocarpa*
CF-287448	*Pseudoseptoria*	*obscura*	*Dothideales*	*Rosmarinus eriocalyx*
CF-281030	*Pulvinula*	sp.	*Pezizales*	*Retama sphaerocarpa*
CF-287464	*Pyrenochaeta*	sp.	*Pleosporales*	*Genista umbellata*
CF-090337	*Rhizoctonia*	sp.	*Cantharellales*	*Anthyllis cystisoides*
CF-287466	*Rosellinia*	sp.	*Xylariales*	*Artemisia barrelieri*
CF-285766	*Scclerostagonospora*	sp	*Pleosporales*	*Lygeum spartum*
CF-090364	*Seiridium*	sp.	*Amphisphaeriales*	*Sedum sediforme*
CF-285463	*Selenophoma*	*juncea*	*Dothideales*	*Salsola oppositifolia*
CF-285774	*Selenophoma*	*juncea*	*Dothideales*	*Salsola oppositifolia*
CF-277101	*Selenophoma*	sp.	*Dothideales*	*Spartium junceum*
CF-090317	*Selenophoma*	sp.	*Dothideales*	*Artemisia barrelieri*
CF-285346	*Septoria*	*arundinacea*	*Capnodiales*	*Phragmites australis*
CF-285751	*Septoria*	sp.	*Capnodiales*	*Centaurea dracunculifolia*
CF-090353	*Lepteutypa*	*cupressi*	*Xylariales*	*Sedum sediforme*

CF-287457	*Setomelanomma*	sp.	*Pleosporales*	*Lycium intricatum*
CF-288954	*Sordaria*	sp.	*Sordariales*	*Thymus zygis subs gracilis*
CF-090778	*Stagnospora*	sp	*Pleosporales*	*Asparagus horridus*
CF-281556	*Stagnospora*	sp.	*Pleosporales*	*Stipa tenacissima*
CF-090378	*Stagnospora*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-090359	*Stagnospora*	sp.	*Pleosporales*	*Sedum sediforme*
CF-090235	*Stagnospora*	sp.	*Pleosporales*	*Suaeda vera*
CF-090258	*Stagnospora*	sp.	*Pleosporales*	*Asparagus horridus*
CF-090776	*Stemphylium*	sp	*Pleosporales*	*Genista umbellata*
CF-090346	*Stemphylium*	sp.	*Pleosporales*	*Onoris ramosissina*
CF-090850	*Stemphylium*	sp.	*Pleosporales*	*Onoris ramosissina*
CF-090781	*Stemphylium*	sp.	*Pleosporales*	*Asparagus horridus*
CF-090250	*Stemphylium*	sp.	*Pleosporales*	*Genista umbellata*
CF-090279	*Tamaricicola*	sp.	*Pleosporales*	*Limonium insigne*
CF-288916	*Tamaricicola*	sp.	*Pleosporales*	*Limonium majus*
CF-288959	*Tamaricicola*	sp.	*Pleosporales*	*Limonium majus*
CF-091926	*Teratosphaeria*	sp.	*Capnodiales*	*Genista umbellata*
CF-090304	*Teratosphaeria*	sp.	*Capnodiales*	*Launaea arborescens*
CF-287456	*Teratosphaeria*	sp.	*Capnodiales*	*Lycium intricatum*
CF-091928	*Toxicocladosporium*	sp.	*Capnodiales*	*Asparagus horridus*
CF-277739	*Trichothecium*	*roseum*	*Hypocreales*	*Retama sphaerocarpa*
CF-285757	*Trichothecium*	*roseum*	*Hypocreales*	*Dorycnium pentaphyllum*
CF-282017	*Tympanis*	sp.	*Helotiales*	*Juniperus oxycedrus*
CF-290943	*Unidentified*	Fungus	*not determined*	*Genista umbellata*
CF-290928	*Unidentified*	Fungus	*not determined*	*Limodorum abortivum*
CF-290929	*Unidentified*	Fungus	*not determined*	*Limodorum abortivum*
CF-290937	*Unidentified*	Fungus	*not determined*	*Retama sphaerocarpa*
CF-287441	*Unidentified*	Fungus	*not determined*	*Ononis fruticosa*
CF-288924	*Unidentified*	Fungus	*not determined*	*Ononis tridentata*
CF-288925	*Unidentified*	Fungus	*not determined*	*Ononis tridentata*
CF-287475	*Unidentified*	Fungus	*not determined*	*Centaurea dracunculifolia*
CF-090748	*Unidentified*	Fungus	*not determined*	*Thymelaea hirsuta*
CF-090210	*Unidentified*	Fungus	*not determined*	*Ziziphus lotus*
CF-090222	*Unidentified*	Fungus	*not determined*	*Ziziphus lotus*
CF-090758	*Unidentified*	Fungus	*not determined*	*Ziziphus lotus*
CF-282344	*Unidentified*	Fungus	*not determined*	*Dittrichia viscosa*
CF-092179	*Unidentified*	Fungus	*not determined*	*Nicotiana glauca*
CF-091954	*Unidentified*	Fungus	*not determined*	*Onoris ramosissina*
CF-091948	*Unidentified*	Fungus	*not determined*	*Cistus albidus*
CF-287472	*Unidentified*	Fungus	*not determined*	*Asparagus horridus*
CF-090273	*Unidentified*	Fungus	*not determined*	*Limonium insigne*
CF-091924	*Unidentified*	Fungus	*not determined*	*Nerium oleander*
CF-091929	*Unidentified*	Fungus	*not determined*	*Tamarix canariensis*
CF-285763	*Ustilago*	sp.	*Ustilaginales*	*Launaea arborescens*
CF-090221	*Verrucocladosporium*	sp.	*Capnodiales*	*Ziziphus lotus*
CF-279251	*Volutella*	sp.	*Hypocreales*	*Retama sphaerocarpa*
CF-288937	*Xenodidymella*	sp.	*Pleosporales*	*Sonchus crassifolius*
CF-288955	*Xenodidymella*	sp.	*Pleosporales*	*Thymus zygis subs gracilis*
CF-288957	*Xenodidymella*	sp.	*Pleosporales*	*Thymus zygis subs gracilis*
CF-090345	*Xenodidymella*	sp.	*Pleosporales*	*Onoris ramosissina*
CF-092173	*Xenodidymella*	sp.	*Pleosporales*	*Ballota hirsuta*
CF-290936	*Xylaria*	sp.	*Xylariales*	*Retama sphaerocarpa*
CF-286680	*Xylaria*	sp.	*Xylariales*	*Lygeum spartum*
CF-287438	*Xylaria*	sp.	*Xylariales*	*Ononis fruticosa*
CF-091951	*Xylaria*	sp.	*Xylariales*	*Rhamnus lycioides*
CF-285461	*Xylaria*	sp.	*Xylariales*	*Thymelaea hirsuta*
CF-090770	*Xylaria*	sp.	*Xylariales*	*Genista umbellata*

Apendix

Supplementary Table 2. Fungal strains included in the phylogenetic analysis (**newly isolated strains** from plants collected in arid zones of Andalucía are **in bold**).

Species	Strain code [a]	Substrate	Origin	Genbank accesion numbers[b]	
				ITS	28S
***Cryptococcus* sp.**	**CF-285748**	***Beta macrocarpa***	**El Margen salt marsh, Granada, Spain**	**MG065727**	
***Coniothyrium* sp.**	**CF-091953**	***Anthyllis cihsoides***	**Sierra Alhamilla, Almeria, Spain**	**MG065728**	
***Coniothyrium* sp.**	**CF-090243**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**	**MG065729**	
Coniothyrium palmarum	CBS 400.71	*Chamaerops humilis*	Italy	AY720708	JX681084
Coniothyrium carteri	CBS 105.91	*Quercus robur*	Germany	JF740181	KF251712
***Coniothyrium* sp.**	**CF-090330**	***Cistus albidus***	**Sierra Alhamilla, Almeria, Spain**	**MG065730**	
***Coniothyrium* sp.**	**CF-090362**	***Sedum sediforme***	**Sierra Alhamilla, Almeria, Spain**	**MG065731**	
Coniothyrium multiporum	CBS 353.65	saline soil	India	JF740187	JF740268
Coniothyrium telephii	CBS 188.71	air	Filand	JF740188	GQ387599
Fungal endophyte	SNP 431	*Parkinsonia microphylla*	Saguaro National Park, Arizona, USA	KP335607	
***Coniothyrium* sp.**	**CF-285356**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**	**MG065732**	
Hazslinszkyomyces aloes	CBS 136437	*Aloe dichotoma*	Western Cape Province, South Africa	KF777142	
***Hazslinszkyomyces* sp.**	**CF-090320**	***Artemisia sp.***	**Tabernas desert, Almeria, Spain**	**MG065733**	
Coniothyrina agaves	CBS 470.69	*Agave americana*	El Arenal, Mallorca, Spain	JX681075	
Neophaeosphaeria filamentosa	CBS 102202	*Yucca rostrata*	Mexico	JF740259	GQ387577
Camarosporium arezzoensis	MFLUCC 14-0238	*Cytisus* sp.	Italy	KP120926	KP120927
Camarosporium aureum	MFLUCC 14-0620	*Cotinus coggygria*	Russia	KP744436	KP744478
Camarosporium robiniicola	MFLUCC 13-0527	*Robinia pseudoacacia*	Italy	KJ562214	KJ589412
Camarosporium uniseriatum	MFLUCC 15-0444	*Celtis occidentalis*	Russia	KU697613	KU697614
***Camarosporium* sp.**	**CF-285350**	***Euzomodendron bourgeanum***	**Tabernas desert, Almeria, Spain**	**MG065734**	
Camarosporium spartii	MFLUCC 13-0548	Cytisus sp.	Italy	KJ562215	KJ589413
Camarosporium clematidis	MFLUCC 13-0336	*Clematis vitalba*	Italy	KJ562213	
Fungal endophyte	SNP 009	*Larrea tridentata*	Saguaro National Park, Arizona, USA	KP335215	
***Pseudoamarosporium* sp.**	**CF-285347**	***Helianthemum almeriense***	**Tabernas desert, Almeria, Spain**	**MG065735**	
Pseudocamarosporium propinquum	MFLUCC 13-0544	*Salix* sp.	Italy	KJ747049	KJ813280
Pseudocamarosporium lonicerae	MFLUCC 13-0532	*Lonicera* sp.	Italy	KJ747047	KJ813278
Pseudocamarosporium piceae	MFLUCC 14-0192	*Picea excels*	Italy	KJ747046	KJ803030
Pseudocamarosporium tilicola	MFLUCC 13-0550	*Pinnus nigra*	Italy	KJ747050	KJ813281
Pseudocamarosporium corni	MFLUCC 13-0541	*Cornus sanguinea*	Italy	KJ747048	KJ813279
***Pseudocamarosporium* sp.**	**CF-090324**	***Artemisia sp.***	**Tabernas desert, Almeria, Spain**	**MG065736**	
***Pseudocamarosporium* sp.**	**CF-090338**	***Anthyllis cihsoides***	**Sierra Alhamilla, Almeria, Spain**	**MG065737**	
Camarosporium brabeji	CBS 123026	*Protea* sp.	South Africa	EU552105	
Paracamarosporium hawaiiensis	CBS 1200225	*Sophora chrysophylla*	Hawaii, USA	DQ885897	
Paracamarosporium psoraleae	CBS 136628	*Psoralea pinnata*	Western Cape Province, South Africa	KF777143	KF777199
***Paracamarosporium* sp.**	**CF-090248**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**	**MG065738**	

Paramarosporium leucadendri	CBS 123027	*Brabejum stellatifolium*	South Africa		EU552106
Paracamarosporium tamaricis	MFLUCC 15-0495	*Tamarix gallica*	Italy	KU900327	KU900299
Paracamarosporium fagi	CPC 24892	*Fagus sylvatica*	Germany	KR611887	KR611905
Fungal endophyte	SNP 291	*Parkinsonia microphylla*	Saguaro National Park, Arizona, USA		KP335478
Fungal sp.	ARIZ AZ0149	*Juniperus deppeana*	Coronado National Forest, Arizona, USA		HM122902
***Camarosporium* sp.**	**CF-090314**	***Artemisia barrelieri***	**Tabernas desert, Almeria, Spain**		**MG065739**
Kalmusia sarothamni	CBS 113833	*Cytisus sarothamni*	Sweden	KF796675	KF796671
***Kalmusia* sp.**	**CF-091181**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**		**MG065740**
Kalmusia variispora	95SA2	*Quercus brantii*	Iran	KY783414	KY825094
Kalmusia ebuli	CBS 123120	*Populus tremula*	France		KF796674
Kalmusia italica	MFLUCC 13-0066	*Spartium junceum*	Italy	KP325440	KP325441
Fungal sp.	ARIZ AZ0553	*Juniperus deppeana*	Coronado National Forest, Arizona, USA		HM123272
Fungal sp.	ARIZ AZ0644	*Quercus rugosa*	Coronado National Forest, Arizona, USA		HM123362
Xenodidymella applanata	CBS 102634	*Rubus idaeus*	Netherlands	GU237726	GU237997
***Xenodidymella* sp.**	**CF-092173**	***Bellota hirsuta***	**Sierra Alhamilla, Almeria, Spain**		**MG065741**
***Xenodidymella* sp.**	**CF-090345**	***Onoris ramosissina***	**Sierra Alhamilla, Almeria, Spain**		**MG065742**
***Xenodidymella* sp.**	**CF-288955**	***Thymus zygis subs gracillis***	**El Margen salt marsh, Granada**		**MG065743**
***Xenodidymella* sp.**	**CF-288937**	***Sonchus crassifolius***	**El Margen salt marsh, Granada**		**MG065744**
Neodidymelliopsis cannabis	CBS 121.75	*Urtica dioica*	Netherlands	GU237761	GU237972
Neodidymelliopsis xanthina	CBS 383.68	*Delphinium* sp.	Netherlands	NR_135994	GU238157
***Neodidymelliopsis* sp.**	**CF-286681**	***Cynanchum acutum***	**El Margen salt marsh, Granada**		**MG065745**
Microsphaeropsis olivacea	CBS 303.68	*Ligustrum vulgare*	Netherlands		JX681101
Boeremia exigua var exigua	CBS 431.74	*Solanum tuberosum*	Netherlands	FJ427001	JX681074
***Neomicrosphaeropsis* sp.**	**CF-090262**	***Tamarix canariensis***	**Tabernas desert, Almeria, Spain**		**MG065746**
***Neomicrosphaeropsis* sp.**	**CF-288942**	***Tamarix canariensis***	**El Margen salt marsh, Granada**		**MG065747**
***Neomicrosphaeropsis* sp.**	**CF-090785**	***Tamarix canariensis***	**Tabernas desert, Almeria, Spain**		**MG065748**
Neomicrosphaeropsis tamaricicola	MFLUCC 14-0443	*Tamarix gallica*	Italy	KU900322	KU729851
***Neomicrosphaeropsis* sp.**	**CF-288902**	***Arthrocneum macrostachyum***	**El Margen salt marsh, Granada**		**MG065749**
***Neomicrosphaeropsis* sp.**	**CF-285742**	***Arthrocneum macrostachyum***	**El Margen salt marsh, Granada**		**MG065750**
***Neomicrosphaeropsis* sp.**	**CF-288907**	***Frankenia pulvirulenta***	**El Margen salt marsh, Granada**		**MG065751**
***Neomicrosphaeropsis* sp.**	**CF-285767**	***Lycnium intricatum***	**Torre Garcia, Almeria, Spain**		**MG065752**
Didymella exigua	CBS 183.55	*Rumex arifolius*	France	NR_135936	EU754155
***Didymella* sp.**	**CF-288938**	***Sonchus crassifolius***	**El Margen salt marsh, Granada**		**MG065753**
Ascochyta medicaginicola	CBS 533.66	*Medicago sativa*	Netherlands		EU167575
Leptosphaerulina australis	CBS 317.83	*Eugenia aromatica*	Indonesia	GU237829	EU754166
Leptosphaerulina australis	NBRC 33240	house dust	Japan		NBRC33240
Leptosphaerulina trifolii	NBRC 7250	-	-		NBRC7250
Leptosphaerulina argentinensis	CBS 569.94	*Lonicera periclymenum*	Netherlands		AY849947
Ascochyta phacae	CBS 184.55	*Phaca alpina*	Switzerland		EU167570

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***Ascochyta* sp.**	**CF-090372**	***Sedum sediforme***	**Sierra Alhamilla, Almeria, Spain**		**MG065754**
Ascochyta rabiei	CBS 237.37	Cicer arietinum	Bulgaria		EU167600
***Ascochyta* sp.**	**CF-090376**	***Bellota hirsuta***	**Sierra Alhamilla, Almeria, Spain**		**MG065755**
***Ascochyta* sp.**	**CF-090240**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065756**
***Ascochyta* sp.**	**CF-091946**	***Lycium intricatun***	**Tabernas desert, Almeria, Spain**		**MG065757**
***Ascochyta* sp.**	**CF-091945**	***Launaea arboresceus***	**Tabernas desert, Almeria, Spain**		**MG065758**
***Ascochyta* sp.**	**CF-287439**	***Ononis fruticosa***	**El Margen salt marsh, Granada**		**MG065759**
***Ascochyta* sp.**	**CF-090252**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065760**
***Ascochyta* sp.**	**CF-090321**	***Artemisia barrelieri***	**Tabernas desert, Almeria, Spain**		**MG065761**
***Ascochyta* sp.**	**CF-090305**	***Launaea arborescens***	**Tabernas desert, Almeria, Spain**		**MG065762**
***Ascochyta* sp.**	**CF-090840**	***Launaea arborescens***	**Tabernas desert, Almeria, Spain**		**MG065763**
***Ascochyta* sp.**	**CF-285366**	***Rosmarinus eriocalyx***	**Tabernas desert, Almeria, Spain**		**MG065764**
***Ascochyta* sp.**	**CF-285355**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**		**MG065765**
***Ascochyta* sp.**	**CF-287454**	***Rosmarinus eriocalyx***	**Tabernas desert, Almeria, Spain**		**MG065766**
***Ascochyta* sp.**	**CF-287450**	***Rosmarinus eriocalyx***	**Tabernas desert, Almeria, Spain**		**MG065767**
***Ascochyta* sp.**	**CF-287451**	***Rosmarinus eriocalyx***	**Tabernas desert, Almeria, Spain**		**MG065768**
Ascochyta viciae-pannonicae	CBS 254.92	*Vicia pannonica*	Czechoslovakia	EU167559	KT389702
Ascochyta pisi var pisi	CBS 108.26	-	-		EU167557
***Phoma* sp.**	**CF-092164**	***Lygeum spartum***	**Tabernas desert, Almeria, Spain**		**MG065769**
Neosetophoma samarorum	CBS 139.96	grass	Netherlands	FJ427062	KF251665
Ascomycota sp.	ARIZ PAAsh314	*Fraxinus velutina*	Arizona, USA		JN120365
Phaeosphaeria nigrans	NBRC 33095	*Phragmites karka*	Japan		NBRC33095
Neosetophoma italica	MFLUCC 13-0388	*Iris germanica*	Italy	KP711356	KP711361
***Neosetophoma* sp.**	**CF-282001**	***Retama sphaerocarpa***	**Albuñuelas, Granada, Spain**		**KU295580**
***Neosetophoma* sp.**	**CF-288948**	***Teucrium pollium sbs gracillium***	**El Margen salt marsh, Granada**		**MG065770**
***Phoma* sp.**	**CF-090323**	***Artemisia barrelieri***	**Tabernas desert, Almeria, Spain**		**MG065771**
***Phoma* sp.**	**CF-091947**	***Suaeda vera***	**Tabernas desert, Almeria, Spain**		**MG065772**
Pyrenochaeta lycopersici	CBS 267.59	*Lycopersicon esculentum*	Netherlands	JF740261	GQ387612
Foliophoma fallens	CBS 167.78	*Olea europaea*	New Zealand	KY940772	GU238074
Foliophoma fallens	**CF-090782**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**		**MG065773**
***Libertasomyces* sp.**	**CF-090382**	***Bellota hirsuta***	**Sierra Alhamilla, Almeria, Spain**		**MG065774**
***Libertasomyces* sp.**	**CF-092175**	***Bellota hirsuta***	**Sierra Alhamilla, Almeria, Spain**		**MG065775**
Libertasomyces platani	CPC 29609	*Platanus* sp.	New Zealand	KY173416	KY173507
Libertasomyces myopori	CPC 27354	*Myoporum serratum*	South Africa	KX228281	KX228332
Libertasomyces sp.	CF-092176	*Bellota hirsuta*	Sierra Alhamilla, Almeria, Spain		**MG065776**
Neoplatysporoides aloicola	CBS 139901	Aloe sp.	Tanzania	KR476719	KR476754
Alternaria alternata	**CF-090395**	***Arthrocneum macrostachyum***	**Cabo de gata salt marsh, Almeria, Spain**		**MG065777**
Alternaria alternata	**CF-090318**	***Artemisia barrelieri***	**Tabernas desert, Almeria, Spain**		**MG065778**
Alternaria alternata	CBS 115200	*Citrus x tangelo*	South Africa	KP124352	KP124504

Alternaria alternata	CBS 118814	*Solanum lycopersicum*	USA	KP124357	KP124509
Alternaria alternata	**CF-288903**	***Arthrocneum macrostachyum***	**El Margen salt marsh, Granada**		**MG065779**
Alternaria alternata	**CF-090322**	***Artemisia barrelieri***	**Tabernas desert, Almeria, Spain**		**MG065780**
Alternaria alternata	CBS 102600	*Citrus reticulata*	USA	KP124331	KP124483
Alternaria alternata	**CF-288939**	***Suaeda vera***	**El Margen salt marsh, Granada**		**MG065781**
***Alternaria* sp.**	**CF-090254**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**		**MG065782**
Alternaria gaisen	CBS 118488	*Pyrus pyrifolia*	Japan	KP124427	KP124581
Alternaria arborescens	CBS 102605	*Lycopersicon esculentum*	California	NR_135927	KC584253
Alternaria eichhorniae	CBS 119778	*Eichhornia crassipes*	Indonesia	KP124426	KP124580
***Alternaria* sp.**	**CF-090752**	***Thymelaea hirsuta***	**Los Almorades, Almeria, Spain**		**MG065783**
***Alternaria* sp.**	**CF-090365**	***Sedum sediforme***	**Sierra Alhamilla, Almeria, Spain**		**MG065784**
Alternaria terricola	CBS 202.67	wheat field soil	Northern Utah	NR_103600	KC584365
Alternaria	**CF-288921**	***Ononis tridentata***	**El Margen salt marsh, Granada**		**MG065785**
Alternaria nobilis	CBS 116490	*Dianthus caryophyllus*	New Zealand	KC584208	KC584291
Alternaria infectoria	CBS 210.86	*Triticum aestivum*	England	NR_131263	KC584280
Alternaria ethzedia	CBS 197.86	*Brassica napus*	Switzerland	NR_135928	KC584274
***Alternaria* sp.**	**CF-288904**	***Arthrocneum macrostachyum***	**El Margen salt marsh, Granada**		**MG065786**
***Alternaria* sp.**	**CF-282008**	***Retama sphaerocarpa***	**Albuñuelas, Granada, Spain**		**MG065787**
***Alternaria* sp.**	**CF-288922**	***Ononis tridentata***	**El Margen salt marsh, Granada**		**MG065788**
***Alternaria* sp.**	**CF-288961**	***Sonchus crassifolius***	**El Margen salt marsh, Granada**		**MG065789**
***Alternaria* sp.**	**CF-090246**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065790**
***Alternaria* sp.**	**CF-090261**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**		**MG065791**
***Alternaria* sp.**	**CF-288960**	***Frankenia pulvirulenta***	**El Margen salt marsh, Granada**		**MG065792**
Alternaria penicillata	CBS 116607	*Papaver rhoeas*	Austria	KC584229	KC584322
Dendryphion papaveris	NBRC 9282	*Papaver somniferum*	-		NBRC9282
Dendryphion papaveris	NBRC 9801	*Papaver somniferum*	Nagano, Japan		NBRC9801
Alternaria solani	CBS 116651	*Solanum tuberosum*	USA	KC584217	KC584306
Alternaria cumini	**CF-090366**	***Sedum sediforme***	**Sierra Alhamilla, Almeria, Spain**		**MG065793**
Alternaria cumini	CBS 121329	*Cuminum cyminum*	India	KC584191	KC584267
***Stemphylium* sp.**	**CF-090250**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065794**
***Stemphylium* sp.**	**CF-090346**	***Onoris ramosissina***	**Sierra Alhamilla, Almeria, Spain**		**MG065795**
***Stemphylium* sp.**	**CF-090776**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065796**
***Stemphylium* sp.**	**CF-090781**	***Asparagus horridus***	**Tabernas desert, Almeria, Spain**		**MG065797**
Paradendryphiella salina	CBS 142.60	*Spartina* sp.	Southampton, England	DQ411540	KF156158
Paradendryphiella salina	**CF-285749**	***Centaurea dracunculifolia***	**El Margen salt marsh, Granada**		**MG065798**
Pleospora herbarum var herbarum	CBS 191.86	*Medicago sativa*	India	KC584239	JX681120
Pleospora herbarum	**CF-090771**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065799**
Pleospora herbarum	NBRC 7404	-	-		NBRC7404
Pleospora herbarum	**CF-285761**	***Inula crithmoides***	**Punta Entinas, Almeria, Spain**		**MG065800**

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Pleospora herbarum	**CF-288936**	***Sonchus crassifolius***	**El Margen salt marsh, Granada**		**MG065801**
Pleospora herbarum	**CF-090249**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065802**
Comoclathris sedi	MFLUCC 13-0763	*Rosa* sp.	Italy	KP334717	KP334707
***Comoclathris* sp.**	**CF-090266**	***Lygeum spartum***	**Tabernas desert, Almeria, Spain**		**MG065803**
***Comoclathris* sp.**	**CF-287447**	***Rosmarinus eriocalyx***	**Tabernas desert, Almeria, Spain**		**MG065804**
***Comoclathris* sp.**	**CF-090792**	***Lygeum spartum***	**Tabernas desert, Almeria, Spain**		**MG065805**
***Comoclathris* sp.**	**CF-091934**	***Lygeum spartum***	**Tabernas desert, Almeria, Spain**		**MG065806**
***Comoclathris* sp.**	**CF-091944**	***Launaea arboresceus***	**Tabernas desert, Almeria, Spain**		**MG065807**
***Comoclathris* sp.**	**CF-285379**	***Fagonia cretica***	**Tabernas desert, Almeria, Spain**		**MG065808**
***Comoclathris* sp**	**CF-282003**	***Retama sphaerocarpa***	**Albuñuelas, Granada, Spain**		**MG065809**
***Comoclathris* sp**	**CF-090361**	***Sedum sediforme***	**Sierra Alhamilla, Almeria, Spain**		**MG065810**
***Comoclathris* sp**	**CF-090763**	***Nerium oleander***	**Tabernas desert, Almeria, Spain**		**MG065811**
Comoclathris spartii	MFLUCC 13-0214	*Spartium junceum*	Italy	KM577159	KM577160
***Comoclathris* sp.**	**CF-091922**	***Ziziphus lotus***	**Los Almorades, Almeria, Spain**		**MG065812**
***Comoclathris* sp.**	**CF-090267**	***Lygeum spartum***	**Tabernas desert, Almeria, Spain**		**MG065813**
***Tamaricicola* sp.**	**CF-288959**	***Limonium majus***	**El Margen salt marsh, Granada**		**MG065814**
***Tamaricicola* sp.**	**CF-288916**	***Limonium majus***	**El Margen salt marsh, Granada**		**MG065815**
***Tamaricicola* sp.**	**CF-090279**	***Limonium insigne***	**Tabernas desert, Almeria, Spain**		**MG065816**
Tamaricicola muriformis	MFLUCC 15-0488	*Tamarix gallica*	Italy	KU752187	KU561879
Tamaricicola muriformis	MFLUCC 15-0490	*Tamarix gallica*	Italy	KU752189	KU729856
Decorospora gaudefroyi	NBRC 32144	*Salicornia herbacea*	Lake Notoro, Hokkaido, Japan		NBRC32144
Decorospora sp.	ATCC MYA-3203	*Sarcocornia perennis*	Red Bank, Northampton Co., VA, USA.	FJ914870	FJ914897
***Phoma* sp.**	**CF-288917**	***Lygeum spartum***	**El Margen salt marsh, Granada**		**MG065817**
***Phoma* sp.**	**CF-090826**	***Thymus hyemalis***	**Tabernas desert, Almeria, Spain**		**MG065818**
***Phoma* sp.**	**CF-090829**	***Thymus hyemalis***	**Tabernas desert, Almeria, Spain**		**MG065819**
***Phoma* sp.**	**CF-090291**	***Thymus hyemalis***	**Tabernas desert, Almeria, Spain**		**MG065820**
Neocamarosporium sp.	**CF-090392**	***Arthrocneum macrostachyum***	**Cabo de gata salt marsh, Almeria, Spain**		**MG065821**
***Neocamarosporium* sp.**	**CF-288932**	***Salsola vermiculata***	**El Margen salt marsh, Granada**		**MG065822**
***Neocamaroporium* sp.**	**CF-288928**	***Salsola vermiculata***	**El Margen salt marsh, Granada**		**MG065823**
***Neocamarosporium* sp.**	**CF-288962**	***Arthrocneum macrostachyum***	**El Margen salt marsh, Granada**		**MG065824**
***Neocamarosporium* sp.**	**CF-090841**	***Salsola genistoides***	**Tabernas desert, Almeria, Spain**		**MG065825**
***Neocamarosporium* sp.**	**CF-285744**	***Atriplex glauca***	**El Margen salt marsh, Granada**		**MG065826**
***Neocamarosporium* sp.**	**CF-285775**	***Suaeda vera***	**El Margen salt marsh, Granada**		**MG065827**
***Neocamarosporium* sp.**	**CF-285377**	***Suaeda vera***	**Tabernas desert, Almeria, Spain**		**MG065828**
***Neocamarosporium* sp.**	**CF-288934**	***Salsola vermiculata***	**El Margen salt marsh, Granada**		**MG065829**
***Neocamarosporium* sp.**	**CF-090309**	***Suaeda vera***	**Tabernas desert, Almeria, Spain**		**MG065830**
Neocamarosporium chichastianum	**CF-285348**	***Salsola papIllosa***	**Tabernas desert, Almeria, Spain**		**MG065831**
Neocamarosporium chichastianum	CBS 137502	saline soil	Iran	KP004455	KP004483
Neocamarosporium chersinae	CPC 27298	Dead angulate tortoise shell	South Africa	KY929153	KY929182

***Neocamarosporium* sp.**	**CF-090312**	***Suaeda vera***	**Tabernas desert, Almeria, Spain**		**MG065832**
***Neocamarosporium* sp.**	**CF-285373**	***Salsola papollosa***	**Tabernas desert, Almeria, Spain**		**MG065833**
***Neocamarosporium* sp.**	**CF-285768**	***Lycnium intricatum***	**Torre Garcia, Almeria, Spain**		**MG065834**
***Neocamarosporium* sp.**	**CF-090390**	***Arthrocneum macrostachyum***	**Cabo de gata salt marsh, Almeria, Spain**		**MG065835**
***Neocamarosporium* sp.**	**CF-287455**	***Lycium intricatum***	**Torre Garcia, Almeria, Spain**		**MG065836**
***Dimorphosporicola* sp.**	**CF-090768**	***Suaeda vera***	**Tabernas desert, Almeria, Spain**		**MG065837**
Dimorphosporicola tragani	CBS 570.85	*Traganum nudatum var. microphyll*	Mauritania	KU728497	KU728536
Dimorphosporicola tragani	**CF-090383**	***Arthrocneum macrostachyum***	**Cabo de gata salt marsh, Almeria, Spain**		**MG065838**
Chaetosphaeronema hispidulum	CBS 826.88	soil	Israel		EU754145
***Neocamarosporium* sp.**	**CF-090393**	***Arthrocneum macrostachyum***	**Cabo de gata salt marsh, Almeria, Spain**		**MG065839**
***Neocamarosporium* sp.**	**CF-288930**	***Salsola vermiculata***	**El Margen salt marsh, Granada**		**MG065840**
***Neocamarosporium* sp.**	**CF-285746**	***Atriplex glauca***	**El Margen salt marsh, Granada**		**MG065841**
***Neocamarosporium* sp.**	**CF-285745**	***Atriplex glauca***	**El Margen salt marsh, Granada**		**MG065842**
***Neocamarosporium* sp.**	**CF-285747**	***Beta macrocarpa***	**El Margen salt marsh, Granada**		**MG065843**
Chaetodiplodia sp.	CBS 453.68	*Halimione portulacoides*	Netherlands		DQ678054
***Neocamarosporium* sp.**	**CF-285349**	***Anabasis articulata***	**Tabernas desert, Almeria, Spain**		**MG065844**
***Neocamarosporium* sp.**	**CF-285372**	***Anabasis articulata***	**Tabernas desert, Almeria, Spain**		**MG065845**
***Neocamarosporium* sp.**	**CF-090335**	***Anthyllis cihsoides***	**Sierra Alhamilla, Almeria, Spain**		**MG065846**
***Neocamarosporium* sp.**	**CF-285368**	***Anabasis articulata***	**Tabernas desert, Almeria, Spain**		**MG065847**
***Neocamarosporium* sp.**	**CF-090759**	***Nerium oleander***	**Tabernas desert, Almeria, Spain**		**MG065848**
Neocamarosporium goegapense	CBS 138008	*Mesembryanthemum* sp.	Northen Cape Province, South Africa	KJ869163	KJ869220
Neocamarosporium goegapense	**CF-090399**	***Chamaerops humilis***	**Cabo de gata salt marsh, Almeria, Spain**		**MG065849**
***Neocamarosporium* sp.**	**CF-285352**	***Atriplex glauca***	**El Margen salt marsh, Granada**		**MG065850**
***Neocamarosporium* sp.**	**CF-090227**	***Suaeda vera***	**Tabernas desert, Almeria, Spain**		**MG065851**
***Neocamarosporium* sp.**	**CF-090284**	***Salicornia sp.***	**Tabernas desert, Almeria, Spain**		**MG065852**
***Neocamarosporium* sp.**	**CF-090843**	***Salsola genistoides***	**Tabernas desert, Almeria, Spain**		**MG065853**
***Neocamarosporium* sp.**	**CF-090773**	***Genista umbellata***	**Tabernas desert, Almeria, Spain**		**MG065854**
Phaeosphaeriaceae sp.	DF-R-7	*Kochia scoparia*	QingDao, China	KU991885	KU991909
Neocamarosporium betae	CBS 523.66	*Beta vulgaris*	Netherlands	FJ426981	EU754179
Neocamarosporium betae	CBS 109410	*Beta vulgaris*	unknown	KY940790	EU754178
Neocamarosporium calvescens	CBS 344.78	*Atriplex hastata*	Netherlands		EU754132
***Neocamarosporium* sp.**	**CF-090842**	***Salsola genistoides***	**Tabernas desert, Almeria, Spain**		**MG065855**
Neocamarosporium calvescens	CBS 432.77	*Obione portulacoides*	Netherlands	GU230752	JF740267

[a] ATCC, American Type Culture Collection, University Boulevard Manassas VA, USA; CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; CF, Fundación MEDINA Private Fungal Collection, Granada, Spain; CPC. Culture collection of Pedro Crous, housed at CBS, Netherlands; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; NBRC, Biological Resource Center, National Institute of Technology and Evolution, Tokyo, Japan[b] Accession numbers of sequences newly generated in this study are indicated in bold. 28S, large subunit of the nrDNA; ITS, internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDN

Apendix

Supplementary Table 3. Categorization of active isolates according to target strain. Chemical dereplication of fungal metabolites (otherwise specified). A (*Aspergillus fumigatus*), C (*Candida albicans*), M (*Magnaporthe grisea*), Ca (*Colletotrichum acutatum*) and H (HepG2).

