HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Secondary Metabolites from Fungi: Strategies of Activation of Silent Biosynthetic Pathways, Structure Elucidation and Bioactivity

Inaugural dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

Sergi Herve AKONE
from Douala, Cameroon

Düsseldorf, 07. 2016

from the institute for Pharmaceutical Biology and Biotechnology
at the Heinrich Heine University Düsseldorf

Published by permission of the
Faculty of Mathematics and Natural Sciences at
Heinrich Heine University Düsseldorf

Supervisor: Prof. Dr. Peter Proksch
Co-supervisor: Prof. Dr. Rainer Kalscheuer

Date of the oral examination: 22.08. 2016

Devoted to my parents

Bitty Bruno and Bitty Justine née Tondi,

my beautiful angels

Kylyan Handy Piadan Akone and Ayden Brunin Akone Bitty,

My wife

Carine Annick Ma'Alam à Ndioro

CONTENTS

Abstract

Since the discovery of penicillin, fungi stand as an inexhaustible source of novel chemotypes and pharmacophores. A particular interest is given to fungi that are referred to endophytic fungi which are fungi inhabiting internal tissues of plant without causing any visible symptoms of disease. In the past two decades, endophytic fungi have been recognized, as an important source of natural bioactive products with potential application in many areas such as medicine. Since the ‘‘gold” bioactive compound paclitaxel (Taxol®) was discovered from the endophytic fungus *Taxomyces andreanae*, an increasing interest has been given to the study of fungal endophytes as potential producers of novel and biologically active compounds. This is reinforced by the fact that almost all vascular plants that have been examined were found to harbor endophytic fungi and it is estimated that there are over one million fungal endophytes existing in nature. However, it appears that the potential of fungal capability to produce bioactive natural compound has been underexplored as only a subset of fungal bioactive compounds is usually obtained under standard laboratory conditions.

This dissertation mainly focuses on strategies to increase fungal potential to produce new bioactive compounds through the activation of silent biosynthetic pathways with a particular interest in cytotoxic and antibiotic activities. We applied co-cultivation of endophytic fungi with bacteria to increase their chemical diversity. Moreover, the treatment of fungi with small molecules epigenetic modifiers such as the DNA methyltransferase inhibitors (DMATs) or the histone deacetylases inhibitors (HDACs) was undertaken for the activation of silent fungal genes.

This dissertation consists of the following four parts that have already been published or submitted for publication:

Inducing secondary metabolites production by the endophytic fungus Chaetomium sp. through bacterial co-culture and epigenetic modification

Co-cultivation of the endophyte *Chaetomium* sp. with the bacterium *Bacillus subtilis* on solid rice medium resulted in the accumulation of a 1:1 mixture of 3- and 4-hydroxybenzoic acid methyl esters (**1** and **2** respectively) (8.3-fold) compared to axenic controls while the polyketides acremonisol A (**3**), SB236050 (**4**), and SB238569 (**5**) were slightly influenced or not detected. In addition, seven compounds, including isosulochrin (**6**)

and protocatechuic acid methyl ester (**7**), as well as five new natural products (**8**-**12**) were only detected in the co-cultures. The treatment of *Chaetomium* with the epigenetic modifier suberoylanilide hydroxamic acid or 5-azacytidine resulted in an enhanced accumulation of compound (**6**). All the compounds were evaluated for their antibacterial and cytotoxic activities. Compound **5** showed strong cytotoxicity against the mouse lymphoma L5178Y cell line with an IC_{50} value of 1 μM, as well as weak antibacterial activity against *B. subtilis* with an MIC value of 53 μM.

Cytosporins F–K, new epoxyquinols from the endophytic fungus Pestalotiopsis theae

Chemical investigation of an endophytic fungus, *Pestalotiopsis theae*, isolated from the leaves of *Turraeanthus longipes* (Meliaceae) collected in Cameroon, resulted in the isolation of six new epoxyquinols, cytosporins F–K (**2**–**7**), together with the known cytosporin D (**1**). The structures of the new compounds were unambiguously determined by analysis of the 1D, 2D NMR, and HRMS spectra. Cytosporins G–K (**3**–**7**) are the first cytosporins with a hydroxyl substituted C7 side chain, while cytosporins F–I (**2**–**5**) contain a 13-acetoxyl group that was not reported previously. A plausible biosynthetic pathway for the cytosporin derivatives is proposed.

Unguisin F, a new cyclic peptide from the endophytic fungus Mucor irregularis

The new cyclic heptapeptide unguisin F (**1**) and the known congener unguisin E (**2**) were obtained from the endophytic fungus *Mucor irregularis*, isolated from the medicinal plant *Moringa stenopetala*, collected in Cameroon. The structure of the new compound was unambiguously determined on the basis of one- and twodimensional NMR spectroscopy as well as by high-resolution mass spectrometry. The absolute configuration of the amino acid residues of **1** and **2** was determined using Marfey's analysis. Compounds **1** and **2** were evaluated for their antibacterial and antifungal potential, but failed to display significant activities.

2-Pentenedioic acid derivatives from a soil-derived fungus Gongronella butleri

Eight new 2-pentenedioic acid derivatives (**1**–**8**), and one known congener, 2-decanyl-2-pentenedioic acid (**9**), were isolated from a soil-derived fungus *Gongronella butleri* collected in Cameroon. The structures of the new compounds were established by spectroscopic methods including 1D, 2D NMR, and MS. The isolated compounds feature a 2-pentenedioic acid core structure substituted by a 2-alkyl chain that has even number of carbon atoms (C6, C8, and C10) with or without an oxygenated substituent. These compounds were screened for their cytotoxic and antibacterial potentials; however, the tested compounds displayed no significant activities.

Zusammenfassung

Seit der Entdeckung des Penicillins bilden Pilze eine schier unerschöpfliche Quelle für neuartige Verbindungsklassen und Pharmakophoren. Es besteht dabei ein besonderes Interesse an den sogenannten endophytischen Pilzen, welche innerhalb von Pflanzengewebe leben, ohne hierbei ersichtliche Krankheitssymptome zu verursachen. Endophytische Pilze wurden innerhalb der letzten zwei Jahrzehnte als eine wichtige Quelle für neuartige bioaktive Naturstoffe mit vielfältigen potentiellen Anwendungsgebieten wie der Medizin identifiziert. Seit der Entdeckung der "goldenen" bioaktiven Verbindung Paclitaxel (Taxol®) im endophytischen Pilz *Taxomyces andreana*, ist das Interesse an der Forschung mit endophytischen Pilzen, als potentielle Produzenten neuartiger bioaktiver Verbindungen, gewachsen. Dieses wurde durch die Entdeckung bestärkt, dass nahezu alle bisher untersuchten Gefäßpflanzen endophytische Pilze beherbergen und nach Schätzungen mehr als seine Million endophytische Pilze in der Natur existieren. Allerdings scheint es, als sei das Potential der Pilze zur Produktion bioaktiver Verbindungen bisher untererforscht, da unter den Standard Laborbedingungen nur ein Bruchteil der möglichen bioaktiven Pilzverbindungen erhalten wird.

Diese Dissertation konzentriert sich hauptsächlich auf Strategien, durch die Aktivierung stiller Biosynthesewege, das Potential der Pilze neue bioaktive Verbindungen zu produzieren zu verbessern, besonderes Interesse gilt hierbei der zytotoxischen und antibiotischen Aktivität. Zur Erhöhung der chemischen Diversität wurden Co-Kultivierungsansätze zwischen Pilzen und Bakterien angewandt. Außerdem wurden die Pilze zur Aktivierung stummer Gene mit niedermolekularen Agenzien zur epigenetischen Modifikation behandelt, wie DNA-Methyltransferase-Inhibitoren (DMATs) oder Histon-Deacetylase-Inhibitoren (HDACs).

Diese Dissertation besteht aus den folgenden vier Teilen, welche bereits publiziert oder zur Publikation eingereicht wurden:

Induktion der Sekundärmetabolit Produktion des endophytischen Pilzes Chaetomium sp. durch bakterielle Co-Kultivierung und epigenetische Modifikation

Der endophytische Pilz *Chaetomium* sp. wurde aus den, in Kamerun gesammelten, frischen, gesunden Blättern von *Sapium ellipticum* isoliert. Der EtOAc Extrakt von, auf

festem Reismedium kultiviertem, *Chaetomium* sp. Lieferte eine 1:1 Mischung aus 3- und 4-Hydroxybenzoesäure Methylester (**1** und **2**), zusätzlich zu den bekannten Polyketiden Acreminisol A (**3**), SB236050 (**4**) und SB238569 (**5**). Die Mischkultur aus *Chaetomium sp.* mit dem Bakterium *Bacillus subtilis* auf festem Reismedium, führte zu einer, im Vergleich mit der Reinkulturkontrollen 8,3-fach höheren Akkumulation von **1** und **2**. Zusätzlich wurden weitere sieben Verbindungen, einschließlich Isosulochrin (**6**) und Protocatechusäure Methylester (**8**), sowie weitere fünf neue Naturstoffe (**8** - **12**), ausschließlich in der Co-Kultur nachgewiesen. Die Struktur der neuen Verbindungen wurde durch den Einsatz von 1D und 2D NMR, sowie HRESIMS, eindeutig bestimmt. Die Behandlung von *Chaetomium* sp. mit den epigenetischen Modifikationagenzien Suberoylanilid-Hydroxamsäure und 5-Azacytidin führte zu einer verstärkten Akkumulation von Verbindung **6**, welche ebenfalls in der Co-Kultur von *Chaetomium sp.* und *Bacillus subtilis* beobachet wurde, allerdings nicht in der Reinkulturkontrolle. Alle Verbindungen wurden bezüglich ihrer antibakteriellen und zytotoxischen Aktivität evaluiert. Verbindung **5** zeigte starke Zytotoxizität gegenüber der Maus-Lymphom-Zelllinie L5178Y mit einem IC_{50} Wert von 1µM und außerdem schwache antibakterielle Aktivität gegenüber *B. subtilis* mit einer MIC von 53µM.

Cytosporin F-K, neue Epoxyhydrochinone aus dem endophytischen Pilz Pestalotiopsis theae

Die chemische Untersuchung des endophytischen Pilzes *Pestalotiopsis theae*, welcher aus den Blättern, der in Kamerun gesammelten, *Turraeanthus longipes* (Meliceae), isoliert wurde, führte zur Isolation der sechs neuen Epoxyhydrochine Cytosporin F-K (**2**-**7**) und dem bekannten Cytosporin D (**1**). Die Struktur der neuen Verbindungen wurde durch den Einsatz von 1D und 2D NMR, sowie HRESIMS, eindeutig aufgeklärt. Cytosporin G-K (**3**-**7**) sind die ersten Cytosporine mit einer hydroxilierten C7 Seitenkette, während Cytosporin F-I (**2**-**5**) eine 13 Acetoxygruppe besitzen die bisher nicht beschrieben wurde.

Unguisin F, ein neues zyklisches Peptid aus dem endophytischen Pilz Mucor irregularis

Aus dem endophytischen Pilz *Mucor irregularis*, welcher aus der in Kamerun gesammelten Medizinalpflanze *Moringa stenopetala* isoliert wurde, wurden das neue zyklische Heptapeptid Unguisin F (**1**) und die verwandte bekannte Verbindung Unguisin E (**2**) erhalten. Die Struktur der neuen Verbindung wurde durch den Einsatz von zweidimensionaler NMR, sowie der Hochauflösungsmassenspektrometrie, eindeutig bestimmt Die absolute

Konfiguration der Aminosäurebausteine von **1** und **2** wurden durch den Einsatz der Marfey Analyse bestimmt. Verbindung **1** und **2** wurden bezüglich ihrer antibakteriellen und zytotoxischen Eigenschaften getestet, zeigten aber keine signifikante Aktivität.

2-Pentendisäure Derivate aus dem Bodenpilz Gongronella butleri

Acht neue 2-Pentendisäure Derivate (**1**-**8**) und eine verwandte bekannte Verbindung 2-Decanyl-pent-2-endisäure (**9**) wurden aus dem Pilz *Gongronella butleri* erhalten, welcher aus einer in Kamerun genommenen Bodenprobe stammt. Die Struktur der neuen Verbindungen wurde durch den Einsatz spektroskopischer Methoden einschließlich 1D, 2D NMR und MS bestimmt. Die isolierten Verbindungen besitzen ein 2-Pentendisäuregrundgerüst, welches an Position 2 mit einer Alkylkette substituiert ist, welche eine geradzahlige Anzahl an Kohlenstoffatomen trägt (C6, C8 und C10) und einen sauerstoffhaltigen Substituenten tragen kann. Diese Verbindungen wurden bezüglich ihres zytotoxischen und antibakteriellen Potentials untersucht, sie wiesen allerdings keine signifikante Aktivität auf.

Chapter 1

General Introduction

1.1 Natural Products in Traditional Medicine

Natural products, including secondary metabolites, are defined as chemical substances produced by living organisms. Secondary metabolites are formed as a peculiar offshoot through specific biogenetic pathways and are not involved in the primary metabolism of the cell, including growth and development (Gurnani *et al.*, 2014). Secondary metabolites, unlike primary metabolites, such as proteins and carbohydrates, are not essential for growth or reproduction of an organism. Instead, they are the result of the adaptation of an organism to its surrounding environment or are produced as a defense mechanism against predators or herbivores (Colegate and Molyneux, 2007; Dewick, 2002). As a result, secondary metabolites, with their unique biosynthesis and diverse characteristic chemical structures, have appeared to be an inexhaustible source of novel chemotypes and pharmacophores and have been used as therapeutic agents for thousands of years (McMurry *et al.*, 2010).

1.1.1 Medicinal Plants used in Folklore

Traditional medicine dates back practically to the existence of human civilization. Even though medicine has been extensively developed over the past years, this progress has been rooted in traditional medicine and therapies, prevailing worldwide for thousands of years. The use of natural products from medicinal plants has been described from Cuneiform scripts in Mesopotamia since 2600 B.C. (Cragg and Newman, 2005). These records indicate that up to 1000 plant-based medicines were prepared during that time. Plants were initially used in the form of crude drugs, such as tinctures, teas, poultices, powders and other diverse herbal formulations for the treatment of inflammation, influenza, coughing, and parasitic infestations, among others (Gurib-Fakim, 2006; Samuelson, 2004).

This knowledge of plant-based medicine was passed down from generation to generation by trial and error over centuries and significantly influenced the development of different traditional systems of medicine (Kinghorn, 2011). For example, the plant *Alhagi maurorum* Medik (Camel thorn) produces a sweet material from the stems and leaves (Duke *et al.*, 2008). This is known as "*manna*" and consists mainly of melezitose and sucrose. It has been documented by Ayurvedic people that this material aids in the treatment of anorexia,

dermatosis, and leprosy (Duke *et al.*, 2008). Nowadays, the Konkani people use the plant for the treatment of asthma through smoking (Duke *et al.*, 2008). Another example includes the plant *Ligusticum scoticum* L. which grows in Northern Europe and Eastern North America. This plant was eaten raw in the morning to protect from daily infections (Dillenius, 1724); and its root was used to treat flatulence (Martin, 1934), and as an aphrodisiac (Beith, 1995).

Plant-based medicine has been documented in many parts of the world. In India, the medicinal use of plants is recorded in the Rig Veda, which is considered as one of the oldest repository of human knowledge written between 4500 and 1600 BC. In China, the "Tang Herbal" recorded in 659 A.D. describes 850 drugs (Cragg and Newman, 2013). In Africa, Egyptians have also documented the uses of various herbs in 1500 B.C. (Brahmachari, 2011). In Europe, the Greek botanist Pedanius Dioscorides compiled the use of traditional medicine in a five-volume book entitled "*De Materia Medica*" in A.D. 60 (Cragg and Newman, 2001). Nowadays, many people, especially in developing countries, still rely on crude drug preparation from plants used in traditional medicine for the treatment of a plethora of health problems (Chang and But, 1986; Dev, 1999).

1.1.2 Other Sources of Medicinal Natural Products used in Folklore

Lichens have been used as a raw material for perfumes and cosmetics, and as medicines in Chinese and Egyptian civilizations (Purvis, 2000). One of the well known examples is *Usnea dillenius* ex Adanson, which in ancient times was used in traditional medicine to cure diseases of the scalp (Purvis, 2000). Another, example is *Parmelia omphalodes* (L.) Acharius that was traditionally used scattered on stockings before journeys in order to prevent inflammation of the feet (MacFarlane, 1924; Cameron, 1900). Interestingly, it was also used in Ireland as a cure for bad sores from burns and cuts (Allen and Hatfield, 2004).

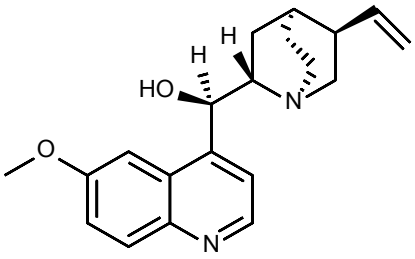
Fungi, such as *Piptoporus betulinus* growing on birches have also been used in traditional medicine (Swanton, 1916). Indeed, they were steamed to produce charcoal, which was used as an antiseptic and disinfectant (Swanton, 1916). Moreover, the fungus *Agaricus campestris* L. (field mushroom) was stewed in milk to soothe throat cancer (Hatfield, 1994).

1.2 Impact of Natural Products in Drug Discovery

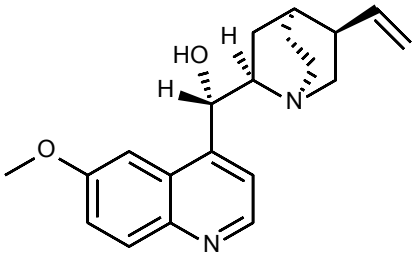
1.2.1 Bioactive Compounds from Plants in Drug Discovery

Over the centuries humans have relied on plant-based medicine for health problems. The chemicals present in plants are classified into several groups, such as alkaloids, terpenoids, and phenolics. (Harborne, 1984; Ramawat and Merillon, 2008). It is estimated that about 25 % of the drugs prescribed worldwide originate from plants.

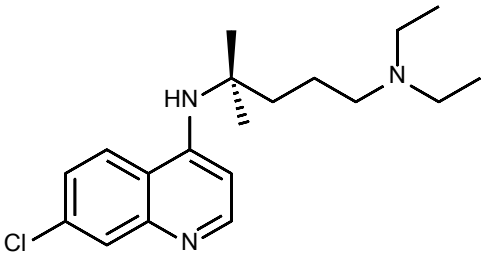
Many important drugs have been produced by plants, including quinine and quinidine from *Cinchona* sp., which are used for the treatment of malaria. Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. One of the most dangerous species, *P. falciparum*, which is mainly found in sub-saharan Africa, accounts for almost 90% of all malaria cases worldwide (Hay *et al.*, 2004). The pathogen is transmitted to humans through *Anopheles* mosquito species, and the disease especially affects children aged less than 5 years, as their level of acquired immunity to the parasite is often weak (Müller and Hyde, 2010). Many synthetic drugs have been produced based on the natural product quinine and its stereoisomer quinidine, both possessing a quinoline ring, including chloroquine and amodiaquine. All these compounds inhibit heme polymerization and hemozoin formation (Kuhn *et al.*, 2007; Klonis *et al.*, 2010), leading to parasite death (Fitch, 2004). However, emergence of parasites, which are resistant to antimalarial agents, such as quinine derivatives, encouraged the search for new antimalarial agents. As a result, artemisinin, a sesquiterpene lactone, was isolated from the sweet wormwood *Artemisia annua*, a Chinese plant traditionally used for the treatment of malaria (Cui and Su, 2009). Since 2006, the World Health Organisation (WHO) has promoted a policy for treating *P. falciparum* based on artemisinin combination therapies (Bosman and Mendis, 2007). It was reported that artemisinin, inhibits heme polymerization and hemozoin formation, as in the case of quinine (Kannan *et al.*, 2002).



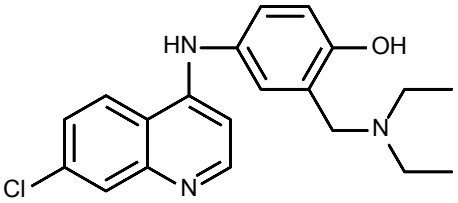
Quinine



Quinidine

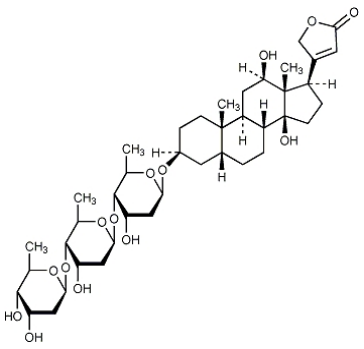


Chloroquine



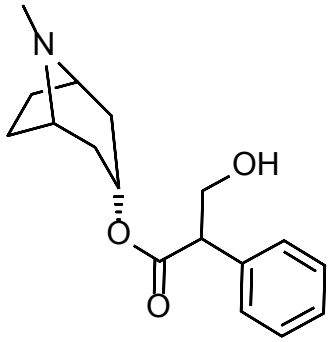
Amodiaquine

Another important compound produced by plants is digoxin, a cardiac glycoside that was isolated from *Digitalis* sp. and is used for the treatment of heart failure (Figueiredo and Machado, 2010). This drug slows the ventricular rate in sinus rhythm by increasing the parasympathetic tone in patients with heart failure (Figueiredo and Machado, 2010).



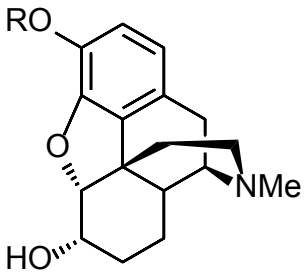
Digoxin

Hyoscyamine, isolated from *Atropa belladonna (*Asano *et al.*, 2013), was the first drug used for the treatment of bradycardia. It has also been used as mydiatric and as antidote for poisoning by organophosphate insecticides (Grynkiewicz and Gadzikowska, 2008).



Atropine (racemic form of hyoscyamine)

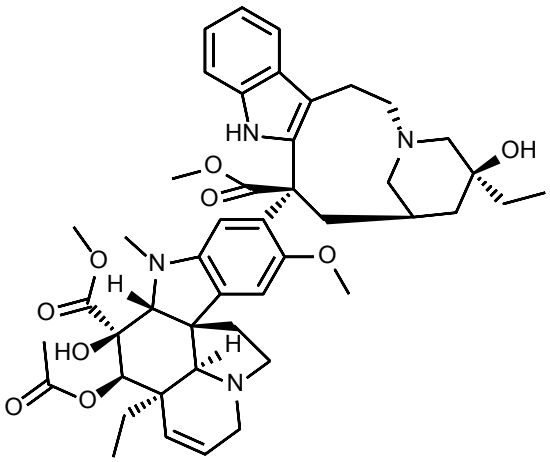
Other famous drugs produced by plants are the analgesics morphine and codeine, which were isolated from *Papaver somniferum* L. (opium poppy). Codeine and morphine are pain relief therapeutics, belonging to the opioid family (Thorn *et al.*, 2009). In ancient times, poppy extracts were medicinally used by Sumerians and Greeks, while Arabs reported the addictive properties of opium (DerMarderosian and Beutler, 2002).



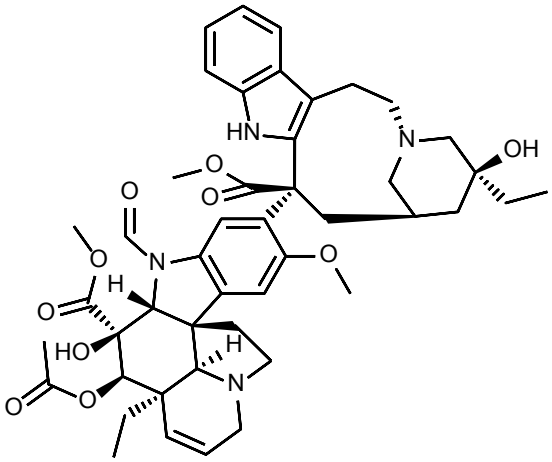
R=H Morphine

R=Me Codeine

It has been reported that 60% of antitumor and anti-infectious drugs already on the market or under clinical trials originate from nature (Shu, 1998). Examples are vincristrine and vinblastine isolated from *Catharanthus roseus* that are used in antitumor therapy as antineoplastic agents (Keglevich *et al.*, 2012). Both compounds inhibit mitosis by blocking the microtubules of the mitotic apparatus (Wilson *et al.*, 1975). Moreover, vincristine binds to DNA and chromatin (Mohammadgholi *et al.*, 2013; Zhu *et al.*, 2004). This binding alters the structure of chromatin, thus perturbing histone-DNA interaction (Mohammadgholi *et al.*, 2013).