		Exclusive Resin					Both					Exclusive No Resin					
Strain	**Taxonomy**	A	C	M	Ca	H	A	C	M	Ca	H	A	C	M	Ca	H	**LC-MS Dereplication**
CF-285778	*Albifimbria verrucaria*	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	illudin C2, roridin A &H, verrucarin A&B
CF-277739	*Trichothecium roseum*	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	trichothecene
CF-285757	*Trichothecium roseum*	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	trichothecene
CF-285465	*Coprinus cinereus*	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	skyrin, lagopodin B
CF-091944	*Comoclathris* sp.	0	0	0	0	0	0	0	1	0	0	1	1	0	1	1	phomasetin, altersetin
CF-285780	*Neosetophoma* sp.	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	globosuxanthone A
CF-285756	*Pseudodiplodia* sp.	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	globosuxanthone A, emodin
CF-282003	*Comoclathris* sp.	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	phomasetin, altersetin
CF-287447	*Comoclathris* sp.	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	phomasetin, altersetin
CF-285362	*Dendryphion* sp.	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	phomasetin
CF-287464	*Pyrenochaeta* sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	$C_{29}H_{41}NO_5$ (previously described in plants)
CF-090752	*Alternaria* sp.	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	dextrusin B, B4, terpestacin
CF-279239	*Coniochaeta* sp.	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	Curvicolilde A/B, xylactam, $C_{26}H_{42}O_5$ (peviosly described in plants)
CF-091924	*unidentified*	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	posible antibiotic YW 3548, $C_{47}H_{75}N_7O_{12}$ and $C_{46}H_{73}N_7O_{12}$ (no match in DNP)
CF-091922	*Comoclathris* sp.	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	illudin C3, $C_{26}H_{43}NO_6$ (previously described in plants)
CF-091951	*Xylaria* sp.	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	nectriapyrone, cytochalasin C/D/M/Q
CF-279244	*Coprinopsis episcopalis*	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	lagopodin A & B
CF-282344	*unidentified*	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	phomasetin
CF-091933	*Pleospora* sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	phomasetin, altersetin
CF-090383	*Dimorphosporicola traganii*	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	cerulenin
CF-090835	*Preussia* sp.	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	di-hydro-bi-chloro-geodin, TMC 120 B & C
CF-090227	*Neocamarosporium* sp.	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	leptosphaeridione
CF-090766	*Comoclathris* sp.	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	possible coriolide
CF-288925	*unidentified*	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	hormonemate, possible coriolide
CF-090379	*Camarosporium* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	naematolin, naematolone, roridin L2
CF-286681	*Neodidymelliopsis* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	waol A
CF-285753	*Pseudodiplodia ruticola*	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	violaceol, $C_{22}H_{24}O_{11}S$ (no match in DNP)
CF-285754	*Pseudodiplodia ruticola*	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	possible 2-(3,4-Epoxy-5-heptenoyl)-5-methylpyrrole, $C_{22}H_{24}O_{11}S$ (no match in DNP)
CF-090353	*Lepteutypa cupressi*	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	MDN-210 ($C_{17}H_{24}O_4$), pestahivin
CF-090758	*unidentified*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	possible coriolide
CF-090357	*Leptosphaeria hispanica*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	massarigenin A, cordyol C, dehydromassarilactone D, phomasetin
CF-090361	*Comoclathris* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	2-(2-Hydroxy-5-metgosyphenoxy) acrylic acid, $C_{18}H_{30}O_4S$ (no math in DNP), possible coriolide

CF-287440	*Aureobasidium* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	$C_{48}H_{78}O_{18}$ (previously described from plants)
CF-090213	*Eutypa consobrina*	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	MDN-209 ($C_{17}H_{26}O_4$), MDN-210 ($C_{17}H_{24}O_4$), MDN-211 ($C_{10}H_{12}O_4$)
CF-090748	*unidentified*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	possible cerebroside B
CF-281556	*Stagnospora* sp.	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	secalonic acid C, possible alternethanoxin A
CF-090324	*Pseudocamarosporium* sp.	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	calbistrin A
CF-285462	*Fusarium equiseti*	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	equisetin
CF-090393	*Neocamarosporium* sp.	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	possible solanopyrone B
CF-285355	*Ascochyta* sp.	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	cephalochromin and cercosporamide
CF-285768	*Neocamarosporium* sp.	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	terrecyclic acid A
CF-090792	*Comoclathris* sp.	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	phomasetin, altersetin
CF-288952	*Phaeosphaeria* sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	possible brefeldin A and mycophenolic acid
CF-287454	*Ascochyta* sp.	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	cercosporamide
CF-090351	*Hormonema carpetanum*	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	$C_{30}H_{54}O_{14}$ (no match in DNP)
CF-285358	*Phaeotheca triangularis*	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	possible coriolide $C_{18}H_{30}O_2$
CF-285461	*Xylaria* sp.	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	antibiotic TKR2648
CF-285752	*Cryptococcus* sp.	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	$C_{15}H_{20}O_3$
CF-090267	*Comoclathris* sp.	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	possible coriolide and nematolin
CF-287457	*Setomelanomma* sp.	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	11,12-dihydroxyeudesm-4-en-3-one, possible coriolide
CF-288933	*Preussia australis*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	$C_{18}H_{33}NO$ (previously described in plants)
CF-277101	*Selenophoma juncea*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	heptelidic acid (avocettin)
CF-090350	*Anthostomella* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	heptidelic and hydroheptidelic acid
CF-090359	*Stagnospora* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	11,12-dihydroxyeudesm-4-en-3-one, diorcinol F, altersetin, phomasetin
CF-090782	*Foliophoma* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	$C_{39}H_{27}NO_8S$ (no match in DNP)
CF-090785	*Neomicrosphaeropsis* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	possible ACTG Toxin C
CF-090828	*Lophiostoma* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	oxasetin, cytochalasin, $C_{22}H_{22}O_8$ (previously described in plants)
CF-092173	*Xenodidymella sp.*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	possible coriolide, phomasetin
CF-092175	*Libertasomyces sp.*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	possible naematolin
CF-092179	*unidentified*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	$C_{29}H_{33}N_3O_6$ (previously described in plants)
CF-278520	*Phoma* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	curvicolide A/B, $C_{26}H_{42}O_5$ (peviosly described in palnts)
CF-279248	*Anthostomella* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	$C_{26}H_{42}O_5$ (peviosly described in palnts)
CF-285767	*Neomicrosphaeropsis* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	phomasetin
CF-286679	*Chaetomium* sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	possible sphaeropsidin B, $C_{26}H_{42}O_5$ (peviosly described in palnts)
CF-090249	*Pleospora herbarum*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	possible coriolide
CF-090313	*Morinia* sp.	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	MDN-209 ($C_{17}H_{26}O_4$)
CF-090838	*Preussia grandispora*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	mellein
CF-285373	*Neocamarosporium* sp.	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	spirostaphylotrichin (C, D, H o G)
CF-290943	*unidentified*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	brefeldin A, $C_{16}H_{22}O_8$ possible macrosphelide
CF-287465	*Aureobasidium pullulans*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	possible exophilin A, $C_{40}H_{74}O_{13}$ (no match in DNP)
CF-285363	*Phaeosphaeria* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	elsinochrome D, possible phaeosphaerin C

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CF-090221	*Verrucocladosporium* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	$C_{19}H_{30}O_9$ (previously described in plants), cyclo(Pro-Phe)
CF-090254	*Alternaria* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	$C_{12}H_{19}NO_3$ (previous described in plants)
CF-090330	*Coniothyrium sp.*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	brefeldin A, $C_{13}H_{17}NO_2$ (previously descrined in plants)
CF-090371	*Morinia pestalozzioides*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	moriniafungin
CF-090372	*Ascochyta* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	$C_{48}H_{78}O_{18}$ (previously described in plants)
CF-090382	*Libertasomyces* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	naematolin, cytosporone D, cordyol E
CF-090768	*Dimorphosporicola* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	cerulenin
CF-091931	*Chaetothyriales* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	tentative ilicicolin H and ascochlorin
CF-278800	*Dothiorella* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4-Hydroxymellein
CF-285741	*Neomicrosphaeropsis* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	phomasetin
CF-285749	*Paradendryphiella salina*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	waol A
CF-287476	*Fusarium* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	fusaric acid, 3-Hydroxymellein
CF-288910	*Aureobasidium pullulans*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	$C_{26}H_{43}NO_6$ and $C_{20}H_{38}O_7$ (previously described in plants)
CF-285750	*Cryptococcus* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	lovastatin acid
CF-285759	*Entodesmium* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	pycnidione, $C_{33}H_{40}O_7$ (no match in DNP)
CF-285364	*Pleiochaeta* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	phomasetin
CF-090228	*Neoamarosporium* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	phomasetin
CF-285758	*Ascochyta* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	emodin, possible pestalamide A, $C_{22}H_{21}O_8$ (no match in DNP)
CF-288957	*Xenodidymella* sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	hormonemate, $C_{30}H_{54}O_{14}$ (no match in DNP)
CF-285350	*Camarosporium* sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	$C_{17}H_{31}NO_3$ and $C_{16}H_{29}NO_4$ (previously described in plants)
CF-285357	*Preussia similis*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	pleofungin A
CF-285375	*Preussia australis*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	$C_{18}H_{33}NO$ (previously described in plants)
CF-285376	*Basifimbria* sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	cyclo (phenylalanylprolyl)
CF-285755	*Pseudodiplodia ruticola*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	possible 6-methyl-9-heptadecenoic acid, $C_{22}H_{24}O_{11}S$ (no match in DNP)
CF-287456	*Teratosphaeria* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3-indolylacetic acid
CF-090284	*Neoamarosporium* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	leptosphaerodione
CF-091947	*Phoma sp.*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	massarigenin A, hormonemate
CF-285378	*Preussia* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	$C_{21}H_{33}N_3O_2$ (previously described in plants)
CF-285751	*Septoria* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	$C_{26}H_{43}NO_6$ and $C_{27}H_{45}NO_6$ (previously described in plants)
CF-285760	*Cryptococcus* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	$C_{18}H_{32}N_8O_{10}$ (no match in DNP)
CF-285765	*Paraconiothyrium* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	$C_{36}H_{22}O_6$ (no match in DNP)
CF-285774	*Selenophoma juncea*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	possible phomotone, norteanuazonic acid
CF-285777	*Dothiorella* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	dechlorogriseofulvin, griseofulvin
CF-285772	*Preussia* sp.	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	di-hydro-bi-chloro-geodin, asterric acid
CF-285773	*Preussia* sp.	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	cerulenin analog, asterric acid, $C_{18}H_{32}N_8O_{10}$ (No match in DNP)
CF-090317	*Selenophoma* sp.	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	$C_{26}H_{43}NO_6$ (previously described in plants), cyclo(phenylalanylprolyl)
CF-288939	*Alternaria* sp.	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	nortenuazonic and tenuazonic acid
CF-288959	*Tamaricicola* sp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	cyclo (phenylalanylprolyl), possible phenopyrrozin
CF-092176	*Libertasomyces* sp.	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	possible naematolin, $C_{24}H_{30}O_5$

CF-282335	*Homortomyces tamaricis*	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	cephalochromin, TAN 2483 A and B
CF-090258	*Stagnospora* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	secalonic acid C, 11,12-dihydroxyeudesm-4-en-3-one, iludinic acid
CF-279249	*Cainia* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	cyclo (phenylalanylprolyl)
CF-285365	*Phoma* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	cercosporamide
CF-285743	*Epicoccum* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	tenuazonic acid, mellein, phomasetin
CF-285771	*Nigrospora* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	possible taurocholic acid
CF-287451	*Phoma* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	mycophenolic acid
CF-090778	*Stagnospora* sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	11,12-dihydroxyeudesm-4-en-3-one, possible gonytolide F
CF-279243	*Leptospora* sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	possible chrysoxanthone
CF-279245	*Pseudopithomyces chartarum*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	possible antibiotic LL-N 313ε
CF-285353	*Dothiora* sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	hormonemate
CF-285371	*Microascus trigonosporus*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	petasol
CF-090323	*Phoma* sp.	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	antibiotic PF 1163A
CF-090387	*Camarosporium* sp.	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	possible coriolide
CF-288938	*Didymella* sp.	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	cyclo (phenylalanylprolyl), $C_{39}H_{27}NO_8S$ (no match in DNP)
CF-282337	*Ascochyta* sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	possible sequalestatin H1, $C_{37}H_{52}O_{15}$ (previously described in plants)
TOTAL		17	7	13	6	11	22	9	13	4	26	22	7	13	10	30	

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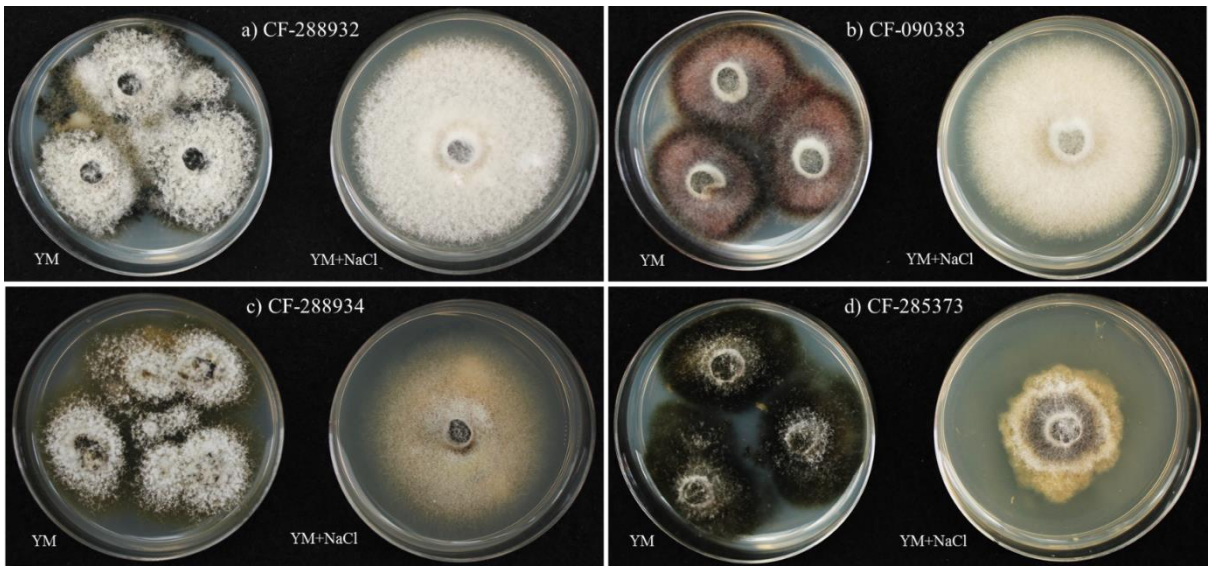


Supplementary Figure 2 a-d. Different isolates of *Neocamarosporium* spp grown on yeast malt agar (YM) and yeast malt agar supplemented with NaCl at 3% (YM+NaCl) during 14 days at 22ºC and 70% of relative humidity.

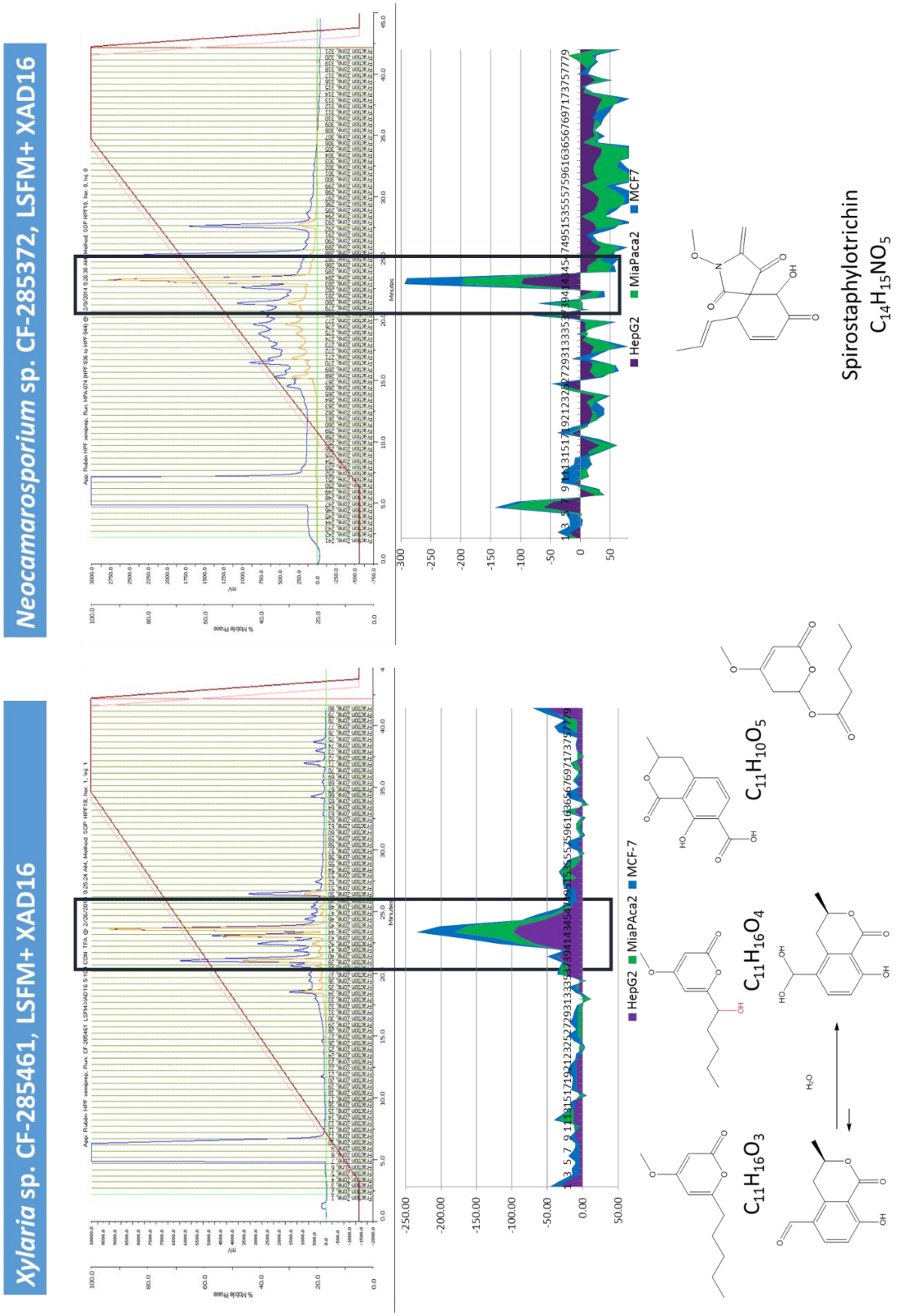
Supplementary Table 4. List of collected plant specimens.

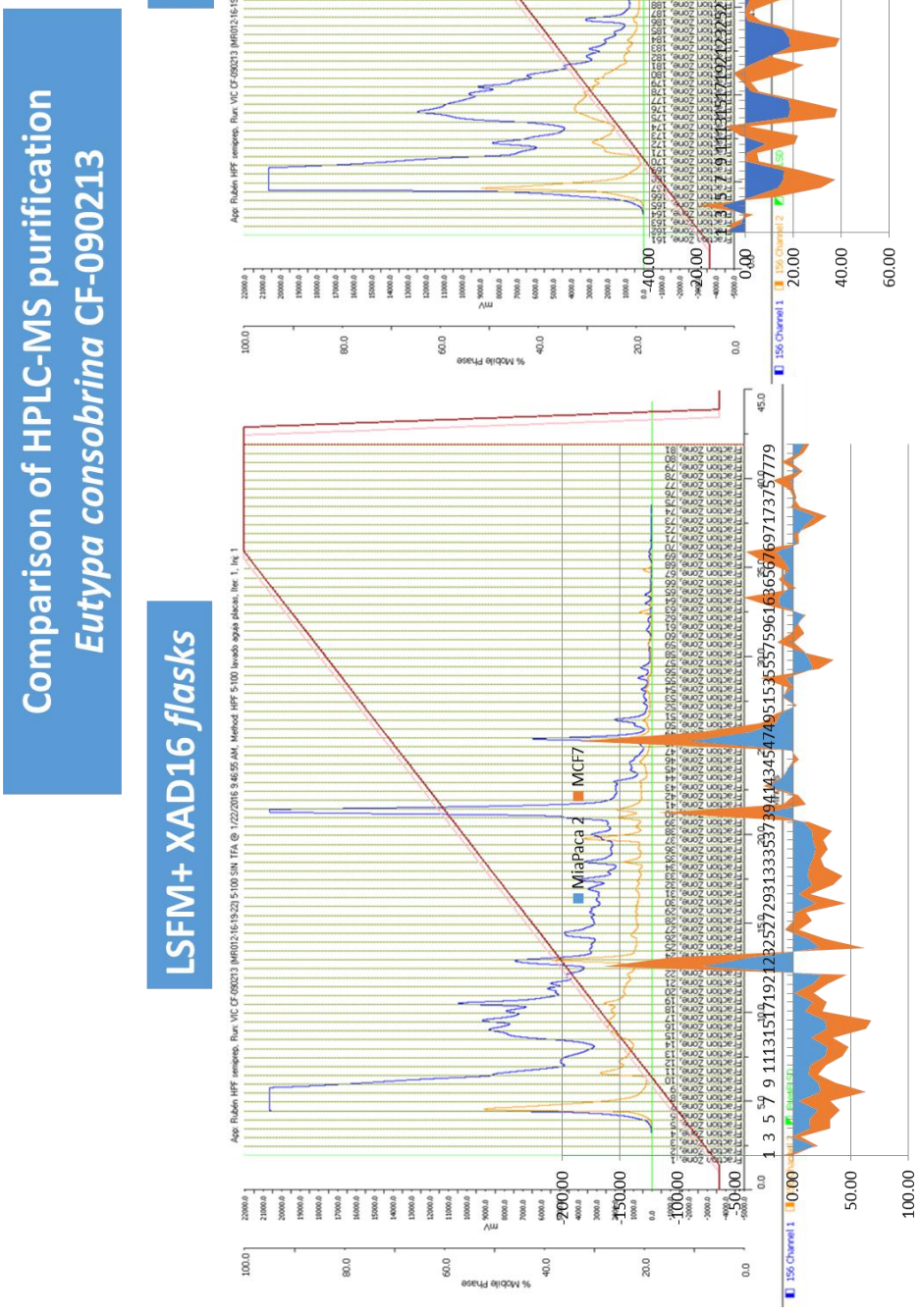
Plant Species	Collection place	Ecology	Termo-phile	Xero-phile	Gypso-phile	Halo-phile	Salt Tolerant
Anabasis articulata	Tabernas desert (Almeria)	Desert	x	x		x	
Anthyllis cystisoides	Sierra de la Alhamilla (Almeria)	Semiarid	x				
Anthyllis terniflora	Tabernas desert (Almeria)	Desert	x	x			
Artemisia barrelieri	Tabernas desert (Almeria)	Desert		x			
Arthrocnemum macrostachyum	Cabo de Gata (Almeria)	Salt mars	x	x		x	
Asparagus horridus	Tabernas desert (Almeria)	Desert	x	x			
Atriplex glauca	El Margen (Granada)	Salt mars	x	x		x	
Ballota hirsuta	Sierra de la Alhamilla (Almeria)	Semiarid		x			
Beta macrocarpa	El Margen (Granada)	Salt mars	x			x	
Centaurea dracunculifolia	El Margen (Granada)	Salt mars	x			x	
Chamaerops humilis	Cabo de Gata (Almeria)	Salt mars	x				
Cistus albidus	Sierra de la Alhamilla (Almeria)	Semiarid		x			
Cynanchum acutum	El Margen (Granada)	Salt mars	x			x	
Dittrichia viscosa	Venta de Fraile (Granada)	Semiarid					
Dorycnium pentaphyllum	Punta Entinas (Almeria)	Semiarid					
Euzomodendron bourgeanum	Tabernas desert (Almeria)	Desert	x			x	
Fagonia cretica	Tabernas desert (Almeria)	Desert	x			x	
Frankenia pulverulenta	El Margen (Granada)	Salt mars	x			x	
Frankenia corymbosa	Tabernas desert (Almeria)	Desert	x			x	
Genista umbellata	Tabernas desert (Almeria)	Desert	x				
Gypsophila tomentosa	El Margen (Granada)	Salt mars	x		x	x	
Helianthemum almeriense	Tabernas desert (Almeria)	Desert	x				x
Limbarda crithmoides	Punta Entinas (Almeria)	Semiarid	x			x	
Juniperus oxycedrus	Alfacar (Granada)	Semiarid		x			
Launaea arborescens	Tabernas desert (Almeria)	Desert	x	x			
Lepidium subulatum	El Margen (Granada)	Salt mars			x		

Limodorum abortivum	Barranco de los Pinos (Granada)	Semiarid	x				
Limonium insigne	Tabernas(Almeria)	Desert	x			x	
Limonium majus	El Margen (Granada)	Salt mars				x	
Lycium intricatum	Torre Garcia (Almeria)	Semiarid	x				x
Lygeum spartum	Tabernas(Almeria)	Desert		x			x
Macrosyringion longiflorum	El Margen (Granada)	Salt mars		x			
Moricandia foetida	Tabernas (Almeria)	Desert	x				x
Nerium oleander	Tabernas(Almeria)	Desert	x	x			
Nicotiana glauca	Rodalquilar (Almeria)	Semiarid	x				
Ononis tridentata	El Margen (Granada)	Salt mars			x		x
Ononis fruticosa	El Margen (Granada)	Salt mars		x			
Onoris ramosissina	Sierra de la Alhamilla (Almeria)	Semiarid				x	
Opuntia ficus-indica	Dilar (Granada)	Semiarid		x			
Otanthus maritimus	Torre Garcia (Almeria)	Semiarid	x			x	
Phragmites australis	El Margen (Granada)	Salt mars	x				x
Pinus halepenssis	Sierra de la Alhamilla (Almeria)	Semiarid	x				
Retama sphaerocarpa	Tabernas (Almeria)	Semiarid	x				
Rhamnus lycioides	Sierra de la Alhamilla (Almeria)	Semiarid		x			
Rosmarinus eriocalyx	Tabernas (Almeria)	Desert	x	x			
Salicornia ramossissima	Tabernas(Almeria)	Desert	x			x	
Salsola genistoides	Tabernas (Almeria)	Desert				x	
Salsola oppositifolia	Tabernas (Almeria)	Desert				x	
Salsola papillosa	Tabernas (Almeria)	Desert				x	
Salsola vermiculata	El Margen (Granada)	Salt mars				x	
Sedum sediforme	Sierra de la Alhamilla (Almeria)	Semiarid		x			
Sonchus crassifolius	El Margen (Granada)	Salt mars				x	
Spartium junceum	Fuente de Hervidero (Granada)	Semiarid		x			
Stipa tenacissima	Albuñuelas (Granada)	Semiarid		x			
Suaeda vera	Tabernas desert (Almeria)	Desert				x	
Tamarix canariensis	Tabernas desert (Almeria)	Desert	x	x		x	
Teucrium capitatum sbsp. gracillimum	El Margen (Granada)	Salt mars		x			
Thymelaea hirsuta	Los Almorades (Almeria)	Semiarid		x			
Thymus hyemalis	Tabernas desert (Almeria)	Desert	x	x			x
Thymus zygis subsp. gracilis	El Margen (Granada)	Salt mars		x			
Withania frutescens	Almerimar (Almeria)	Semiarid	x	x			x
Ziziphus lotus	Torre Garcia (Almeria)	Semiarid		x			x
Zygophyllum fabago	Torre Garcia (Almeria)	Semiarid	x			x	

Apendix

Bioassay-guided purification of representative active extracts. Semipreparative HPLC fractions *vs.* antitumoral activities and corresponding elucidated active compounds.




Comparison of HPLC-MS purification
Eutypa consobrina CF-090213
LSFM+ XAD16 *flasks*
LSFM *flasks*
MiaPaca2
MCF7
% Mobile Phase
mV
minutes

Apendix

Structure elucidation of new natural products

MDN-0209 (**1**) was isolated as a yellow pale oil. A molecular formula of $C_{17}H_{26}O_4$ was determined from HRESIMS data (*m/z* 277.1800 ($[M + H - H_2O]^+$; calcd. for $C_{17}H_{25}O_3^+$, 277.1798), requiring five double bond equivalents. The ^1H, ^{13}C-NMR and (**Table S1**) HSQC data, accounted for the presence in the molecule of three double bonds (δ_C 134.0, C-3; δ_C 131.5, C-4; δ_H 5.23, δ_C 118.8, CH-8; δ_C 136.2, C-9; δ_H 6.42, δ_C 126.9, CH-12; δ_H 6.08, δ_C 134.9, C-13). Two allylic methylenes (δ_H 2.83 and 2.37, δ_C 30.9, CH_2-7; δ_H 2.15, δ_C 36.6, CH_2-14), one aliphatic triplet methyl group (δ_H 0.94, δ_C 14.0, CH_3-16), two allylic singlet methyls (δ_H 1.67, δ_C 18.1, CH_3-10; δ_H 1.74, δ_C 26.0, CH_3-11) and one aliphatic methylenes (δ_H 1.47, δ_C 23.5, CH_2-15) were also observed. Additionally, several oxygenated carbons were present in the molecule: one quaternary carbon (δ_C 61.1, C-1), three methines (δ_H 4.43, δ_C 67.6, CH-2; δ_H 4.61, δ_C 65.2, CH-5; δ_H 3.21, δ_C 59.5, CH-6) and one methylene (δ_H 4.39 and 4.15, δ_C 59.9, CH_2-17). COSY (**Figure S1**) and HMBC (**Figure S2**) spectra helped to construct the planar structure, which contained one epoxide and one six carbons ring, completing the total number of unsaturations. The presence of the epoxide was justified by the shielded chemical shift of carbons C-1 and C-6 respect to the rest of oxygenated carbons. An *E* configuration was proposed for the double bond C-12/C-13 based on the value of the H-12/H-13 coupling constant (*J* = 15.7 Hz). The NOESY interactions observed between protons H-7 and H-10, and those between H-8 and H-11 secured the identity of the two allylic methyl groups. The relative configuration around the six members ring was supported by key NOESY correlations between protons H-2, H_2-7 and H-6, indicating that all of them are on the same face of the molecule. Finally, the absolute configuration at C-2 and C-5 was determined applying the modified Mosher's method (1) to the R- and S-MTPA esters of both secondary hydroxyl groups. Although the ^1H gave an irregular distribution of $\Delta\delta^{SR}$ values, the configuration could eventually be determined by analysis of the $\Delta\delta^{SR}$ distribution for the ^{13}C signal (**Figure S10**), indicating an *S* configuration in both centers.

MDN-0210 (**2**) was isolated as a brown pale oil. A molecular formula of $C_{17}H_{24}O_4$ was determined from HRESIMS (*m/z* 293.1747 ($[M + H]^+$; calcd. for $C_{17}H_{25}O_4^+$, 293.1747), requiring six degrees of unsaturation. Two protons less in the molecular formula of **2** with respect to **1** and the analysis of the ^1H and ^{13}C-NMR spectra (**Table S1**) indicated that the hydroxyl group at C-5 in **1** was oxidized to a carbonyl group in **2**. The HMBC correlation between H-6 (δ_H 3.30) and C-5 (δ_C 198.1) supported this change (**Figure S12**). The rest of the planar structure was confirmed by COSY (**Figure S11**) and HMBC spectra, and proved to be similar to compound **1**. On the other hand, the NOESY interactions observed between protons 2, 7 and 6 supported the relative configuration proposed and the absolute configuration was assumed to be the same as in compound **1**.

MDN-0211 (**3**) was isolated as a white amorphous solid. Its HRESIMS analysis gave a molecular ion a *m/z* 235.0574 ($[M + Na]^+$; calcd. for $C_{10}H_{12}NaO_5^+$, 235.0577), which was consistent with the molecular formula $C_{10}H_{12}O_5$, requiring five double bond equivalents. The ^1H and ^{13}C-NMR spectra (**Table S2**) showed signals for two methoxy groups (δ_H 3.81, δ_C 56.6, CH_3-9; δ_H 3.88, δ_C 57.1, CH_3-10), one oxygenated methylene (δ_H 5.06 and 4.42, δ_C 71.5, CH_2-8), one double oxygenated methine (δ_H 6.12, δ_C 107.6, CH_3-1) and one aromatic ring (δ_H 6.57, δ_C 98.1, CH-4; δ_C 118.6, C-2; δ_C 149.6, C-3; δ_C 150.7, C-5; δ_C 135.1, C-6; δ_C 129.5, C-7). The analysis of HMBC spectrum (**Figure S20**) determined the connectivity of these units. The methoxy protons of OCH_3-9 and OCH_3-10 had an HMBC correlation to C-3 and C-5, respectively, on the aromatic moiety. HMBC correlations from H_2-8 to C-1 and from H-1 to C-8 and the chemical shift of these hydrogen and carbon atoms in the ^1H and ^{13}C NMR spectra secured the presence of an oxygen bridge between both carbon atoms and hence of a heteroaromatic ring in the structure of **3**. Finally, the chemical shift of C-6 (δ_C 135.1) was indicative of the presence of a hydroxyl group at this carbon, completing the initially determined chemical formula. The absence of specific rotation in the sample confirmed the existence of a mixture of enantiomers in this sample. Most probably, compound **3** is formed by nucleophilic attack of a primary hydroxyl group at C-8 to an aldehyde carbonyl group at C-1 equally favored in both faces of the carbonyl group.

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MDN-0209: pale yellow oil; $[\alpha]^{26}_{D}$ -48.22(c 0.19w/v%, CH_2Cl_2); UV (DAD) λ_{max} 240nm; IR (ATR) υ cm^{-1}: 3357, 3041, 2959, 2927, 2872, 2728, 1671, 1453, 1377, 1263, 1143, 1089, 1005, 963, 841. HRMS *m/z* 277.1800 $[M+H-H_2O]^+$ (calcd. for $C_{17}H_{25}O_3^+$, 277.1798); 312.2171 $[M+NH_4]^+$ (calcd. for $C_{17}H_{30}NO_4^+$, 312.2169); 611.3555 $[2M+Na]^+$ (calcd. for $C_{34}H_{52}NaO_8^+$, 611.3554); for ^1H and ^{13}C NMR data, see Table S1.

Table S1. ^1H (500MHz) and ^{13}C (125MHz) NMR Data for Compounds 1 and 2 in CD_3OD

Position	**1**		**2**	
	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
1	61.1, C		66.2, C	
2	67.6, CH	4.43, s	66.7, CH	4.77, s
3	134.0, C		151.1, C	
4	131.5, C		131.2, C	
5	65.2, CH	4.61, s	198.1, C	
6	59.5, CH	3.21, br t (1.7)	60.2, CH	3.30, s
7a	30.9, CH_2	2.83, dd (15.2, 7.4)	29.9, CH_2	2.94, dd (15.4, 7.8)
7b		2.37, dd (15.2, 7.4)		2.45, dd (15.4, 7.2)
8	118.9, CH	5.23, tt (7.4, 1.4)	118.2, CH	5.22, tm (7.5, 1.4)
9	136.2, C		137.1, C	
10	18.1, CH_3	1.67, s	18.0, CH_3	1.67, br s
11	26.0, CH_3	1.74, s	25.9, CH_3	1.75, br s
12	126.9, CH	6.42, d (15.7)	122.0, CH	6.06, m
13	134.9, CH	6.08, dt (15.7, 7.0)	140.1, CH	6.05, m
14	36.6, CH_2	2.15, c (7.0)	36.8, CH_2	2.14, m
15	23.5, CH_2	1.47, m (7.4)	23.3, CH_2	1.46, m (7.3)
16	14.0, CH_3	0.94, t (7.4)	14.0, CH_3	0.94, t (7.5)
17a	59.9, CH_2	4.39, d (12.3)	60.4, CH_2	4.47, d (13.4)
17b		4.15, d (12.3)		4.32, d (13.4)

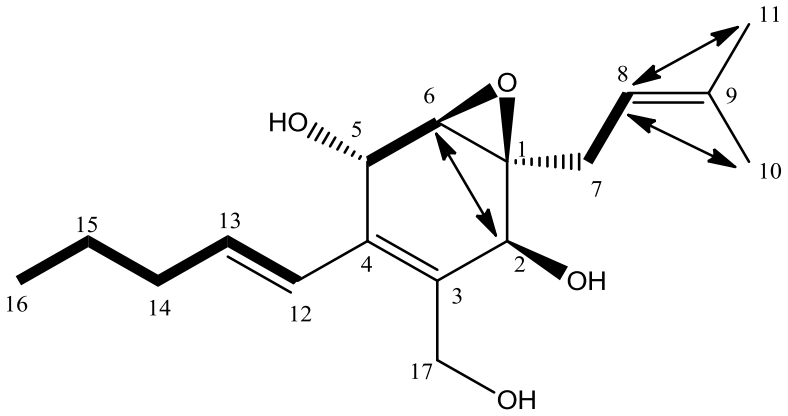


Figure S1. Key ^{1}H-^{1}H COSY (bold lines, along range H↔H) correlations for compound **1**

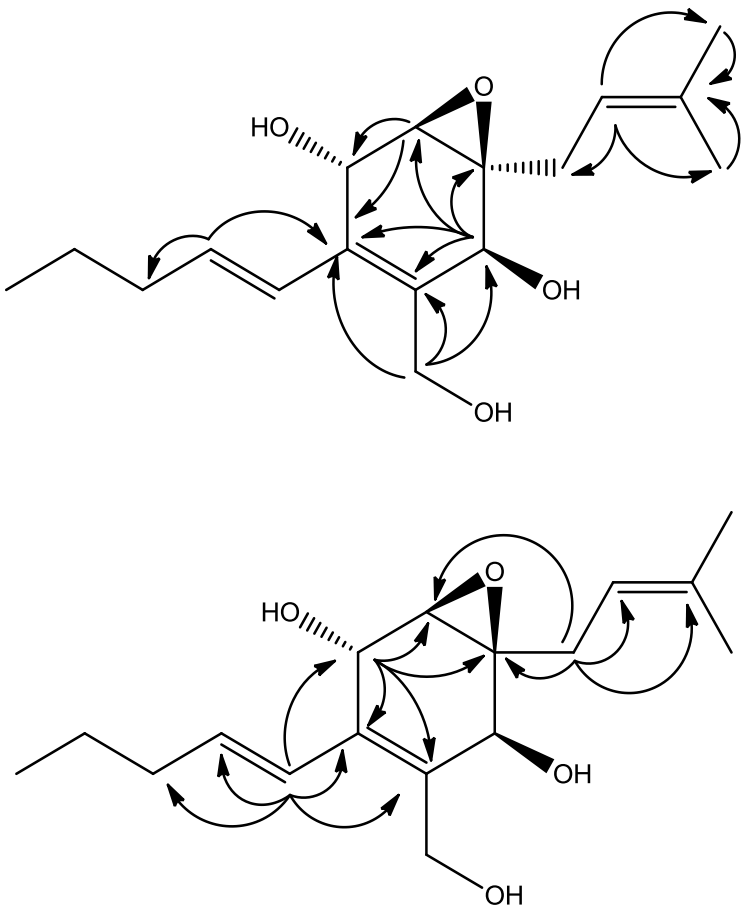


Figure S2. Key HMBC (H→C) correlations for compound **1**

Apendix

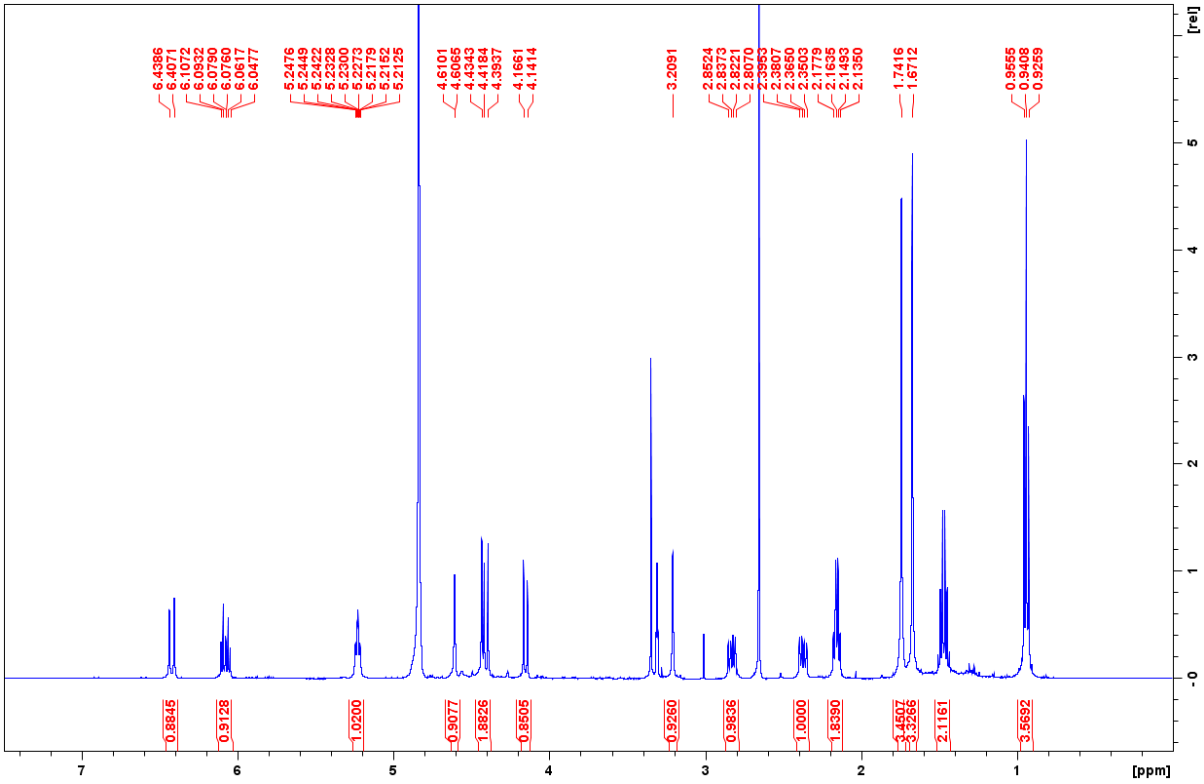


Figure S3. ^{1}H NMR spectrum (500MHz) of **1** in CD_3OD.

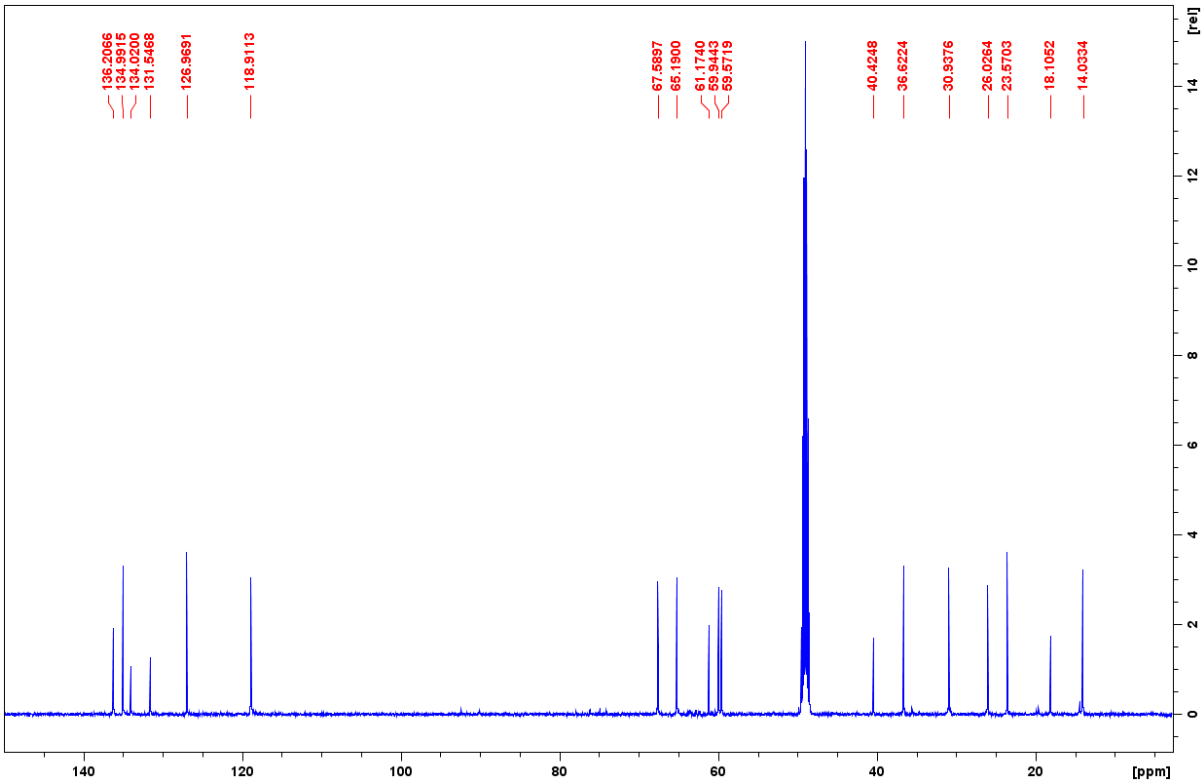


Figure S4. ^{13}C NMR spectrum (125MHz) of **1** in CD_3OD.

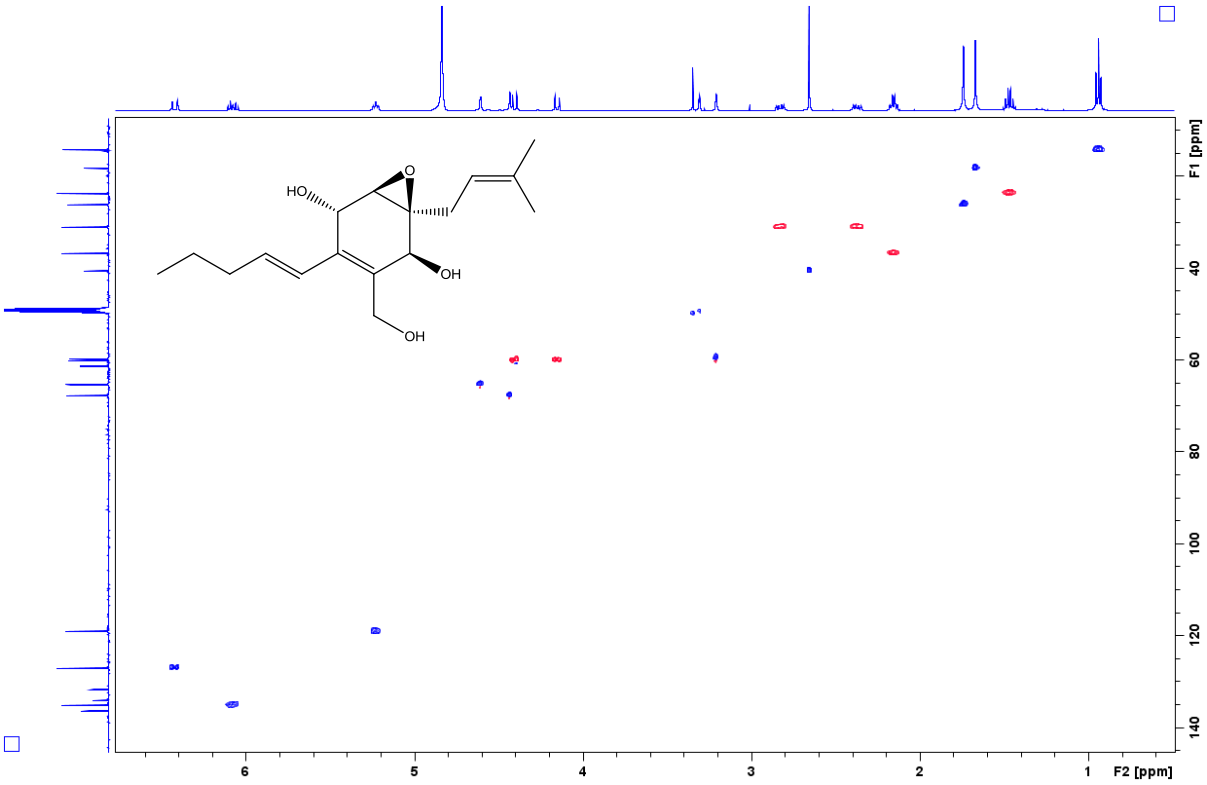


Figure S5. HSQC spectrum (500MHz) of **1** in CD_3OD.

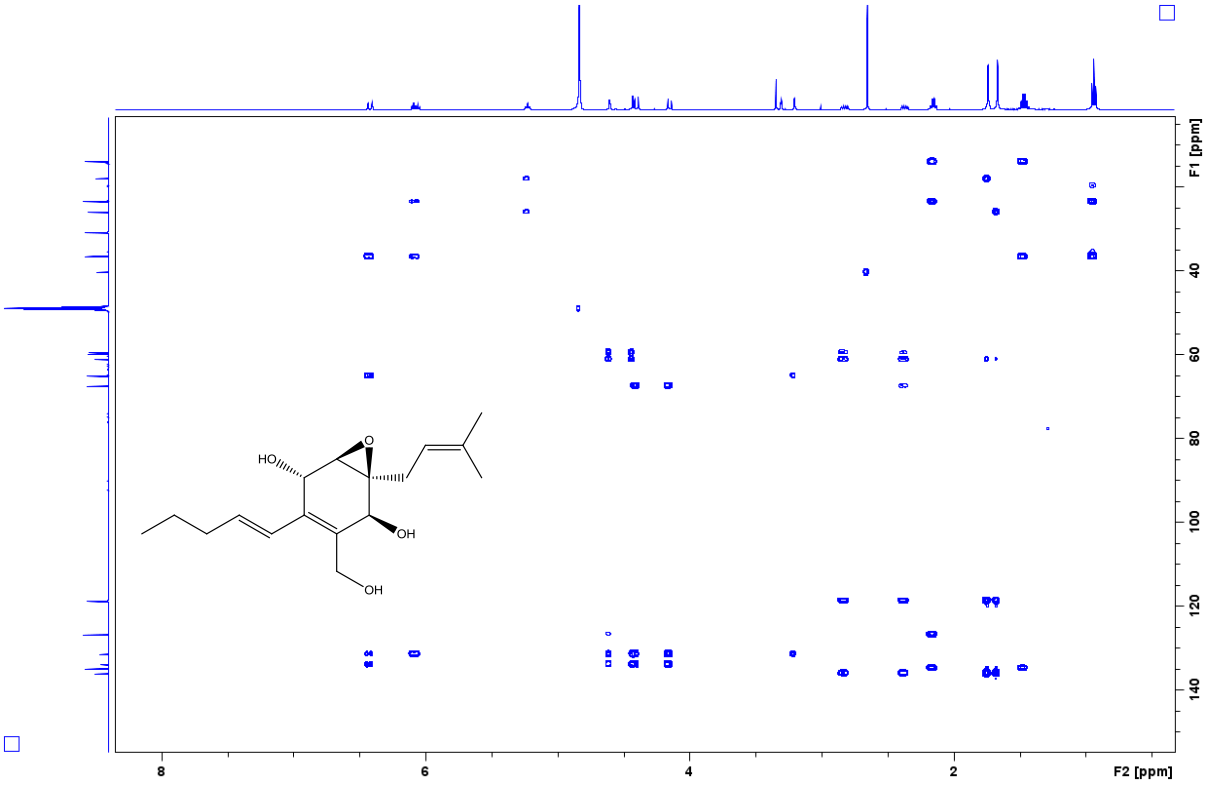


Figure S6. HMBC spectrum (500MHz) of **1** in CD_3OD.

Apendix

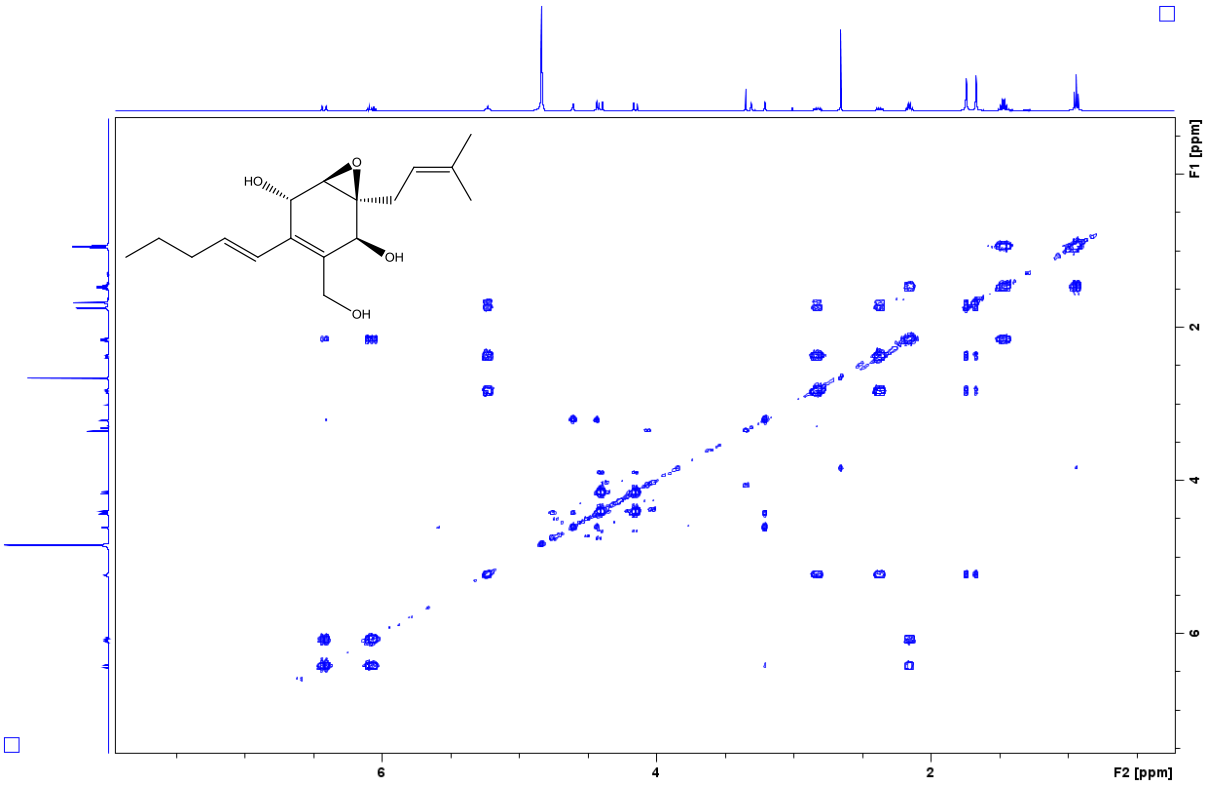


Figure S7. COSY spectrum (500MHz) of **1** in CD_3OD.

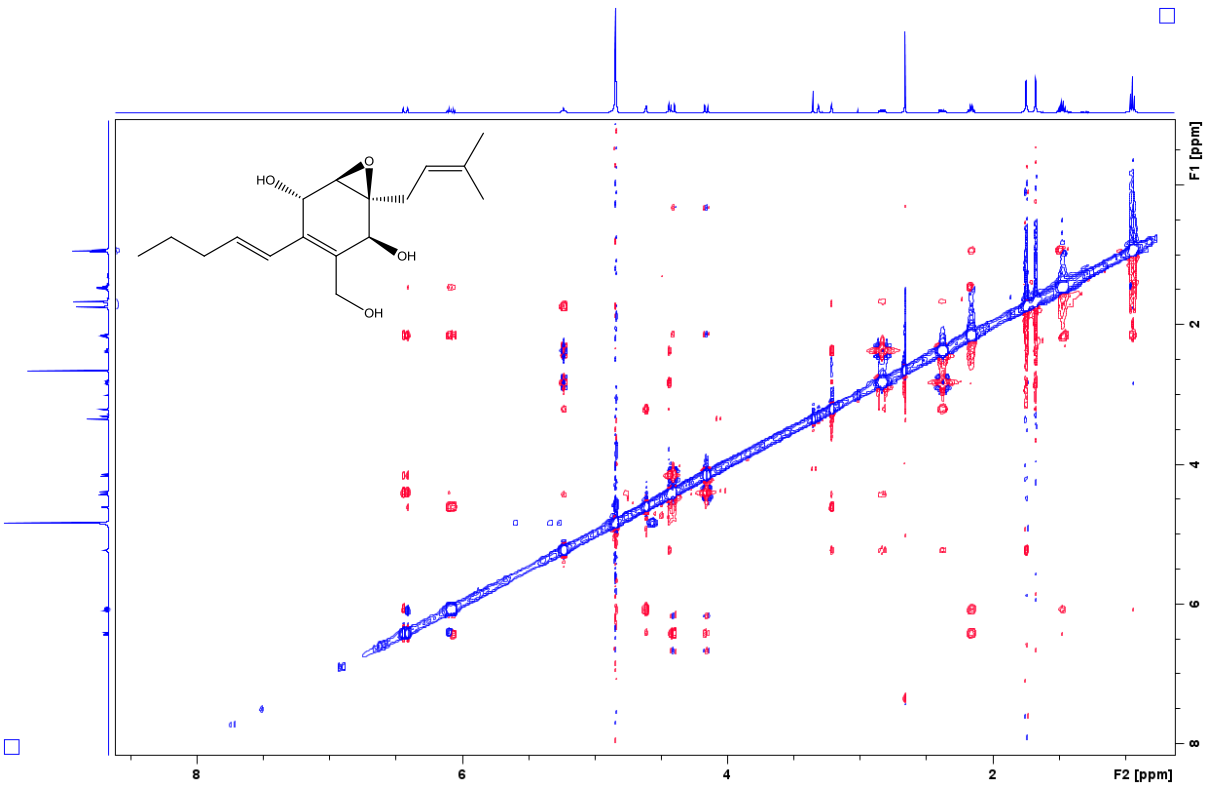


Figure S8. NOESY spectrum (500MHz) of **1** in CD_3OD.

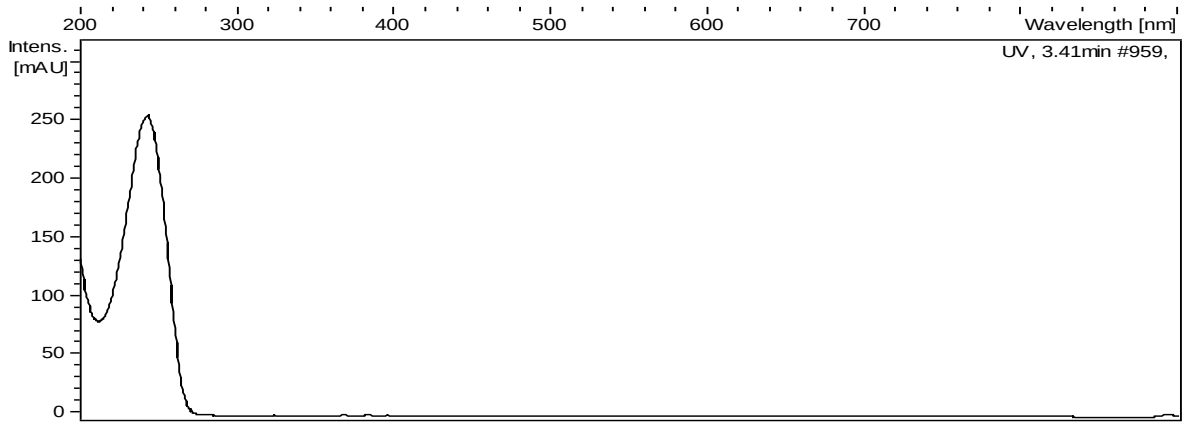


Figure S9. UV spectrum of **1**

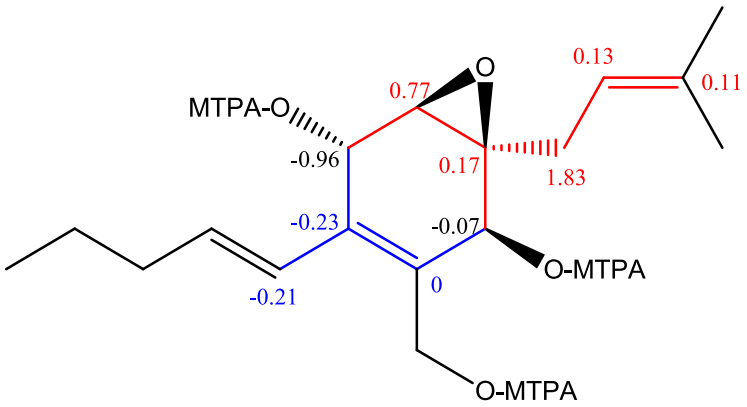


Figure S10. $\Delta\delta^{SR}$ values of MTPA-esters of compound **1**

MDN-0210 (2): White and amorphous solid; $[\alpha]^{26}_{D}$ 95.62 (c 0.04w/v%, CH_2Cl_2); UV (DAD) λ_{max} 210nm, 280nm; IR (ATR) υ cm^{-1}: 3420, 2959, 2928, 2872, 1672, 1453, 1377, 1025, 969, 841. HRMS *m/z* 275.1641 $[M+H-H_2O]^+$ (calcd. for $C_{17}H_{23}O_3^+$, 275.1642); 293.1747 $[M+H]^+$ (calcd. for $C_{17}H_{25}O_4^+$, 293.1747); 310.2007 $[M+NH_4]^+$ (calcd. for $C_{17}H_{28}NO_4^+$, 310.2013); 607.3234 $[2M+Na]^+$ (calcd. for $C_{34}H_{48}NaO_8^+$, 607.3241); for ^1H and ^{13}C NMR data, see Table S1.

Apendix

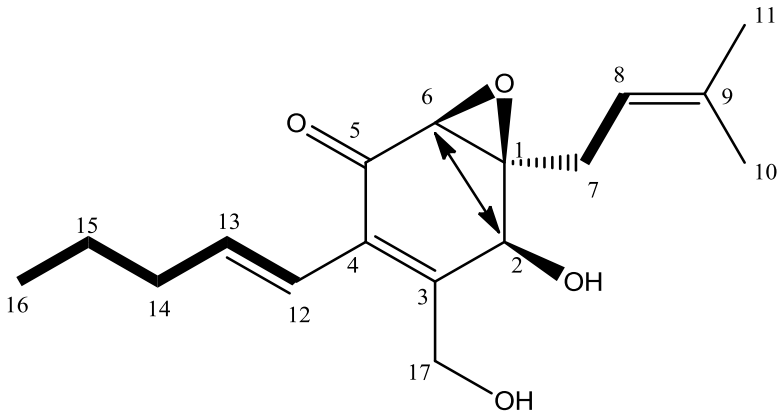


Figure S11. Key ^{1}H-^{1}H COSY (bold lines, and long-range H↔H) correlations for compound **2**.

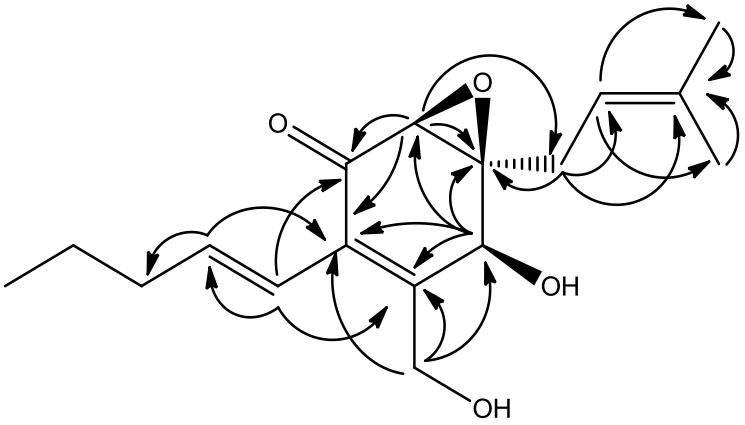


Figure S12. Key HMBC (H→C) correlations for compound **2**.

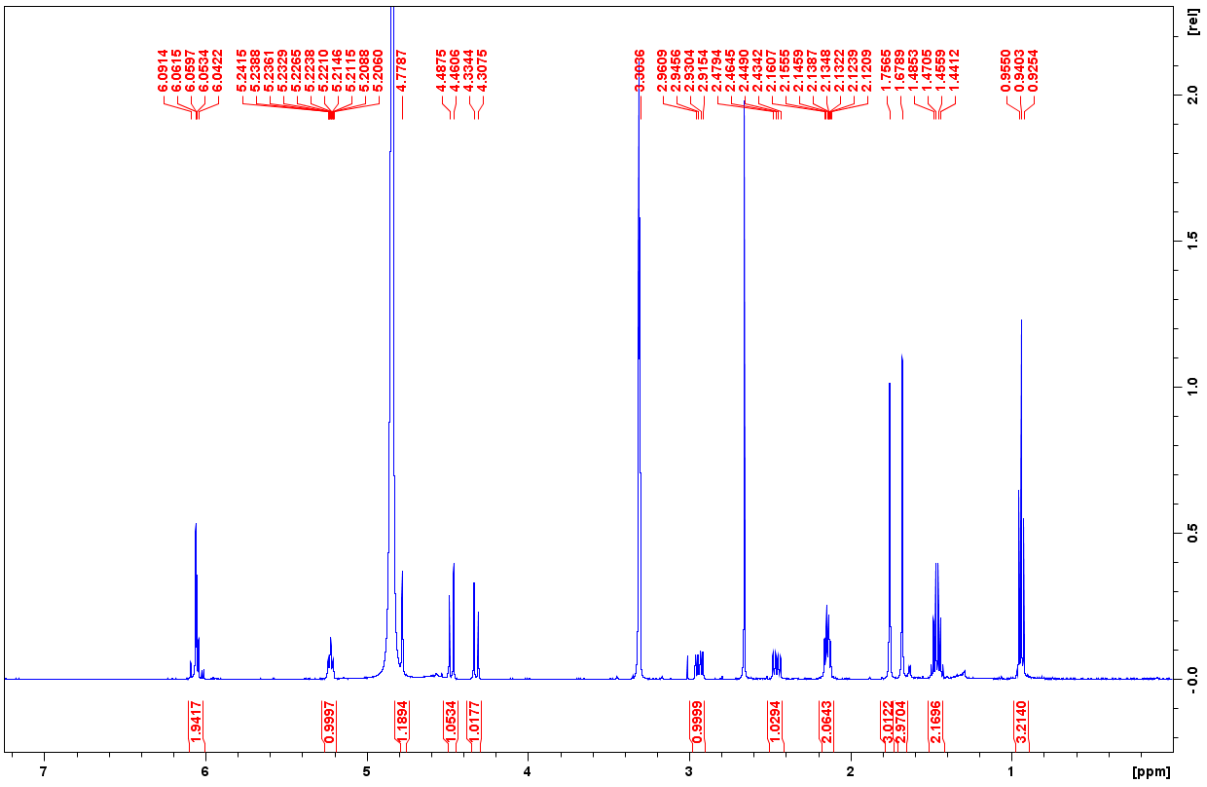


Figure S13. ^{1}H NMR spectrum (500MHz) of **2** in CD_3OD.