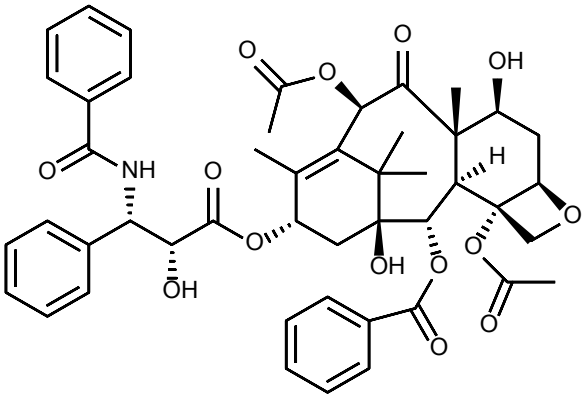


Vinblastine

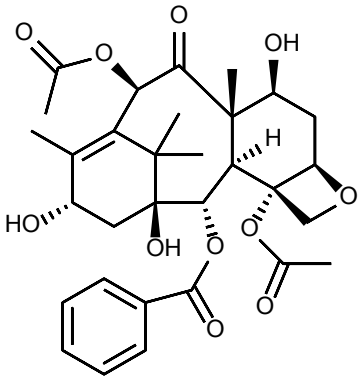


Vincristine

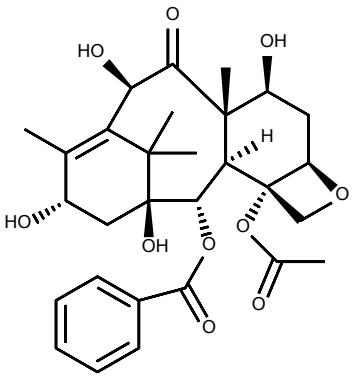
One of the most important plant-derived anticancer drugs is the diterpenoid alkaloid paclitaxel (Taxol®), which was isolated from the bark of *Taxus brevifiola* (Cragg, 1998). Paclitaxel has been appoved by the FDA since 1992 for the treatment of metastatic carcimona in ovarian, breast, and non-small cell lung cancer (Zhong, 2002; Malik *et al.*, 2011). Paclitaxel belongs to the group of taxanes, which inhibit cell proliferation by binding to the surface of microtubules and specifically to β-tubulin, thus, leading to its polymerization (Yeung *et al.*, 1999; Kovacs *et al.*, 2007). However, paclitaxel supply from *Taxus brevifiola* is very limited, if we consider the slow growth of the latter, and the low yield of isolation product amounting to 0.01 % of the dried weight of the bark (Yukimune *et al.*, 1996). To solve this problem, total synthesis of paclitaxel was successfully applied, but it was not feasible at the industrial scale (Vidensek *et al.*, 1990; Holton *et al.*, 1994; Nicolaou *et al.*, 1994). Nevertheless, semisynthesis through its precursors, such as baccatin III and 10-deaceyltbaccatin, which are naturally found in needles and twigs of the plant, in higher yields than paclitaxel, is applied nowadays for paclitaxel production (Dewick, 2002; Denis *et al.*, 1988; Guo *et al.*, 2006). Another promising approach is through plant cell culture under optimized conditions (Malik *et al.*, 2011; Zhong, 2002; Yukimune *et al.*, 1996). In addition, the application of hydroponic growth of seedlings has recently been reported (Tafreshi *et al.*, 2011).



Paclitaxel

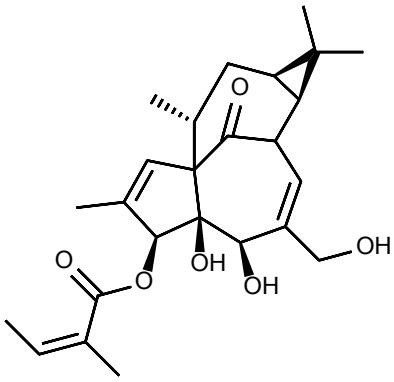


Baccatin III

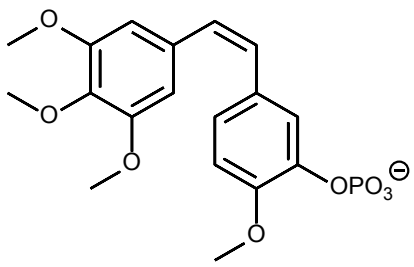


10-Deacetylbaccatin III

Other antitumor compounds include ingenol 3-*O*-angelate from the sap of *Euphorbia peplus*, a promising candidate for the treatment of skin cancer (Kedei *et al.*, 2004; Ogbourne *et al.*, 2004), and the stilbene combretastatin A-4 phosphate, an anti-angiogenic agent from the South African Bush willow *Combretum caffrun*. The latter compound is currently under Phase II clinical trials (Cragg and Newman, 2005; Holwell *et al.*, 2001; Dias *et al.*, 2012).



Ingenol 3-O-angelate



Combretastatin A-4 phosphate

1.2.2 Bioactive Compounds from Microorganisms in Drug Discovery

Prokaryotic and eukaryotic microorganisms, especially fungi and terrestrial actinomycetes, have been the source of a wide array of potent and effective medicines in the modern era (Harvey, 2008). The use of microorganisms as a source of natural products began with the discovery of penicillin G from the fungus *Penicillium notatum* (Mann, 1994) and streptomycin from the bacterium *Streptomyces griseus* (Hinshaw *et al.*, 1946). Nowadays, microorganisms continue to be reliable sources of novel lead compounds. This is mainly due to the fact that microorganisms, unlike plants, can be cultured under laboratory conditions and biotechnologically exploited for large-scale production of the compounds of interest.

1.2.2.1 Bioactive Natural Products from Bacteria

Soil-derived bacteria are well known to produce clinically used antimicrobial agents, including gentamicin from *Micromonospora purpurea*, rifamycin from *Amycolatopsis mediterranei*, and chloramphenicol from *Streptomyces venezuelae*. Vancomycin was isolated from the bacterium *Amycolatopsis orientalis* and displayes activity against several gram-positvive and gram negative bacteria, including penicillin-resistant strains. Vancomycin was approved by the FDA in 1958 for the treatment of severe bacterial infections (Butler, 2004). Salinosporamide A was discovered from the marine actinomycete *Salinospora tropica* and it is known to exhibit its anticancer activity through inhibition of the 20S proteasome (Fenical, and Jensen, 2006). Salinosporamide A is currently under clinical studies (Fenical *et al.*, 2009). However, isolation of bioactive metabolites from the soil-derived bacteria is hindered due to difficulties with culturing the vast majority of these species.

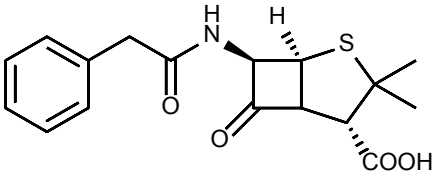
1.2.2.2 Fungi-Derived Natural Products as Pharmaceutical Drugs

The fungal kingdom contains numerous organisms, including mushrooms, rusts, smuts, puffballs, mold or yeast that play remarkable economic and ecological roles (Gurnani *et al.*, 2014). Mushrooms have been used for the production of food and alcoholic beverages (yeast), for medication in traditional medicine, as well as for cultural purposes (Dias *et al.*, 2012). The study of microfungi has led to the discovery of many compounds that are used as antibiotics, antifungal, immunosuppressive, or cholesterol lowering agents.

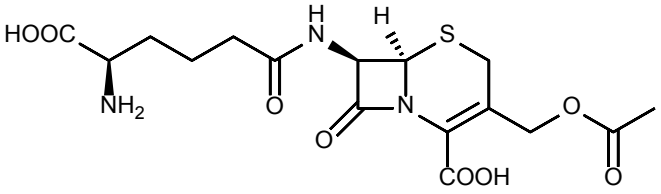
One of the most famous drugs is the β-lactam antibiotic penicillin G isolated from the fungus *Penicillium notatum* in 1929 by Alexander Fleming (Mann, 1994). The discovery of penicillin G followed by clinical studies in the early 1940s revolutionized drug discovery research (Sengupta *et al.*, 2013). Since then, many companies worldwide started the assembly

of a large collection of microorganisms aiming to discover new antibiotics (Wainwright, 1990). Penicillin G was introduced as an antibacterial agent to the market in the early 1950s. The discovery of penicillin G led to the identification and development of other β-lactam antibiotics, such as cephalosporins, monobactams and carbapenems (Papp-Wallace *et al.*, 2011), which incorporate a four-membered β-lactam ring in their structures. These compounds act through inhibition of bacterial cell wall synthesis by binding to the transpeptidase domain of penicillin binding proteins (PBPs) (Zervosen *et al.*, 2012). Four years after the introduction of penicillin G on the market, penicillin-resistant strains of *Staphylococcus aureus* that secrete β-lactamases were identified (Rammelkamp and Maxon, 1942). These enzymes hydrolyse the β-lactam ring of penicillins leading to loss of their antibiotic activity. Interestingly, cephalosporin C, a ring-expanded version of penicillin, which was isolated from *Cephalosporium* sp. (Newton and Abraham, 1956), currently renamed *Acremonium chrysogenum* (Liu *et al.*, 2010; Brakhage, 1998), presented a wide spectrum of activity, including the inhibition of *Staphylococcus aureus* resistant to penicillin (Florey *et al.*, 1956). The first cephalosporin-type therapeutic agent with oral administration is cephalexin introduced in the market in 1970 (Newman and Cragg, 2010). Currently, there are nine known β-lactam derivatives, including two cephalosporins, six carbapenems and one penem that are in clinical trials (Fabbretti *et al.*, 2011).

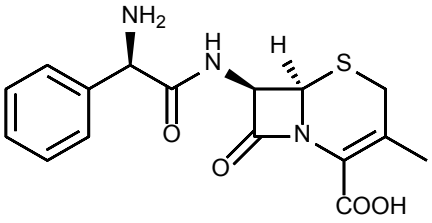
The terpenoid fusidic acid is another antibacterial agent produced by the fungus *Fusidium coccineum*, which was first isolated from monkey feces (Verbist, 1990) and is available on the market since 1962. Fusidic acid inhibits bacterial protein synthesis through interference with elongation factor G (Musmade *et al.*, 2013).



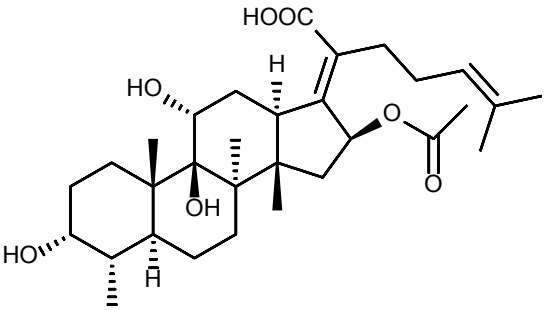
Penicillin G



Cephalosporin C



Cephalexin

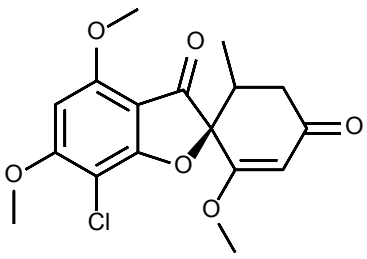


Fusidic acid

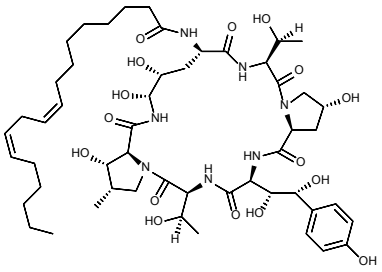
Fungi also produce drugs used as antifungal agents. The polyketide griseofulvin, one of the first antifungal natural products found in filamentous fungi, was isolated from *Penicillium griseofulvum* in 1939 (Oxford *et al.*, 1939; Grove and McGowan, 1947) and is used for the treatment of dermatophytosis (Williams *et al.*, 1958; Bent and Moore, 1966). This natural product has also been reported from 16 different fungi belonging to the genus *Penicillium*, as well as from *Aspergillus lanosus* (Larsen *et al.*, 2005). Griseofulvin was reported to bind nucleic acids (Osment, 1969; Gull and Trinci, 1973; Grisham *et al.*, 1973), as well as tubulin, thus inhibiting tubulin polymerization (Chaudhuri *et al.*, 2001; Keates, 1981). In 2006, the anticancer activity of griseofulvin and some of its analogs were demonstrated (Oda, 2006) through the prevention of mitosis during cell division (Rebacz *et al.*, 2007).

Following the discovery of griseofulvin, other important antifungal drugs were identified from fungi, including echinocandin B and pneumocandin B_0, which were isolated from *Aspergillus nidulans* (Nyfeler and Keller-schierlein, 1974) and from *Glarea lozoyensis* (Masurekar *et al.*, 1992), respectively. Echinocandins that are currently in clinical use or in development are cyclic hexapeptides bearing an *N*-linked acyl lipid side-chain (Kurtz and Rex, 2001; Lucas *et al.*, 1996). Echinocandin B served as the lead compound for the semisynthesis of anidulafungin (Eraxis ®), whereas pneumocandin B_0 is the lead compound for caspofungin (Cancidas ®). The echinocandins are non-competitive inhibitors of β-(1.3)-D-

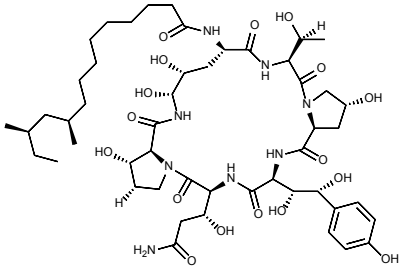
glucan synthase, a complex enzyme responsible of the formation of glucan polymers; therefore leading to fungal cell wall damage (Denning, 2003).



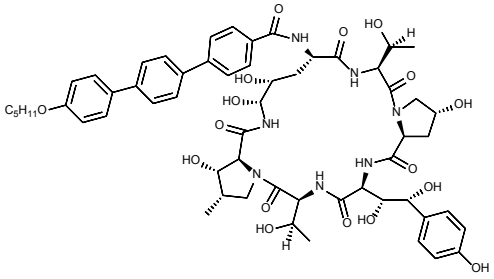
Griseofulvin



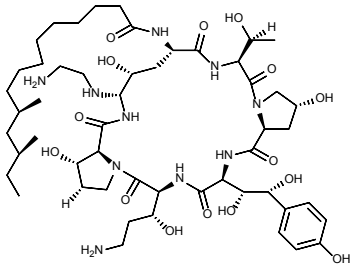
Echinocandin B



Pneumocandin B_0



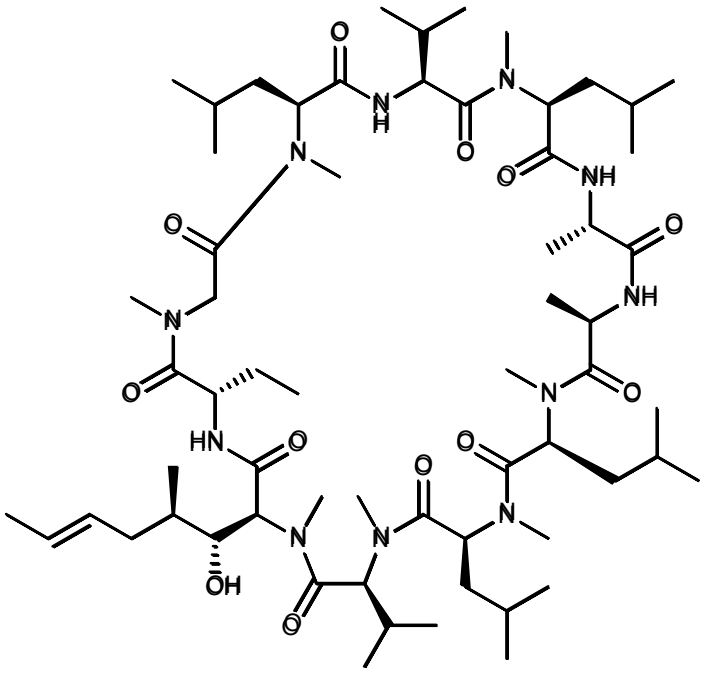
Anidulafungin



Caspofungin

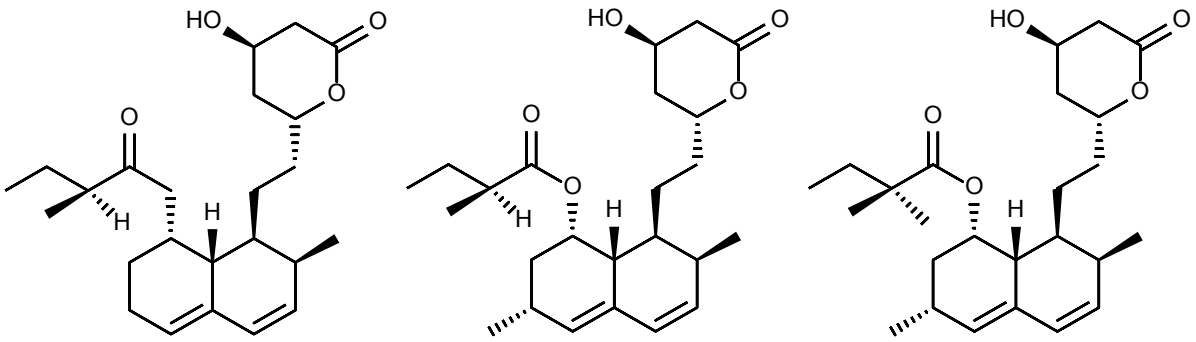
The organ transplant medicine was revolutionized by the discovery of the lipophilic cyclic undecapeptide cyclosporine (cyclosporine A, CsA), produced by the fungus *Hypocladium inflatum* (Borel *et al.*, 1994). Cyclosporin is immunosuppressant and has been widely used for the treatment of transplant rejection (Matsuda and Koyasu, 2000). One of the pathways involved in its mechanism of action is the calcineurin/NFAT pathway. Indeed, cyclosporine forms a complex with cyclophilin leading to the inhibition of the phosphatase activity of calcineurin, which regulates the activation of NFAT transcription factors (Crabtree, 1989; Matsuda and Koyasu, 2000). The NFAT group is involved in the transcriptional activation of the genes encoding IL-2, IL-4 and CD40L, and therefore cyclosporine is classified as a specific inhibitor of T cell activation (Allison, 2000; Su *et al.*, 1994; Matsuda *et al.*, 1998). In addition to its immunosuppressant activity, cyclosporine is used in the treatment of severe psoriasis (Pereira *et al.*, 2006). Furthermore, cyclosporine has been reported to be a potent inhibitor of HIV-1 and HCV replication (Hatziioannou *et al.*, 2005; Paeshuyse *et al.*, 2006) and it has been used as a lead structure for the development of potent antiviral compounds against HCV, such as alisporivir (Debio 025) that currently is under phase III clinical studies (Hubler *et al.*, 2000; Crabbe *et al.*, 2009). Interestingly, the antimalarial

activity of cyclosporin was also reported as it showed inhibition against *Plasmodium falciparum* (Bell *et al.*, 1994) and *P. vivax* (Kocken *et al.*, 1996).



Cyclosporin A

Another important group of fungal metabolites are the statins. These compounds inhibit 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which catalyses the *de novo* synthesis of cholesterol (Rao *et al.*, 2011), and therefore have been used as cholesterol-lowering drugs (Seenivasan *et al.*, 2008). Statins have been shown to reduce cardiovascular morbidity and mortality in patients with cardiovascular disease. There are seven statins currently approved for clinical use for the treatment of broad spectrum dyslipidemia (Rao *et al.*, 2011). The first reported statin, mevastatin, was isolated from *Penicillium citrinum*. After this discovery, lovastatin was purified by *Monascus ruber* cultures (Negishi *et al.*, 1986) and the industrial process for its production was set-up in 1980 using *Aspergillus terreus* (Bucklan *et al.*, 1989). Simvastatin, a leading statin in the market is currently produced as a semisynthetic derivative of lovastatin (Gulyamova *et al.*, 2014). Both compounds differ at the C-8 position of their side chain, with lovastatin and simvastatin bearing a 2-methylbutyrate and a 2,2-dimethylbutyrate moiety, respectively. However, simvastatin has also been recently reported as a natural compound produced by *Aspergillus terreus* (Gulyamova *et al.*, 2014).



Mevastatin Lovastatin Simvastatin

1.3 Endophytic Fungi

The term endophyte was introduced by De Bary and was applied to all microorganisms that reside in internal tissues of plants (Bary, 1866). Similarly, microfungi that live within plant tissues, without causing any immediate overt negative effects, are termed endophytic fungi (Aly *et al.*, 2011; Bacon and White, 2000), however, they may turn pathogenic during host senescence (Rodriguez and Redman, 2008). Endophytes are ubiquitous in terrestrial plants and are found within healthy tissues of plants (Schulz *et al.*, 1993). The transmission of endophytes to their host may occur either horizontally or vertically through airborne spores or seeds respectively (Hartley and Gange, 2009). Endophytes can be found in the internal tissues of stems, petioles, roots and leaves of plants (Petrini, 1991), whereas different tissues may yield different communities of endophytes (Hyde and Soytong, 2008). The population of endophytes is variable from plant to plant and may even differ according to the climatic conditions of the same region (Nair and Padmavathy, 2014). The diversity of endophytic fungi decreases linearly from tropical areas to northern boreal forests. Moreover, the endophyte communities from higher latitudes have proved to harbor relatively few fungal species representing many classes, whereas the tropical areas were found to be dominated by a small number of fungal classes, but by a very large number of different endophytic species (Arnold *et al.*, 2007). The majority of endophytes isolated so far includes ascomycetes and their anamorphs as a major group; basidiomycetes have been less frequently encountered as endophytes, due to the fact that they are mainly considered as plant pathogens (Alexopoulos *et al*., 1996; Frohlich and Hyde, 1999; Rundjindamai *et al.*, 2008; Hyde and Soytong, 2008). Endophytes are categorized into two classes including grass-inhabiting (clacivitalean) and non grass-inhabiting (clacivipitalean) (Hyde and Soytong, 2008). Regarding the role of endophytes, they are thought to intereact with their host in a mutualistic relationship mainly

based on the increase of host resistance against pathogens or herbivores, and therefore they are often termed as "acquired plant defence" (Carroll, 1988; Clay, 1988). Indeed, in the case of grass-inhabiting endophytes, they are known to produce loline alkaloids to protect the plant against herbivores (Siegel and Bush, 1997). Non-grass inhabiting endophytes increase resistance of host plants to herbivores, pathogens, drought, plant diseases or even enhance plant growth (Fröhlich and Hyde, 1999; Sieber, 2007). Similarly, host plants provide a spatial structure, nutrition, and protection to endophytes (Clay, 1988; Madej and Clay, 1991).

It has been reported that one or more endophytic fungi are hosted among each of the nearly 300.000 plants existing on Earth (Strobel and Daisy, 2003). This large biodiversity also implies a large chemical diversity as it is known that endophytic fungi are rich sources of organic compounds that are useful in medicine and agriculture. Emergence of resistant pathogens to the currently existing drugs, life threatening diseases, and complication in patients with organ transplantation has reinforced the exploration of bioactive secondary metabolites from microorganisms, including endophytic fungi.

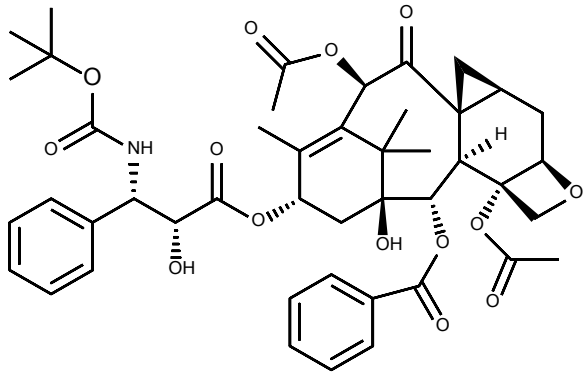
1.3.1 Endophytes as a Store House of Lead Structures Derived from Plants

Endophytes are living in the inner tissues of plants, where they contribute to their hosts by producing a plethora of compounds. It is assumed that during the long period of co-evolution, endophytic fungi got adapted to microenvironments gradually through genetic variation, including the uptake of plant DNA segments into their genomes or insertion of their DNA segments into the plant genome, allowing endophytes to biosynthesize similar compounds as their hosts and *vice versa* (Zhao *et al.*, 2010). Therefore, many compounds first isolated from plants were also found to be produced by endophytic fungi, the latter having the advantage of large-scale production.

1.3.2 Endophytic Fungal Metabolites as Anticancer Agents

Paclitaxel is one of the most exciting plant-derived anticancer drugs discovered in the past decades. It was first reported from the bark of the pacific yew *Taxus brevifolia* (Kingston, 2011) and was also found in other *Taxus* species (Schiff *et al.*, 1979; Khosroushahi *et al.*, 2006). Paclitaxel has been approved as a drug against ovarian and breast cancer (Cragg and Newman, 2013). However, the production of paclitaxel from the bark is limited. For the isolation of 1g of taxol approximately 700.000 kg of yew bark were stripped from of three mature trees (100 year old) (Khosroushahi *et al.*, 2006; Joyce, 1993). A way to solve this problem was found after the discovery in 1993 of an endophytic fungus *Taxomyces*

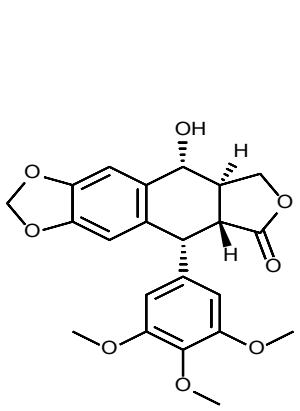
andreanae from *Taxus brevifolia* that was capable to produce paclitaxel (Stierle *et al.*, 1993). Since then, paclitaxel has been discovered as a secondary metabolite of many other endophytic fungi (Wang *et al.*, 2000; Zhang *et al.*, 2009). This discovery indicated that endophytic fungi might also be used for the production of valuable plant natural products. However, the low yield of paclitaxel through fungal culture prevents its industrial production by employing this method. Recently, paclitaxel has served as a lead compound for the synthesis of larotaxel, currently in phase III clinical studies for the treatment of breast and pancreatic cancers (Kingston, 2009).