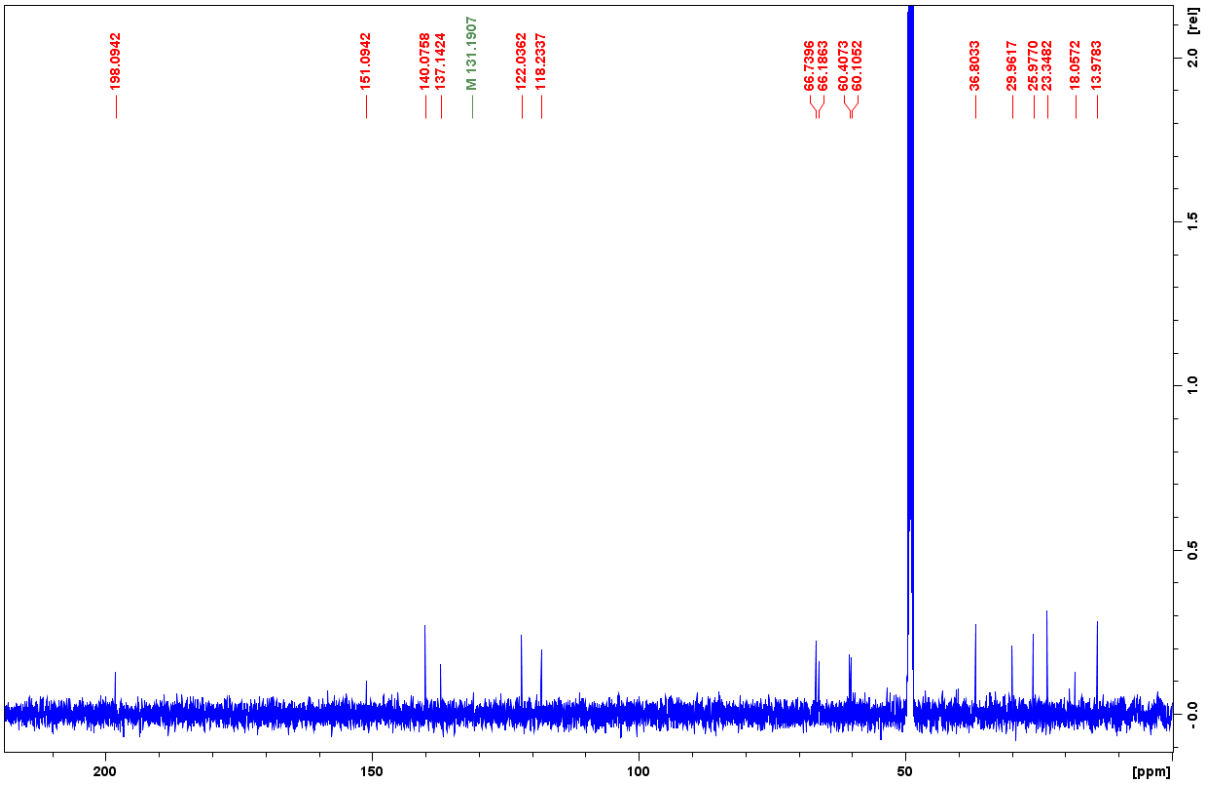


Figure S14. ^{13}C NMR spectrum (125MHz) of **2** in CD_3OD.

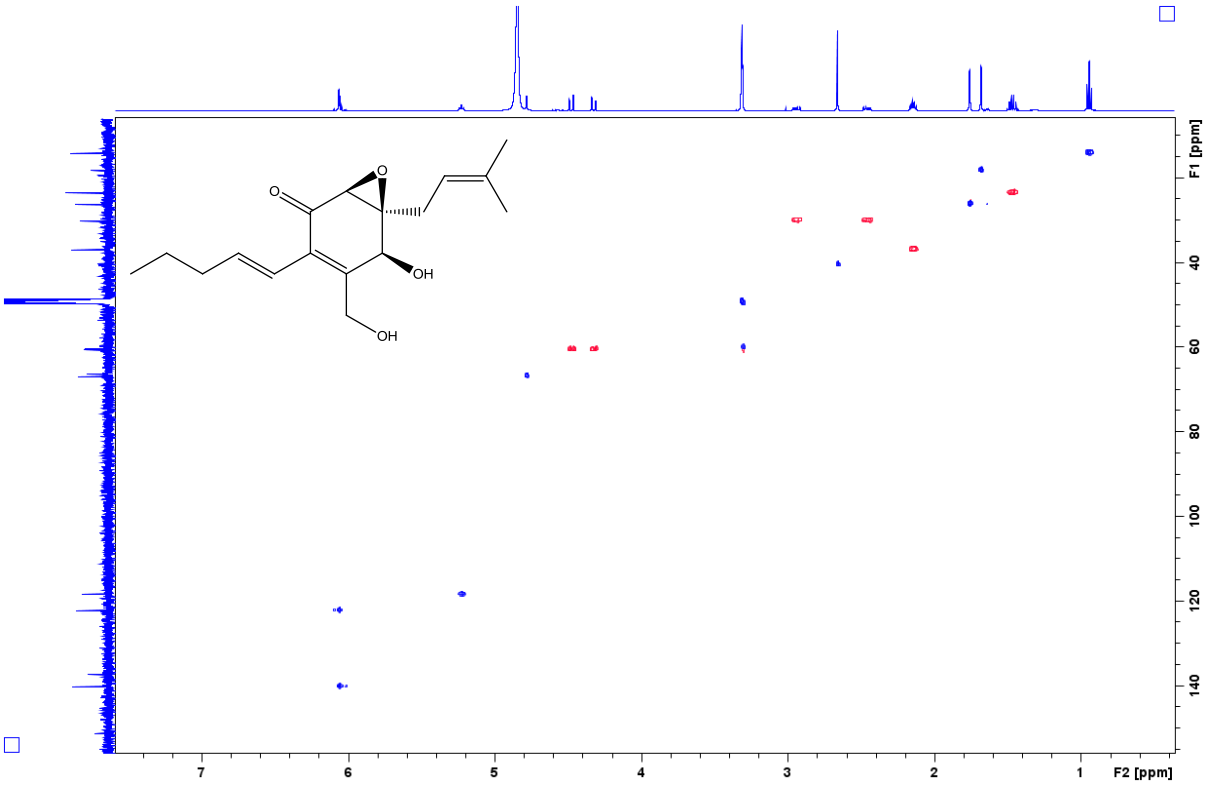


Figure S15. HSQC spectrum (500MHz) of **2** in CD_3OD.

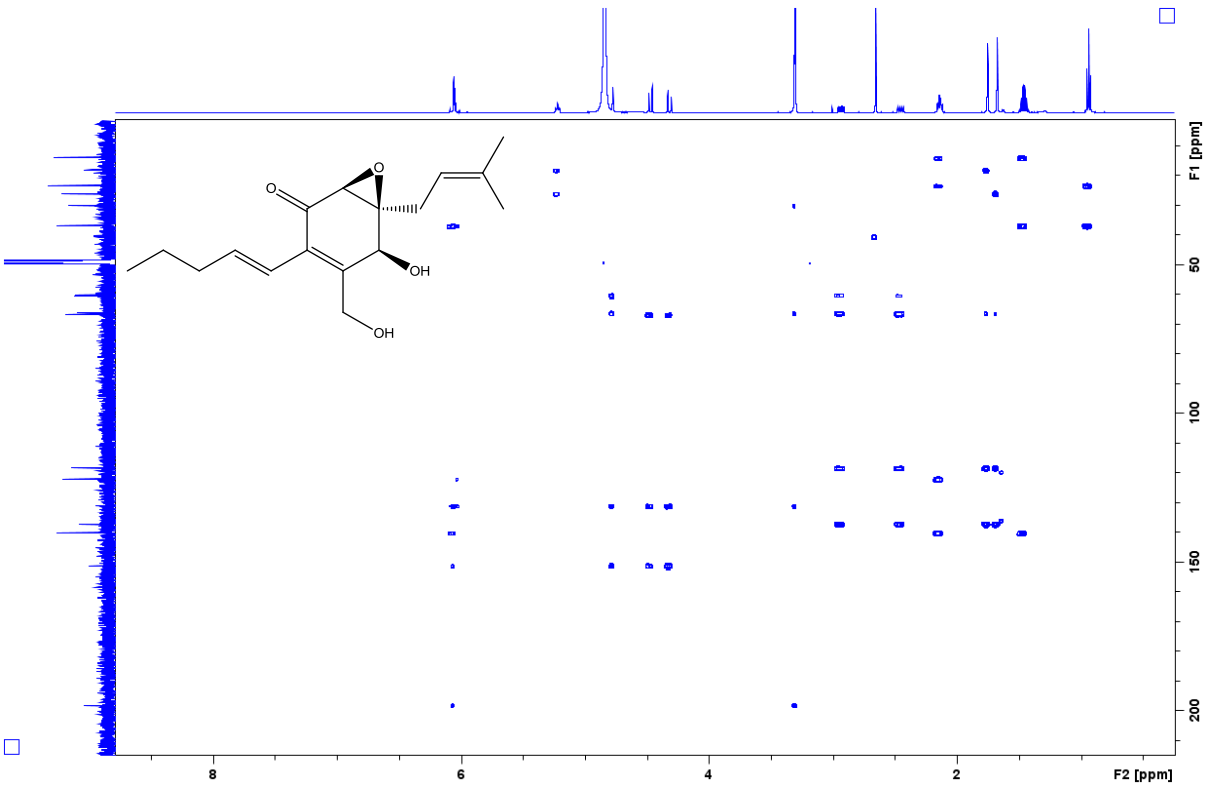


Figure S16. HMBC spectrum (500MHz) of **2** in CD_3OD.

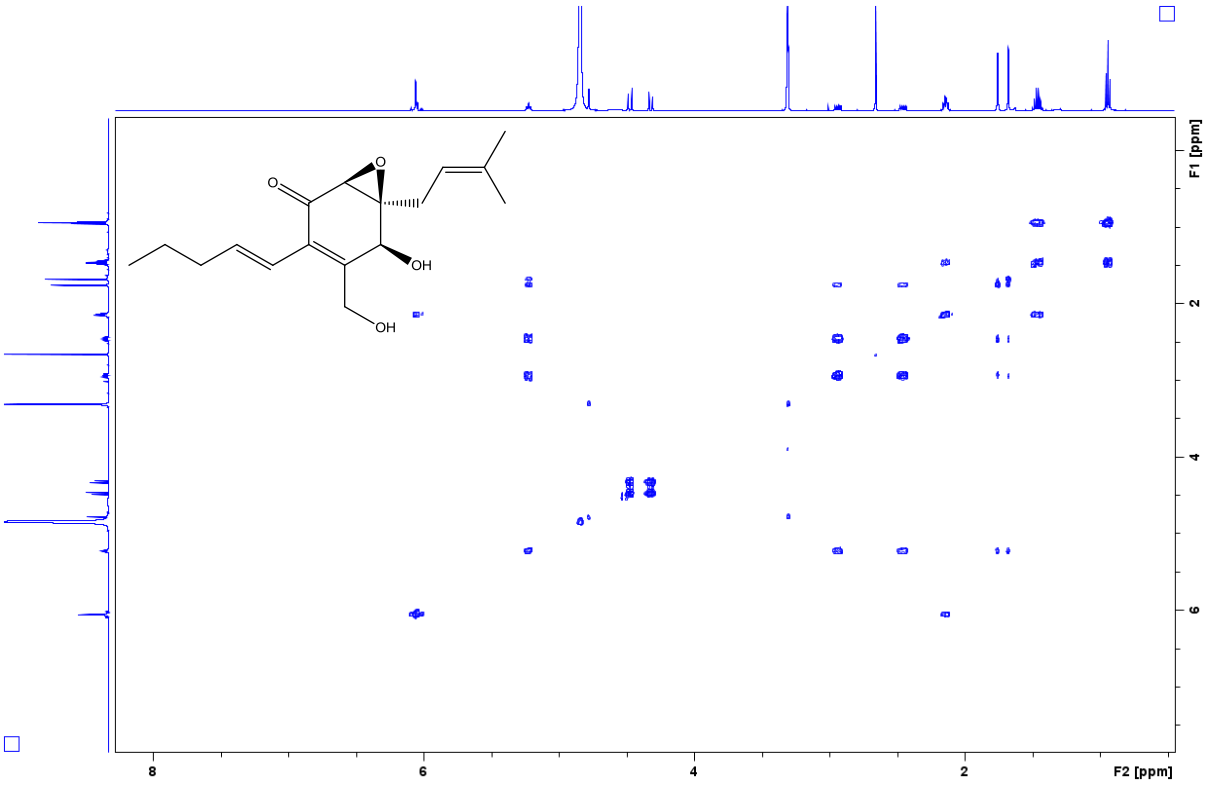


Figure S17. COSY spectrum (500MHz) of **2** in CD_3OD.

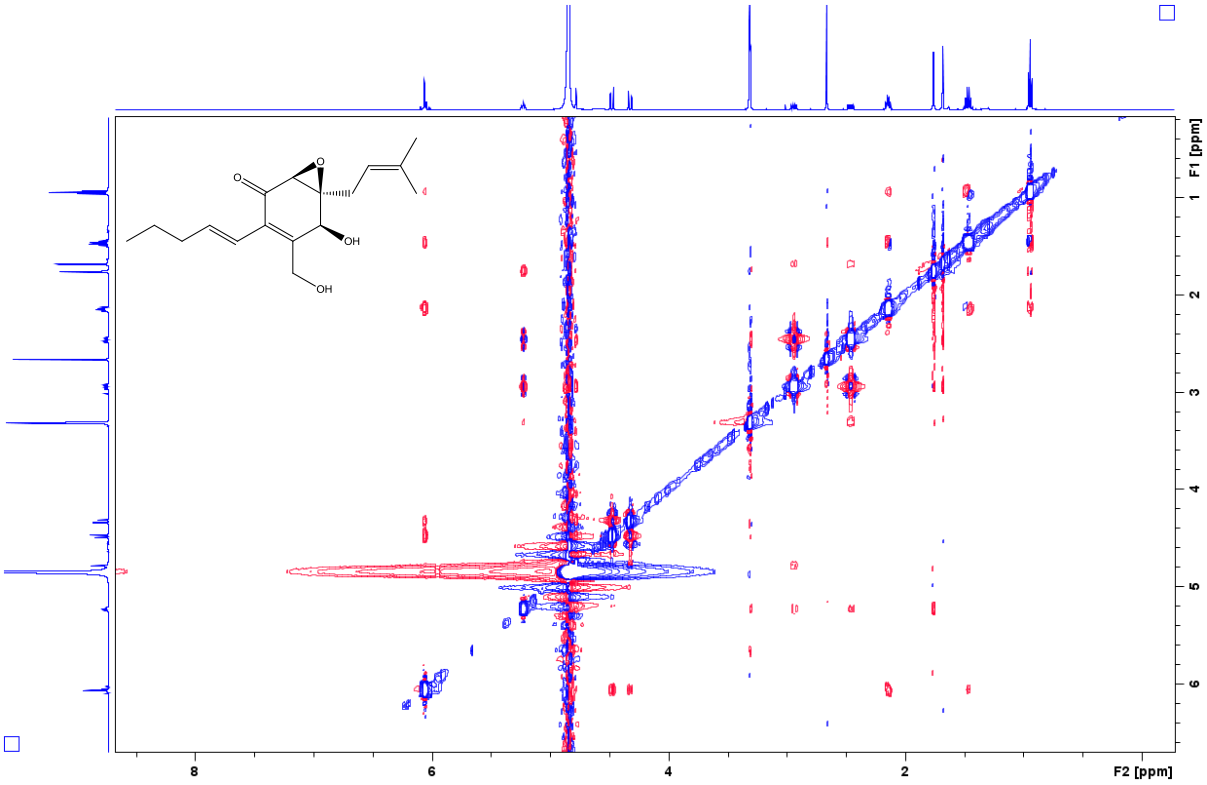


Figure S18. NOESY spectrum (500MHz) of **2** in CD_3OD.

Apendix

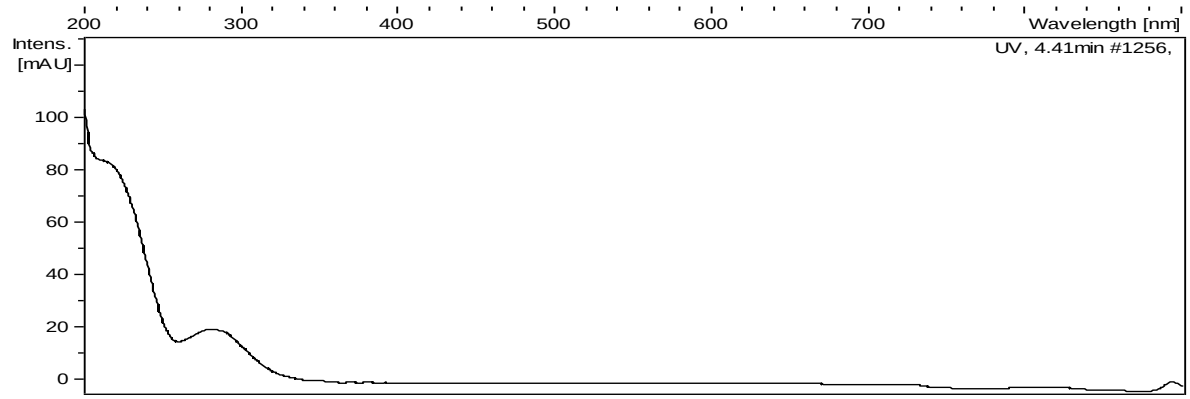


Figure S19. UV spectrum of **2**

MDN-0211 (3): White and amorphous solid; $[\alpha]^{25}_{D}$ 0.00 (c 0.16w/v%, CH_3OH); UV (DAD) λ_{max} 230nm, 280nm; IR (ATR) υ cm^{-1}: 3363, 2943, 2839, 1643, 1505, 1454, 1438, 1245, 1210, 1133, 1086, 1024. HRESIMS *m/z* 195.0655 $[M+H-H_2O]^+$ (calcd. for $C_{10}H_{11}O_4^+$, 195.0652); 235.0574 $[M+Na]^+$ (calcd. for $C_{10}H_{12}NaO_5^+$, 235.0577); 447.1257 $[2M+Na]^+$ (calcd. for $C_{20}H_{24}NaO_{10}^+$, 447.1262); for ^1H and ^{13}C NMR data, see Table S3:

Table S3. ^1H (500 MHz) and ^{13}C (125 MHz) NMR Data for Compound 1 in MeOD

Position	δ_C, type	δ_H, mult. (*J* in Hz)
1	107.6, CH	6.12, d (1.96)
2	118.6, C	
3	149.6, C	
4	98.1, CH	6.57, s
5	150.7, C	
6	135.1, C	
7	129.5, C	
8a	71.5, CH2	5.06, dd (12.8, 1.48)
8b		4.42, d (12.8)
9	56.6, CH3	3.81, s
10	57.1, CH3	3.88, s

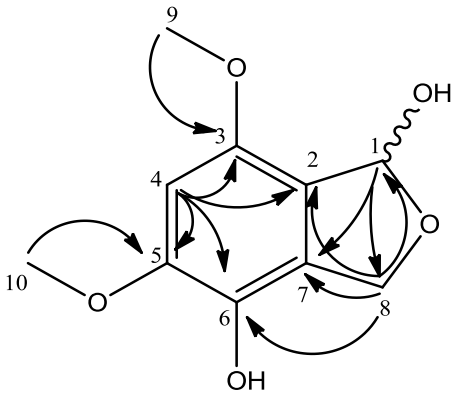


Figure S20. Key HMBC (H→C) correlations for compound **3**

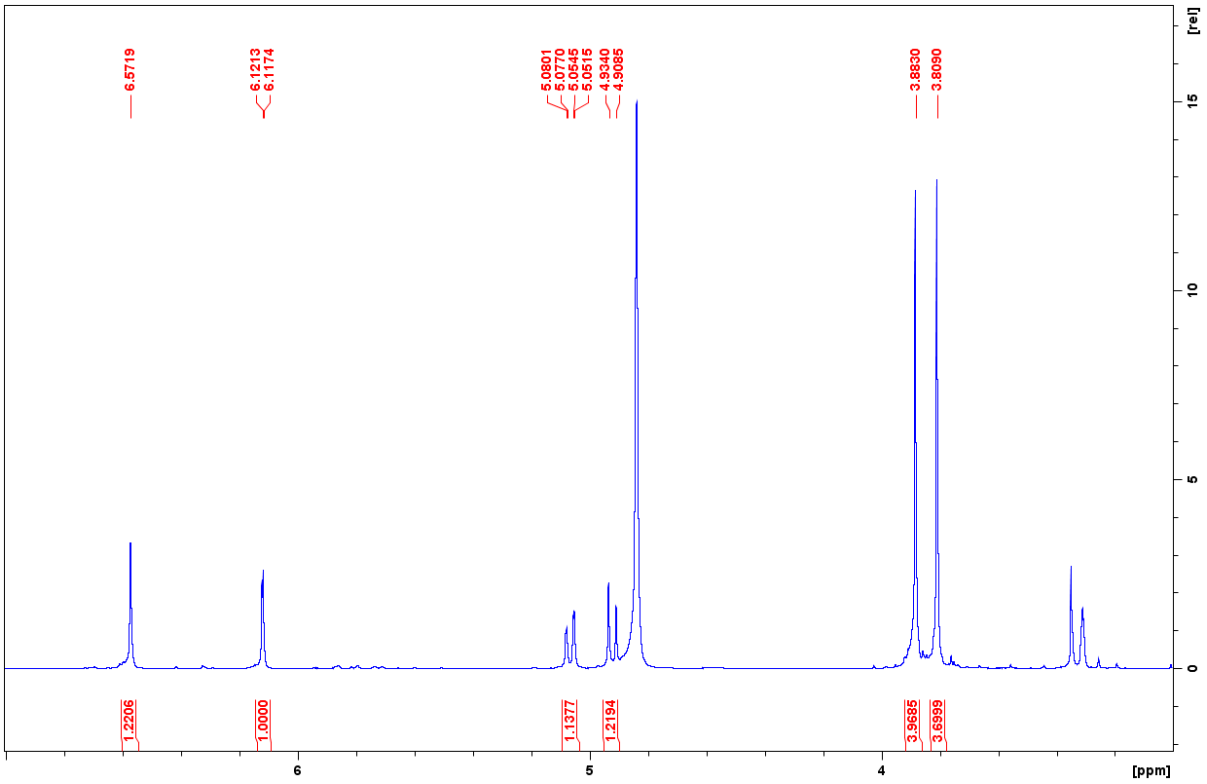


Figure S21. ^{1}H NMR spectrum (500MHz) of **3** in CD_3OD.

Apendix

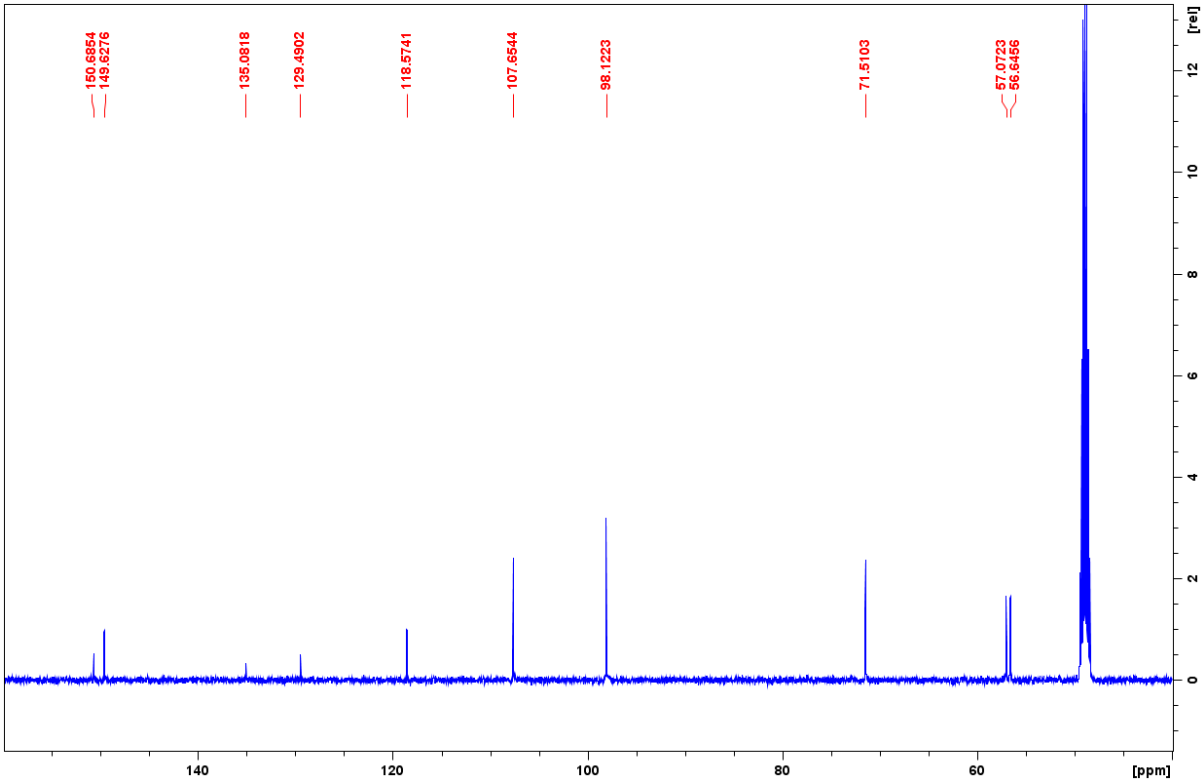


Figure S22. ^{13}C NMR spectrum (125MHz) of **3** in CD_3OD.

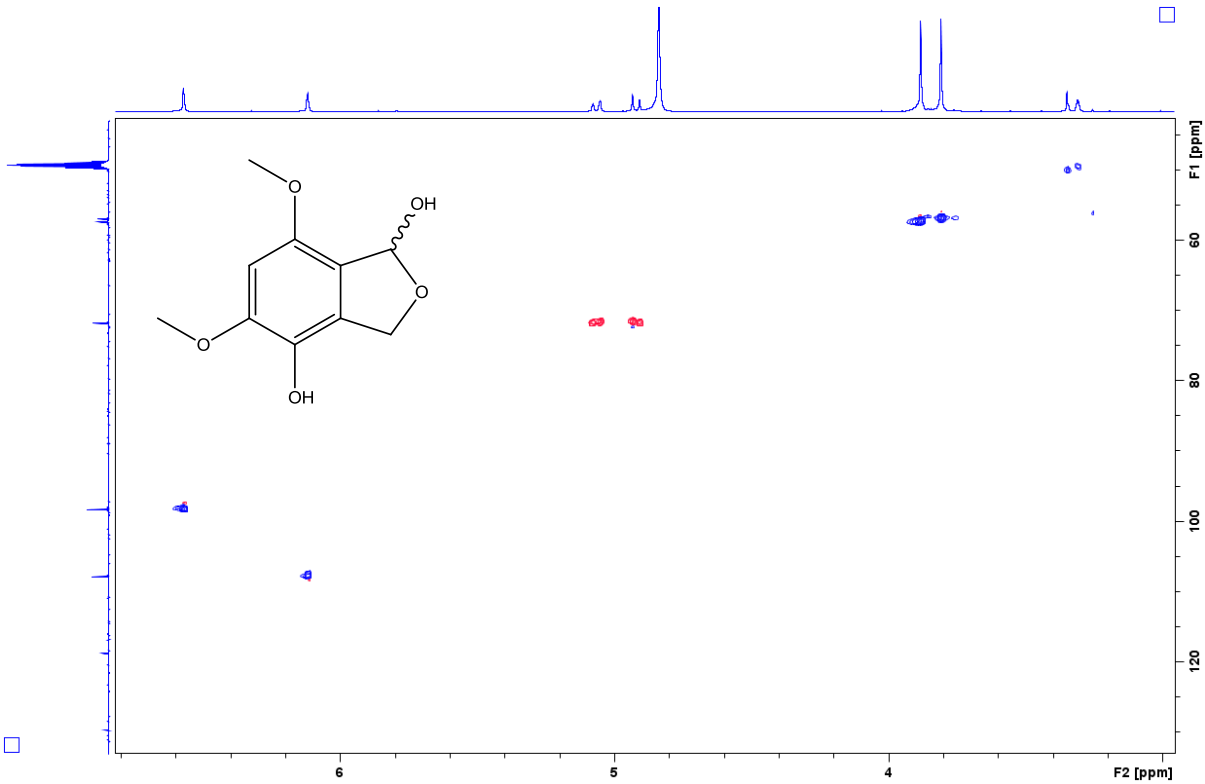


Figure S23. HSQC spectrum (500MHz) of **3** in CD_3OD.

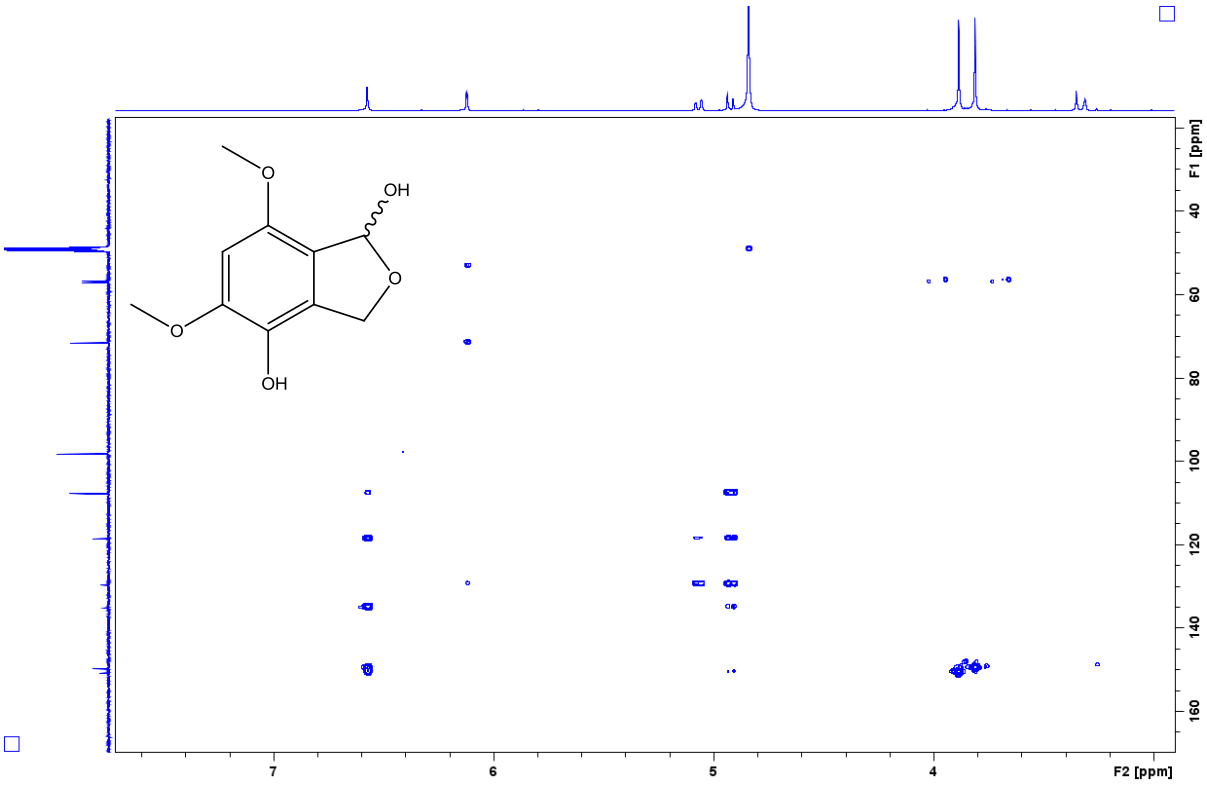


Figure S24. HMBC spectrum (500MHz) of **3** in CD_3OD.

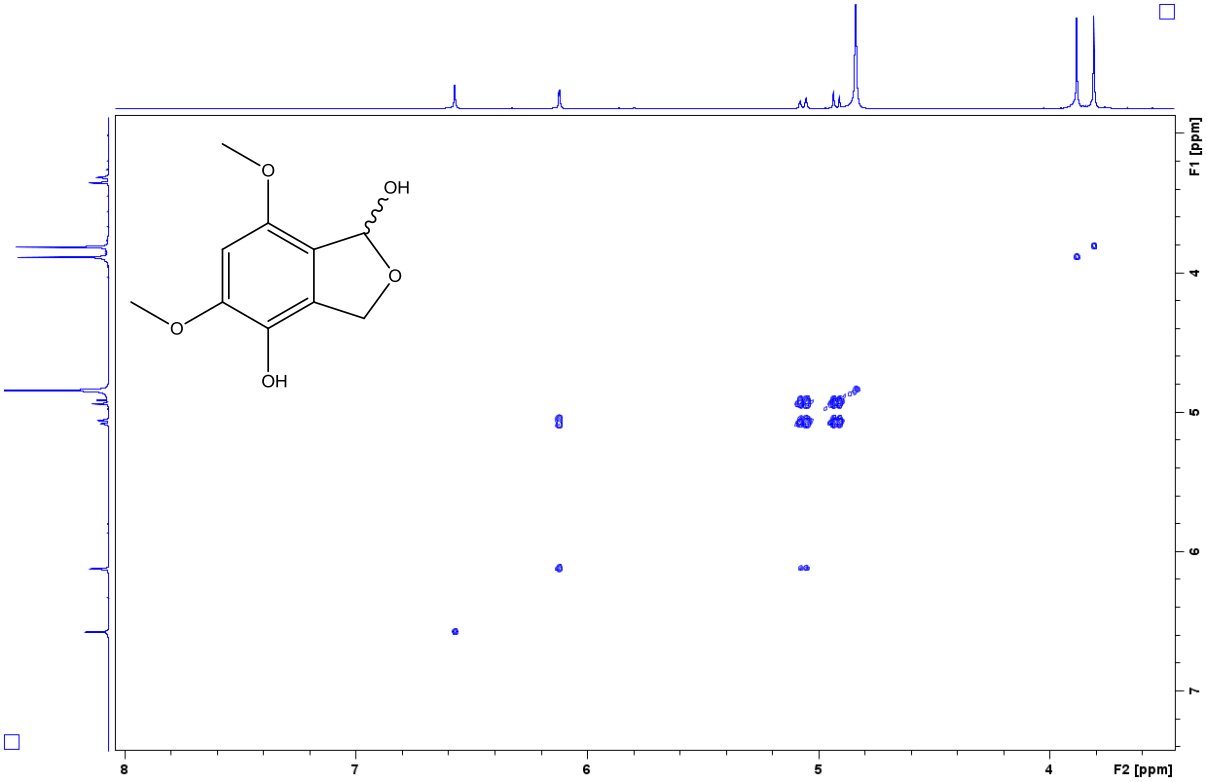


Figure S25. COSY spectrum (500MHz) of **3** in CD_3OD.

Apendix

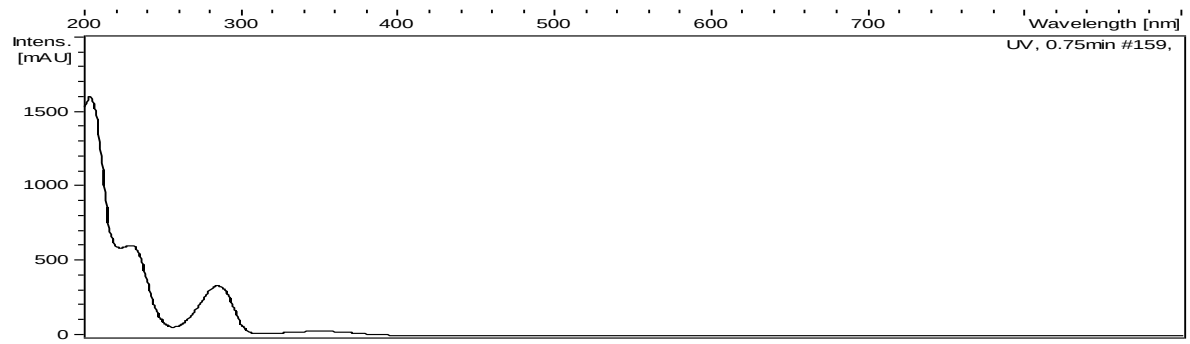


Figure S26. UV spectrum of **3**.

Reference for Supporting Information Chapter 1.

1. Seco J.M., Quiñoá E. and Riguera R. The Assignment of Absolute Configuration by NMR. *Chemical Reviews* **104**, 17-117 (2004).

APENDIX CHAPTER 2: Biodiversity and chemotaxonomy of *Preussia* isolates from Iberian Peninsula

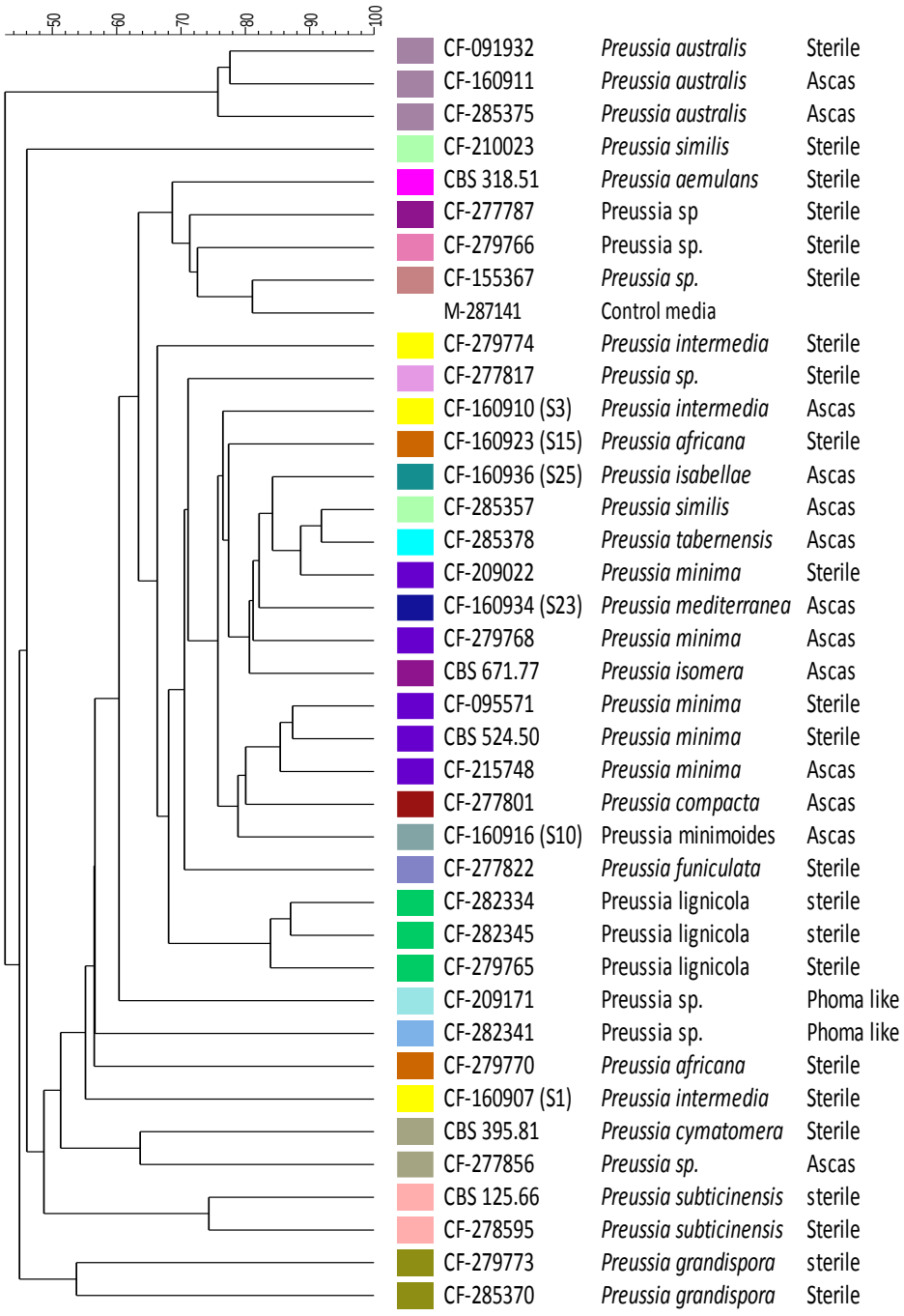


Figure S1. Similarity clustering dendrogram for different species of Preussia analyzed, based on their secondary metabolites m/z signals from positive ion mode LC-LRMS profiling. Absolute values of LC-MS detection intensity, Person correlation coefficient and UPGMA statistics were used to determine the similarity indexes.

Apendix

APENDIX CHAPTER 3: Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity

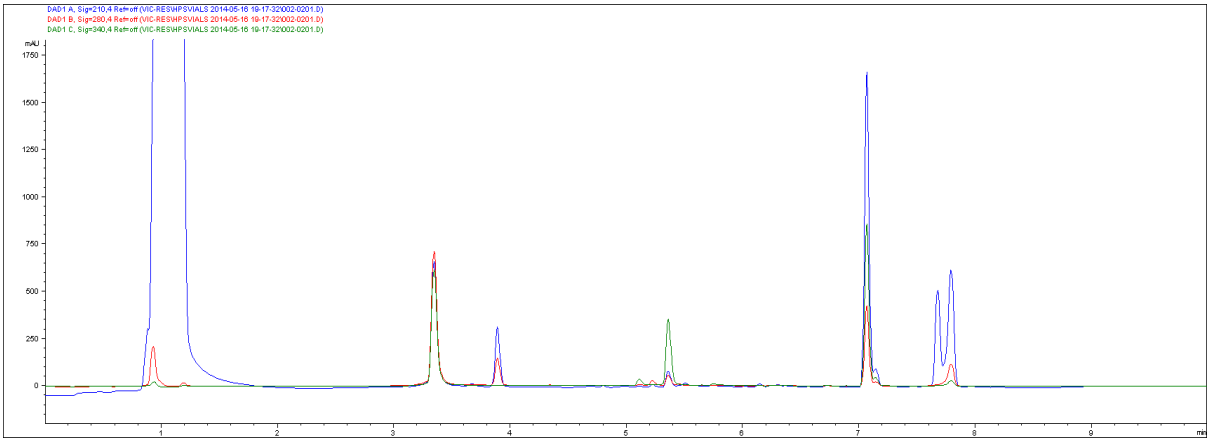


Figure S1. Standars in MeOH at 100mg/L 210-280-340 nm

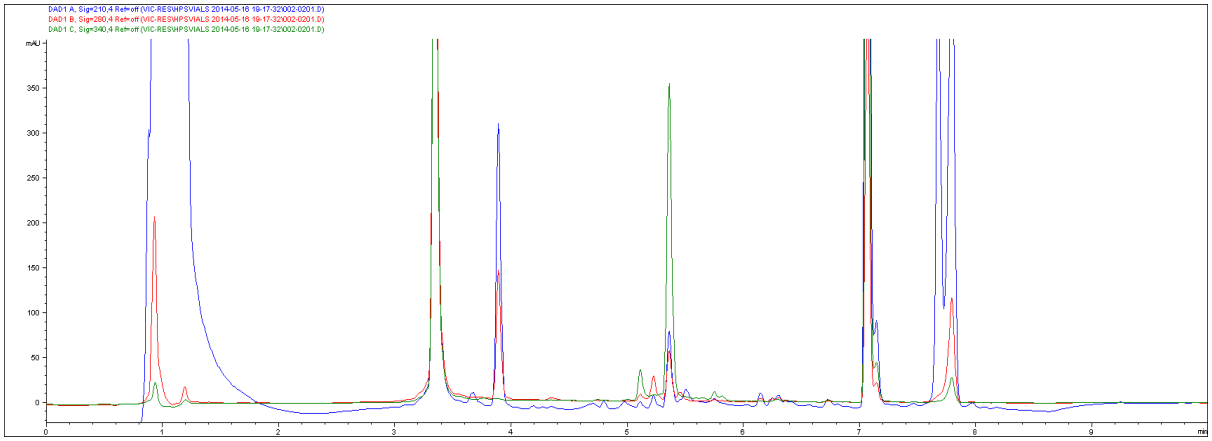


Figure S2. Standars in MeOH at 100mg/L 210-280-340 nm Zoom

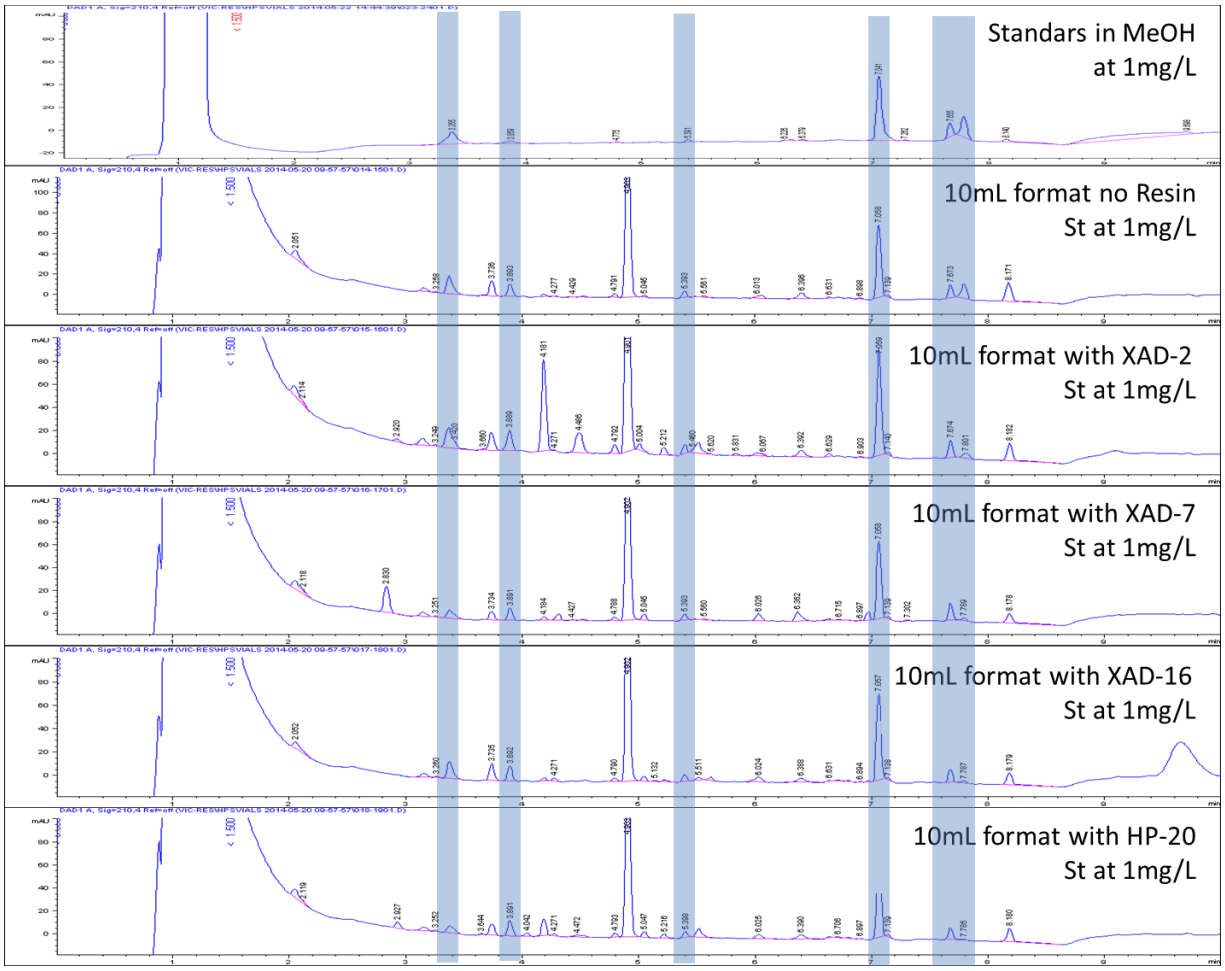


Figure S3. Standars in MeOH at 100mg/L 210-280-340 nm of different resins and conditions.

APENDIX CHAPTER 4. Multicomponent analysis of the differential induction of secondary metabolites profiles in fungal endophytes.

Isolation and identification of metabolites

The mycelium and fermentation broth (600 mL) was extracted by adding acetone (600 mL) and shaking at 220 rpm for 2 h. After filtration, the acetone extract was concentrated under reduced pressure to a final volume of 600 mL (100% water). The aqueous residue was loaded onto a SP207ss resin column (65g, 32 × 100 mm) and eluted with an acetone/H_2O stepped gradient (10/90 for 6 min, 20/80 for 6 min, 40/60 for 6 min, 60/40 for 6 min, 80/20 for 6 min and 100/0 for 12 min, 10 mL/min, 20 mL/fraction) to give nineteen fractions. Target metabolites were identified by LCMS in these fractions. An aliquot (300 µL) of fraction FS010 was subjected to semi-preparative reversed phase HPLC (Zorbax SB-C_{18} column, 9.4 × 250 mm, 5 µm, 3.6 mL/min, UV detection at 210 and 280 nm) eluting with CH_3CN: H_2O, linear gradient from 5 to 100% CH_3CN in 63 min to yield a mixture of compounds **9** and **12** (t_R 26 min) and compound **10** (t_R 30 min). Fractions FS011 to FS014 were pooled and subjected to preparative reversed-phase HPLC (Zorbax SB-C_{18} PrepHT, 21.2 × 250 mm, 7 µm, 16 mL/min, UV detection at 210 and 280 nm) using H_2O + 0.1% TFA (solvent A) and CH_3CN + 0.1% TFA (solvent B), isocratic conditions of 5% B for 5 min and then a linear gradient from 5% to 100% B in 43 min to yield a fraction containing compound **11** (t_R 28 min) and major impurities.

A molecular formula of $C_9H_6N_4$ was assigned to compound **9** after analysis of its ESI-TOF spectrum (*m/z* 171.0657 $[M+H]^+$, calc. for $C_9H_7N_4^+$, 171.0664). The presence of a singlet at 8.95 ppm in the ^{1}H-NMR spectra of compound **9** along with two doublets at 8.18 ppm (H-6) and 8.60 ppm (H-9) and two triplets each integrating for one proton at 7.97 ppm (H-7) and 8.08 ppm (H-8) were assigned to the aromatic protons of a phtalazine moiety. In addition, a singlet observed at 9.40 ppm was assigned to the H-13 proton. The MS/MS analysis of compound **9** showed a quasimolecular ion at 144.0551 corresponding to $C_8H_6N_3^+$, due to the loss of the carbon and one of the nitrogen atoms of the triazol moiety, and consistent with the proposed structure of [1,2,4]triazolo[3,4-a]phthalazine.

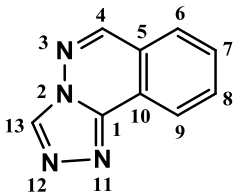
Compound **10** displayed a pseudomolecular ion at *m/z* 185.0819 (calc. for $[M+H]^+$ 185.0827) corresponding to a molecular formula of $C_{10}H_8N_4$. It displayed similar chemical shifts as compound **9** for the protons and carbons of the phthalazine moiety. The major difference between the ^{1}H NMR spectra of both compounds was the absence of the proton at 9.40 ppm in the spectrum of **9** and the presence of a methyl group at 2.81 ppm in the spectrum of **10**. The chemical shift of the methyl group in the carbon spectrum (9.7 ppm) indicated its linkage to a carbon that was identified as C-13. A cross peak between the methyl group and a carbon at 150.12 ppm observed in the HMBC spectrum supported this evidence. According to the MS/MS results, compound **10** displayed the same ion at 144.0559 as compound **9**, suggesting the same fragmentation pattern that is consistent with a structure of 3-methyl-[1,2,4]triazolo[3,4-a]phthalazine for this compound.

Compound **11** was assigned a molecular formula of $C_{13}H_{14}N_4$ by ESI-TOF (*m/z* 227.1280, calc. for $[M+H]^+$ 227.1286). The NMR spectra of this compound were very similar to those

of compounds **9** and **10**, with the major difference being in the absence of the methyl group present in **10** and the presence of a doublet at 3.13 ppm accounting for methylene protons, together a multiplet corresponding to a proton in the aliphatic region. Additionally, this proton was coupled in the COSY spectrum to two aliphatic methyl groups and confirmed the presence of an isobutyl moiety in the structure. Finally this compound also presented a quasimolecular ion at *m/z* 144.0555 corresponding to $C_8H_6N_3^+$ in a MS/MS measurement, confirming the same structural pattern as for compounds **9** and **10**, and the structural proposal.

Compounds **9** and **10** have been previously reported as metabolites obtained by transformation of hydralazine by rat liver microsomes (LaCagnin *et al.*, 1986) as well as main products formed in human saliva and under gastric conditions when this drug was administered to patients suffering from hypertension (Noda *et al.*, 1979).

The identification of compound **12** was straightforward from the molecular formula and proton and 2D-NMR spectra. Compound **12** displayed a pseudomolecular ion at *m/z* 245.1282 (calc. for $[M+H]^+$ 245.1206) corresponding to a molecular formula of $C_{14}H_{16}N_2O_2$ suggesting a diketopiperazine nature for the compound. The presence of a proline moiety was inferred from signals attributed to the presence of three broad methylene multiplets in the proton spectrum (δ_H 3.31-3.35 and 3.51-3.57 (C-3), δ_H 1.60-1.69 and 1.88-1.93 (C-4) and δ_H 1.60-1.69 and 2.01-2.06 (C-5)) and by H-H correlations observed in the COSY experiment. Proton in alpha for proline amino acid presents a chemical shift of 2.60-2.63 ppm that is significantly different to that expected for an amino acid alpha proton. This upfield shift could be due to a conformational arrangement of the aromatic ring that leaves this proton closer to its inner part. Analysis of the NMR spectra also indicated that phenylalanine was the second amino acid residue, showing signals of methylene benzylic protons at δ_H 2.99 and 3.19 respectively (C-10) attached to a monosubstituted aromatic ring (δ_H 7.18-7.31). A proton at 4.20 ppm is attributed to the alpha proton for phenylalanine residue.



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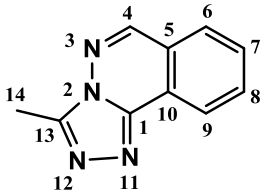

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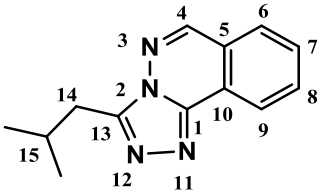


11

Table NMR. ^{1}H-NMR Data of Hydralazine and Compounds **9**, **10** and **11**.[a]

Position	Hydralazine	9	10	11
	δ_H, m (*J* in Hz)	**δ_H, m (*J* in Hz)**	**δ_H, m (*J* in Hz)**	**δ_H, m (*J* in Hz)**
1	--	--	--	--
2	--	--	--	--
3	--	--	--	--
4	8.78, s	8.95 s	8.93 s	8.95 s
5	--	--	--	--
6	8.16-8.18, m	8.18, dd (8.0, 1.1)	8.16, brd (8.0)	8.18, brd (7.5)
7	8.07-8.10, m	7.97, td (8.0, 1.1)	7.94, td (8.0, 1.2)	7.96, brt (7.5)
8	8.16-8.18, m	8.08, td (8.0, 1.1)	8.05, td (8.0, 1.1)	8.07, brt (7.5)
9	8.41, dd (8.2, 0.9)	8.60, td (8.0, 1.1)	8.54, dd (8.0, 0.7)	8.56, brd (7.5)
10	--	--	--	--
11	--	--	--	--
12	--	--	--	--
13	--	9.40, s	--	--
14	--	--	2.81, s	3.13, d (7.2)
15	--	--	--	2.32-2.36, m
16	--	--	--	1.04-1.06, m
17	--	--	--	1.04-1.06, m

[a] Measured in CD_3OD, Chemical shifts (δ) in ppm.

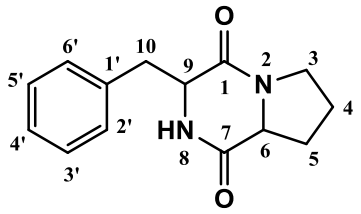
Table NMR. ^{13}C-NMR Data of Hydralazine and Compounds **9**, **10** and **11**.[b]

Position	Hydralazine[a]	9[a]	10	11[a]
	δ_C	**δ_C**	**δ_C**	**δ_C**
1	--	--	144.43	--
2	--	--	--	--
3	--	--	--	--
4	144.94	149.18	150.12	149.97
5	--	--	125.14	--
6	136.96	128.81	130.32	130.05
7	134.84	131.40	132.81	132.67
8	129.31	134.16	135.62	135.46
9	124.47	122.18	123.74	123.48
10	--	--	124.10	--
11	--	--	--	--
12	--	--	--	--
13	--	140.55	150.12	--
14	--	--	9.70	32.20
15	--	--	--	29.85
16	--	--	--	21.22
17	--	--	--	21.22

[a] Signal obtained from HSQC spectrum.

[b] Measured in CD_3OD, Chemical shifts (δ) in ppm

Apendix



12

Table NMR. ^{1}H and ^{13}C-NMR[a] Data of Compounds **12**.[b]

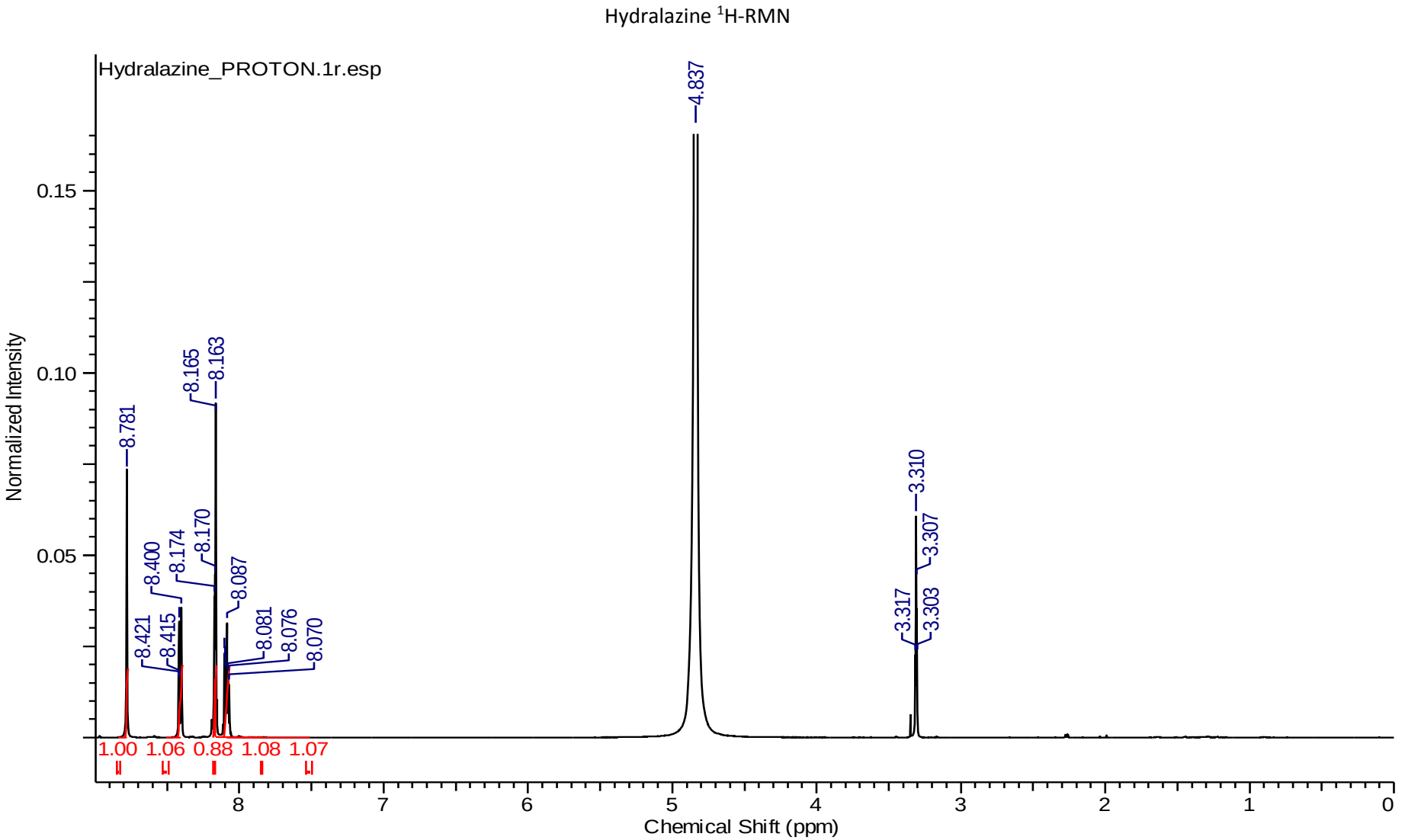
Position	12	
	δ_H, m (*J* in Hz)	δ_C
1	--	--
2	--	--
3a	3.51-3.57, m	44.64
3b	3.31-3.35,[c] m	
4a	1.88-1.93, m	21.02
4b	1.60-1.69, m	
5a	2.01-2.06, m	28.33
5b	1.60-1.69, m	
6	2.60-2.63, m	57.66
7	--	--
8	--	--
9	4.20, t (4.7)	58.30
10a	3.19, dd (13.7, 4.7)	39.50
10b	2.99, dd (13.7,4.7)	
1’	--	--
2’	7.18-7.20, m	129.80
3’	7.30-7.31, m	128.48
4’	7.30-7.31, m	127.26
5’	7.30-7.31, m	127.76
6’	7.18-7.20, m	129.47

[a] Signal obtained from HSQC spectrum.
[b] Measured in CD_3OD, Chemical shifts (δ) in ppm.
[c] Obscured by solvent peak.

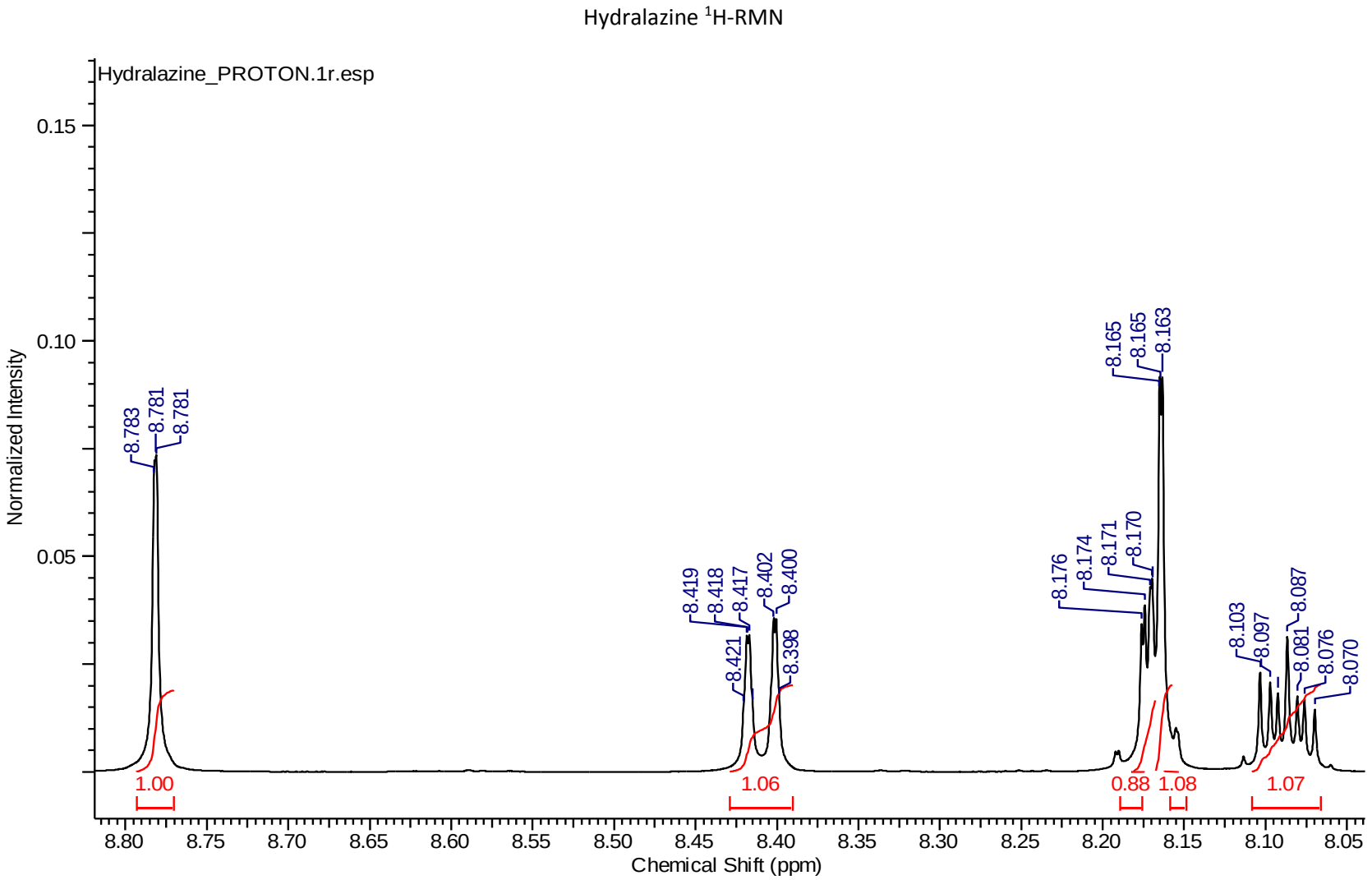
References

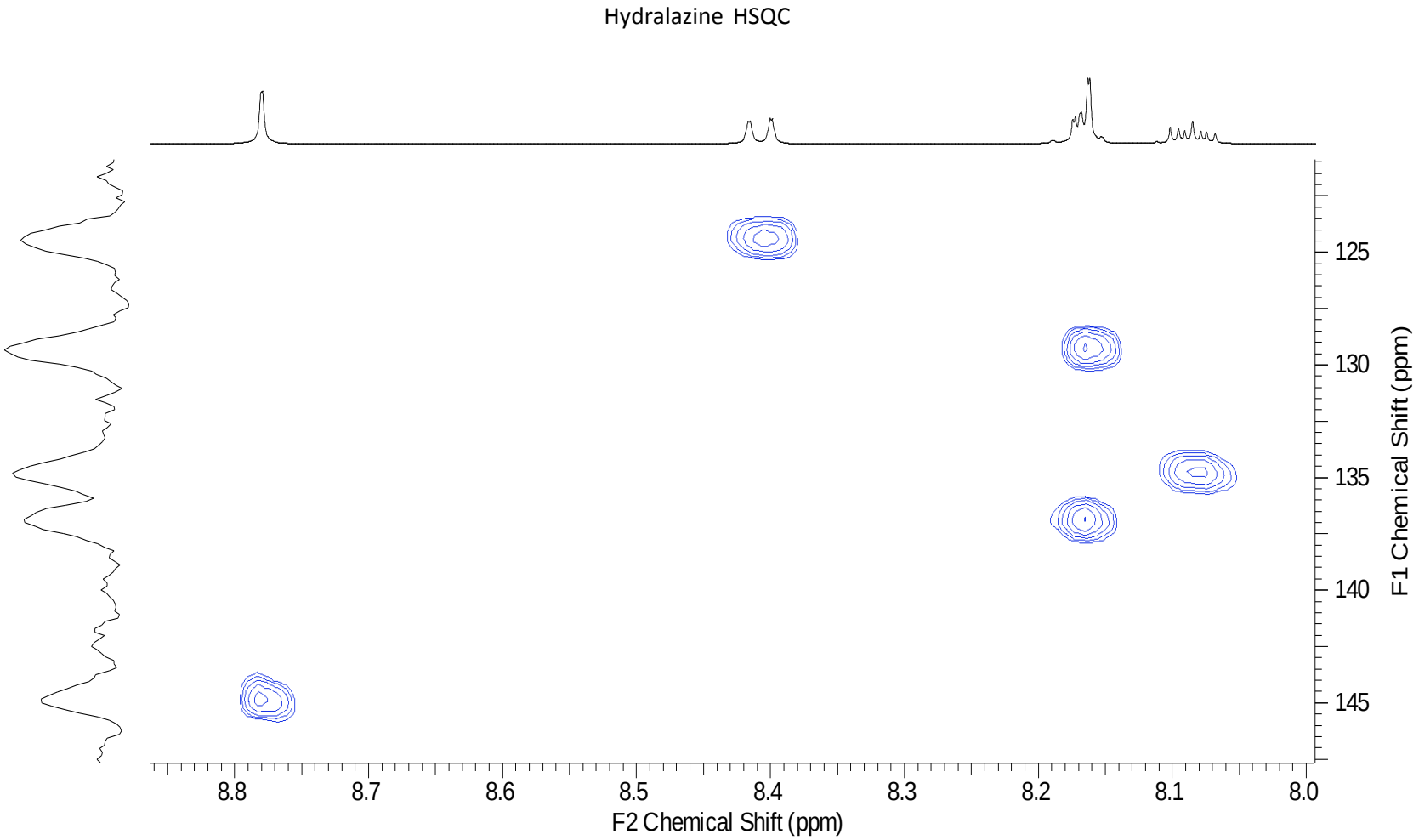
LaCagnin, L.B.; Colby, H.D.; O’Donnell, J.P. The oxidative metabolism of hydralazine by rat liver microsomes. *Drug Metab. Dispos.* **1986**, 14(5):549-554.