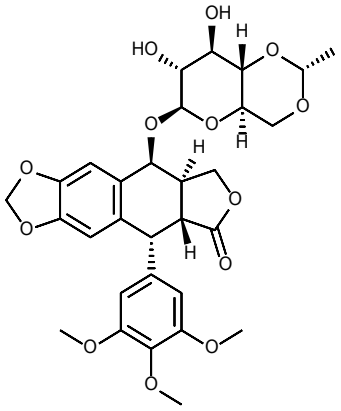
Larotaxel

Another important plant-derived anticancer agent is the pentacyclic quinoline alkaloid camptothecin. Camptothecin which is the third largest anticancer drug based on annual sales was first isolated from the wood of *Camptotheca acuminata* (Wall *et al.*, 1966) and later from *Nothapodytes foetida* (Pu *et al.*, 2013). However, its production from the plant was very low leading to the search of another strategy to increase its availability. Fortunately, the endophytic fungus *Entrophospora infrequens* isolated from the plant *Nothapodytes foetida* likewise produced camptothecin (Puri *et al.*, 2005). Later on, camptothecin was also reported from other endophytic fungi, including *Aspergillus* sp. and *Trichoderma atroviride* from the plant *Camptotheca acuminata* (Pu *et al.*, 2013), *Neurospora* sp. from the plant *Nothapodytes foetida* (Rehman *et al.*, 2008) and *Fusarium solani* from the plant *Camptotheca acuminata* (Kusari *et al.*, 2009). Clinical trials of camptothecin were not concluded as the compound was dropped due to severe bladder toxicity (Cragg and Newman, 2013; Kjeldsen *et al.*, 1992; Sawada *et al.*, 1995). However, the campothecin derivatives topotecan and irinotecan are used in the treatment of ovarian cancer and colorectal cancer, respectively (Mathijssen *et al.*, 2002). Both are known as inhibitors of topoisomerase I, an enzyme involved in DNA replication and RNA transcription (Jonsson *et al.*, 2000; Mathijssen *et al.*, 2002).

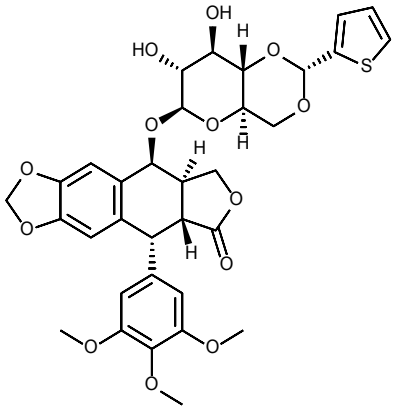
Vinblastine and vincristine are anticancer drugs known as vinca alkaloids that were isolated for the first time from the Madagascar periwinkle *Catharanthus roseus* (Gueritte *et al.*, 2005). These compounds were further obtained from a fungus of the genus *Alternaria* isolated from *Catharanthus roseus* (Guo and Zhang, 1997). Other endophytic fungi were reported for their ability to produce either vinblastine, vincristine or both, including *Fusarium oxysporium* isolated from the same plant (Kumar, 2013). Another example is the aryltetralin-type lignan, podophyllotoxin, produced by the endophytic fungus *Phialocephela fortinii*, which was originally isolated from the plant *Podophyllum peltatum* (Eyberger *et al.*, 2006). Podophyllotoxin has been used as a lead compound for the development of the anticancer drugs etoposide and teniposide (Canel *et al.*, 2000). Podophyllotoxin and it derivatives act through inhibition of topoisomarase II (Guerram *et al.,* 2012; Wigley, 1995).



Podophyllotoxin



Etoposide



Teniposide

1.3.3 Methods to Activate Silent Biosynthetic Pathways in Endophytic Fungi

To date, only a few plants have been investigated for endophytic diversity in depth and their potential to produce bioactive secondary metabolites. Genes responsible for the production of secondary metabolites are organized forming a gene cluster, which is group of closely related genes (Reen *et al.*, 2015).The standard approach for the discovery of novel bioactive secondary metabolites is based on the collection and cultivation of a microbial strain, followed by extraction and bioassay-guided isolation of compounds. However, genomic studies through whole-genome sequencing programs have shown that the number of biosynthetic genes in many fungi, responsible for the production of secondary metabolites, greatly outnumbers the known compounds described so far, as reported for *Aspergillus nidulans* (Brakhage *et al.*, 2008; Netzker *et al.*, 2015). The isolation of a small number of secondary metabolites from an organism compared to its predicted capacity may be due to the fact that under laboratory conditions, the amount of the produced metabolites is low that

cannot be easily detected, or even that several biosynthetic pathways involved in their biosynthesis are not expressed due to the lack of some environmental cues under laboratory conditions. This observation has led to the term of "silent" or "cryptic" genes. Therefore, new methods have been explored to activate these silent biosynthetic pathways that are also termed "cryptic pathways". Interestingly, these approaches have led not only to the discovery of new secondary metabolites, but also to the accumulation of known compounds (Ola *et al.*, 2013). These methods are categorized in two groups, including cultivation-dependent approaches and molecular based techniques (Reen *et al.*, 2015; Scherlach and Hertweck, 2009).

1.3.3.1 Cultivation-Dependent Approaches

OSMAC approach

OSMAC (One Strain MAny Compounds) approach was introduced by Zeeck *et al.*, who reported the ability of *Aspergillus ochraceus* to increase its secondary metabolite diversity by producing 15 additional compounds, when growing under different conditions, including manipulation of the media composition, aeration, shape of culturing flask or temperature (Bode *et al.*, 2002). Recently, this method was employed in the study of the marine fungus *Ascotricha* sp. ZJ-M-5 that led to the isolation of new caryophyllene sesquiterpenes after modification of the culture media (Wang *et al.*, 2014). However, this method showed some limitations related to the strain-specific variations in the quantity of the produced secondary metabolites, as well as due to the ability of fungi to alter metabolite profiles after re-culture (Williams *et al.*, 2008).

In addition to the variation of culture conditions, external factors such as UV radiation, addition of enzyme inhibitors, and heat-shock may lead to the discovery of new bioactive secondary metabolites (Scherlach and Hertweck, 2009). Indeed, the use of the F-actin inhibitor jasplakinolide supplemented in the culture medium of the fungus *Phomopsis asparagi* yielded three new chaetoglobosins that were not detected in the axenic culture of the fungus (Christian *et al.*, 2005). In another study, the cultivation of several strains of *Penicillium* on macerated host tissue media caused the induction of new secondary metabolites (Overy *et al.*, 2006).

Co-cultivation approach

In nature, fungi and bacteria live in complex communities, sharing similar niches, utilizing similar resources, and overcoming the same external cues for survival (Strobel *et al.*, 2004). This dynamic environment is unstable and the presence of different competitive

species lead to interactions that may be beneficial or harmful for the species involved (Netzker *et al.*, 2015). Therefore, mimicking the natural environment in the laboratory, may force direct contact involving competition for space or for limited nutrients, leading to the induction of silent biosynthetic pathways and the production of novel bioactive compounds (Netzker *et al.*, 2015).

Recently, several reviews on co-culture experiments have been published undoubtedly supporting co-cultivation of two or even more microorganisms on solid/liquid media as a powerful tool to activate silent genes (Marmann *et al.*, 2014; Schroeckh *et al.*, 2014; Scherlach and Hertweck, 2009). Fungi and bacteria are present in the plant as endophytes (Strobel *et al.*, 2004); therefore, their co-culture may lead to the discovery of new secondary metabolites. Indeed, recently, co-culture of the endophytic fungus *Fusarium tricintum* with *Bacillus subtilis* yielded new secondary metabolites that were not present in the fungal and bacterial axenic cultures (Ola *et al.*, 2013). Many other co-culture studies including both marine fungi and bacteria have also been reported (Marmann *et al.*, 2014). An example is the co-cultivation of the marine α-proteobacterium *Thalassopia* sp. (CNJ-328) and the fungus *Libertella* sp. affording libertellenones A-D, which showed pronounced cytotoxicity against human adenocarcinoma cells (HCT-116) (Oh *et al.*, 2005). Another example of silent gene activation through co-cultivation is the interaction of *Aspergillus fumigatus* with different bacteria. Accordingly, co-culture of an *Aspergillus fumigatus* strain with *Sphingomonas strainandan* was reported to yield a new diketopiperazine derivative that showed strong antibiotic activity and cytotoxic activity as well (Park *et al.*, 2009).

1.3.3.2 Molecular Based Approaches

Ribosomal Engineering

Ribosomal engineering is another powerful tool that can be applied for the activation of silent genes in fungi (Ochi *et al.*, 2004). This method is based on the modulation of the ribosome by introduction of mutations that confer resistance to anti-ribosomal drugs, such as gentamycin, streptomycin, chloramphenicol, spectinomycin or neomycin. When the fungus is treated with one of these drugs, it develops mutant drug resistance via a point mutation within the ribosomal component (Ochi and Hosaka, 2013). The application of this method in the laboratory requires the availability of the complete genome sequence of fungi. Fortunately, large numbers of fungal genome sequences have been identified, including the species *Aspergillus oryzae* and *Penicillium chrysogenum* (Ma and Fedorova, 2010).

This technique has been successfully applied for the isolation of new secondary metabolites from fungi. Accordingly, introduction of gentamycin resistance into the marine-derived fungus *Penicillium purpurogenum* led to the isolation of antitumor natural products that were not observed in the wild-type culture (Chai *et al.*, 2012). In another experiment, the generation of hygromycin B-resistant mutants of *Monascus pilosus* NBRC 4520 resulted in the enhancement of natural product of this fungus (Gonzalez *et al.*, 1978). These results showed that modulation of the fungal ribosomal function can activate silent genes responsible for the production of new bioactive secondary metabolites or may even enhance the production of interesting known metabolites.

Awakening Activator and Artificial Promoters

This selective method of activation of silent genes is based on the artificial expression of a specific transcription factor gene involved in silencing a gene cluster through the replacement of its promoter by an inducible promoter that may lead to the production of cryptic secondary metabolites (Reen *et al.*, 2015). This method has been undertaken with *Aspergillus nidulans* in two different studies. In the first study, the replacement of the transcription activator promoter by the inducible *alc*A promoter led to the production of the novel polyketide asperfuranone (Chiang *et al.*, 2009). In the second study, transformation of *Aspergillus nidulans* AXB4A2 with a plasmid containing *apd*R under the control of the inducible alcohol dehydrogenase promoter *alc*Ap, generated a mutant capable to produce two new aspyridones A and B that showed moderate cytotoxicity (Bergmann *et al.*, 2007).

Deletion Approach

Deletion has been successfully applied with fungi as a method for activation of silent genes. In a respective study, *Aspergillus nidulans* produced novel pheofungins after deletion of the N-acetyltransferase gene (Scherlach *et al.*, 2011). In another study, deletion of the conserved eukaryotic *cns*E/CSN5 demethylase subunit of the COP9 signalosome resulted in the production of a new antibiotic (Gerke *et al.*, 2012). Interestingly, the csnE/CSN5 gene is commonly found in fungi. Thus, targeting this gene may trigger the production of non-reported fungal bioactive natural products (Gerke *et al.*, 2012).

Epigenetic Modification

It has been shown that gene expression in fungi can be regulated by acetylation, methylation, phosphorylation, and ADP-ribosylation of DNA (Chiang *et al.*, 2011; Cichewicz, 2012). Indeed, many chromatin modifications were found to regulate expression of secondary metabolites (Strahl and Allis, 2000). For example, deletion of the *hdaA* gene encoding an *Aspergillus nidulans* histone deacetylase (HDAC) afforded penicillin and sterigmatocystin (Shwab *et al.*, 2007), whereas deletion of *sumO* increased the production of asperthecin (Szewczyk *et al.*, 2008). These findings suggest that the use of small molecules capable to cause epigenetic modifications may activate silent gene clusters and/or enhance the production of constitutively fungal metabolites. Moreover, this method has the advantage of bypassing complex molecular manipulations and culture-based approaches, thus highlighting its potential in drug discovery (Reen *et al.*, 2015).

1.4 Aim of this Study

Fungi produce a vast diversity of natural products that may find their application in medicine for the treatment of various diseases. The main goal of this study was to investigate fungi from different ecological niches including endophytes and soil for their capacity to produce new secondary metabolites. A particular attention was given to the strategies to stimulate activation of fungal silent biosynthetic pathways.

Chapiter 2 highlights the main focus of this study based on the increase of the endophytic fungal metabolite diversity through the co-cultivation approach with diverse bacteria on solid rice medium (Manuscript submitted). Indeed, during the co-cultivation of *Chaetomium* sp. with viable or autoclaved *Bacillus subtilis*, silent biosynthetic pathways of the endophytic fungus were activated leading to the induction of new natural products along with the enhancement of the accumulation of constitutively present fungal metabolites. Interestingly, some fungal metabolites were not detected in the co-culture. No induction was observed during the co-cultivation with *Streptomyces lividans* or *Mycobacterium smegmatis*. The treatment of the fungus with two epigenetic modifiers revealed that *Bacillus subtilis* triggered histone modifications during the co-cultivation. It is interesting to notice that the new natural dibenzo-α-pyrone derivative induced during the co-cultivation with *Bacillus subtilis* showed a moderate antibacterial activity against *Bacillus subtilis* with MIC of 53 µM while exhibiting a strong cytotoxicity against the mouse lymphoma L5178Y cell line with IC_{50} of 1 µM.

Chapter 3 reports the structure elucidation of new secondary metabolites identified as cytosporine derivatives produced by the endophytic fungus *Pestalotiopsis theae* while chapter 4 reveals the structure elucidation of the new heptapeptide, unguisin F produced by the endophytic fungus *Mucor irregularis*. In the latter chapter we determined the absolute configuration of the new compound along with that of it congener unguisin E reported here for the first time.

In addition, chapter 5 deals with the soil-derived fungus *Gongronela butleri* which produces several new natural compounds found to be 2-pentenedioc acid derivatives.

Chapter 2

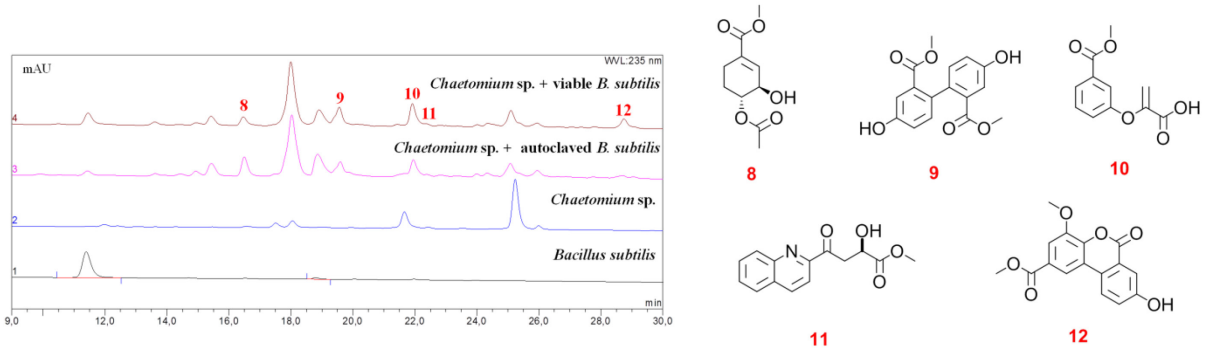
Inducing secondary metabolite production by the endophytic fungus *Chaetomium* sp. through fungal-bacterial co-culture and epigenetic modification

Submitted to "Tetrahedron"

Impact Factor: 2.641,

The overall contribution to the paper: 80% of the first author. The first author involved to all laboratory works as well as the manuscript preparation.

Graphical Abstract



Inducing secondary metabolite production by the endophytic fungus *Chaetomium* sp. through fungal-bacterial co-culture and epigenetic modification

Sergi Herve Akone,[a,b] Attila Mándi,[c] Tibor Kurtán,[c] Rudolf Hartmann,[d] Wenhan Lin,[e] Georgios Daletos,[a*] and Peter Proksch[a*]

[a]Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany

[b]Faculty of Science, Department of Chemistry, University of Douala, P.O. Box. 24157 Douala, Cameroon.

[c]Department of Organic Chemistry, University of Debrecen, Egyetem tér 1, P.O. Box. 400, 4002 Debrecen, Hungary.

[d]Institute of Complex Systems: Strukturbiochemie (ICS-6), Forschungszentrum Julich, Wilhelm-Johnen-Strase, 52428 Julich, Germany

[e]State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Health Science Center, 100191 Beijing, People's Republic of China.

* Author to whom correspondence should be addressed;

E-Mail: georgios.daletos@uni-duesseldorf.de;

Tel.: +49 211 81 14173; fax: +49 211 81 11923

E-Mail: proksch@uni-duesseldorf.de;

Tel.: +49 211 81 14163; fax: +49 211 81 11923

ABSTRACT

Co-culturing of the fungal endophyte *Chaetomium* sp. with the bacterium *Bacillus subtilis* on solid rice medium resulted in an up to 8.3-fold increase in the accumulation of constitutively present metabolites that included a 1:1 mixture of 3- and 4-hydroxybenzoic acid methyl esters (**1** and **2** respectively), and the polyketides acremonisol A (**3**), SB236050 (**4**), and SB238569 (**5**). In addition, seven compounds including isosulochrin (**6**), protocatechuic acid methyl ester (**7**), as well as five new natural products (**8**-**12**) were detected in the co-cultures, but not in axenic fungal cultures. Treatment of *Chaetomium* sp. with the epigenetic modifier suberoylanilide hydroxamic acid or 5-azacytidine resulted in an enhanced accumulation of **6**, which was likewise detected during co-culture. Compound **5** showed strong cytotoxicity against the mouse lymphoma L5178Y cell line with an IC_{50} value of 1 μM, as well as weak antibacterial activity against *B. subtilis* with an MIC value of 53 μM.

Keywords: Co-cultivation; *Chaetomium* sp.; *Bacillus subtilis*; Epigenetic modifiers; natural products

1. Introduction

Microorganisms, including soil-dwelling bacteria and fungi, produce a multitude of secondary metabolites that play an eminent role in drug discovery, such as the blockbuster antibiotic penicillin G produced by *Penicillium notatum*.[1,2] In nature, microorganisms co-exist in complex communities, in which they interact with each other.[3,4] These interactions are mainly based on the production of secondary metabolites that are used as chemical signals for communication and/or competition for limited resources, favoring various defense mechanisms.[5] Therefore, mimicking the natural environment through mixed fermentation of different microorganisms (also called co-cultivation or co-culture) may lead to an enhancement in the production of compounds. In addition, co-cultivation may trigger the expression of silent biosynthetic pathways,[6,7] thus resulting in the accumulation of new natural products.[8,9] During our previous studies, co-cultivation of the endophytic fungus *Fusarium tricinctum* with the bacterium *Bacillus subtilis*, led to an up to 78-fold enhancement in the accumulation of the constitutively present fungal metabolites, along with the induction of new secondary metabolites that were not present in both fungal or bacterial axenic controls.[5] A similar observation was made when co-culturing the soil-dwelling fungus *Aspergillus terreus* with either *B. subtilis* or with *B. cereus*.[10] Besides the co-cultivation approach, treatment of fungi with epigenetic modifiers is another powerful tool for the activation of silent genes.[11] Recently, the addition of the DNA methyltransferase inhibitor 5-azacytidine to the endophytic fungus *Pestalotiopsis crassiuscula* resulted in a change in the secondary metabolite pattern and led to the induction of three new cryptic metabolites.[12]

During our ongoing search for new fungal secondary metabolites, we isolated the endophytic fungus *Chaetomium* sp. from fresh healthy leaves of *Sapium ellipticum* collected in Cameroon. Species of the genus *Chaetomium* have been described as endophytes [13] and are well known to produce a wide range of natural products such as the anticancer compounds chaetocochins A - C, [14] the antibacterial compound furano-polyene 3-*epi*-aureonitol, [15] and the metallo-β-lactamases inhibitors SB236050 and SB238569.[16, 17] Given this broad spectrum of bioactive secondary metabolites, we investigated a mixed-fermentation of *Chaetomium* sp. with the Gram-positive bacteria *B. subtilis* and *Streptomyces lividans*, as well as with *Mycobacterium tuberculosis*. In a further attempt to manipulate the pattern of fungal metabolites, *Chaetomium* sp. was treated with two epigenetic modifiers including the DNA methyltransferase inhibitor 5-azacytidine and the histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA). The isolation and structure elucidation of the new

metabolites (**8**-**12**) (Fig. 1), as well as the biological activities of the isolated compounds are described.

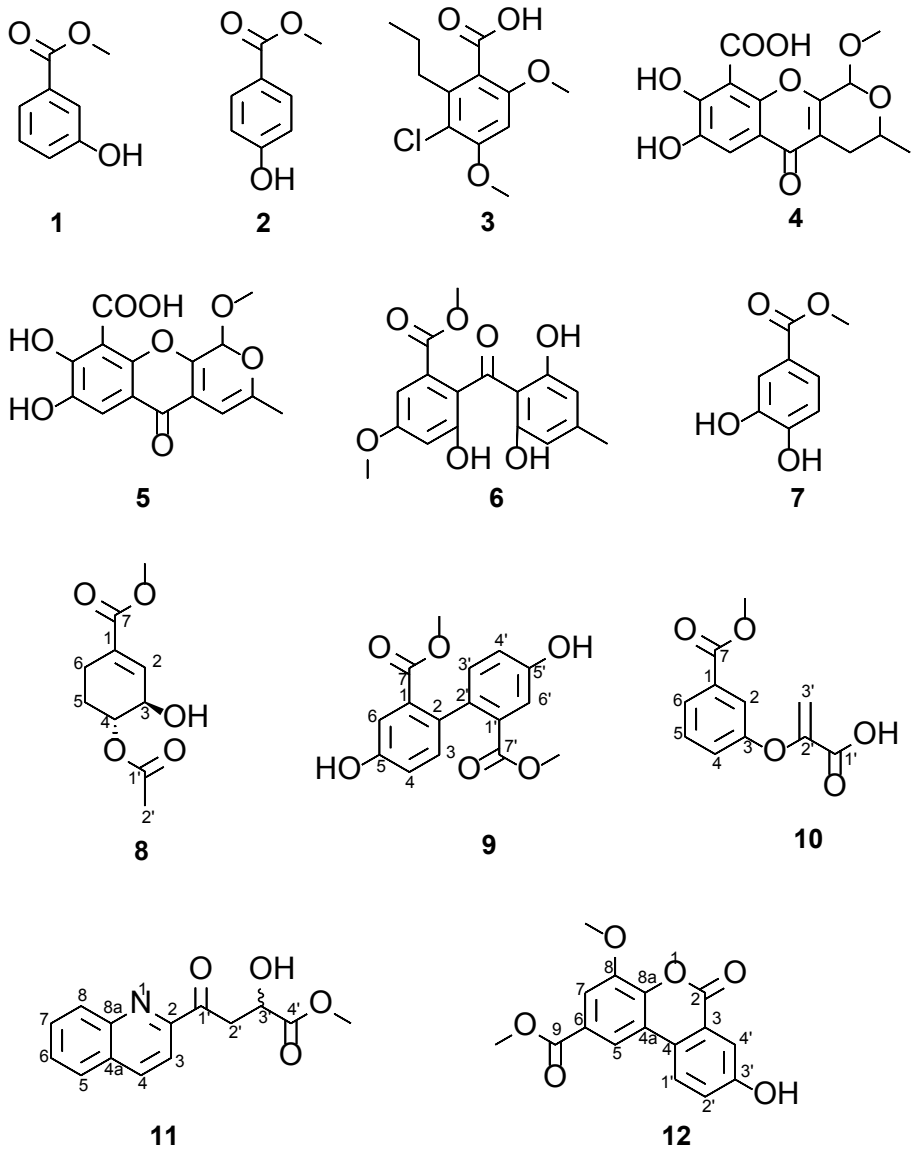


Figure 1. Structures of compounds **1**-**12**.

2. Results and discussion

Chaetomium sp. was isolated from the Cameroonian medicinal plant *Sapium ellipticum*. When *Chaetomium* sp. was cultured axenically on solid rice medium, average yields per culture flask were 2.8 mg for the known acremonisol A (**3**),[18] 13.9 mg for SB236050 (**4**),[16,17] 132.7 mg for SB238569 (**5**),[16,17] and 14.6 mg for the 1:1 mixture of 3- and 4-hydroxybenzoic acid methyl esters (**1** and **2**, respectively)[19,20] (Table 1). Co-cultivation of *Chaetomium* sp. was undertaken with viable or autoclaved cultures of *B. subtilis*. In both

cases, a strong accumulation of the mixture of **1** and **2** was observed, accounting for a 8.3 and 7.4-fold increase, respectively, compared to axenic fungal controls (Table 1). However, the major polyketides SB236050 (**4**) and SB238569 (**5**), which are typical constituents of *Chaetomium* sp., were not detected in co-cultures (Fig. 2). These observations prove that the effects of co-cultivation are not uniform for all fungal metabolites, which is in agreement with our previously reported data.[5,10] In addition, seven compounds, including five new natural products (**8**-**12**), as well as the known isosulochrin (**6**) [21] and protocatechuic acid methyl ester (**7**) [22] were only detected in co-cultures of *Chaetomium* sp. with viable or with autoclaved *B. subtilis* cultures, but were lacking in axenic fungal or bacterial controls (Fig. 2).

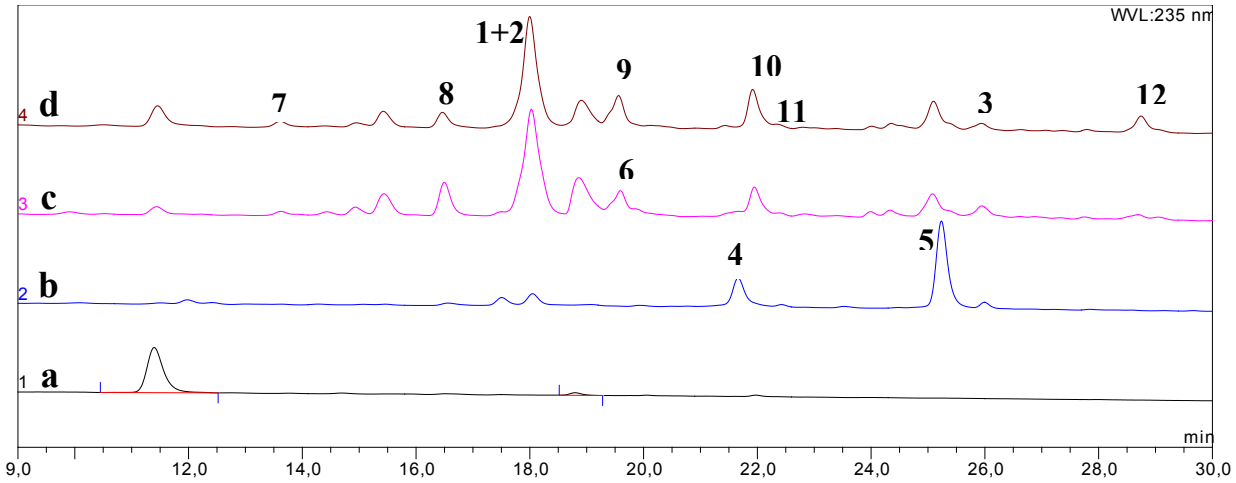


Figure 2. HPLC chromatograms of EtOAc extracts from co-culture experiments (detection at UV 235 nm): (a) *B. subtilis* control, (b) *Chaetomium* sp. control, (c) co-culture of *Chaetomium* sp. with autoclaved *B. subtilis*, (d) co-culture of *Chaetomium* sp. with viable *B. subtilis*.

Table 1: Yield of induced metabolites per flask during co-culture of *Chaetomium* sp. and *Bacillus subtilis* (n=5) vs. axenic controls of *Chaetomium* sp. (n=5).

Compound	Control[a] (mg)	*Chaetomium sp.* vs. *B. subtilis* (mg)	Increase (fold)	*Chaetomium sp.* vs. *autoclaved B. subtilis* (mg)	Increase (fold)
1+**2**	14.58 ± 5.88	121.20 ± 3.60	8.3	108.60 ± 7.20	7.4
3	2.82 ± 1.26	2.58 ± 0.90	0.9	1.86 ± 0.78	0.7
4	13.92 ± 0.07	n.d.		n.d.	
5	132.72 ± 0.47	n.d.		n.d.	
6	n.d.[b]	n.d.		26.82 ± 3.66	
7	n.d.	3.06 ± 0.08		1.02 ± 1.44	
8	n.d	4.98 ± 0.36		32.40 ± 5.52	
9	n.d.	7.20 ± 0.96		n.d.	
10	n.d.	13.98 ± 0.78		9.06 ± 0.18	
11	n.d.	0.72 ± 0.12		n.d.	
12	n.d.	19.20 ± 1.86		6.72 ± 0.36	

[a] *Chaetomium* sp. axenic control. [b] n.d.: not detected

Compound **8** was isolated as a greenish oil. It exhibited a prominent ion peak at *m/z* 215.0913 $[M+H]^+$ in the HRESIMS spectrum, corresponding to the molecular formula $C_{10}H_{14}O_5$. Inspection of the ^{1}H NMR and COSY spectra of **8** revealed the presence of a methoxy group at δ_H 3.71 (3H, s, 7-OCH_3), a methyl group at δ_H 2.00 (3H, s, H_3-2'), as well as a continuous spin system composed of two methylene groups at δ_H 2.37 (2H, m, H_2-6) and 1.95/1.72 (1H, dddd, J = 13.2, 9.8, 8.2, 3.4 Hz, H_{2a}-5; 1H, dddd, J = 13.2, 9.8, 8.2, 6.5 Hz, H_{2b}-5), two oxymethine protons at δ_H 4.80 (1H, ddd, J = 9.0, 6.5, 3.4 Hz, H-4) and 4.27 (1H, dt, J = 9.0, 3.0 Hz, H-3), and an olefinic proton at δ_H 6.72 (1H, dt, J = 3.0, 1.9, H-2) (Table 2). In the HMBC spectrum of **8**, the correlations observed from 7-OMe, H-2, and H_2-6 to C-7 (δ_C 167.4) corroborated the attachment of the methoxy group (7-OMe) at C-7. Moreover, the HMBC correlations from H-4 and H_3-2' to C-1' (δ_C 170.8), suggested the presence of an acetyl group being located at C-4. These data were similar to those reported for methyl 4-*O*-acetyl-4-*epi*-shikimate, [23] the only difference being the absence of the hydroxy group at C-5 in **8**, which is in accordance with the 16 amu molecular weight difference between both

compounds. The large coupling constant between H-3 and H-4 ($J_{3\text{-}4}$ = 9.0 Hz) indicated their trans-diaxial orientation, as reported for methyl 4-*O*-acetyl-4-*epi*-shikimate.[23]

Table 2: NMR data of **8** and **11**.

Position	Shikimeran A (**8**)[a]		Quinomeran (**11**)[b]	
	δ_H	δ_C	δ_H	δ_C[c]
1		131.8, C		
2	6.72, dt (3.0, 1.9)	139.4, C		153.6, C
3	4.27, dt (9.0, 3.0)	68.8, CH	8.10, d (8.4)	118.4, CH
4	4.80, ddd (9.0, 6.5, 3.4)	74.8, CH	8.53, d (8.4)	138.1, CH
4a				130.3, C
5	1.95, dddd (13.2, 9.8, 8.2, 3.4) 1.72, dddd (13.2, 9.8, 8.2, 6.5)	25.5,CH_2	8.08, dd (8.5, 1.5)	128.6,CH
6	2.37, m	23.5, CH_2	7.76, ddd (8.5, 6.9, 1.5)	129.5, CH
7		167.4, C	7.89, ddd (8.5, 6.9, 1.5)	131.0, CH
8			8.21, dd (8.5, 1.5)	130.9, CH
8a				147.7, C
1’		170.8, C		199.0, C
2’	2.00, s	21.2, CH_3	3.80, m	42.5, CH_2
3’			4.83, dd (6.9, 5.4)	67.9, CH
4’				174.5
4’-OCH_3			3.71, s	52.0
7-OCH_3	3.71, s	52.1, CH_3		

[a] Measured in $(CD_3)_2CO$ at 300 (^1H) and 75 (^{13}C) MHz.
[b] Measured in $(CD_3)_2CO$ at 600 (^1H) and 150 (^{13}C) MHz.
[c] Data extracted from HSQC and HMBC spectra.

For the determination of the absolute configuration of **8**, the solution TDDFT-ECD protocol [24] was carried out on the arbitrarily chosen (*3R*,*4R*)-**8** enantiomer. Merk Molecular Force (MMFF) conformational search in $CHCl_3$ resulted in 19 conformers in a 21 kJ/mol energy window. These conformers were reoptimized at B3LYP/6-31G(d) *in vacuo* and B97D/TZVP [25,26] PCM/MeCN levels yielding 4 and 13 low-energy conformers above 2%,

respectively (Fig. 3). ECD spectra calculated at various levels (B3LYP/TZVP, BH&HLYP/TZVP and PBE0/TZVP *in vacuo*) for the individual conformers optimized in the gas-phase reproduced the ECD pattern of the experimental spectra, while the Boltzmann-average spectra did not resemble the experimental ECD. Although all conformers had similar computed ECD pattern at most levels, there was considerable wavelength deviation among the conformers, which caused the mismatch of the Boltzmann-weighted ECDs. Conformers differed in the orientation of the methoxycarbonyl and the acetoxy groups (Figures S4 and S5).

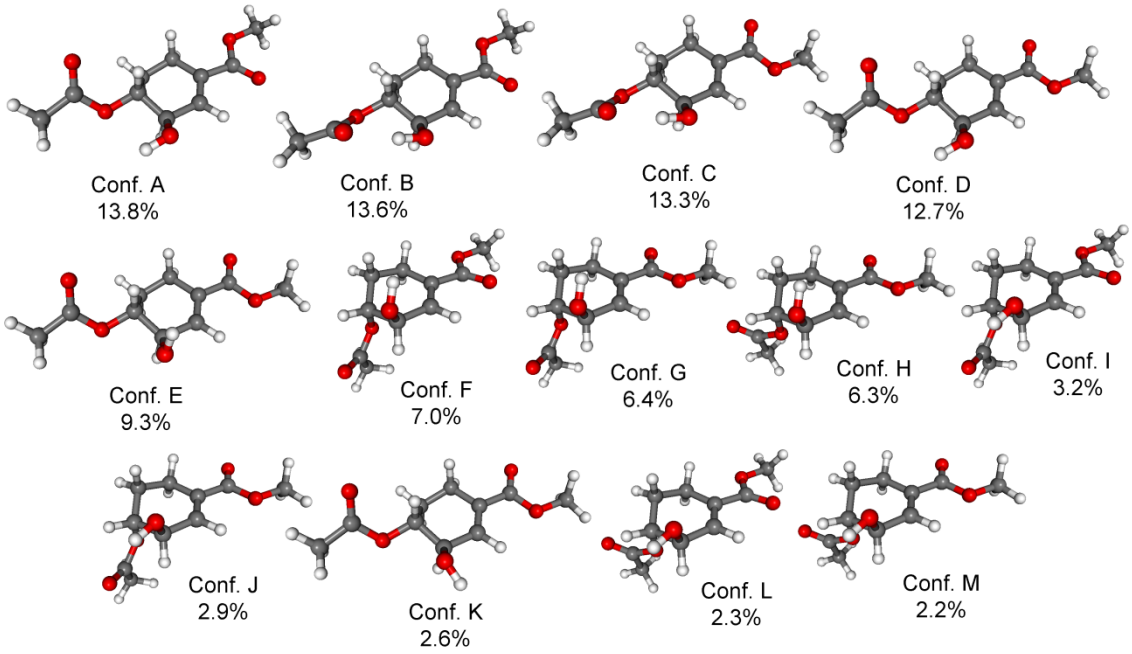


Figure 3. Structures and populations of the low-energy B97D/TZVP PCM/MeCN conformers (≥2%) of (3*R*,4*R*)-**8**.

In contrast, Boltzmann-weighted ECD spectra of (*3R*,*4R*)-**8** computed with PCM for MeCN at the same three levels for the B97D/TZVP PCM/MeCN reoptimized conformers gave nice agreement with the experimental ECD spectrum (Fig. 4) allowing the unambiguous determination of the absolute configuration as (*3R*,*4R*).

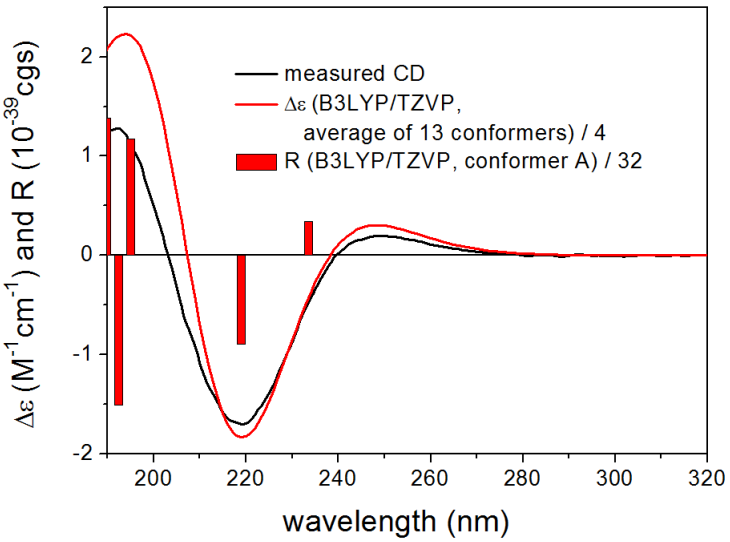


Figure 4. Experimental ECD spectrum of **8** in MeCN compared with the Boltzmann-weighted B3LYP/TZVP PCM/MeCN ECD spectrum of (3*R*,4*R*)-**8** computed for the B97D/TZVP PCM/MeCN conformers. Bars represent the rotational strength of the lowest-energy conformer.

Moreover, to exclude the possibility of **8** being an artefact formed during extraction with EtOAc, shikimic acid was incubated for 72h in EtOAc or MeOH at room temperature; however, no formation of the acetylated or methylated derivative, respectively, was observed by HPLC and LC-MS analysis. Thus, **8** was identified as a new natural product for which we propose the trivial name shikimeran A.

Compound **9** was isolated as a colorless oil. Its molecular formula was established as $C_{16}H_{14}O_6$ based on the prominent ion peak observed at *m/z* 303.0863 $[M+H]^+$ in the HRESIMS spectrum. Inspection of the ^1H and ^{13}C NMR spectra revealed only one set of five proton and eight carbon resonances, respectively (Table 3), indicating that **9** is a symmetrical dimer consisting of two identical monomers. Accordingly, the ^1H NMR spectrum (Table 3) of **9** displayed signals corresponding to six aromatic protons at δ_H 7.35 (2H, dd, J = 2.0, 1.1 Hz, H-6/6') and 7.02 (4H, H-3/3' and H-4/4'), two methoxy groups at δ_H 3.54 (6H, s, 7/7'-OMe), and two hydroxy protons at δ_H 8.65 (2H, br s, 5/5'-OH). In the HMBC spectrum of **2**, the correlations from H-4 (/4') to C-2 (/2') (δ_C 134.9) and C-6 (/6') (δ_C 116.9), from H-3 (/3') to C-5 (/5') (δ_C 157.0) and C-1 (/1') (δ_C 132.4), as well as from H-6 (/6') to C-4 (/4') (δ_C 119.1) and C-2 (/2') revealed the presence of a 1,2,4-trisubstituted phenyl ring (ABX spin system). In addition, the correlations from 5 (/5')-OH to C-4 (/4'), C-5 (/5'), and C-6 (/6') suggested the hydroxyl group being located at C-5 (/5'), adjacent to the aromatic protons H-4 (/4') and H-6

(/6'). This assignment was further corroborated by the ROESY correlations between 5 (/5')-OH and both H-4 (/4') and H-6 (/6'). Similarly, the HMBC correlations from 7/ (7')-OMe and H-6 (/6') to C-7 (/7') (δ_C 168.1) suggested that C-1 (/1') was substituted by a carbomethoxy group. The linkage between the two monomers was found to reside between C-2 and C-2', as H-3 (/3') displayed a strong HMBC correlation to C-2' (/2), allowing us to assign the planar structure of **9**. Notably, **9** was previously described as an intermediate in the total synthesis of aromatic polyesters containing multiple *n*-alkyl side chains.[27] However, to the best of our knowledge, this is the first report of the isolation of **9** from nature. Thus, **9** was identified as a new natural product for which the name bipherin A is proposed.

Table 3: NMR data of **9** measured in $(CD_3)_2CO$ at 300 (^{1}H) and 75 (^{13}C) MHz.

Position	Bipherin A (**9**)	
	δ_H	δ_C
1/1'		132.4, C
2/2'		134.9, C
3/3'	7.02[a]	133.0, CH
4/4'	7.02[a]	119.1, CH
5/5'		157.0, C
6/6'	7.35, dd (2.0, 1.1)	116.9, CH
7/7'		168.1, C
7/7'-OCH_3	3.54, s	51.9, CH_3
5/5'-OH	8.65, br s	

[a] Signal overlap prevents determination of couplings.

Compound **10** was isolated as a yellowish oil. The HRESIMS spectrum exhibited a prominent ion peak at m/z 223.0602 $[M+H]^+$, indicating the molecular formula $C_{11}H_{10}O_5$. The ^{1}H NMR spectrum of **10** revealed the presence of four coupled signals at δ_H 7.77 (1H, dt, J = 8.0, 1.4 Hz, H-6), 7.61 (1H, dd, J = 2.5, 1.4 Hz, H-2), 7.47 (1H, t, J = 8.0 Hz, H-5), and 7.27 (1H, ddd, J = 8.0, 2.5, 1.4 Hz, H-4) (Table 4), indicating a 1,3-disubstituted benzene system. The remaining signals included those of a methoxy group at δ_H 3.90 (3H, s, 7-OCH_3) and two geminal olefinic protons at δ_H 5.83 (1H, d, J = 1.9 Hz, Ha-3') and 5.09 (1H, d, J = 1.9 Hz, Hb-3'), representing typical signals of a disubstituted vinyl group. The HMBC correlations from H-2, H-6, and 7-OCH_3 to C-7 (δ_C 167.8) suggested the presence of a methyl ester group at C-1. Moreover, the HMBC correlations from H_2-3' to C-1' (δ_C 165.7) and C-2' (δ_C 152.1), as well as the deshielded signal of the C-3 (157.8) were indicative of a

monosubstituted acrylic acid moiety, which was connected to C-3 of the benzene ring through an ether bond. Thus, **10** was identified as a new natural product for which the name chorismeron is proposed.

Table 4: NMR data of **10** measured in CD_3OD at 500 (^1H) and 125 (^{13}C) MHz.

Position	Chorismeron (**10**)	
	δ_H	δ_C
1		133.1, C
2	7.61, dd (2.5, 1.4)	119.9, CH
3		157.8, C
4	7.27, ddd (8.0, 2.5, 1.4)	124.4, CH
5	7.47, t (8.0)	131.1, CH
6	7.77, dt (8.0, 1.4)	125.7, CH
7		167.8, C
1’		165.7, C
2’		152.1, C
3’	5.09, d (1.9) 5.83, d (1.9)	107.2, CH_2
7-OMe	3.90, s	53.0, CH_3

Compound **11** was isolated as a brown oil. The HRESIMS spectrum exhibited a prominent ion peak at m/z 260.0915 $[M+H]^+$ consistent with the molecular formula $C_{14}H_{13}NO_4$. The ^1H NMR spectrum of **11** displayed two separate aromatic spin systems; the first consisted of the signals at δ_H 8.21 (1H, dd, *J* = 8.5, 1.5 Hz, H-8), 8.08 (1H, dd, *J* = 8.5 , 1.5 Hz, H-5), 7.89 (1H, ddd, *J* = 8.5, 6.9, 1.5 Hz, H-7), and 7.76 (1H, ddd, *J* = 8.5, 6.9, 1.5 Hz, H-6), typical of a disubstituted aromatic ring (ABCD spin system) (Table 2). The second spin system consisted of two *ortho* protons at δ_H 8.53 (1H, d, *J* = 8.4, Hz, H-4) and 8.10 (1H, d, *J* = 8.4 Hz, H-3) (Table 2). These signals were indicative of a quinoline moiety, as confirmed by the HMBC correlations from H-5 to C-7 (δ_C 131.0), C-8a (δ_C 147.7), and C-4 (δ_C 138.1), from H-8 to C-6 (δ_C 129.5) and C-4a (δ_C 130.3), and from H-4 to C-5 (δ_C 128.6), C-8a, and C-2 (δ_C 153.6) (Fig. 5). The remaining signals of a methylene group resonating at δ_H 3.80 (2H, m, H_2-2’), an oxymethine proton at δ_H 4.83 (1H, dd, *J* = 6.9, 5.4 Hz, H-3’) and a methoxy signal at δ_H 3.71 (3H, s, 4’-OCH_3) were attributed to a 2-hydroxy-4-oxobutanoic acid methyl ester moiety, as supported by the COSY correlations between H_2-2’ and H-3’, as well as by the HMBC correlations from 4’-OCH_3 to C-3’ (δ_C 67.9) and C-4’ (δ_C 174.5), and from both H_2-2’ and H-3’to C-2’ and C-4’ (Fig. 5). Finally, the HMBC correlation from H-3 to C-1’ (δ_C 199.0) corroborated the attachment of the 2-hydroxy-4-oxobutanoic acid methyl ester moiety at C-2 of the quinoline ring. The baseline ECD spectrum of **11** in acetonitrile

indicated that it is a racemic mixture. Thus, **11** was identified as a new natural product for which the name quinomeran is proposed.

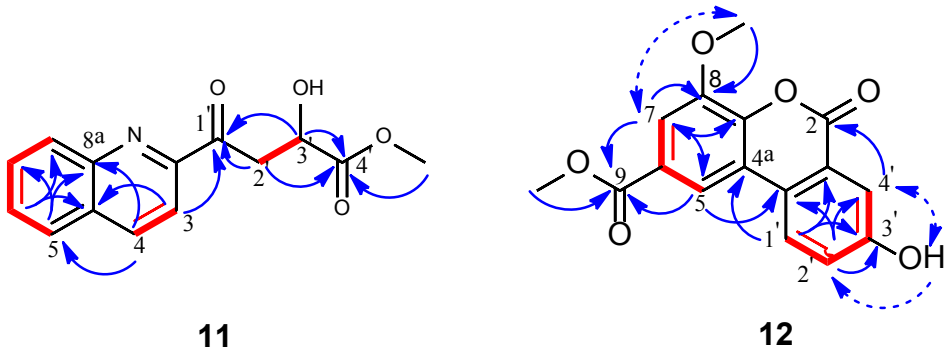


Figure 5. COSY (bold), key ROESY (dashed), and HMBC (plain) correlations of **11** and **12**

Compound **12** was isolated as a yellow amorphous powder. The molecular formula of **12** was determined as $C_{16}H_{12}O_6$ based on the prominent ion peak at m/z 301.0706 $[M+H]^+$. It exhibited UV absorption maxima at 202, 236, and 361 nm, typical for dibenzo-α-pyrone derivatives. Inspection of the ^{1}H NMR (Table 5) of **12** indicated the presence of two meta-coupled protons at δ_H 8.27 (1H, d, J = 1.9 Hz, H-5) and 7.55 (1H, d, J = 1.9 Hz, H-7), as well as an ABX-type spin system, consisting of three aromatic protons at δ_H 8.28 (1H, d, J = 8.8 Hz, H-1'), δ_H 7.37 (1H, dd, J = 8.8, 2.7 Hz, H-2'), and δ_H 7.56 (1H, d, J = 2.7 Hz, H-4'). Additional signals included those of two methoxy groups at δ_H 3.96 (3H, s, 8-OCH_3) and 3.90 (3H, s, 9-OCH_3), and a phenolic hydroxy proton at δ_H 10.55 (1H, s, 3'-OH). Further detailed analysis of the 2D NMR (COSY, HSQC, and HMBC) spectra allowed us to establish the substitution pattern of the dibenzo-α-pyrone skeleton of **12**, as shown in Figure 1. Accordingly, the position of the hydroxy group (3'-OH) was assigned at C-3', as it showed ROESY correlations to H-2' and H-4' (Fig. 5). Moreover, the HMBC correlations from 9-OCH_3, H-7, and H-5 to C-9 (δ_c 165.6) corroborated the presence of a carbomethoxy group and its attachment at C-6. Finally, the remaining methoxy group (8-OCH_3) was assigned at C-8, as supported by the respective HMBC correlation, as well as by its ROESY correlation with H-7 (Figure 2). Thus, **12** was identified as a new natural product for which the name serkydayn is proposed.

Table 5: NMR data of **12** measured in DMSO-*d6* at 300 (^{1}H) and 75 (^{13}C) MHz.

Position	Serkydayn (**12**)	
	δ_H	δ_C
1		
2		159.4, C
3		118.7, C
4		125.1, C
4a		121.9, C
5	8.27, d (1.9)	115.5, CH
6		125.7, C
7	7.55, d (1.9)	110.8, CH
8		147.3, C
8a		142.1, C
1’	8.28, d (8.8)	125.1, CH
2’	7.37, dd (8.8, 2.7)	124.1, CH
3’		158.8, C
4’	7.56, d (2.7)	113.8, CH
9		165.6, C
9-OMe	3.90, s	52.4, CH_3
8-OMe	3.96, s	56.1, CH_3
3’-OH	10.55, s	

The new metabolites **8**, **9**, **10,** and **12** probably originate from *Chaetomium* sp. based on structural analogies with the known metabolites (**1**-**7**), which were obtained from the axenic fungal control. Compound, **11** is the only quinoline derivative in this series of metabolites, which highlights the value of the co-cultivation approach as a powerful tool to activate silent biosynthetic gene clusters in microorganisms. Quinolines produced by fungi have already been reported, [28, 29] and thus the fungal origin of **11** is likely. Interestingly, compounds **8**, **10** and **12** were detected during co-cultivation of *Chaetomium* sp. with viable or autoclaved cultures of *B. subtilis*, whereas compounds **9** and **11** were only detected during co-cultivation of *Chaetomium* sp. with viable *B. subtilis* cultures. These data indicate that the effect of heat sterilized bacterial biomass is not uniform for all fungal metabolites.

It is worth mentioning that the production of fungal metabolites in the co-cultures was found to correlate with the time of preincubation of the solid rice medium with *B. subtilis* prior to inoculation with *Chaetomium* sp.. The strongest effect was observed when the fungus was added 4 days after the rice medium had been inoculated with viable *B. subtilis* (data not shown). During co-cultivation, the fungal growth was slowed down compared to axenic controls of *Chaetomium* sp., but recovered after seven to nine days of co-culture. These data demonstrate an inhibitory effect due to the presence of the bacterium, which is in agreement with our previously reported results.[5]

In a second set of experiments, the endophytic bacterium *Streptomyces lividans* [30] was chosen for co-cultivation with *Chaetomium* sp.. However, no induction of fungal metabolites was detected. Likewise, no induction of fungal metabolites was observed when *Chaetomium* sp. was co-cultured with autoclaved *Mycobacterium smegmatis* (data not shown). These results suggest that the fungal response is specific toward different prokaryotes, as previously also described for other fungi [5, 10].