Noda, A.; Matsuyama, K.; Yen, S.-H.; Sogabe, K. Fate of Hydralazine in Man. I. Reactions under Gastric Conditions. *Chem. Pharm. Bull.* **1979**, 27(11):2820-28


Hydralazine ^{1}H-RMN
Hydralazine_PROTON.1r.esp
Normalized Intensity
0.15
0.10
0.05
8.781
8.421
8.415
8.400
8.174
8.170
8.165
8.163
8.087
8.081
8.076
8.070
4.837
3.317
3.310
3.307
3.303
1.00 1.06 0.88 1.08 1.07
8
7
6
5
4
3
2
1
0
Chemical Shift (ppm)

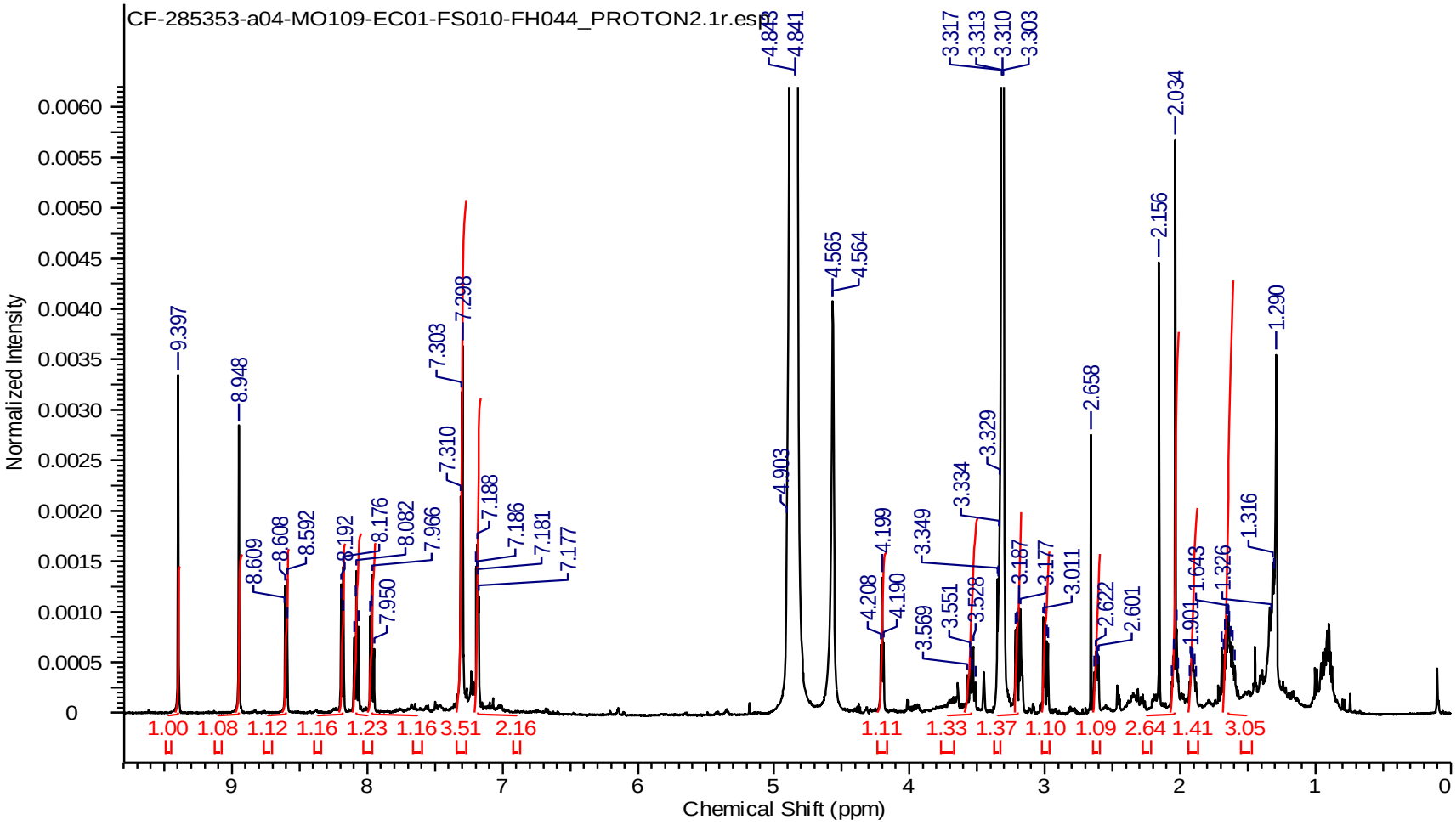
Apendix


Hydralazine ^{1}H-RMN
Hydralazine_PROTON.1r.esp
Normalized Intensity
0.15
0.10
0.05
8.783
8.781
8.781
8.419
8.418
8.417
8.421
8.402
8.400
8.398
8.176
8.174
8.171
8.170
8.165
8.165
8.163
8.103
8.097
8.087
8.081
8.076
8.070
1.00
1.06
0.88
1.08
1.07
8.80
8.75
8.70
8.65
8.60
8.55
8.50
8.45
8.40
8.35
8.30
8.25
8.20
8.15
8.10
8.05
Chemical Shift (ppm)

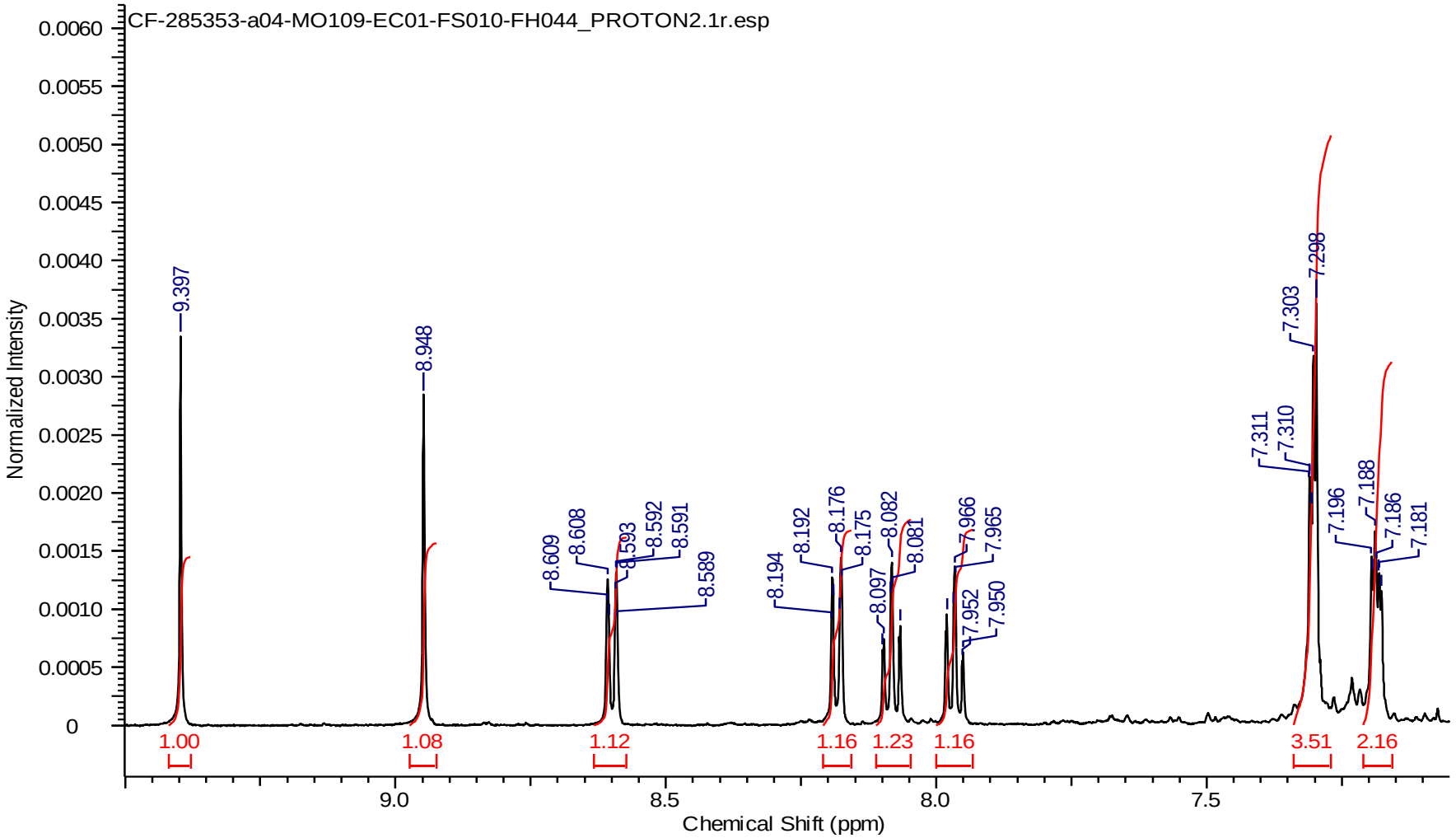

Hydralazine HSQC
125
130
135
140
145
F1 Chemical Shift (ppm)
8.8
8.7
8.6
8.5
8.4
8.3
8.2
8.1
8.0
F2 Chemical Shift (ppm)

Apendix

Compounds **9** and **12** ^{1}H-RMN

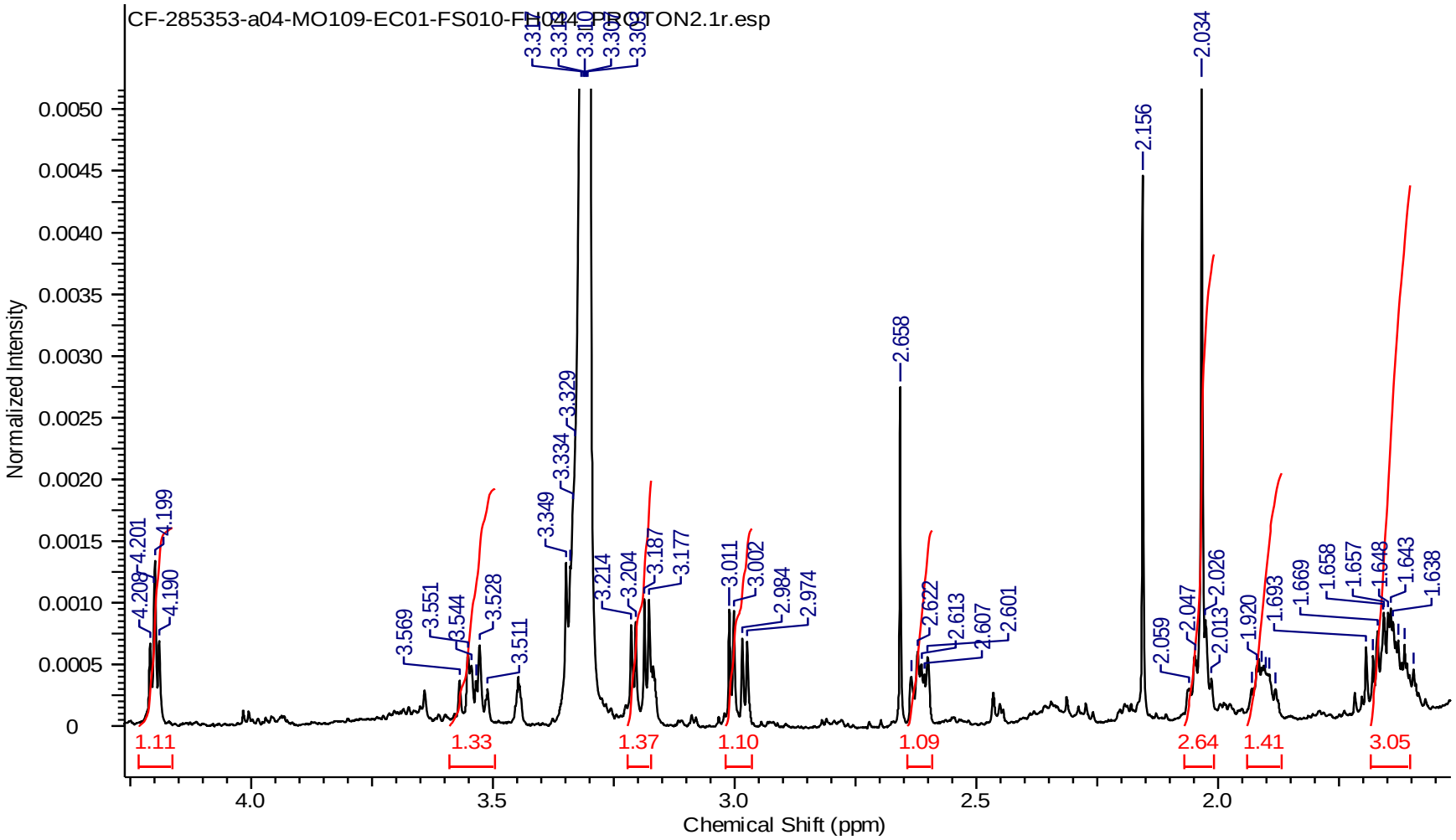


Compounds **9** and **12** ^{1}H-RMN



Apendix

Compounds **9** and **12** ^{1}H-RMN



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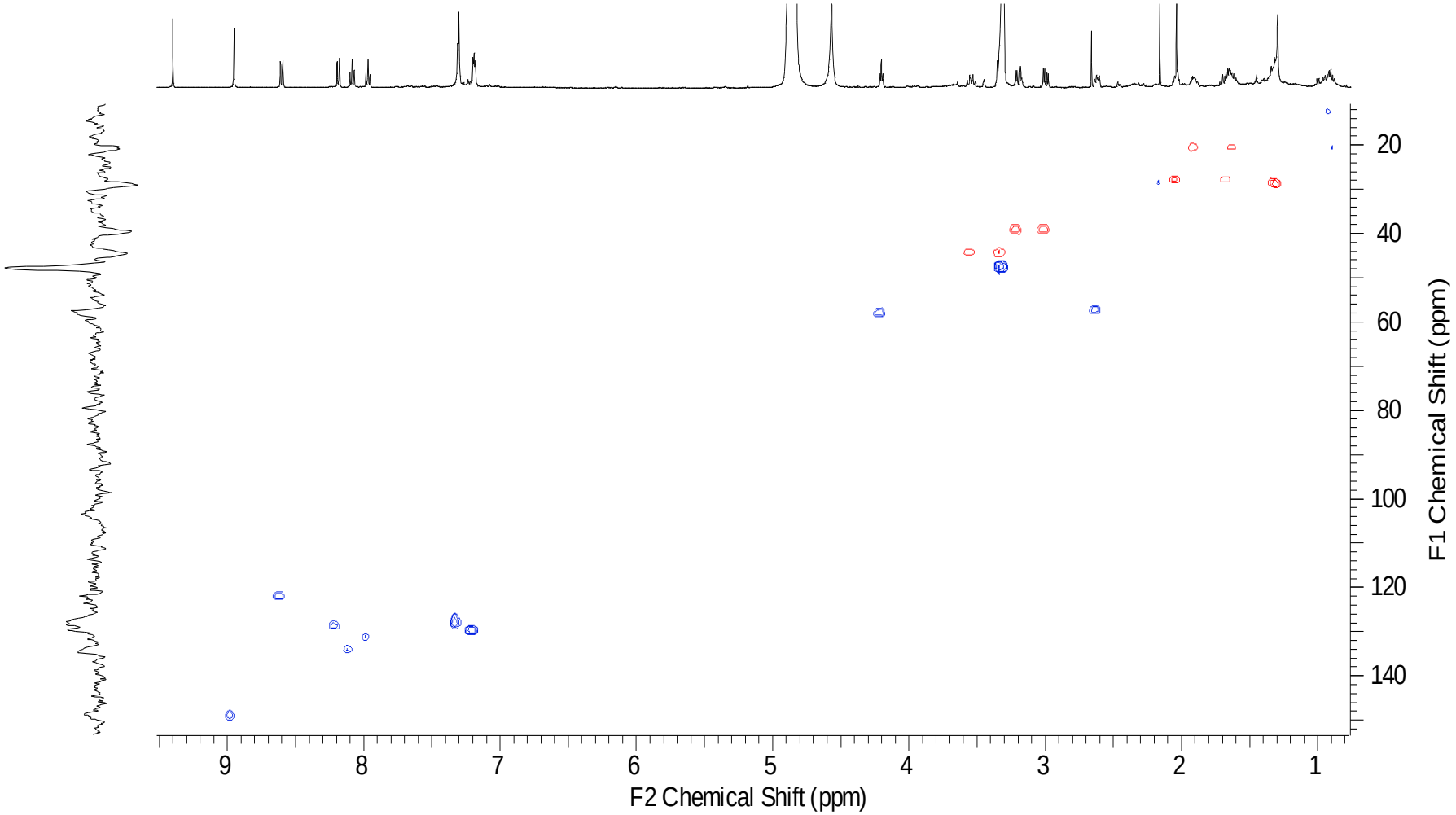

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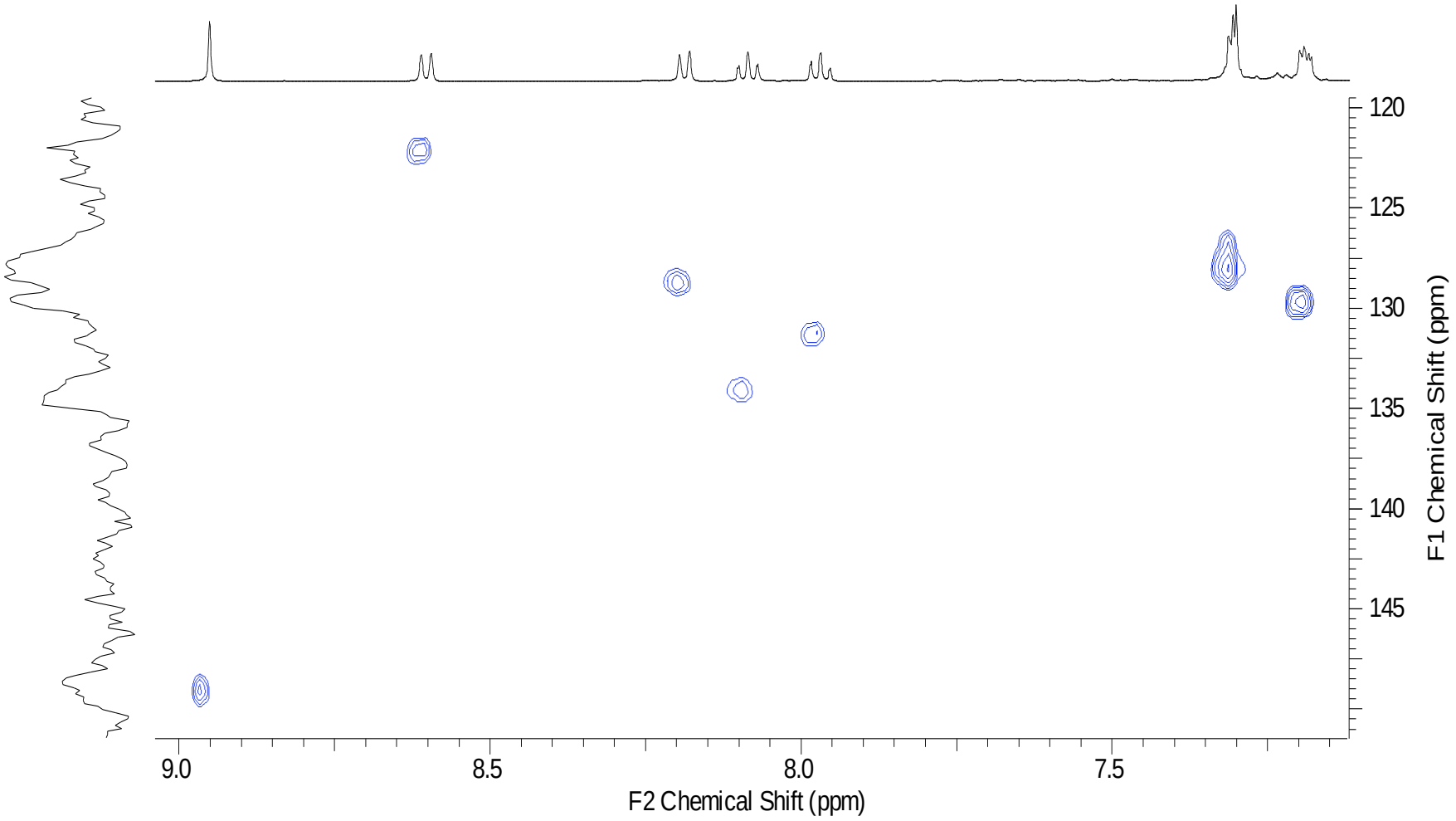
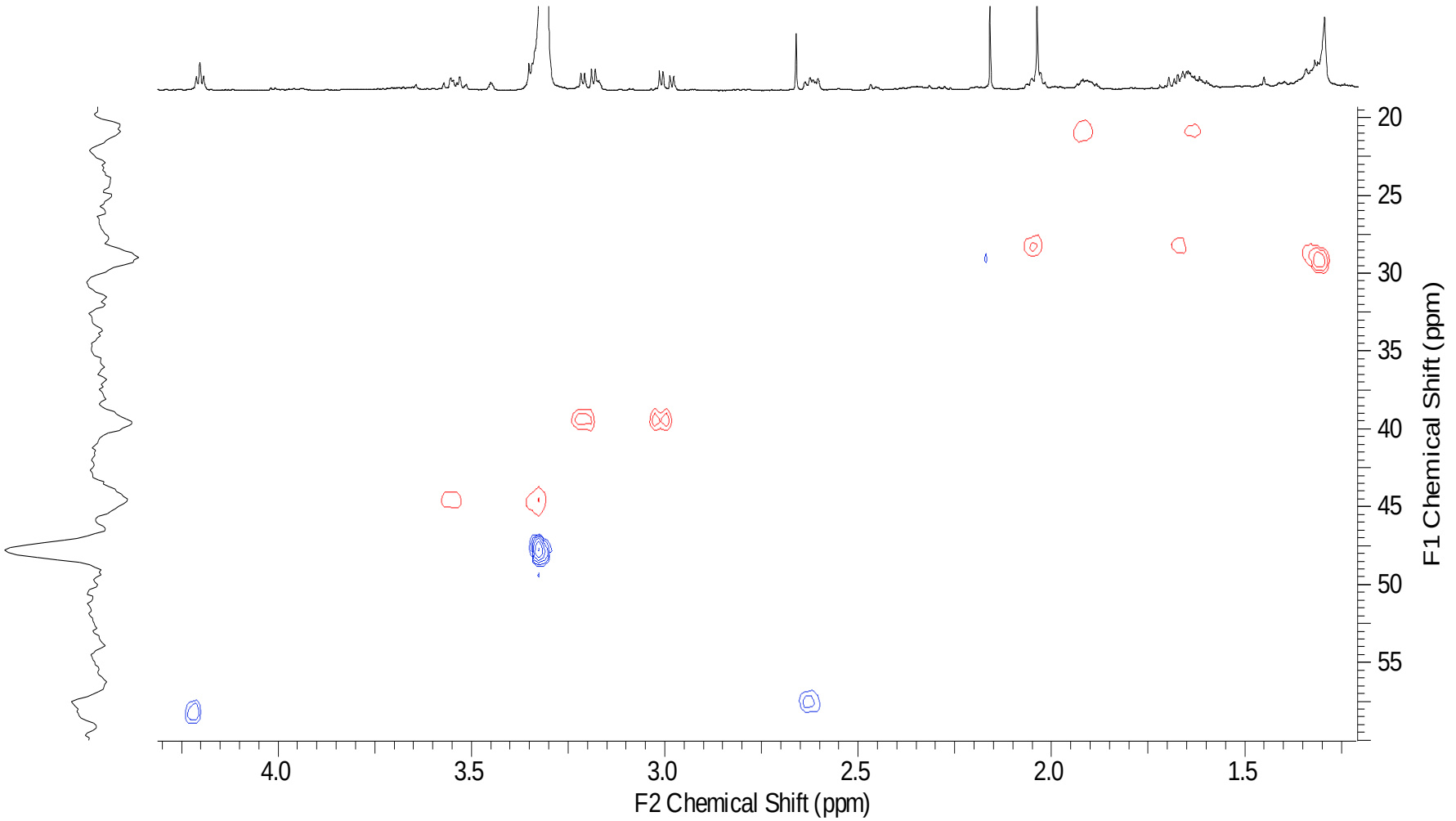
Compounds **9** and **12** HSQC