Taking into consideration that posttranslational modifications of histones influence the pattern of secondary metabolites in filamentous fungi [31], *Chaetomium* sp. was cultured on solid rice medium in the presence of the DNA methyltransferase inhibitor 5-azacytidine or the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). The HPLC chromatographic profiles of the respective extracts were significantly different in comparison to those of the axenic fungal controls, but were very similar to each other and showed the induction of two new peaks, which were not present in the fungal controls (Figure S25). Interestingly, one of the induced peaks was identified as the known isosulochrin (**6**), which was also detected during co-cultivation of *Chaetomium* sp. with *B. subtilis*. Thus, the effect on the accumulation of fungal metabolites during co-cultivation may be partially triggered by histone modifications due to microbial crosstalk.

All compounds isolated in this study were assayed *in vitro* for their antibacterial activities against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Bacillus subtilis* 168 trpC2; the latter being the bacterial strain used for co-cultivation. The compounds were likewise assayed against the Gram-negative bacterium *Acinetobacter baumannii*, as well as against *Mycobacterium tuberculosis*. Compound **12** exhibited weak to moderate activity against *B. subtilis* with an MIC value of 53 μM, whereas the remaining compounds exhibited no activity (MIC > 100 μM). In addition, all compounds were evaluated for their effects on the growth of the mouse lymphoma L5178Y cell line. Compound **12** likewise showed the strongest activity with an IC_{50} value of 1 μM. Compounds **11** and **7** displayed only weak cytoxicity with IC_{50} values of 38.6 and 20.8 μM, respectively. Interestingly, the biosynthesis of **12** was induced only during fungal/bacterial co-cultivation, and thus it could be assumed that *Chaetomium* sp. initiated its production as a stress response to suppress its competitor.

3. Conclusion

In conclusion, the axenic culture of *Chaetomium* sp. grown on solid rice medium yielded five known metabolites (**1** ─ **5**). When *Chaetomium* sp. was grown in mixed

cultures with viable or autoclaved cultures of *B. subtilis*, a strong accumulation of the **1** and **2** was observed compared to axenic fungal or bacterial controls. In addition, five new natural products (**8 ─ 12**) together with **6** and **7** were only detected in the co-cultures. Likewise, induction of **6** was observed when *Chaetomium* sp. was cultured on solid rice medium with the epigenetic modifiers 5-azacytidine or SAHA. Thus, our results highlight the potential of the co-cultivation and epigenetic modification as powerful strategies for triggering the production of cryptic fungal secondary metabolites.

4. Experimental Section

4.1. General Experimental Procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ^{1}H, ^{13}C and 2D NMR spectra were recorded in deuterated solvents on a Bruker ARX 300, Avance III 500, Avance DMX 600 or AV III HD 700 NMR spectrometers. Mass spectra were obtained on a LC-MS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest and high-resolution mass (HRESIMS) spectra were measured on a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed with a Dionex UltiMate3400 SD with a LPG-3400SD Pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient was used (MeOH, 0.1 % HCOOH in H_2O): 0 min (10 % MeOH), 5 min (10% MeOH), 35 min (100 % MeOH), 45 min (100 % MeOH). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; Pump L-7100; Eurosphere-100 C18, 300 mm × 8 mm, Knauer, Germany). Column chromatography included LH-20 Sephadex and Merck MN Silica gel 60 M (0.04-0.063 mm). TLC plates with silica gel F254 (Merck, Darmstadt, Germany) were used to monitor fractions (CH_2Cl_2/MeOH mixtures as mobile phase); detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent. Bacterial growth was monitored by measuring OD_{600} in a Tecan microtiter plate reader (Infinite M200, Tecan).

4.2. Biological material

Chaetomium sp. was isolated from fresh healthy leaves of *Sapium ellipticum* (Euphorbiaceae) collected in the west region of Cameroon in January 2015. The fungus was isolated under sterile conditions from the inner tissue of the leaf according to the procedure

described by Kjer et al. [32] The identification was performed following a molecular biological protocol by DNA amplification and sequencing of the ITS region. The sequence data have been submitted to GenBank, accession number **KU051539**. The bacterial strain panel used on this study included the standard laboratory strains, *B. subtilis* 168 trpC2, [33] *S. lividans* TK24, [34] and *M. smegamatis* (mc^2 155). [35]

4.3. Co-cultivation experiment of *Chaetomium* sp. with *B. subtilis* 168 trpC2

The fungal and bacterial strains were cultivated in Erlenmeyer flask (1 L) containing solid rice media for isolation and characterization of secondary metabolites. Twenty-five Erlenmeyer flasks (five flasks for axenic *Chaetomium* sp., five for co-cultures of *Chaetomium* sp. and *B. subtilis*, five for *Chaetomium* sp. treated with autoclaved *B. subtilis*, five for axenic *B. subtilis* and five for autoclaved *B. subtilis*) containing 60.0 mL of distilled water and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before the fungus and the bacterium were inoculated.

The bacterium *B. subtilis* was grown in lysogeny broth (LB). An overnight culture of this bacterium was used to inoculate prewarmed LB medium (1:20), which was afterwards incubated at 37°C with shaking at 200 rpm to mid exponential growth phase (optical density at 600 nm (OD_{600}) of 0.2-0.4). A 10 mL volume of the bacterial culture was then added to the rice medium, which was further incubated for 4 days at 37°C.

This preincubation was followed by the addition of *Chaetomium* sp. grown on malt agar (three pieces, 1 cm-1 cm) to the rice medium containing viable *B. subtilis*. On the other hand, after 4 days incubation, ten flasks cultured with *B. subtilis* were autoclaved followed by addition of the fungus to five of them, in the same manner like for viable cultures of *B. subtilis*. Both microorganisms were also grown axenically on rice medium. Co-cultures and axenic cultures of *Chaetomium* sp. and *B. subtilis* were kept under static conditions at 23°C until they reached their stationary phase of growth (3 weeks for *Chaetomium* sp. and *B. subtilis*; 5 weeks for co-cultures). For extraction, 300 mL of EtOAc was added to the cultures, and then the resulting mixture was shaken at 140 rpm for 9 h. The cultures were further left overnight and filtered on the following day using a Buchner funnel. The resulting extract was washed with demineralised water and then evaporated under vacuum to remove EtOAc. Each extract was then dissolved in 60 mL of MeOH, and then 15 μL of this was injected into the analytical HPLC column.

4.4. Co-cultivation experiment of *Chaetomium* sp. with *S. lividans* TK24

The co-cultivation of *Chaetomium* sp. with *S. lividans* was done in the same manner as with viable *B. subtilis*. Fifteen Erlenmeyer flasks (five for axenic *Chaetomium* sp., five for *Chaetomium* sp. and *S. lividans* and five for axenic *S. lividans*) containing 60.0 mL of Yeast Malt (YM) medium and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before inoculating the fungus and the bacterium. An overnight culture of *Streptomyces lividans* was used to inoculate prewarmed YM medium (1:20), which was then incubated at 30°C with shaking at 200 rpm to mid exponential growth phase. The corresponding preculture was afterwards incubated in fresh YM medium overnight to reach exponential growth phase. Then 10 mL volume of the bacterial culture was added to the rice medium, which was further incubated for four days at 30°C. After this preincubation, we proceeded in the same manner as described in the co-cultivation experiment of *Chaetomium* sp. and *B. subtilis* 168 trpC2.

4.5. Co-cultivation experiment of *Chaetomium* sp. with *Mycobacterium smegmatis* (mc^2 155)

The fungal and the bacterial strains were cultivated in Erlenmeyer flask (1L) containing solid rice media for isolation and characterization of secondary metabolites. Erlenmeyer flasks containing 60.0 mL of distilled water and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before the fungus and the bacterium were inoculated. *M. smegmatis* was grown in Middelbrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) tyloxapol and 10% (v/v) ADS enrichment (5%, w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride) until exponential growth phase (OD 600 nm of 0.5-0.8). 10 mL volume of the bacterial culture was then added to the rice medium, which was further incubated for 4 days 37°C and finally autoclaved. After this preincubation, we proceeded in the same manner as described in the co-cultivation experiment of *Chaetomium* sp. and *B. subtilis* 168 trpC2.

4.6. Treatment with epigenetic modifiers

The treatment of *Chaetomium* sp. with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) or with DNA methyltranferase inhibitor 5-azacytidine, was carried out to compare the production of cryptic fungal metabolites induced during the co-culture experiments with fungal cultures being treated with epigenetic modifications. Ten flasks (two flasks for axenic *Chaetomium* sp., two flasks for *Chaetomium* sp. and SAHA, two flasks for *Chaetomium* sp. and 5-azacytidine, two flask of SAHA and two

flask of 5-azacytidine) containing 60.0 mL of distilled water and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before inoculation of the fungus and addition of the epigenetic modifiers. Three pieces of *Chaetomium* sp. grown on malt agar were added to the autoclaved rice medium under sterile conditions and incubated for two days. This preincubation was followed by the addition of 10 mL of the solution (Sterilized Water-EtOH 21:4) of each epigenetic modifier (6 mM each) under sterile conditions. Epigenetic and axenic cultures of *Chaetomium* sp. were kept under static conditions at 23°C until they reached their stationary phase of growth (3 weeks for *Chaetomium* sp.; 4 weeks for epigenetic cultures). After this incubation, we proceeded in the same manner as described in the cocultivation experiments of *Chaetomium* sp. and *B. subtilis* 168 trpC2.

4.7. Extraction and isolation

The crude extract obtained from co-cultures of *Chaetomium* sp. with *B. subtilis* (6.0 g) was subjected to vacuum liquid chromatography (VLC) on silica gel employing a step gradient of *n*-hexane-EtOAc then CH_2Cl_2-MeOH to give seven fractions A-G. Fraction B, eluted with *n*-hexane/EtOAc (6:4) was subjected to repeated vacuum liquid chromatography (VLC), using only a step gradient of *n*-hexane-EtOAc to yield nine subfractions B_1-B_9. Subfraction B_2 , eluted with *n*-hexane-EtOAc (9:1, v/v) was further purified using Sephadex LH-20 followed by semipreparative HPLC with MeOH-H_2O (0.1%TFA) to afford the mixture **1+2** (50 mg). Subfraction B_3 eluted with *n*-hexane-EtOAc (8.5:1.5, v/v) was purified by semipreparative HPLC with MeOH-H_2O (0.1%TFA), to yield **11** (1.1 mg) and **7** (1.2 mg). Subfraction B_4, eluted with *n*-hexane-EtOAc (8:2, v/v) was further purified using Sephadex LH-20 followed by semipreparative HPLC with MeOH-H_2O to yield **8** (11 mg), **12** (6 mg) and **6** (4 mg). Subfraction B_5 eluted with *n*-hexane-EtOAc (7.5:2.5, v/v) was treated in the same manner as B_4 to afford **9** (8 mg), **10** (2 mg) and **3** (3.8 mg).

The crude extract of the axenic culture of *Chaetomium* sp. (1.6 g) was also subjected to VLC on silica gel employing a step gradient of *n*-hexane-EtOAc followed by CH_2Cl_2-MeOH to give six fractions A-F. Fraction E eluted with EtOAc was purified by semipreparative HPLC (MeOH-H_2O) to yield **4** (1.9 mg) and **5** (3.1 mg). Compounds **1**[19], **2**[20], **3**[18], **4**[16], **5**[16], **6**[21], **7**[22] were identified by comparing their ^{1}H NMR, LC-MS and UV data with those published.

The crude extracts resulting from co-cultivation of *Chaetomium* sp. with *S. lividans* or with *M. smegmatis* as well as those from the epigenetic modifier experiments were directly

submitted to analytical HPLC and no further work up was undertaken due to their limited amounts.

4.7.1. Shikimeran A (**8**). Greenish oil; $[\alpha]^{20}_{D}$ -58 (*c* 1.01, MeOH); UV (MeOH) λ_{max}: 227 nm; ECD (MeCN,λ [nm] (Δε), *c* = 3.50x10^{-4}M): 250 (0.20), 220 (-1,72), 192 (1,29); ^{1}H and ^{13}C NMR see Table 2; HRESIMS $[M+H]^{+}$ m/z 215.0913 (calcd for $C_{10}H_{15}O_5$, 215.0914).

4.7.2. Bipherin A (**9**). Colorless oil; UV (MeOH) λ_{max}: 233 and 306 nm; ^{1}H and ^{13}C NMR see Table 3; HRESIMS $[M+H]^{+}$ m/z 303.0863 (calcd for $C_{16}H_{15}O_6$, 303.0863).

4.7.3. Chorismeron (**10**)**.** Yellowish oil; UV (MeOH) λ_{max}: 204.6 and 288.8 nm; ^{1}H and ^{13}C NMR see Table 4; HRESIMS $[M+H]^{+}$ m/z 223.0602 (calcd for $C_{11}H_{11}O_5$, 223.0601).

4.7.4. Quinomeran (**11**)**.** Brown oil; $[\alpha]^{20}_{D}$ 0 (*c* 0.3, MeOH); UV (MeOH) λ_{max}: 207, 246 and 296 nm; ^{1}H and ^{13}C NMR see Table 2; HRESIMS $[M+H]^{+}$ m/z 260.0915 (calcd for $C_{14}H_{14}NO_4$, 260.0917).

4.7.5. Serkydayn (**12**)**.** Yellow amorphous powder; UV (MeOH) λ_{max}: 202 (2.74), 236 and 361 nm; ^{1}H and ^{13}C NMR see Table 5; HRESIMS $[M+H]^{+}$ m/z 301.0706 (calcd for $C_{16}H_{13}O_6$, 301.0707).

4.8. Computational section

Mixed torsional/low-frequency mode conformational searches were carried out by means of the Macromodel 9.9.223 software using the Merck Molecular Force Field (MMFF) with an implicit solvent model for $CHCl_3$.[36] Geometry reoptimizations were carried out at the B3LYP/6-31G(d) level *in vacuo* and the B97D/TZVP level [25,26] with the PCM solvent model for MeCN. TDDFT ECD calculations were run with various functional (B3LYP, BH&HLYP, PBE0) and the TZVP basis set as implemented in the Gaussian 09 package with the same or no solvent model as in the preceding DFT optimization step.[37] ECD spectra were generated as sums of Gaussians with 3000 cm^{-1} widths at half-height (corresponding to ca. 15 nm at 220 nm), using dipolevelocity-computed rotational strength values.[38] Boltzmann distributions were estimated from the ZPVE-corrected B3LYP/6-31G(d) energies in the gas-phase calculations and from the B97D/TZVP energies in the solvated ones. The MOLEKEL software package was used for visualization of the results.[39]

4.9. Antibacterial assay

Measurement of MIC values were done by the broth microdilution method according to CLSI guidelines.[40] The direct colony suspension method was used with an inoculum of 5 x 10^5 colony forming units/mL after the last dilution step for inoculum preparation. Compounds were added from stock solution (10 mg/mL in DMSO), resulting in a final DMSO amount of 0.64% at the highest antibiotics concentration tested (64 μg/mL). Serial 2-fold dilutions of antibiotics were prepared with DMSO being diluted along with the compounds.

4.10. Antituberculosis assay

The resazurin dye reduction method was performed as a metabolic assay to evaluate the growth inhibition of *M. tuberculosis*. *M. tuberculosis* cells were grown aerobically at 37°C in Middlebrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) Tyloxapol, and 10% (v/v) ADS enrichment (5%,w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride). Bacteria were precultured until log-phase (OD 600 nm~1) and then seeded at 1 x 10^5 cells per well in a total volume of 100 μL in 96-well round-bottom microtiter plates and incubated with test substances for 6 days. For viability determination, 10 μL of resazurin solution (100 μg/mL, Sima-Alderich) was added per well and incubated for ca. 8h. Then cells were fixed at room temperature for 30 mn after addition of formalin (5%, v/v, final concentration), and fluorescence was measured using a microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to rifampicin-treated (0% growth) and DMSO treated (100% growth) controls.

4.11. Cell viability assay

Cytotoxicity was tested against L5178Y mouse lymphoma cells using an MTT (=3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay and compared to that of untreated controls, as described previously.[41] Experiments were repeated three times and carried out in triplicate. As negative controls, media with 0.1% EGMME/ DMSO were included in the experiments. The depsipeptide kahalalide F, isolated from *Elysia grandifolia* was used as a positive control.

Aknowledgements

The financial support of the Manchot Foundation granted to P.P. is gratefully acknowledged. S.H.A. wants to thank DAAD (Deutscher Akademischer Austauschdienst) for a doctoral scholarship. T.K. and A.M. thank the Hungarian National research Foundation

(OTKA K105871) for financial support and the National Information infrastructure Development Institute (NIIFI 10038) for CPU time. We are indebted to Professor W.E.G. Müller (Johannes Gutemberg University, Mainz, Germany), for cytotoxicity assays. We also thank Professor R. Kalscheuer (Heinrich-Heine Universität Düsseldorf) for antibacterial assays.

References

1. Brakhage, A.A. *Microbiol. Mol. Biol. R.* **1998**, *62*, 547-585.
2. Brakhage, A.A.; V. Schroeckh. *Fungal. Genet. Biol.* **2011**, *48*, 15-22.
3. Strobel, G.; B. Daisy. *Microbiol. Mol. Biol. R.* **2003**, *67*, 491-502.
4. Aly, A.H.; A. Debbab; Proksch, P. *Appl Microbiol Biotechnol*. **2011**, *90*, 1829-1845.
5. Ola, A.R.B.; Thomy, D.; Lai, D.; Brötz-Oesterhelt, H.; Proksch, P. *J. Nat. Prod.* **2013**, *76*, 2094-2099.
6. Rateb, M.E.; Hallyburton, I.; Houssen, W.E.; Bull, A.T.; Goodfellow, M.; Santhanam, R.; Jaspars, M.; Rateb, R.E. *RSC Adv*. **2013**, *3*, 14444-14450.
7. Zuck, K.M.; Shipley, S.; Newman, D.J. *J. Nat. Prod.* **2011**, *74*, 1653-1657.
8. Schroeckh, V.; K.; Nützmann, H.-W.; Shelest, E.; Schmidt-Heck, W.; Schuemann, J.; Martin, K.; Hertweck, C.; Brakhage, A.A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14558-14563.
9. Oh, D.-C; Kauffman, C. A.; , Jensen, P. R. ; Fenical, W. *J. Nat. Prod.* **2007**, *70*, 515-520.
10 Chen, H.; Aktas, N.; Konuklugil, B.; Mandi, A.; Daletos, G.; Lin, W.; Proksch, P. *Tetrahedron Lett.* **2015***, 56*, 5317-5320.
11. Scherlach, K.; C. Hertweck, *Org. Biomol. Chem.* **2009**. *7*, 1753-1760.
12. Yang, X. L.; Huang, L.; Ruan, X. L. *J. Asian. Nat. Prod. Res.* **2014***, 16*, 412-417.
13. Kabbaj, F. Z.; Lu, S.; Faouzi, M. E. A.; Meddah, B.; Proksch, P.; Cherrah, Y.; Altenbach, H.J.; Aly A.H.; Chadli, A.; Debbab, A. *Bioorg. Med. Chem.* **2015**, *23*, 126-131.
14. Li, G.-Y.; Li, B.-G.; Yang, T.; Yan, J.-F.; Liu, G.-Y.; Zhang, G.-L. *J. Nat. Prod.* **2006**, *69*, 1374-1376.
15. Marwah, R. G.; Fatope, M. O.; Deadman, M. L.; Al-Maqbali, Y. M.; Husband, J. *Tetrahedron,* **2007***, 63*, 8174-8180.

16. Payne, D. J.; Hueso-Rodríguez, J. A.; Boyd, H.; Concha, N. O.; Janson, C. A.; Gilpin, M.; Bateson, J.H.; Cheever, C.; Niconovich, N.L.; Pearson, S.; Rittenhouse, S.; Tew, D.; Díez, E.; Pérez, P.; De La Fuente, J.; Rees, M.; Rivera-Sagredo, A. *Antimicrob. Agents. Chemother.* **2002**, *46*, 1880-1886.
17. Lösgen, S; Schlörke, O.; Meindl, K.; Herbst-Irmer, R.; Zeeck, A. *Eur. J. Org. Chem.* **2007**, *21*, 2191-2196.
18. Pontius, A.; Mohamed, I.; Krick, A.; Kehraus, S.; König, G. M. *J. Nat. Prod.* **2008**, *71*, 272-274.
19. Chi-Hwan, L. *J. Agric. Sci.* **2002**, *29*, 91-97.
20. Cao, L.-L.; Tian, H.-Y; Wang, Y.-S; Zhou, X.-F; Jiang, R.-W; Liu, Y.-H. *J. Trop. Oceanogr.* **2015**, *34*, 77-82.
21. Shimada, A.; Takahashi, I.; Kawano, T.; Kimurab, Y. *Z. Naturforsch. B*. **2001**, *56*, 797-803.
22. Miyazawa, M.; Oshima, T.; Koshio, K.; Itsuzaki, Y.; Anzai, J. *J. Agric. Food Chem* **2003**, *51*, 6953-6956.
23. Armesto, N.; Fernández, S.; Ferrero, M.; Gotor, V. *Tetrahedron* **2006***, 62*, 5401-5410.
24. Tian, Z.; Sun, P.; Yan, Y.; Wu, Z.; Zheng,Q.; Zhou, S.; Zhang, H.; Yu, F.; Jia, X. ; Chen, D. ; Mándi, A.;; Kurtán, T.; Liu, W. *Nat. Chem. Biol.*, **2015**, *11*, 259-265.
25. Grimme, S. *J. Comput. Chem.,* **2006**, *27*, 1787-1799.
26. Sun,P.; Xu, D.,X.; Mándi, A.; Kurtán,T.; Li, T., J.; Schulz, B.; Zhang, W. *J. Org. Chem.*, **2013**, *78*, 7030-7047.
27. Wang, D.H.; Cheng, S.Z.; Harris, F.W. *Polymer* **2008**, *49*, 3020-3028.
28. Teichert, A.; Schmidt, J. r.; Porzel, A.; Arnold, N.; Wessjohann, L. *J. Nat. Prod.* **2008**,71, 1092-1094.
29. Abraham, W.-R.; G. Spassov, *Phytochemistry* **1991**, *30*, 371-372.
30. Meschke, H., Walter, S.; Schrempf, H. *Environ. Microbiol.* **2012**, *14*, 940-952.
31. Bok, J. W.; Chiang, Y.-M.; Szewczyk, E.; Reyes-Dominguez, Y.; Davidson, A. D.; Sanchez, J. F.; Lo, H.C.; Watanabe, K.; Strauss, J.; Oakley, B.R.; Wang, C.C.; Keller, N. F. *Nat. Chem. Biol.* **2009**, *5*, 462-464.
32. Kjer, J.; Debbab, A.; Aly, A. H.; Proksch, P. *Nat. Protoc.* **2010**, *5*, 479-490.
33. Anagnostopoulos, C.; Spizizen, J. *J. Bacteriol.* **1961**, *81*, 741.
34. Hopwood, D. A.; Kieser, T.; Wright, H. M.; Bibb, M. J. *Microbiology,* **1983**, *129*, 2257-2269.

35. Cirillo, J. D.; Barletta, R. G.; Bloom, B.; Jacobs, W. *J. Bacteriol.* **1991**, *173*, 7772-7780.

36. MacroModel; Schrödinger, LLC, 2012, http://www.schrodinger.com/MacroModel.

37. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, O.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. Gaussian 09, revision B.01; Gaussian, Inc.: Wallingford, CT, **2010**.

38. Stephens, P. J.; Harada, N.. *Chirality*, **2010**, *22*, 229-233.

39. Varetto, U. MOLEKEL, v. 5.4; Swiss National Supercomputing Centre: Manno, Switzerland, **2009**.

40. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard Ninth ed.; CLSI document M07-A9; Clinical and Laboratory Standards Institute: Wayne, PA, **2012**.

41. Ashour, M.; Edrada R.; Ebel, R.; Wray V.; Wätjen, W.; Padmakumar, K.; Müller, W.E.G.; Lin, W. H., Proksch, P. *J. Nat. Prod.* **2006**, *69*, 1547-1553.

Figure Legends

Figure 1. Structures of compounds **1-12**.

Figure 2. HPLC chromatograms of EtOAc extracts from co-culture experiments (detection at UV 235 nm): (a) *B. subtilis* control, (b) *Chaetomium* sp. control, (c) co-culture of *Chaetomium* sp. with autoclaved *B. subtilis*, (d) co-culture of *Chaetomium* sp. with viable *B. subtilis*.

Figure 3. Structures and populations of the low-energy B97D/TZVP PCM/MeCN conformers (≥2%) of (3*R*,4*R*)-**8**.

Figure 4. Experimental ECD spectrum of **8** in MeCN compared with the Boltzmann-weighted B3LYP/TZVP PCM/MeCN ECD spectrum of (3*R*,4*R*)-**8** computed for the B97D/TZVP PCM/MeCN conformers. Bars represent the rotational strength of the lowest-energy conformer.

Figure 5. COSY (bold), key ROESY (dashed), and HMBC (plain) correlations of **11** and **12**

Supporting Informations

Inducing secondary metabolite production by the endophytic fungus *Chaetomium* sp. through fungal-bacterial co-culture and epigenetic modification

Sergi Herve Akone,[a,b] Attila Mándi,[c] Tibor Kurtán,[c] Rudolf Hartmann,[d] Wenhan Lin,[e] Georgios Daletos,[a*] and Peter Proksch[a*]

[a]Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany

[b]Faculty of Science, Department of Chemistry, University of Douala, P.O. Box. 24157 Douala, Cameroon.

[c]Department of Organic Chemistry, University of Debrecen, Egyetem tér 1, P.O. Box. 400, 4002 Debrecen, Hungary.

[d]Institute of Complex Systems: Strukturbiochemie (ICS-6), Forschungszentrum Julich, Wilhelm-Johnen-Strase, 52428 Julich, Germany

[e]State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Health Science Center, 100191 Beijing, People's Republic of China.

* Author to whom correspondence should be addressed;

E-Mail: georgios.daletos@uni-duesseldorf.de;

Tel.: +49 211 81 14173; fax: +49 211 81 11923

E-Mail: proksch@uni-duesseldorf.de;

Tel.: +49 211 81 14163; fax: +49 211 81 11923

Table of contents

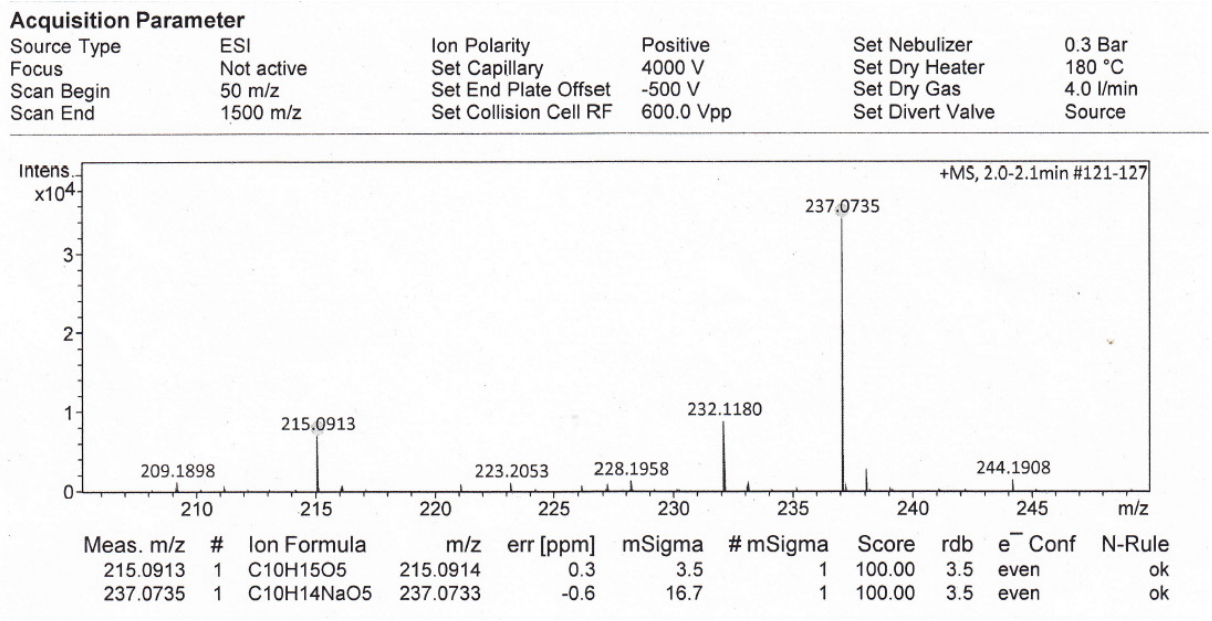


Figure S1. HRESIMS spectrum of compound **8**

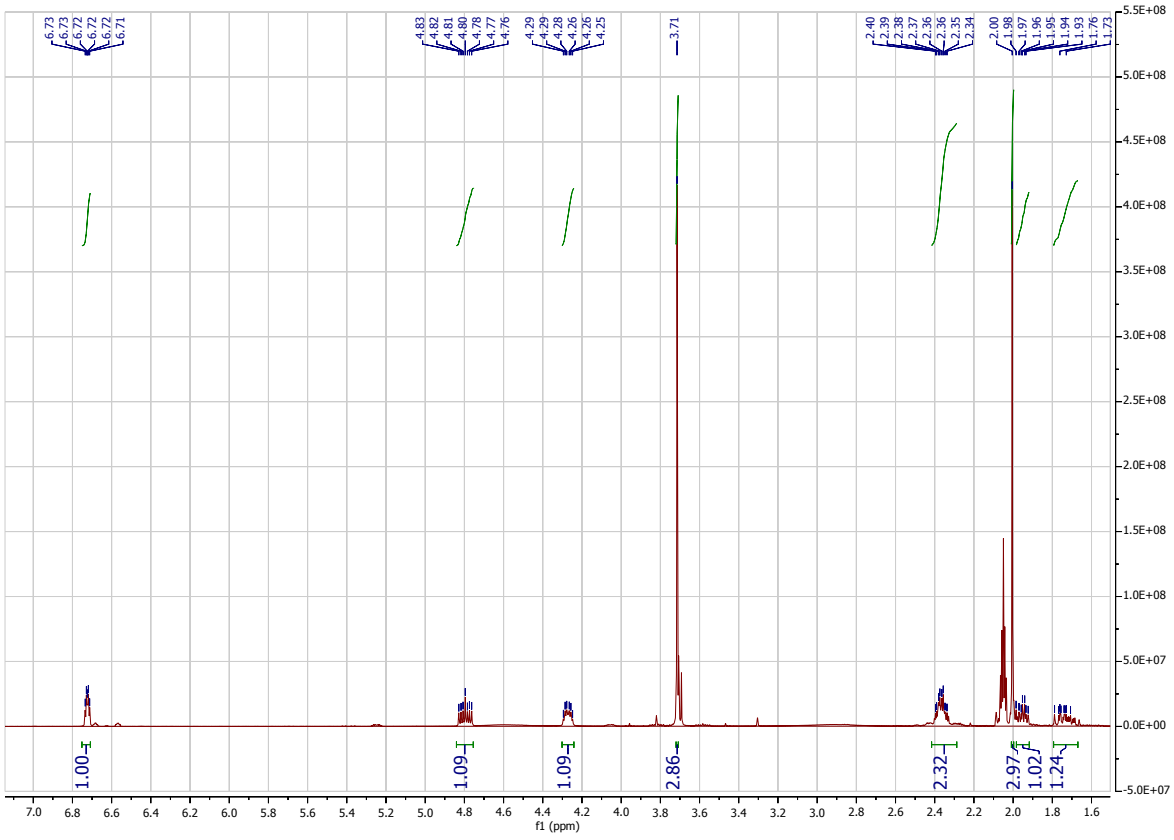


Figure S2. ^{1}H NMR spectrum of compound **8** in $(CD_3)_2CO$ (300 MHz).

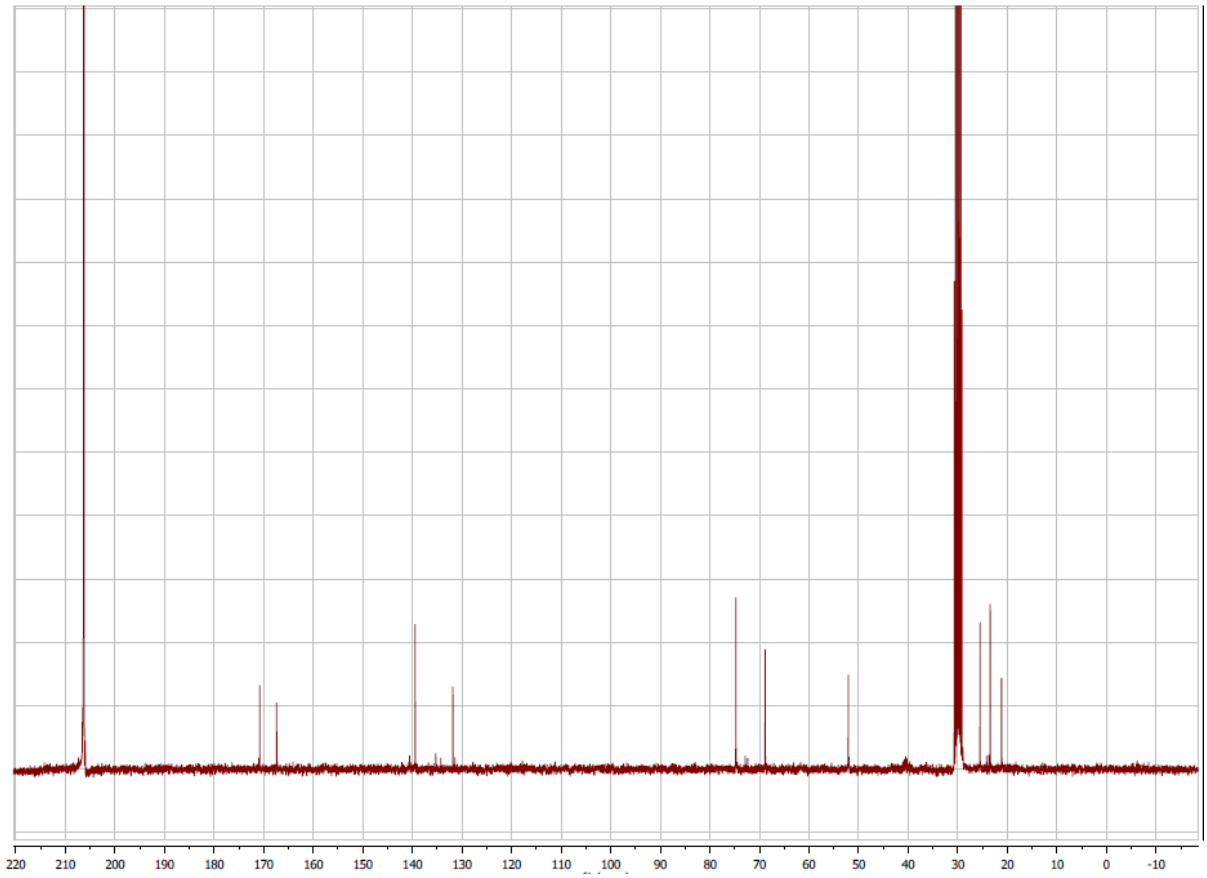


Figure S3. ^{13}C NMR spectrum of **8** in $(CD_3)_2CO$ (75 MHz)

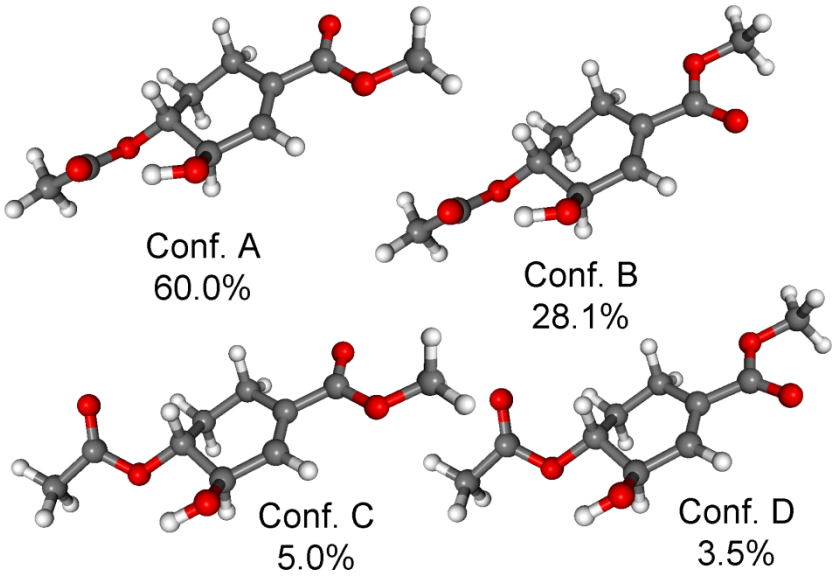


Figure S4. Structures and populations of the low-energy B3LYP/6-31G(d) *in vacuo* conformers (≥2%) of (3*R*,4*R*)-**8**.

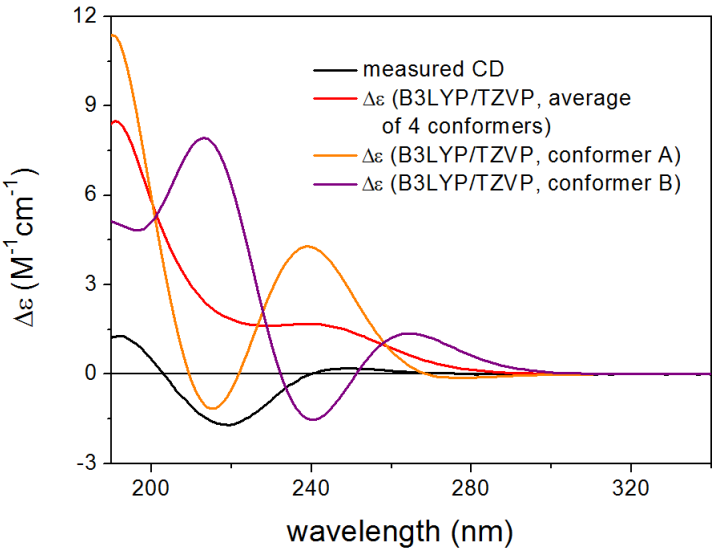


Figure S5. Experimental ECD spectrum of **8** in MeCN compared with the Boltzmann-weighted B3LYP/TZVP *in vacuo* ECD spectrum and those of conformers A and B of (3*R*,4*R*)-**8** computed for the B3LYP/6-31G(d) *in vacuo* conformers. Computed spectra are not shifted.

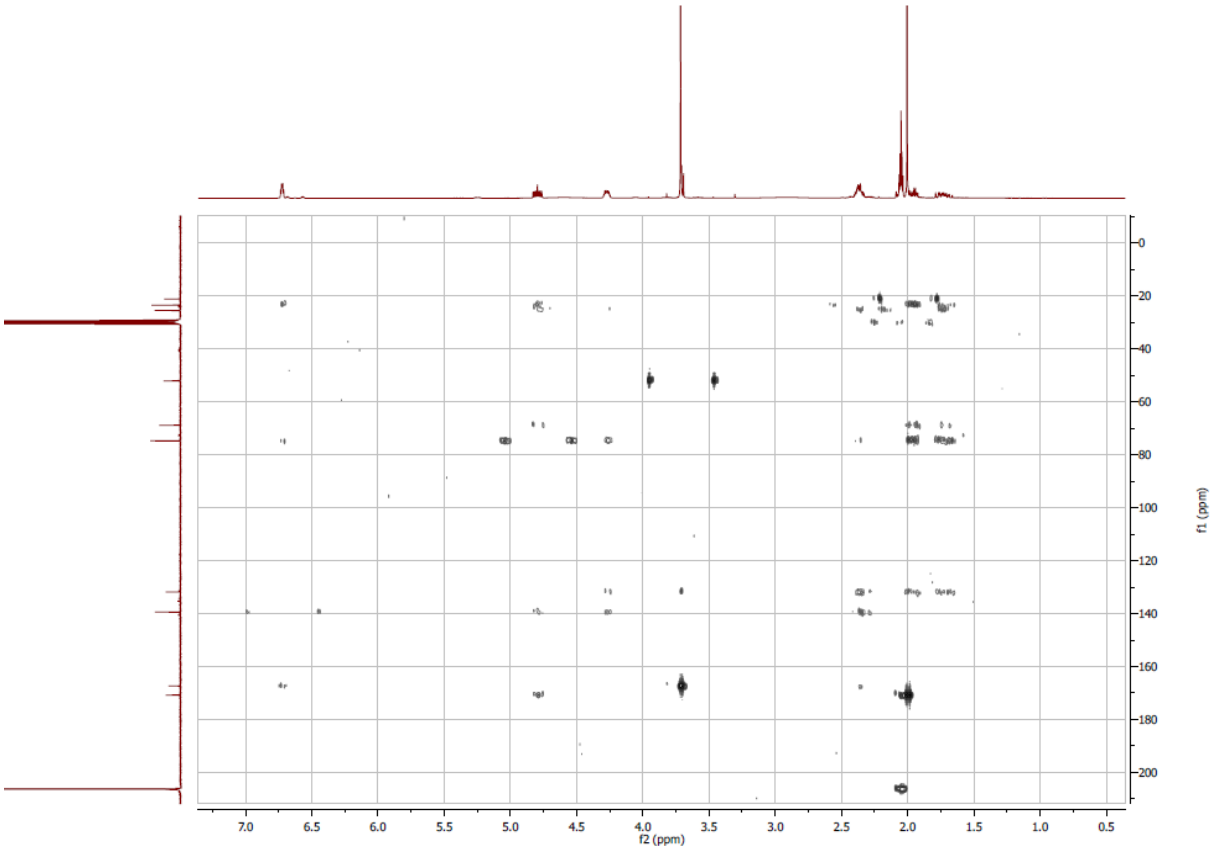


Figure S6. HMBC spectrum of compound **8** (300 MHz and 75 MHz).

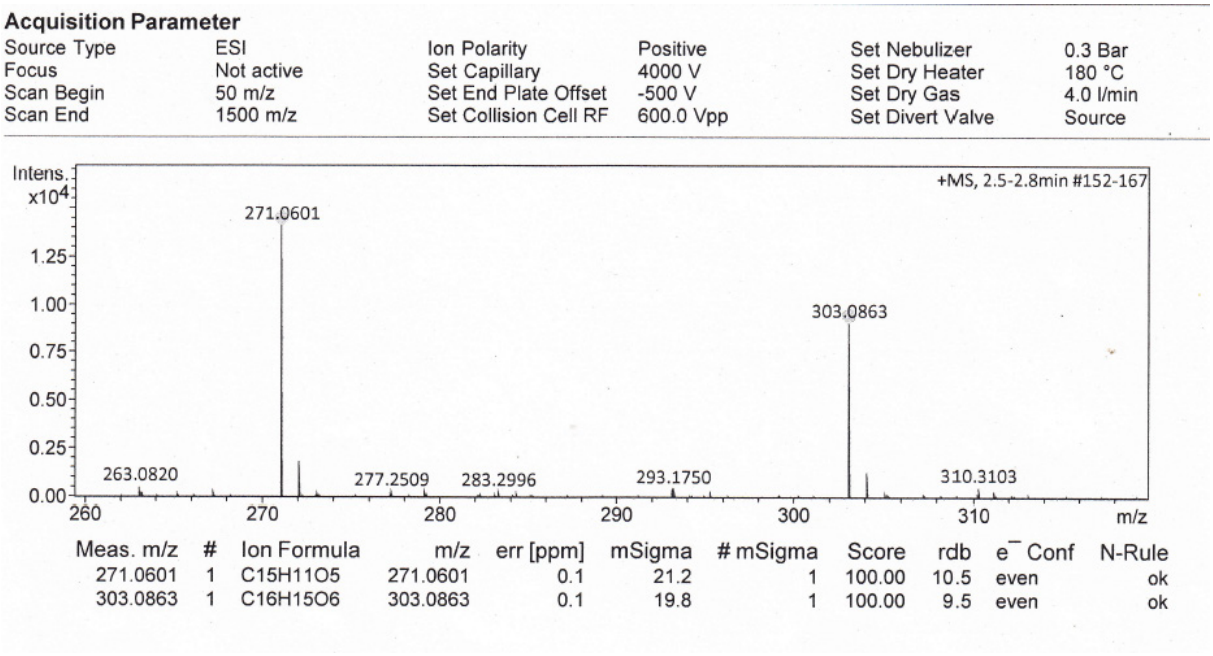


Figure S7. HRESIMS spectrum of compound **9**

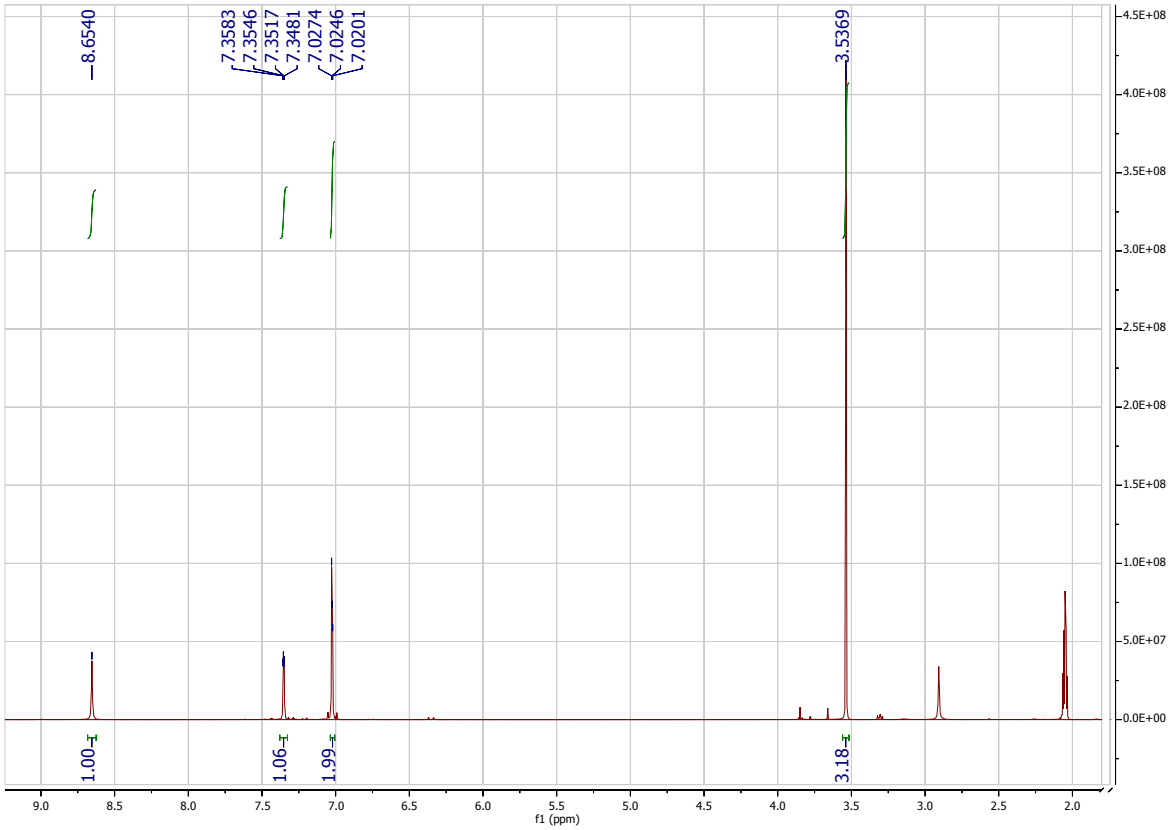


Figure S8. ^{1}H NMR spectrum of compound **9** in $(CD_3)_2CO$ (300 MHz).

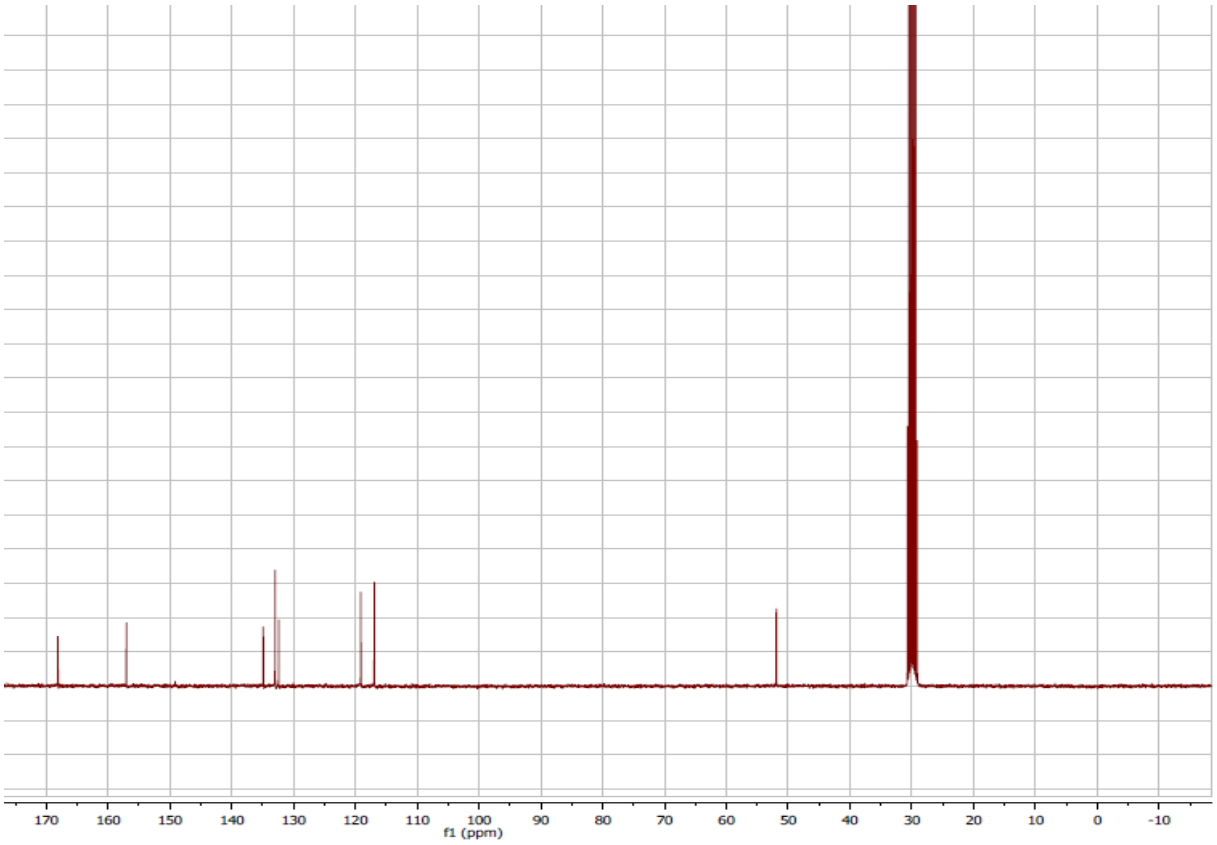


Figure S9. ^{13}C NMR spectrum of compound **9** in $(CD_3)_2CO$ (75 MHz).