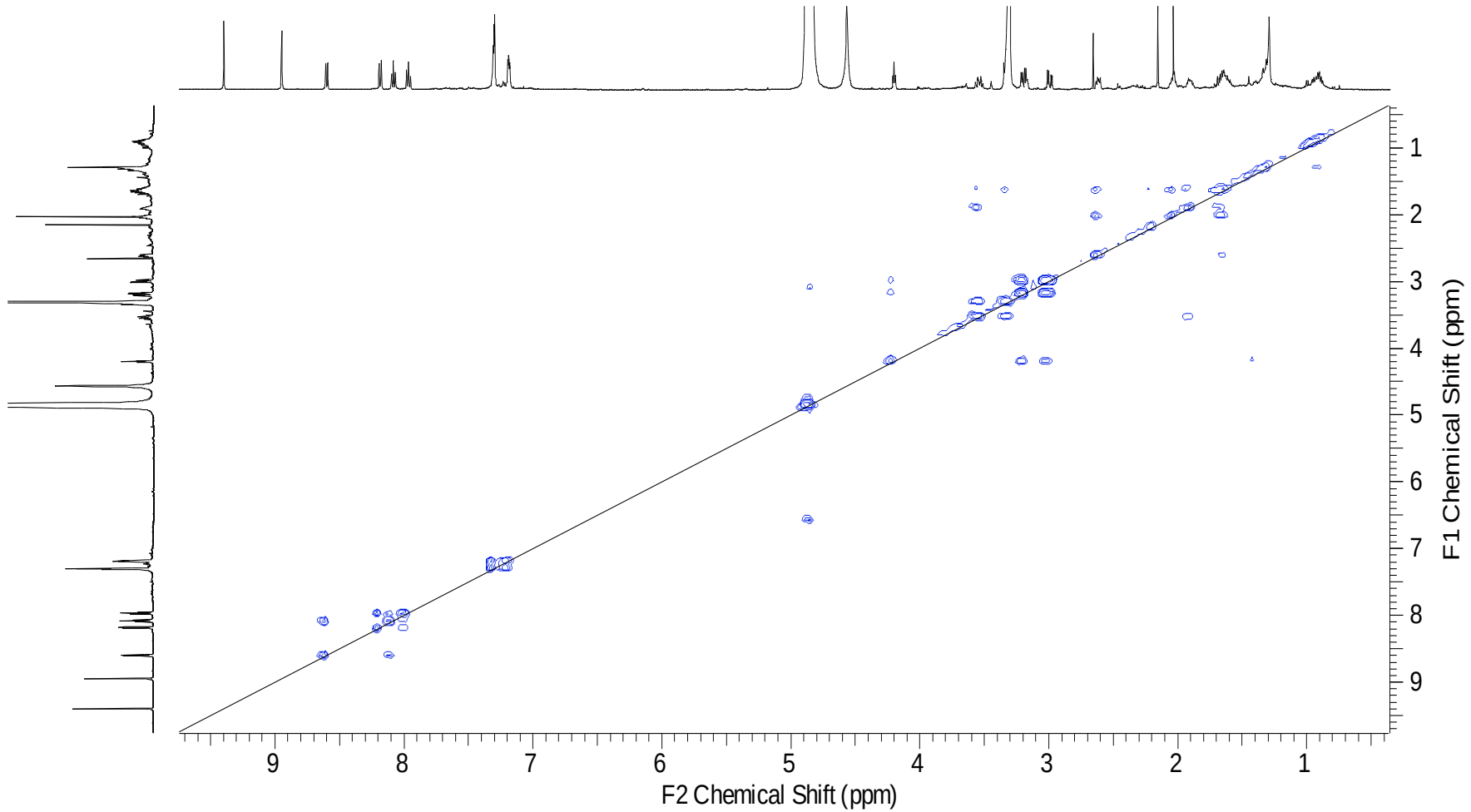


Compounds **9** and **12** HSQC

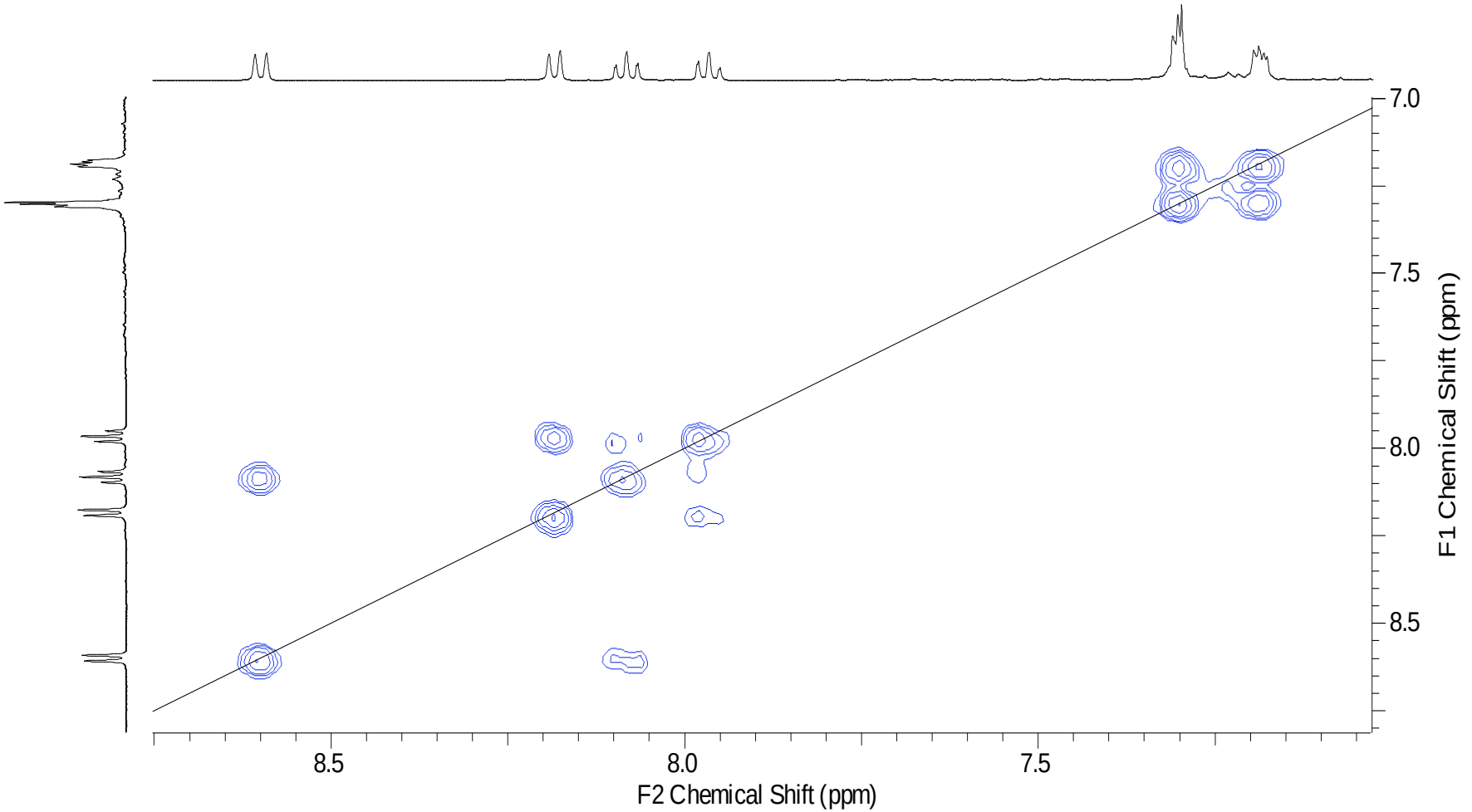


Apendix

Compounds **9** and **12** COSY

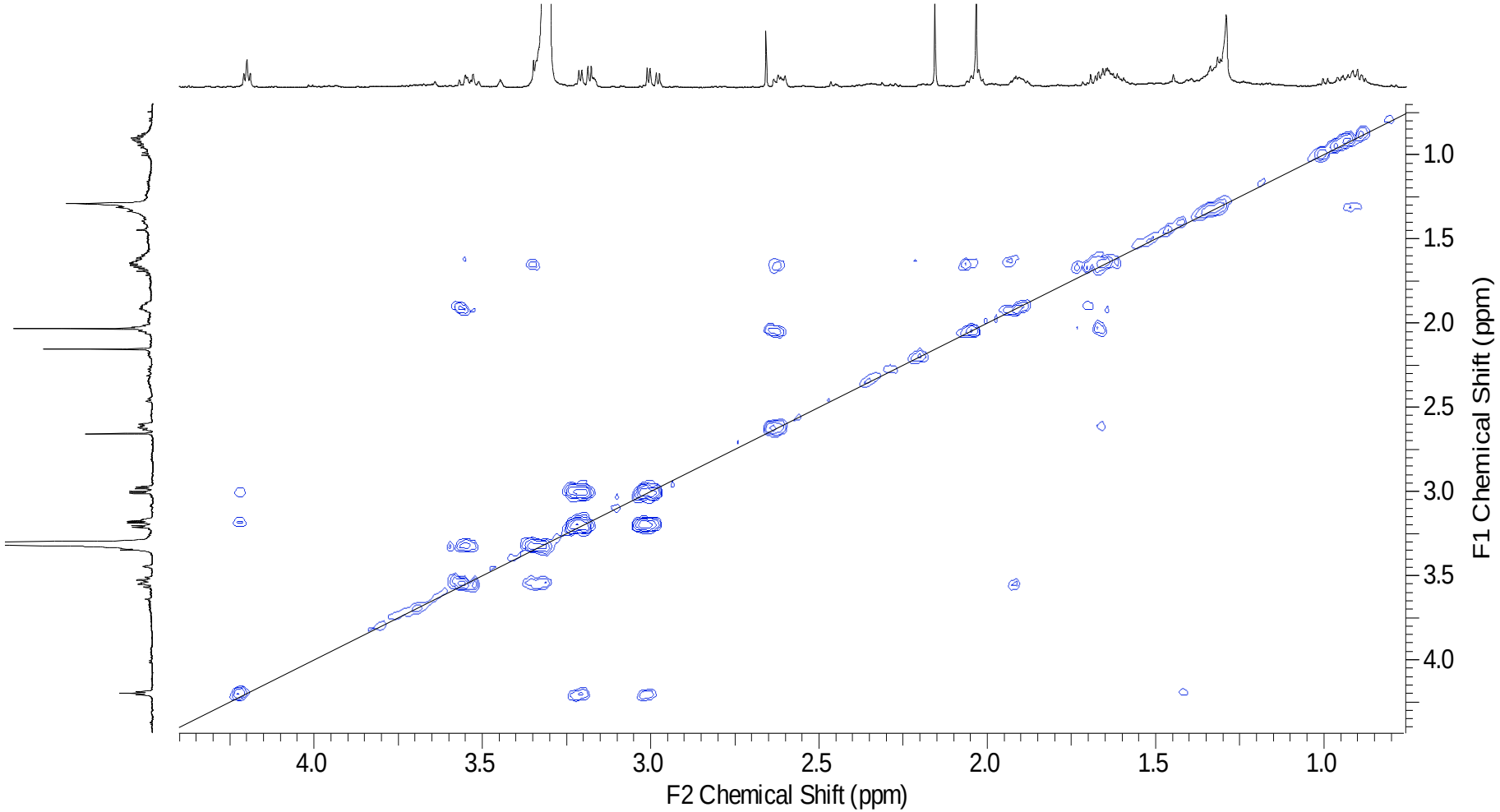


Compounds **9** and **12** COSY

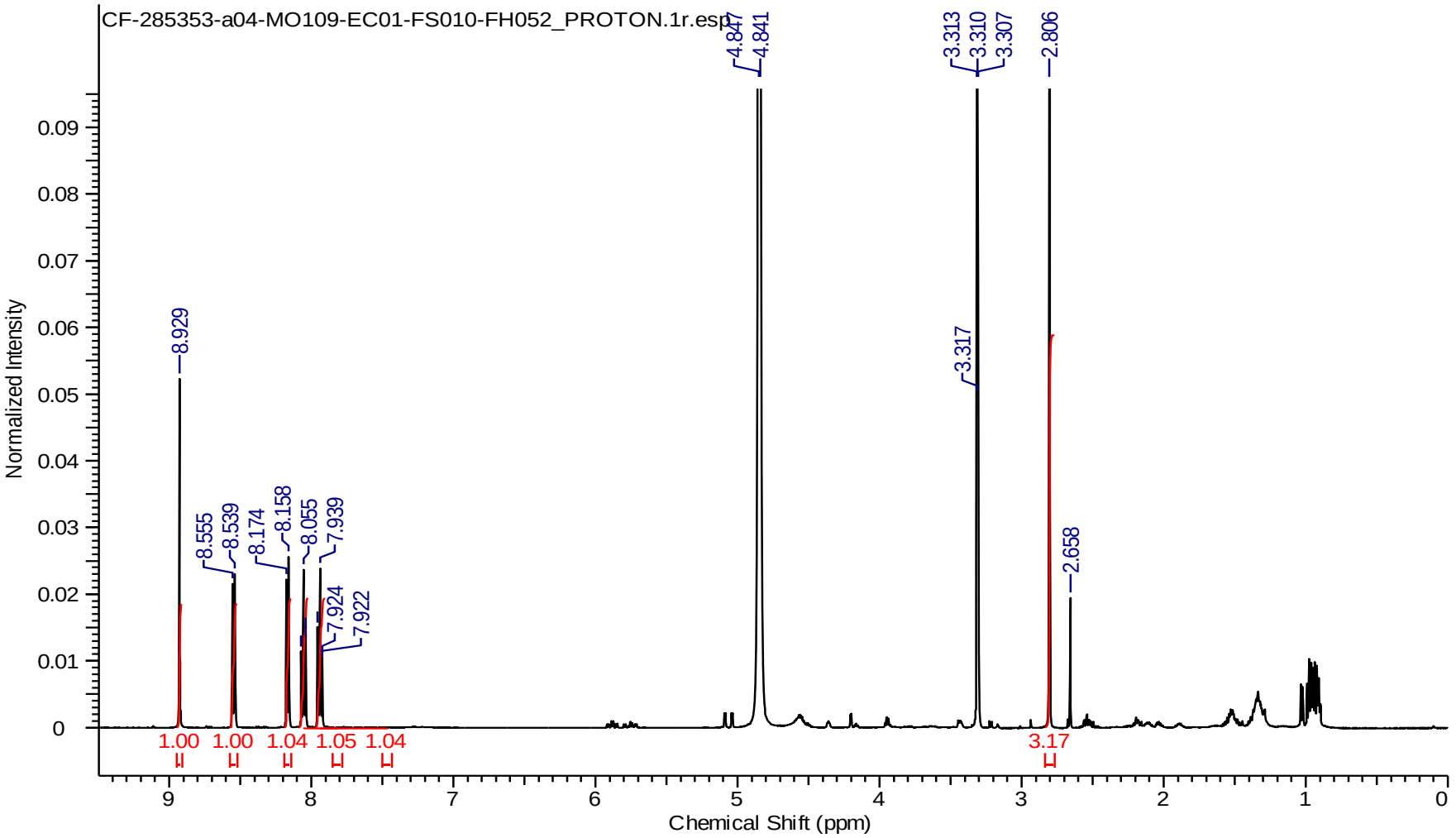


Apendix

Compounds **9** and **12** COSY

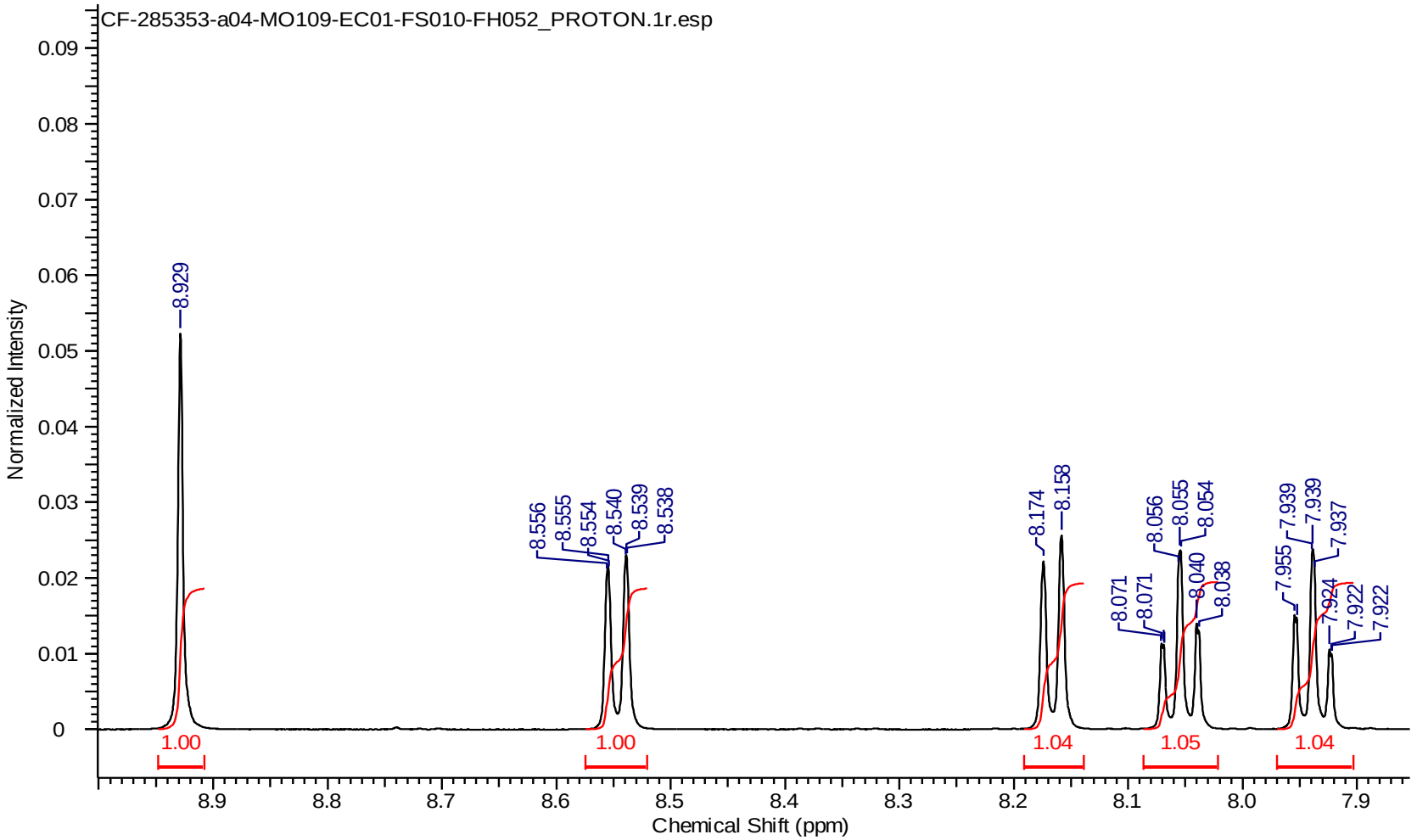


Compound **10** ^{1}H-RMN

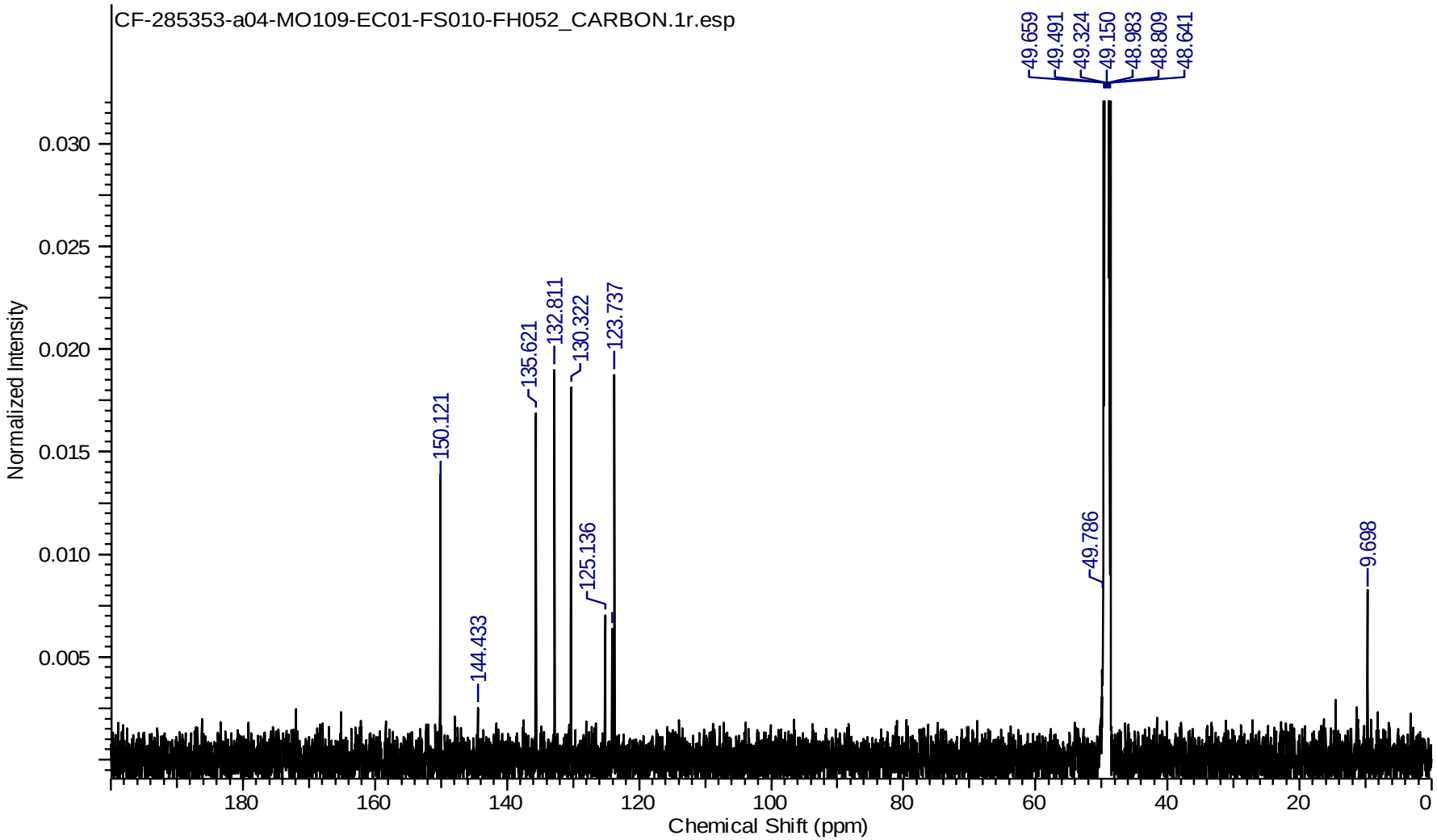


Apendix

Compound **10** ^{1}H-RMN

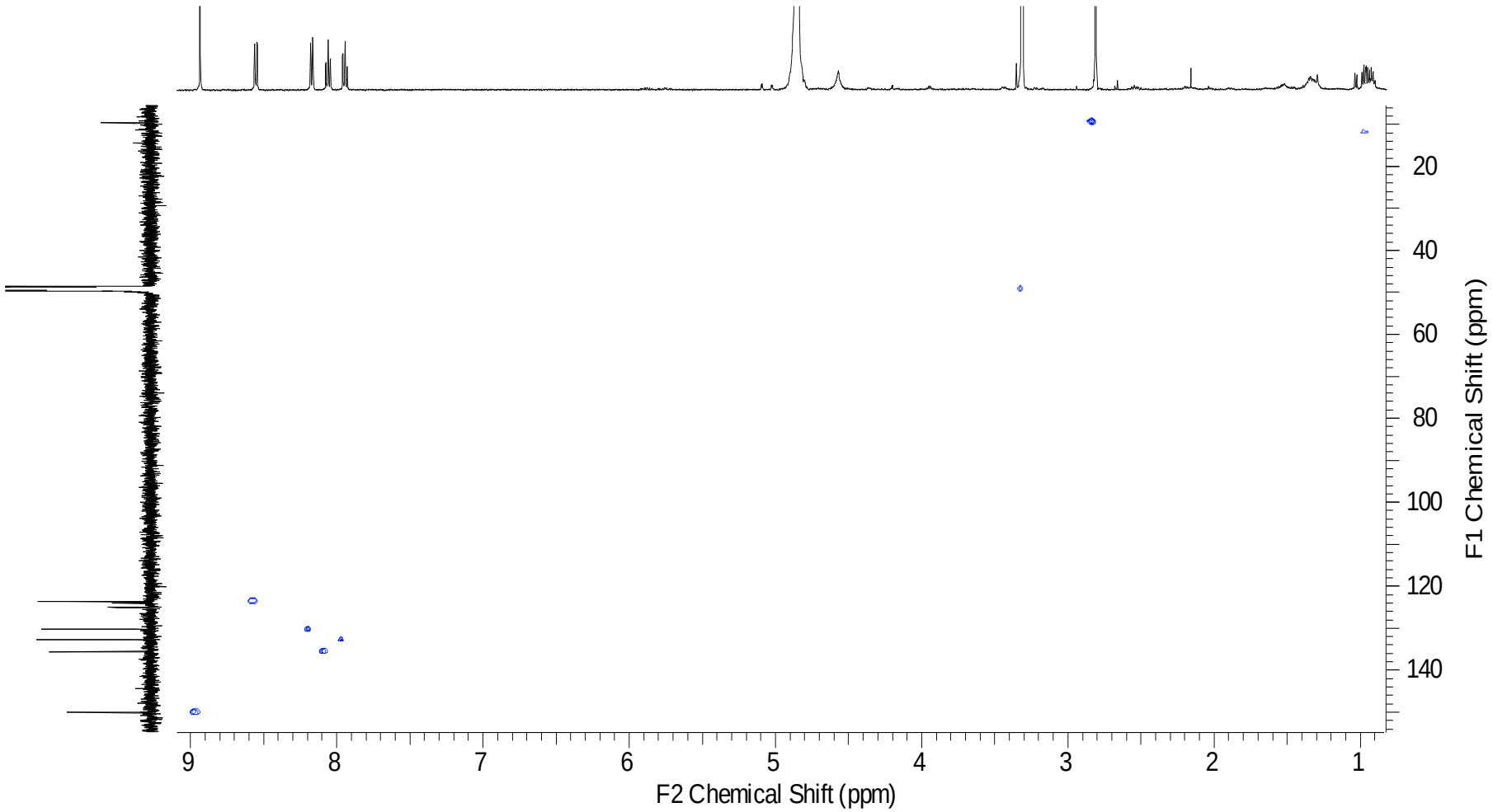


Compound **10** ^{13}C-RMN



Apendix

Compound **10** HSQC



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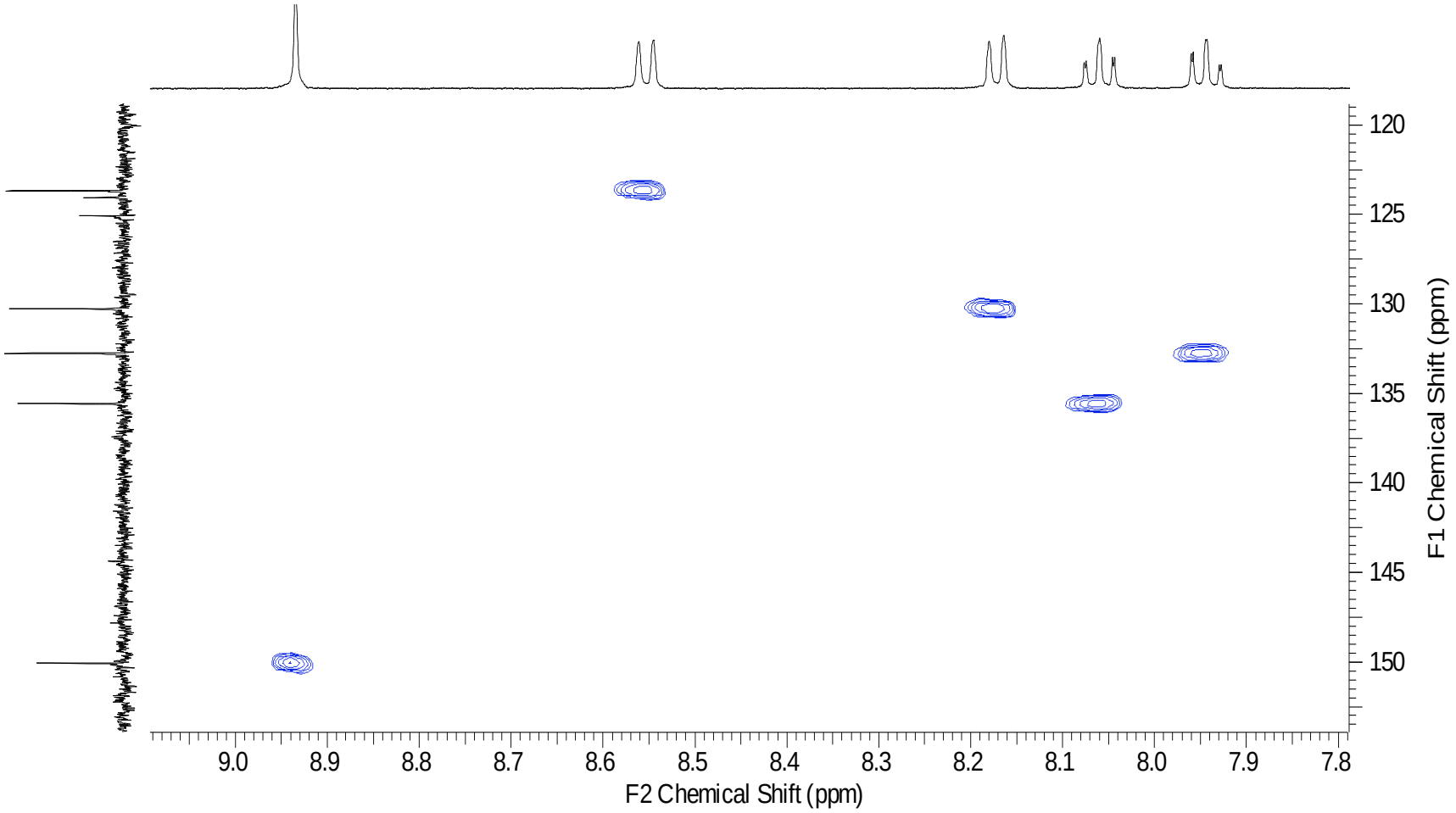

Apendix

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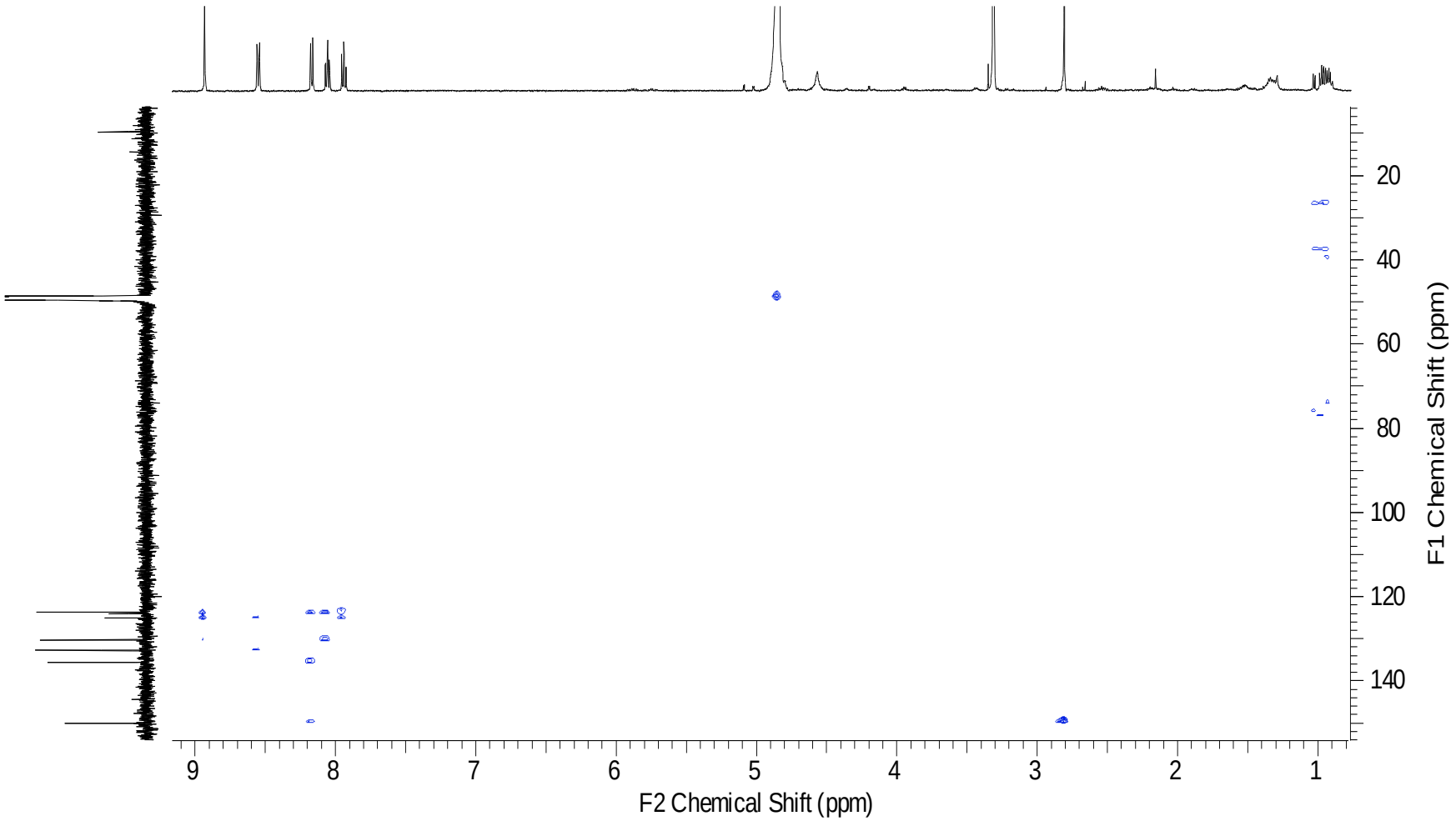
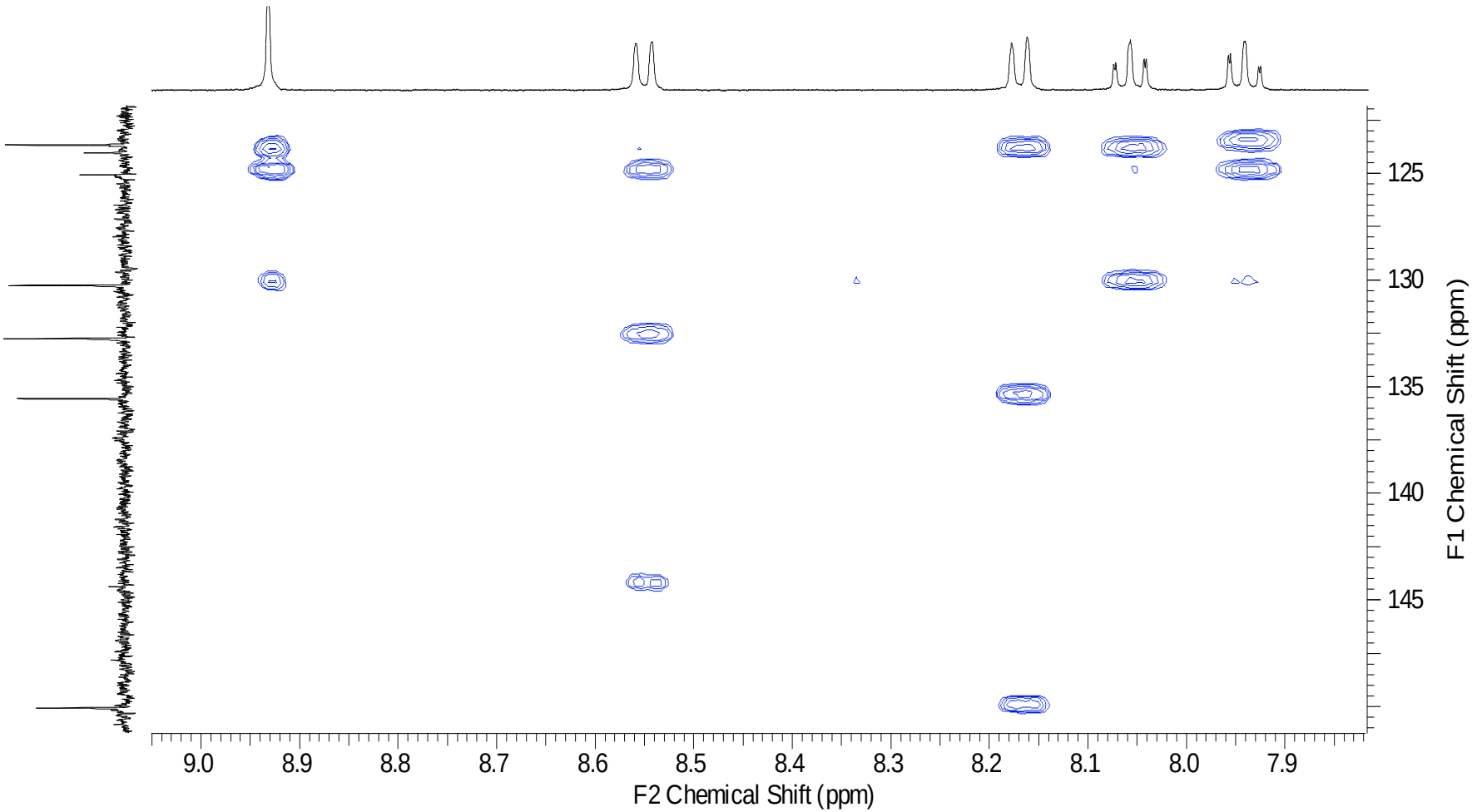