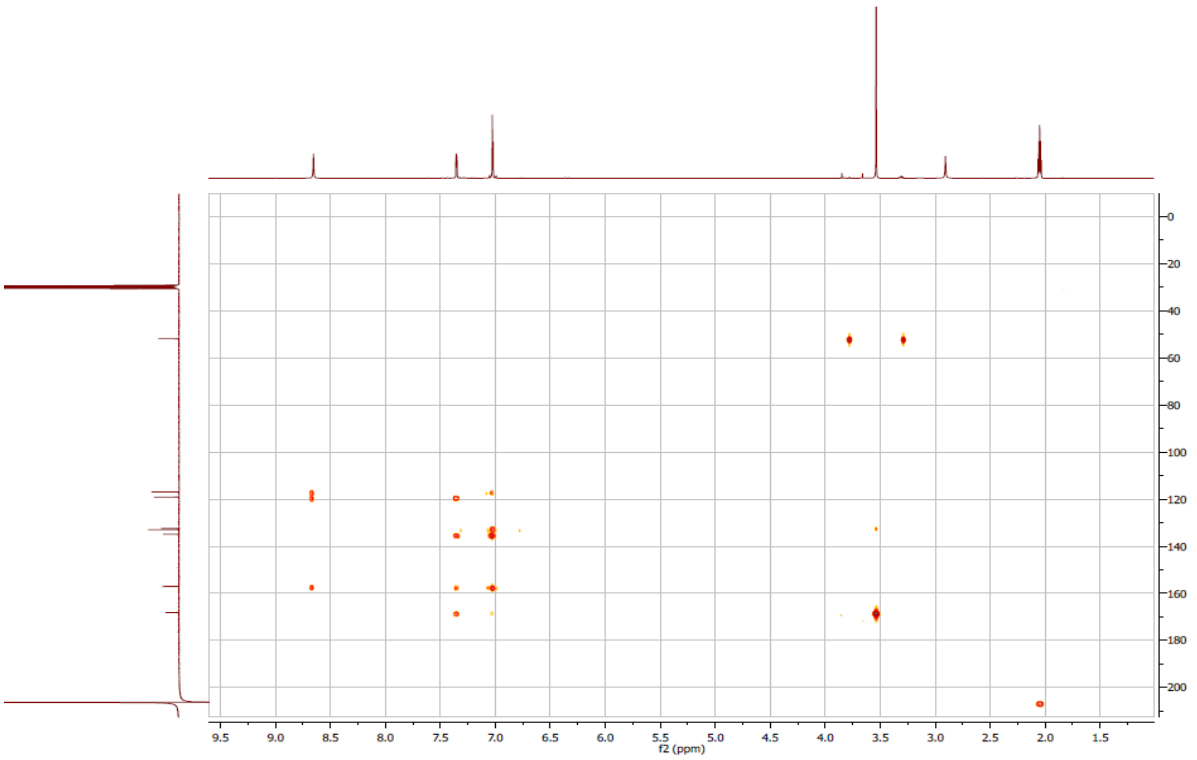


Figure S10. HMBC spectrum of compound **9** in $(CD_3)_2CO$ (300 MHz and 75 MHz).

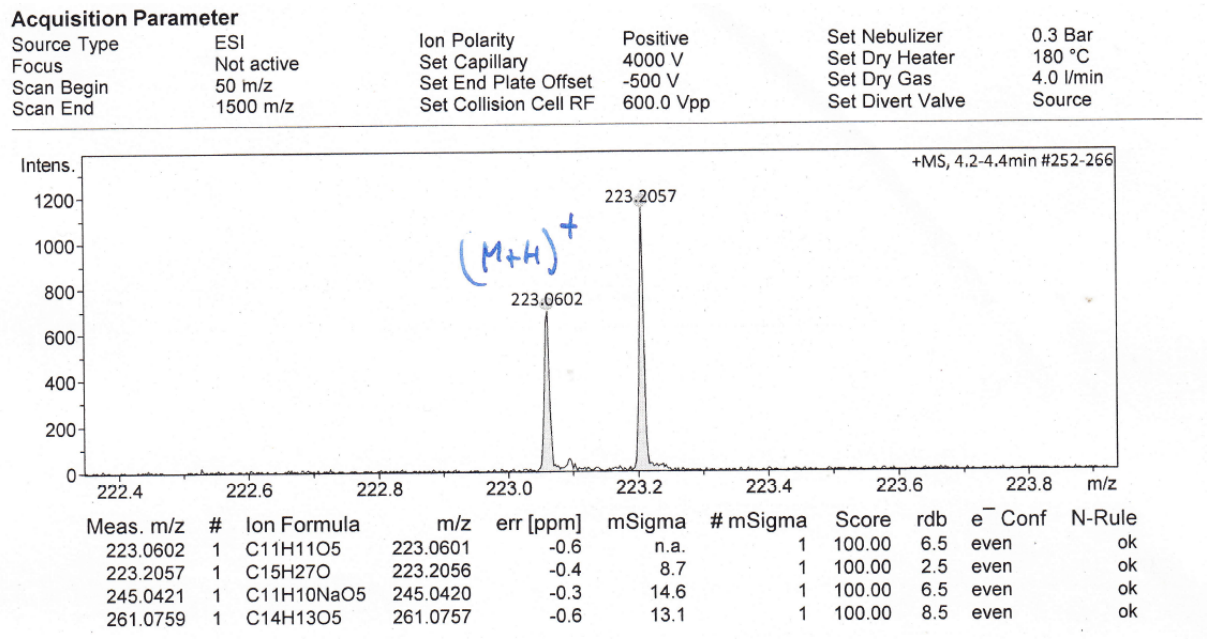


Figure S11. HRESIMS spectrum of **10**.

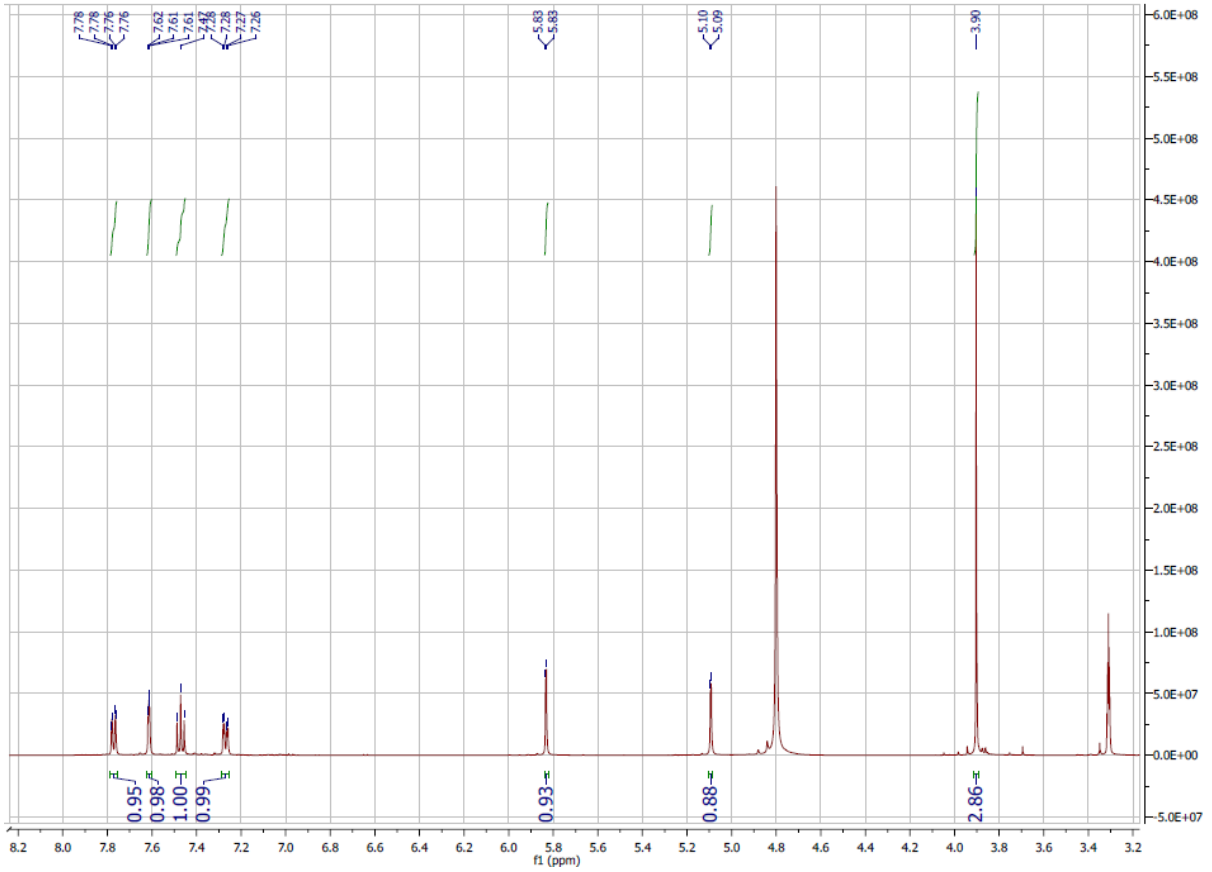


Figure S12. ^{1}H NMR spectrum of **10** in CD_3OD (500 MHz).

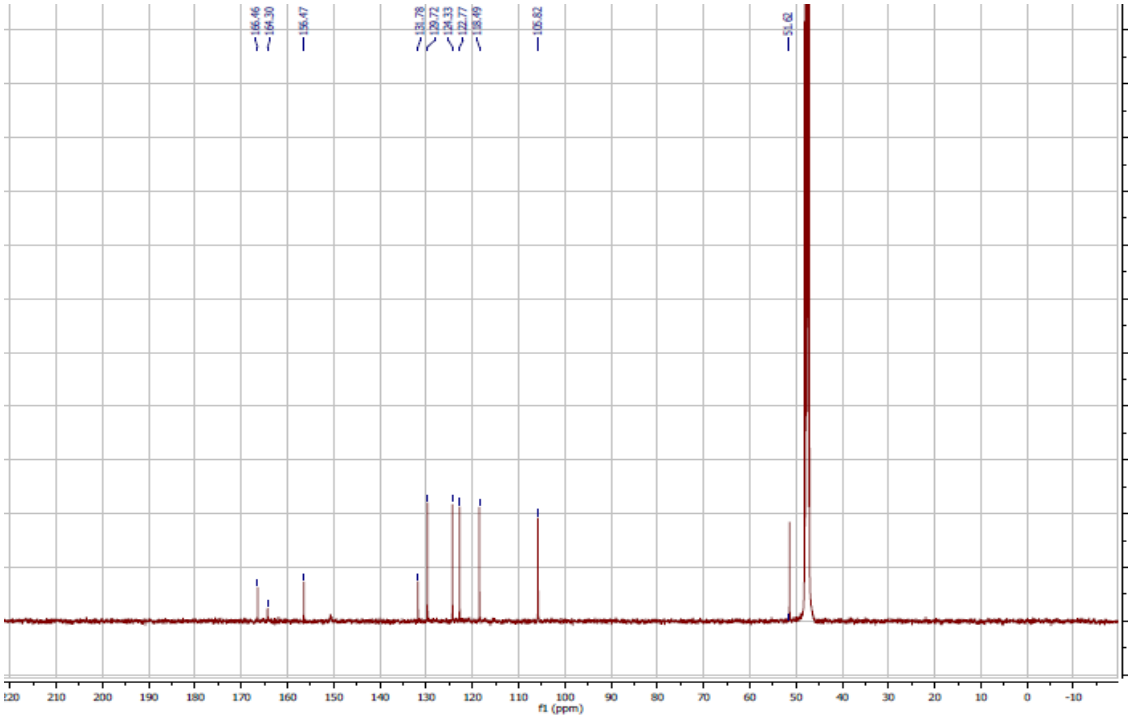


Figure S13. ^{13}C NMR spectrum of **10** in CD_3OD (125MHz).

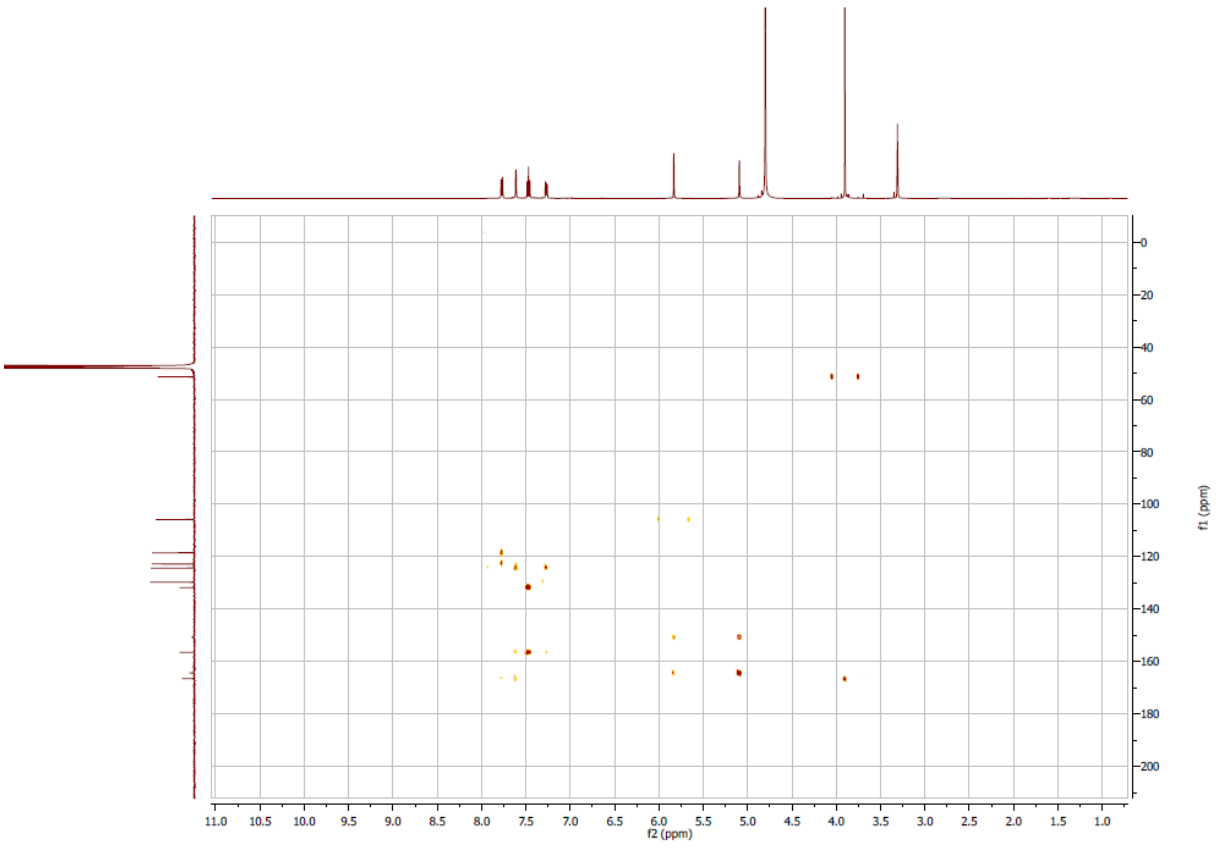


Figure S14. HMBC NMR spectrum of **10** in CD_3OD (500 MHz and 125 MHz).

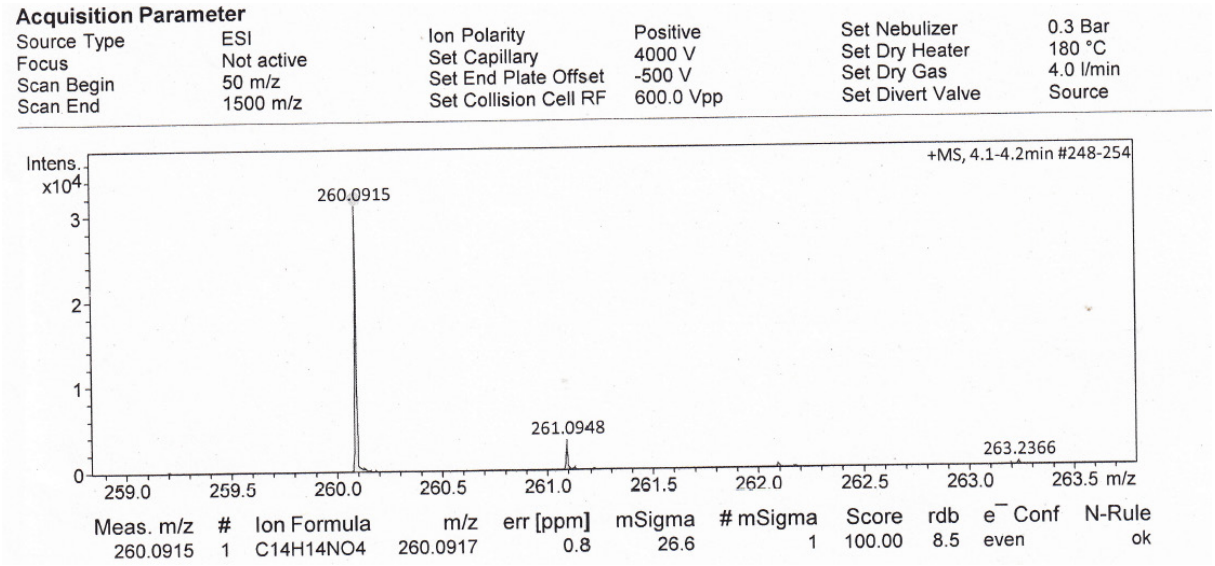


Figure S15. HRESIMS spectrum of compound **11**

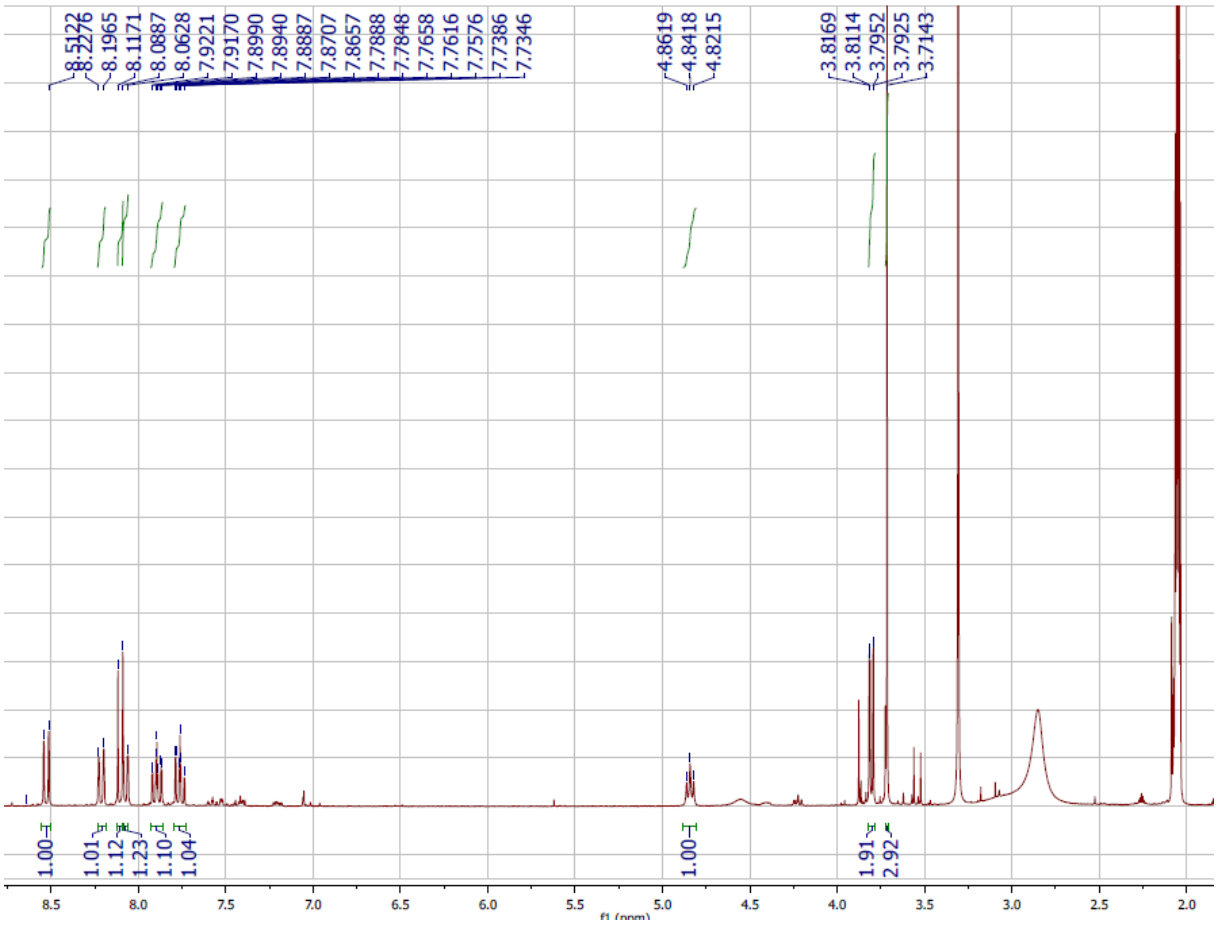


Figure S16. ^{1}H NMR spectrum of compound **11** in $(CD_3)_2CO$ (600 MHz).

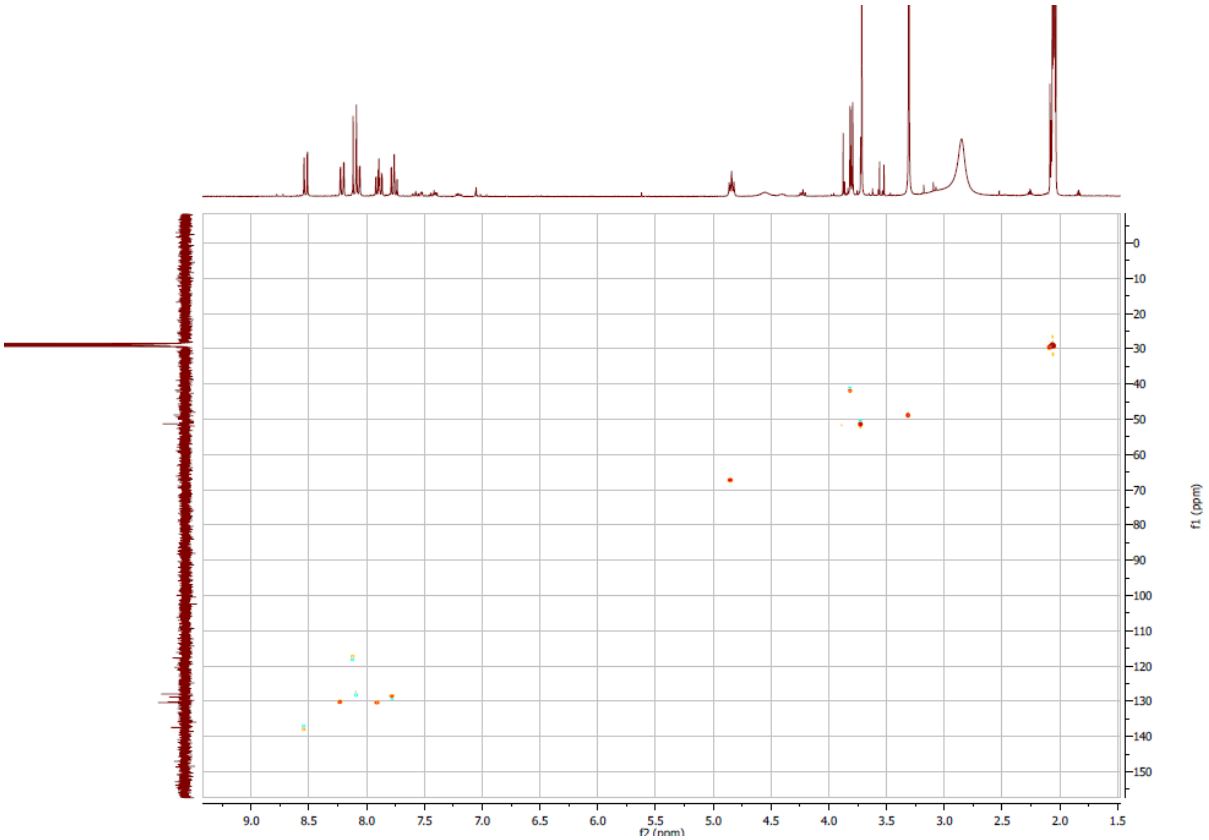


Figure S17. HSQC spectrum of compound **11** in $(CD_3)_2CO$ (600 MHz, and 150.9 MHz).