Compound **10** HMBC



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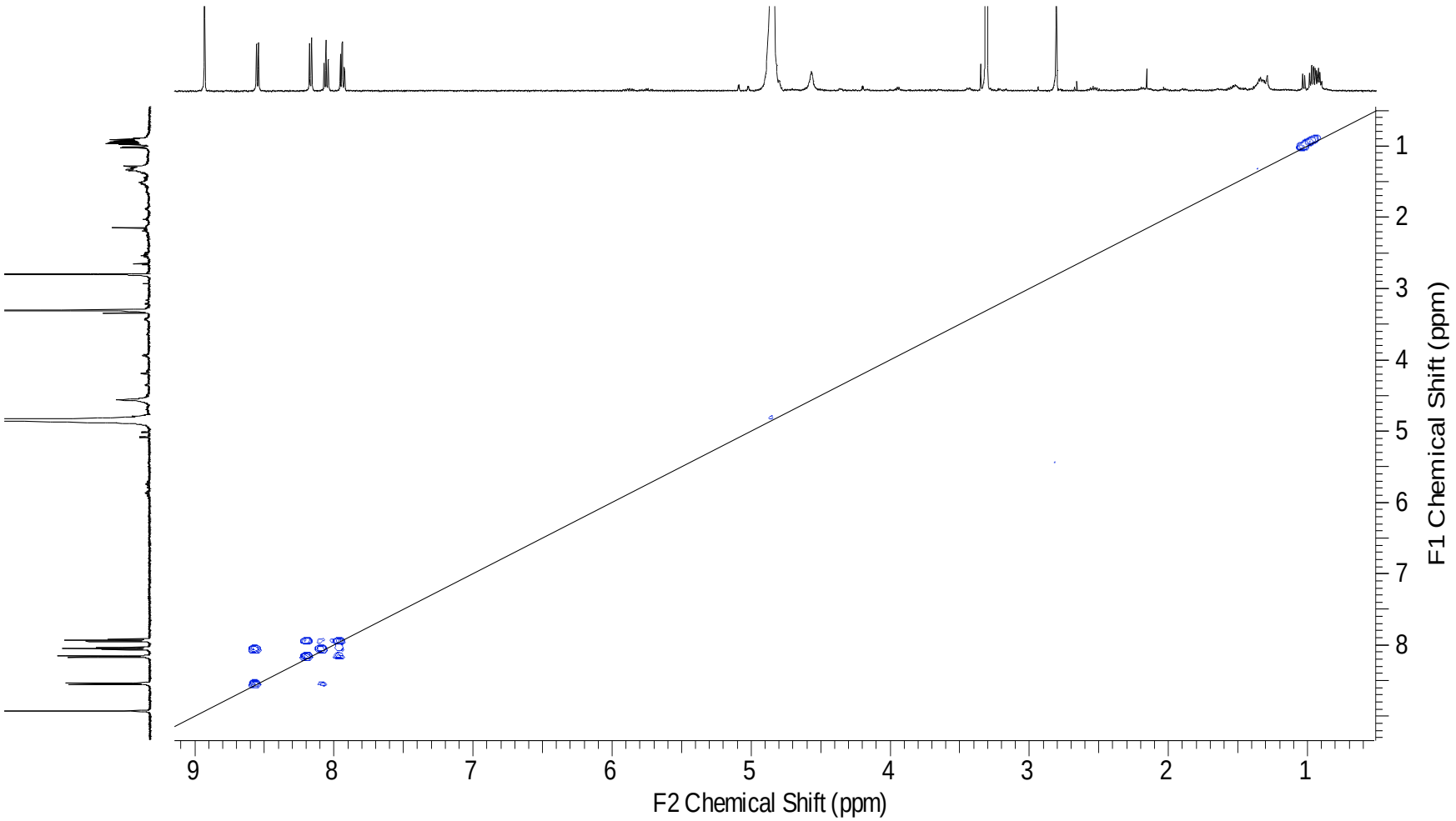
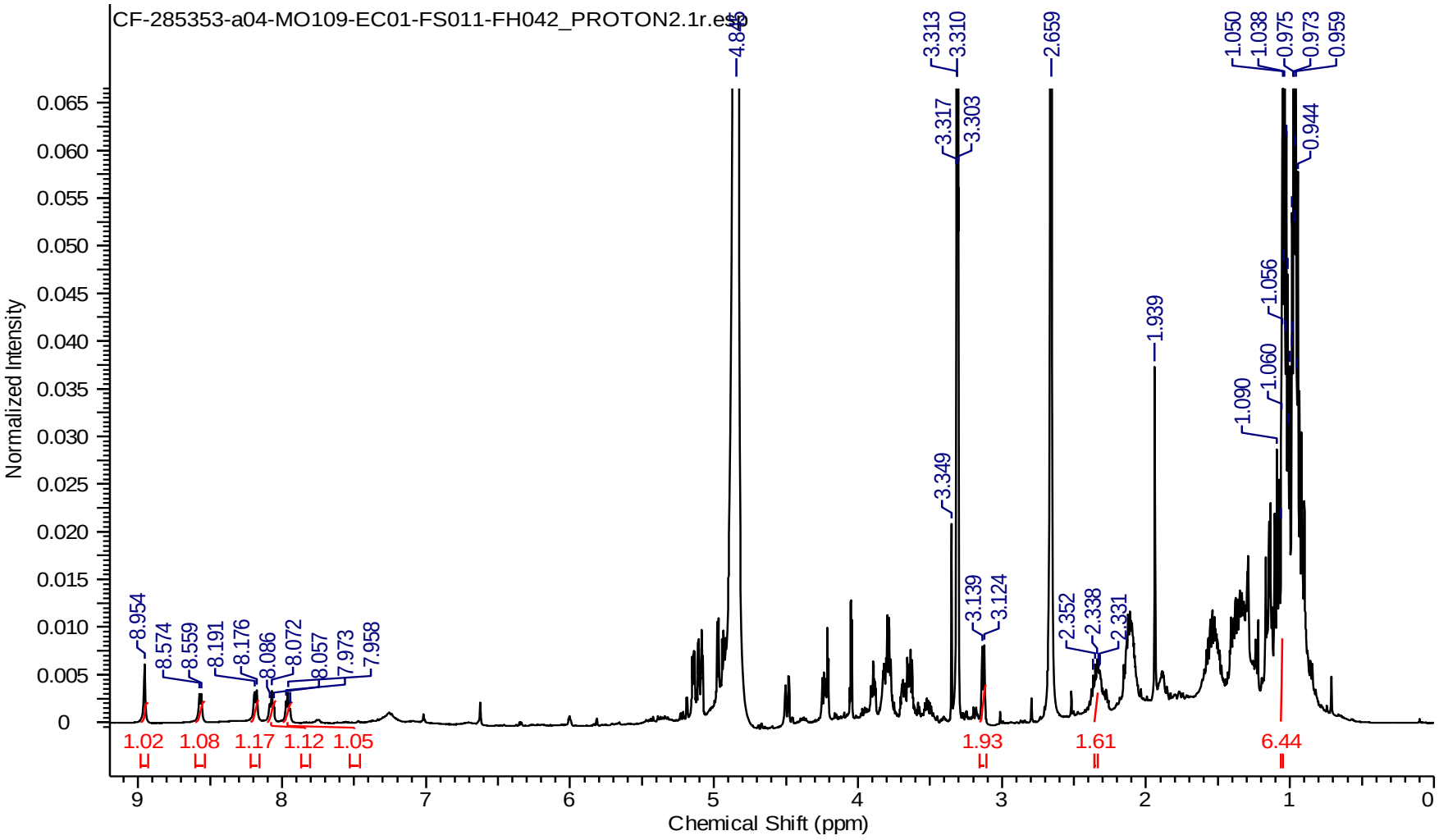
Compound **10** COSY

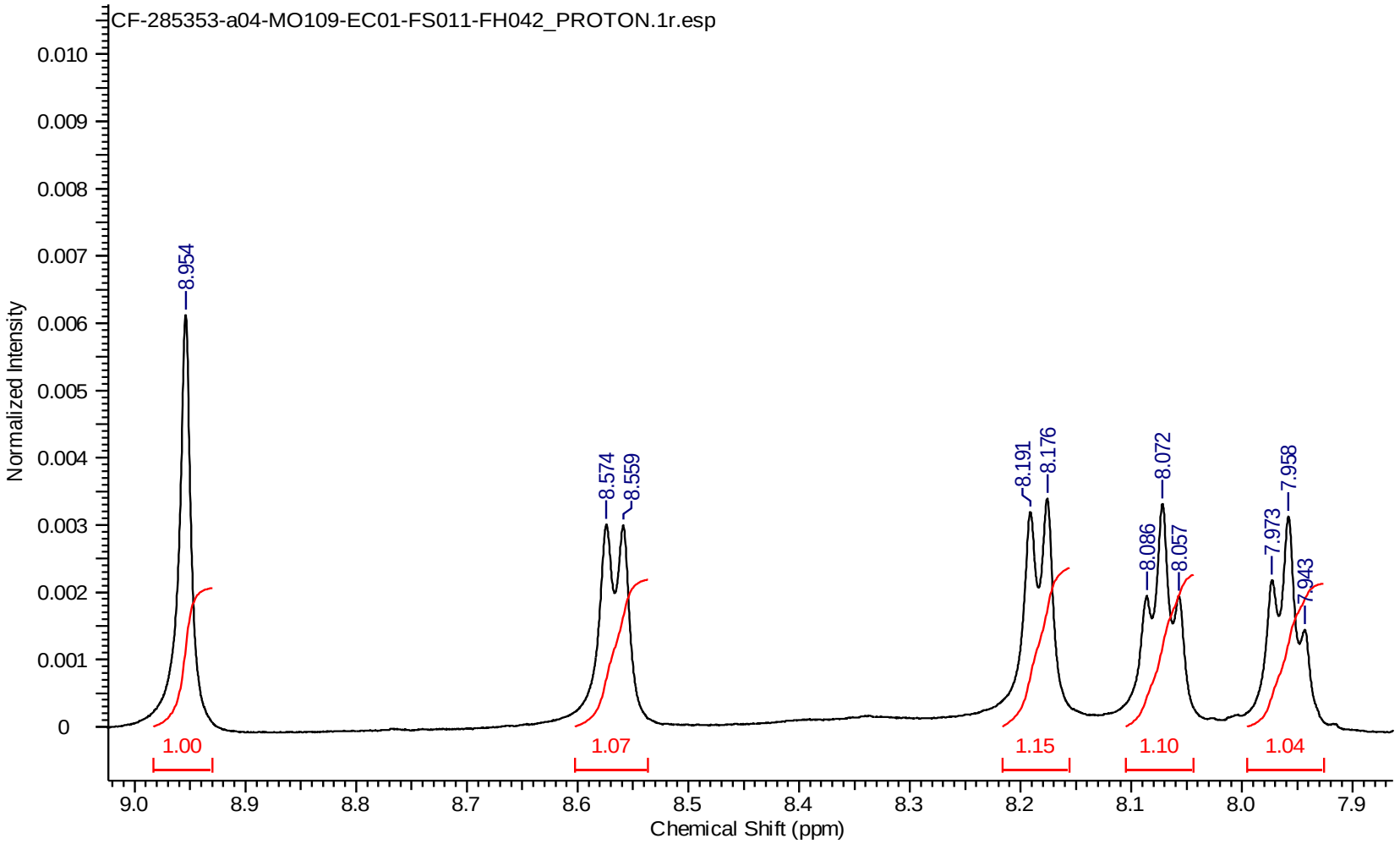


Mixture containing Compound **11** ^{1}H-RMN

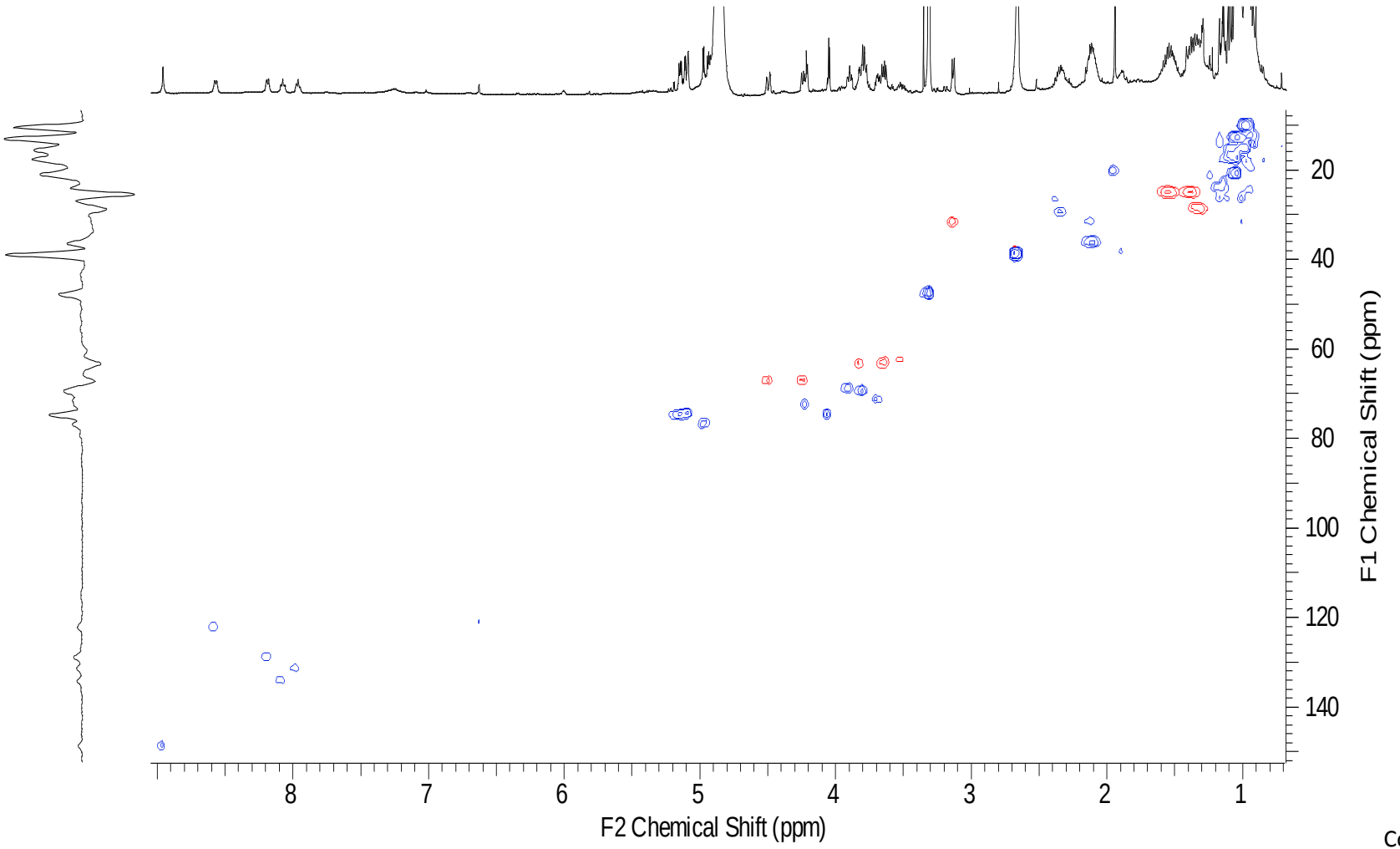


Apendix

Compound **11** ^{1}H-RMN

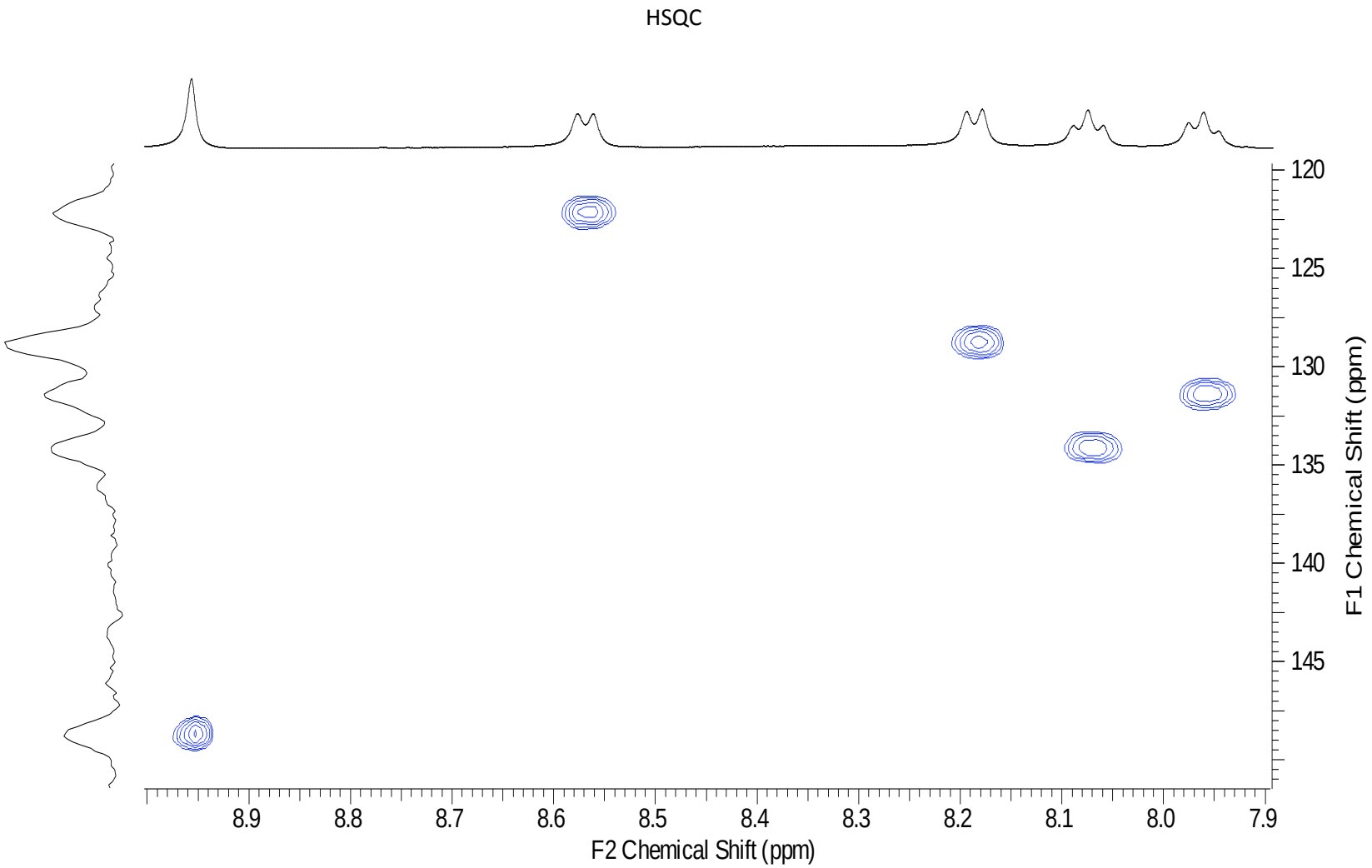


Compound **11** HSQC



Compound **11**

Apendix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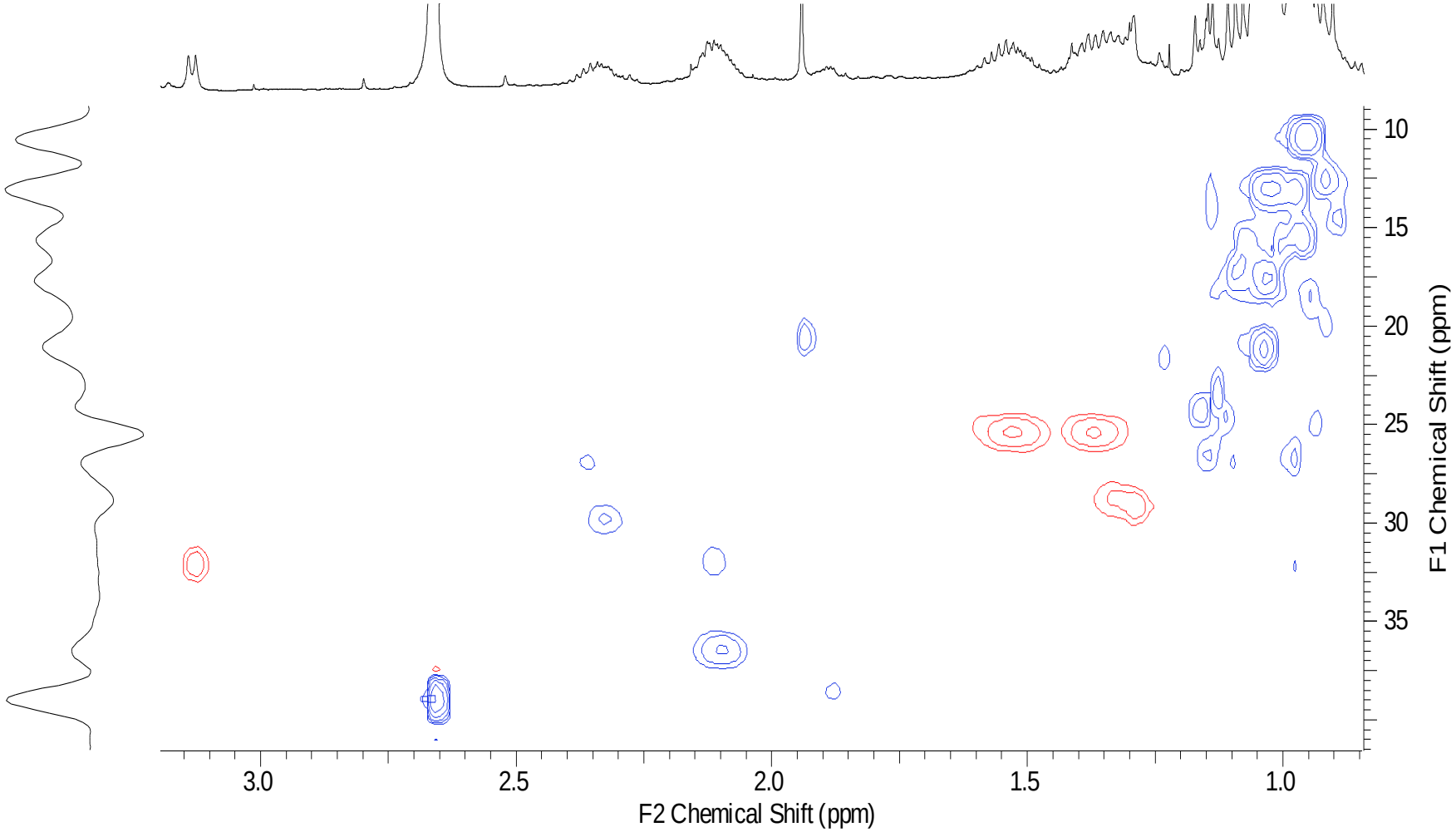
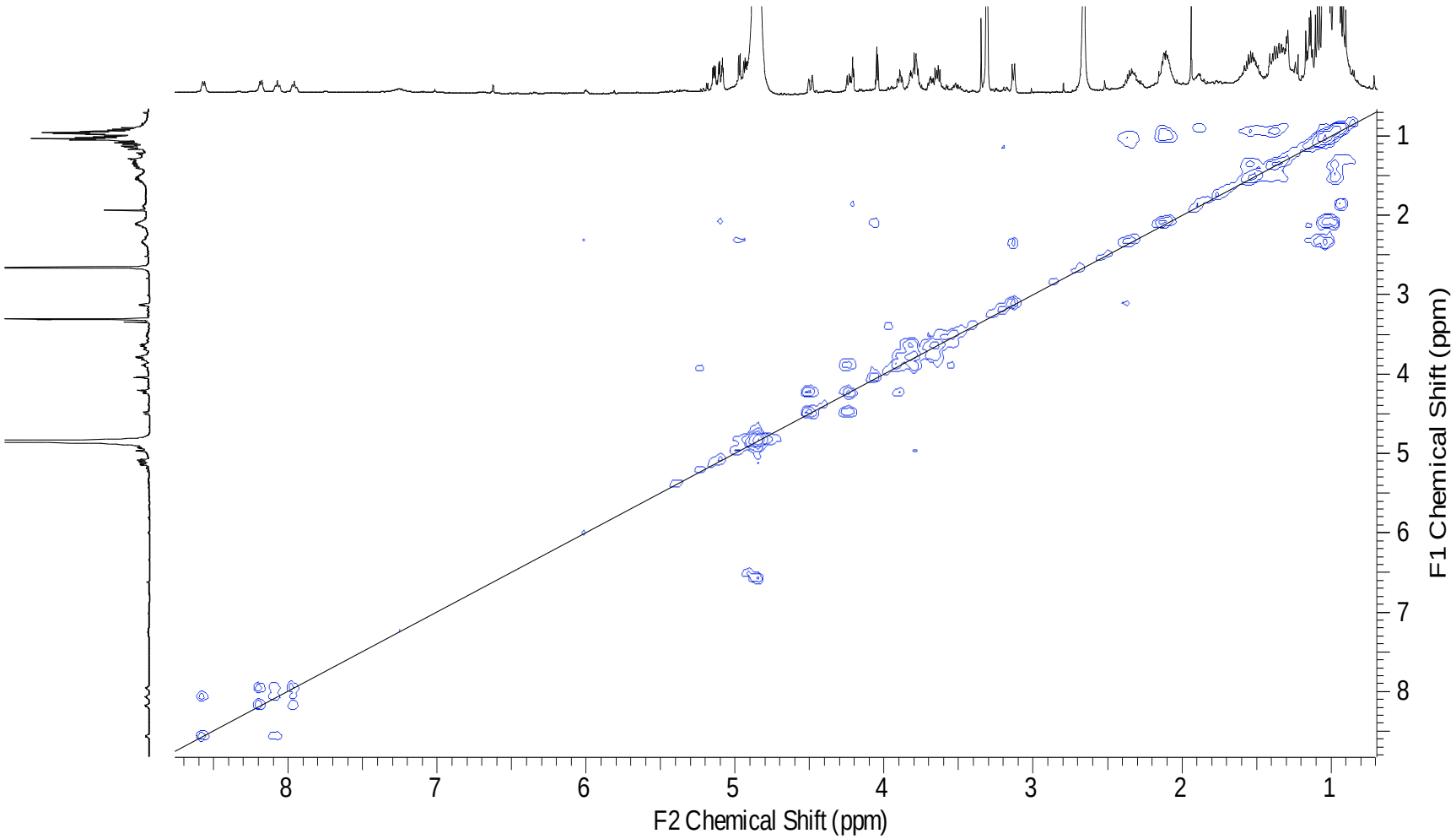
120
125
130
135
140
145
F1 Chemical Shift (ppm)
8.9
8.8
8.7
8.6
8.5
8.4
8.3
8.2
8.1
8.0
7.9
F2 Chemical Shift (ppm)

Compound **11** HSQC

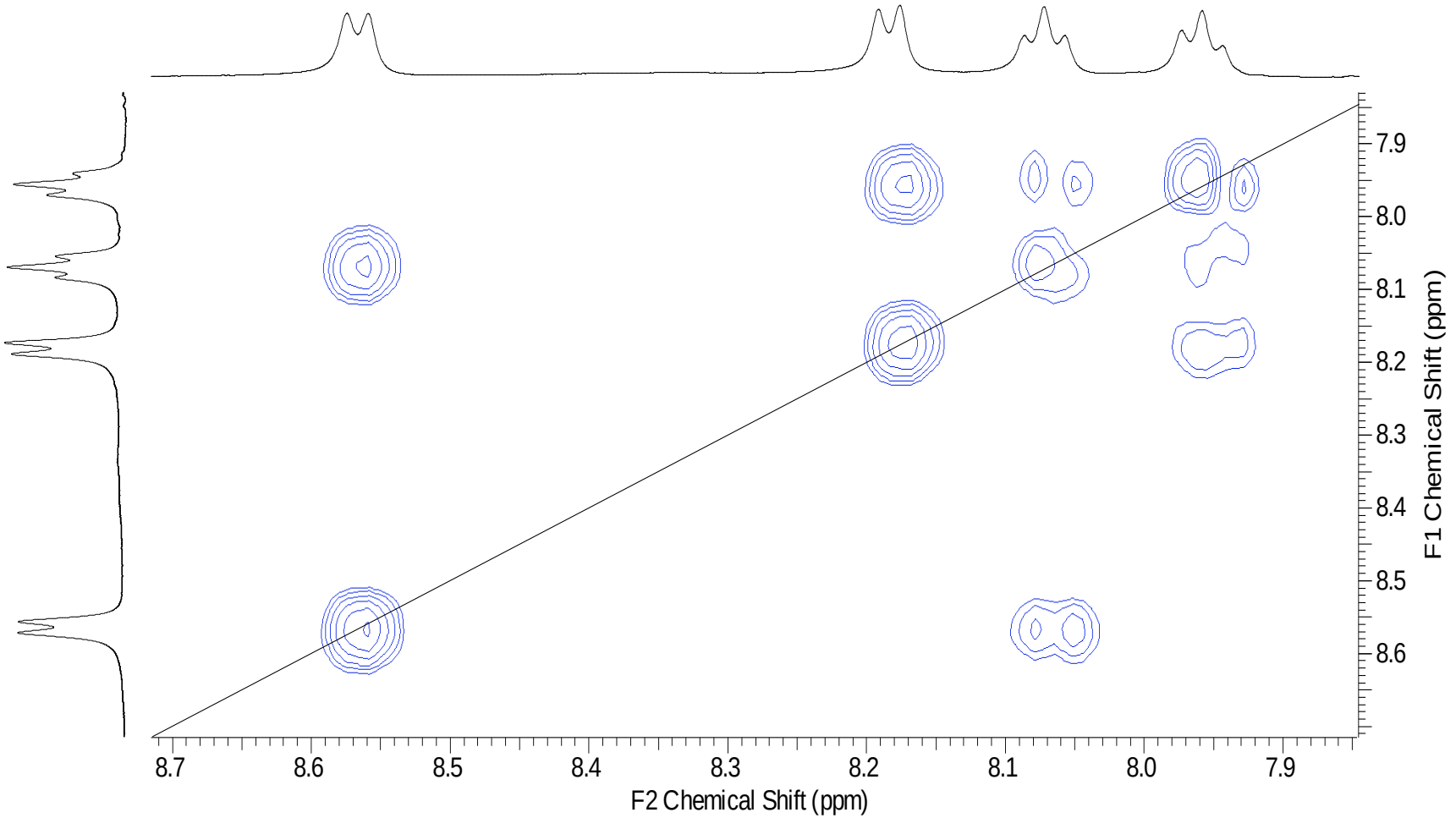


Apendix

Compound **11** COSY



Compound **11** COSY



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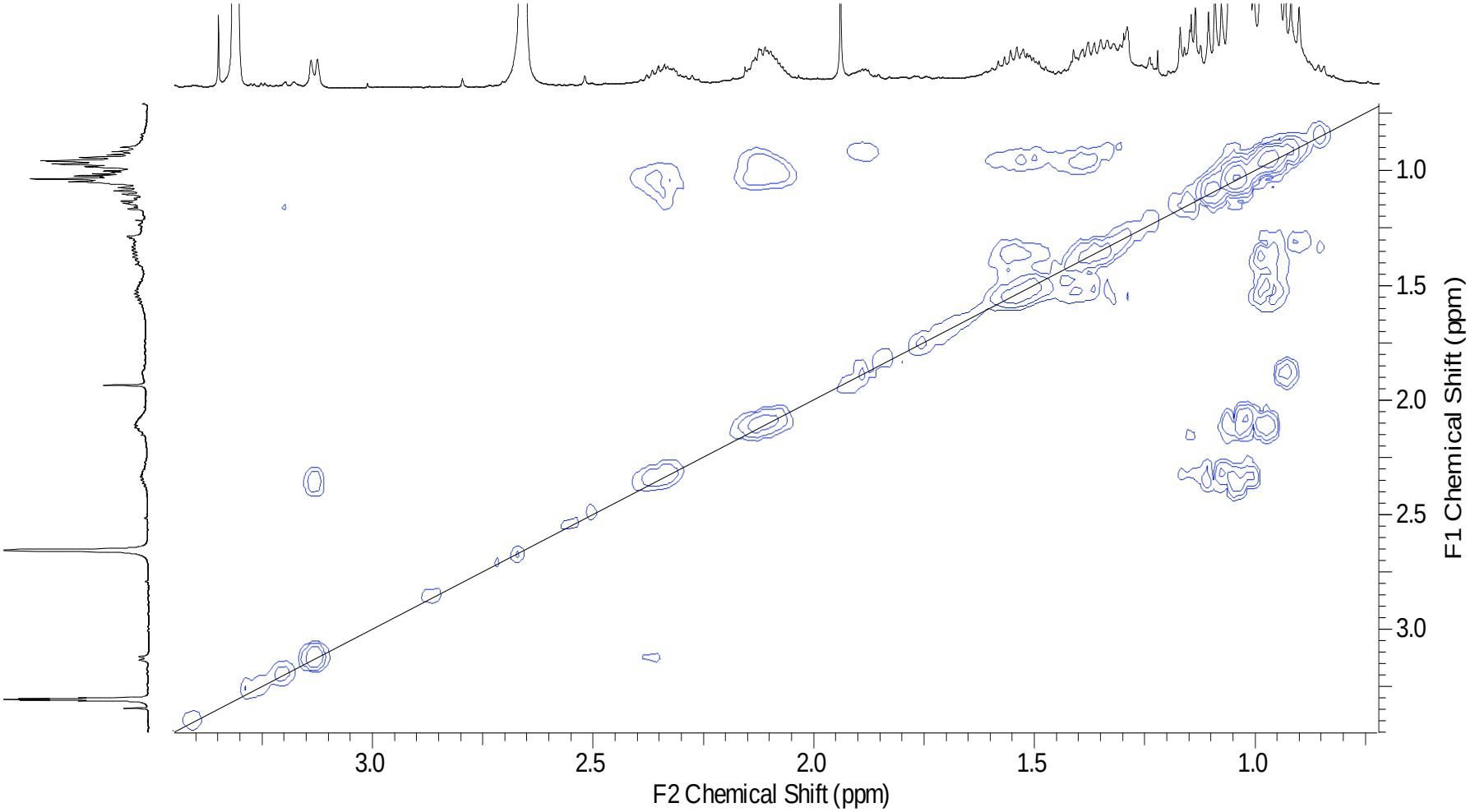
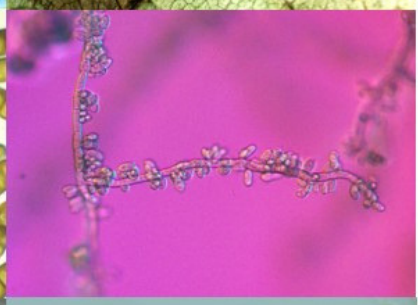
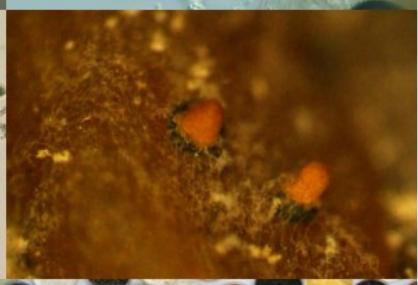
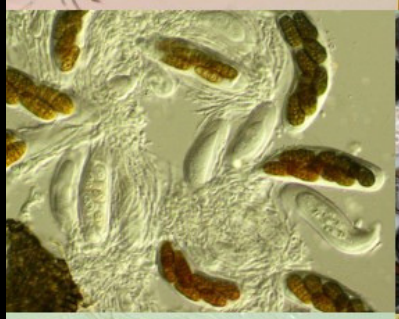
Compound **11** COSY



MEDINA





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