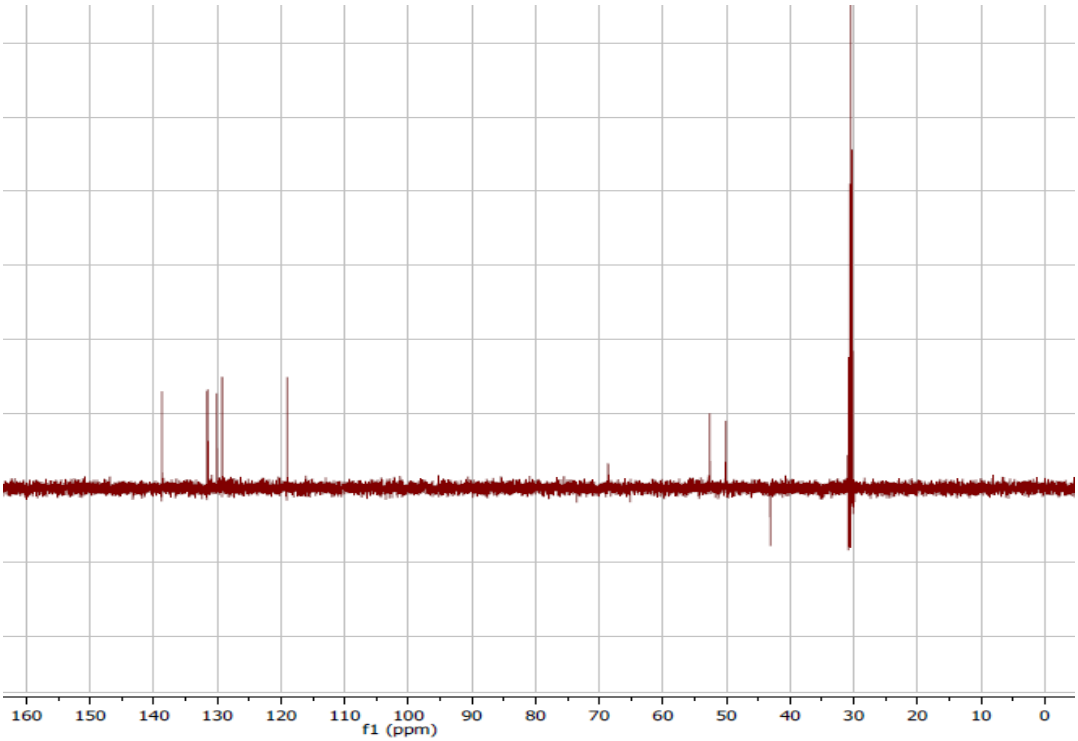


Figure S18. DEPT 135 spectrum of **11** in $(CD_3)_2CO$ (500 MHz).

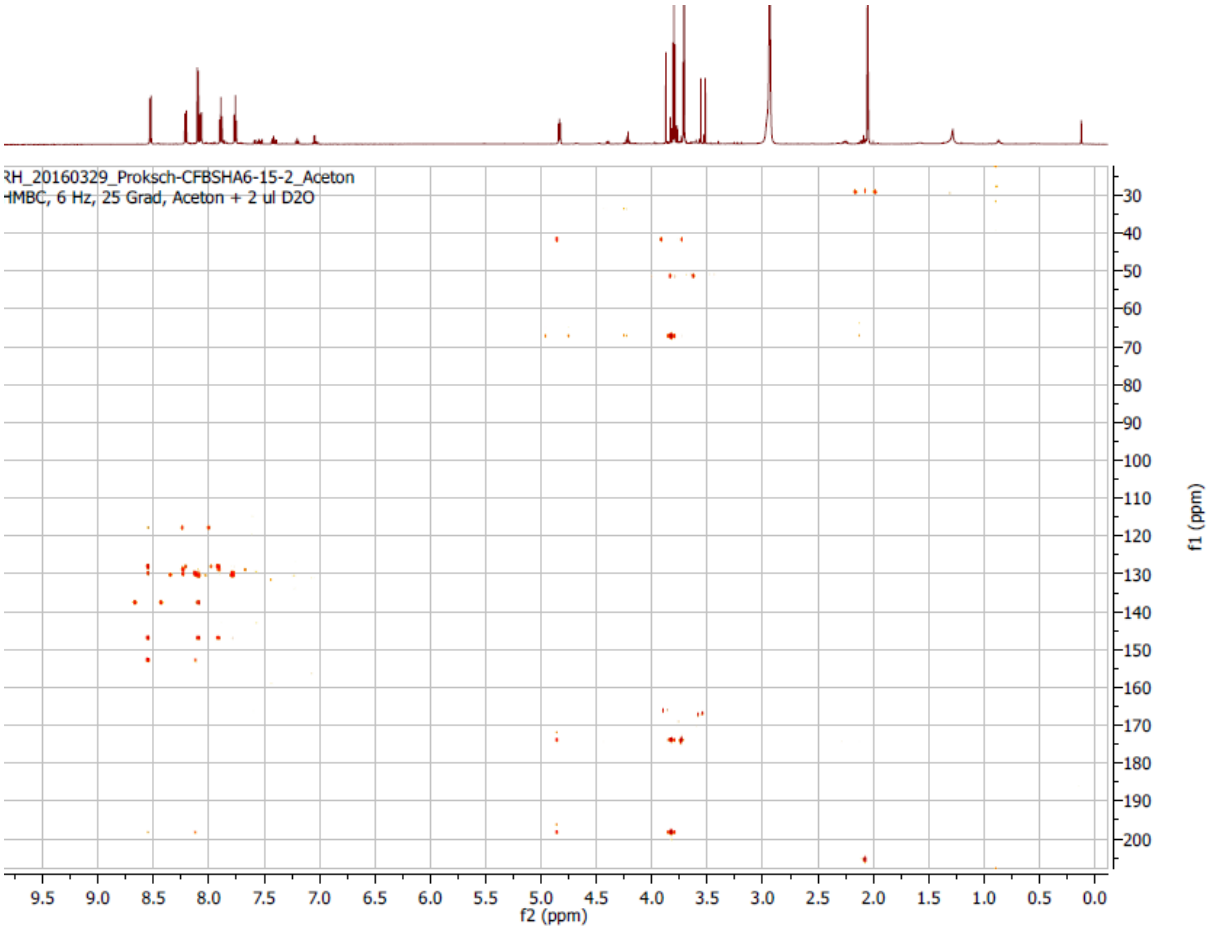


Figure S19. HMBCspectrum of **11** in $(CD_3)_2CO$ (700 MHz and 176 MHz).

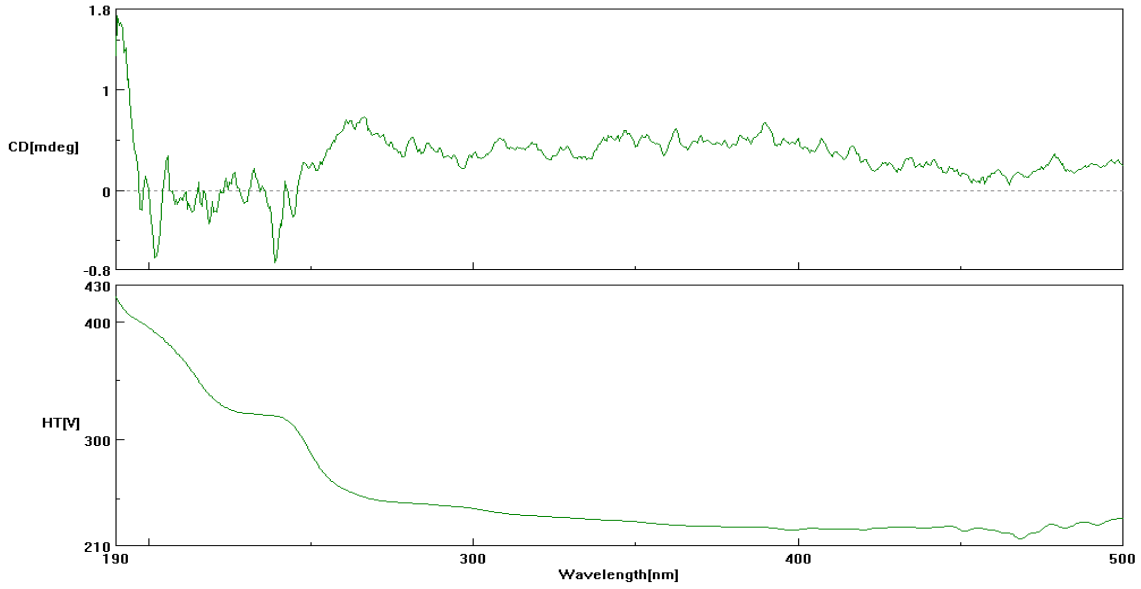


Figure S20. ECD spectrum of **11** in $(CH_3)_2CO$

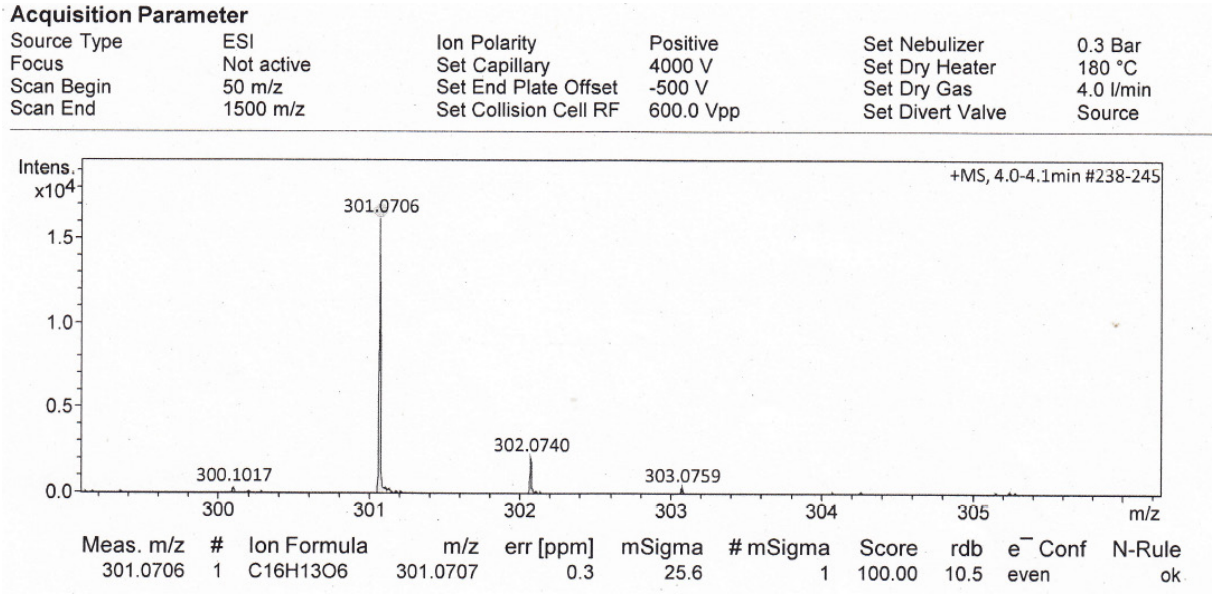


Figure S21. HRESIMS spectrum of compound **12.**

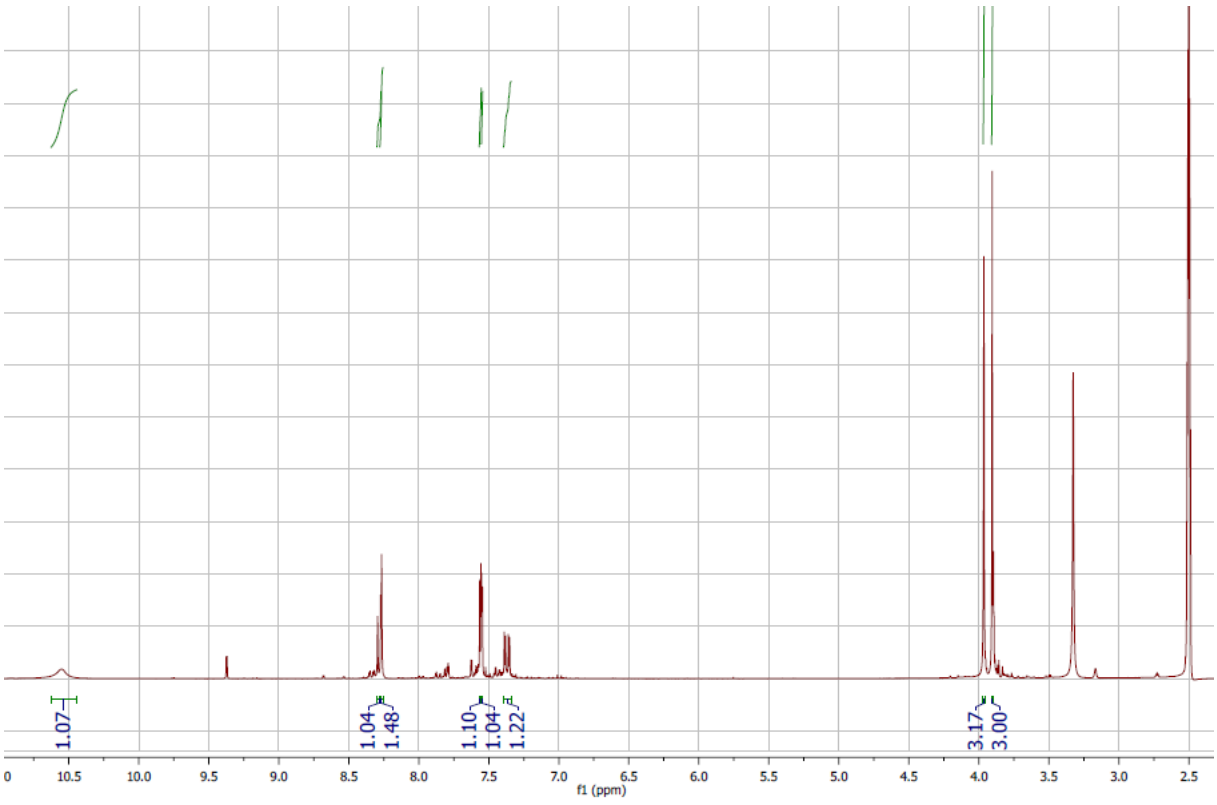


Figure S22. ^{1}H NMR spectrum of **12** in DMSO-d_6 (300 MHz).

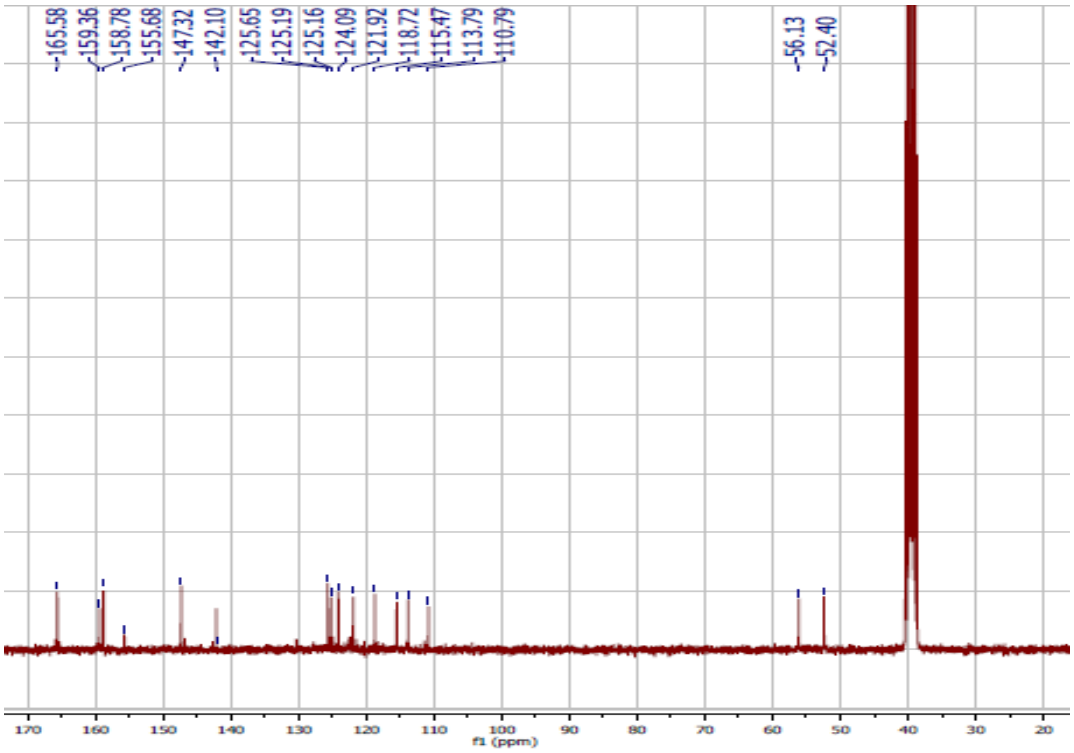


Figure S23. ^{13}C NMR spectrum of **12** in DMSO-d_6 (75.5 MHz).

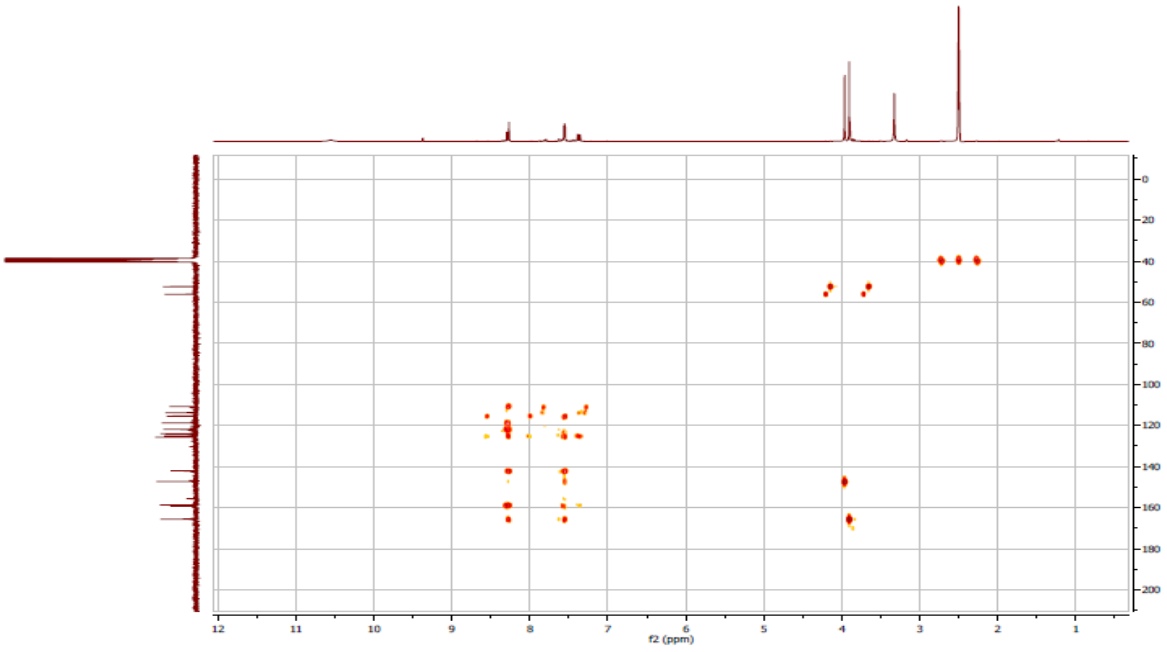


Figure S24. HMBC spectrum of **12** in DMSO-d_6 (300 MHz and 75.5 MHz).

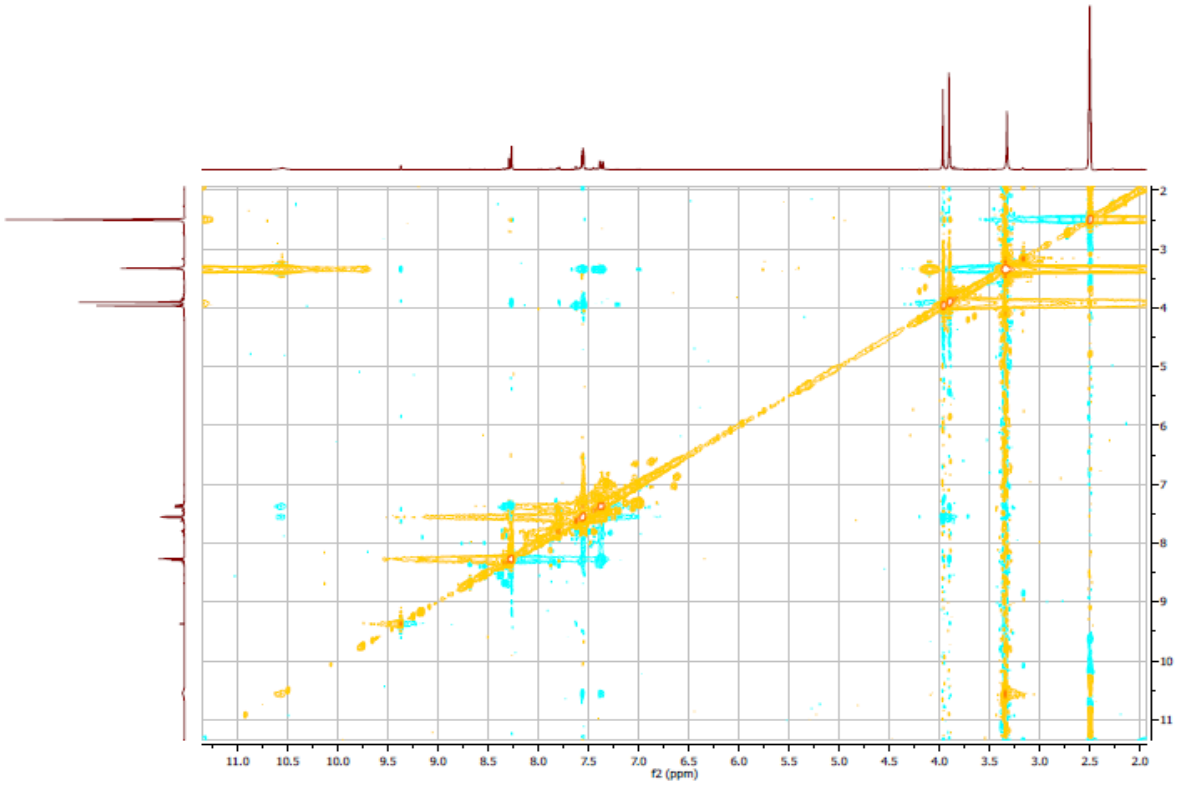


Figure S25. ROESY spectrum of **12** in DMSO-d_6 (300 MHz).

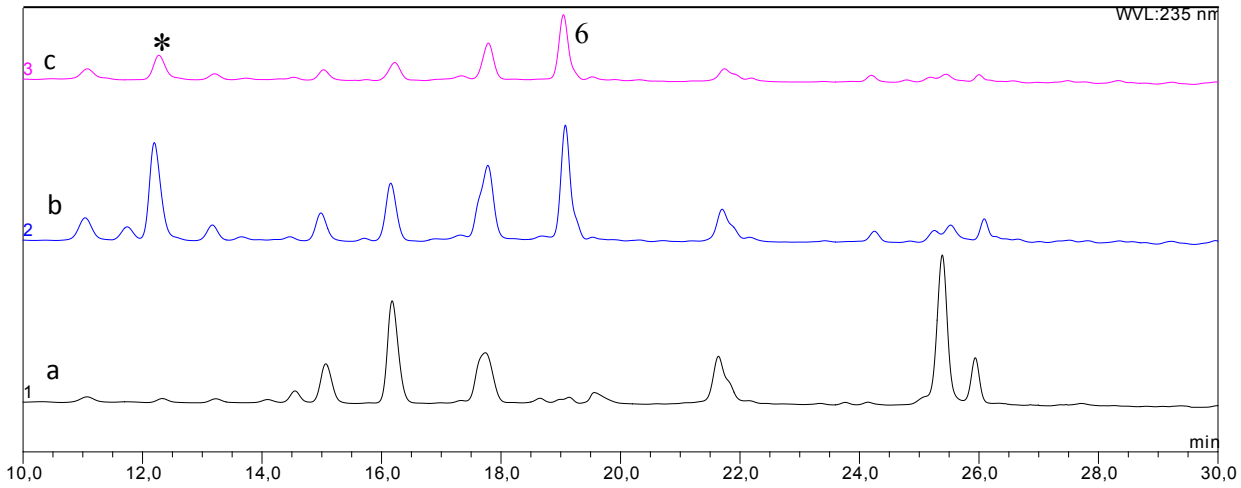


Figure. S26. HPLC chromatograms of EtOAc extracts from epigenetic modifiers experiments (detection at UV 235 nm): (a) *Chaetomium* sp. control, (b) *Chaetomium* sp. treated with SAHA, (c) *Chaetomium* sp. treated with 5-azacytidine (*unidentified metabolite).

Chapiter 3

Cytosporins F–K, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*

Published in “Tetrahedron letters”

Impact Factor: 2.379,

The overall contribution to the paper: 75% of the first author. The first author involved to all laboratory works as well as the manuscript preparation.

Tetrahedron Letters 54 (2013) 6751–6754

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Cytosporins F–K, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*

Sergi Herve Akone [a,b], Mustapha El Amrani [a], Wenhan Lin [c], Daowan Lai [a,*], Peter Proksch [a,*]

[a] Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, Geb. 26.23, 40225 Düsseldorf, Germany
[b] Faculty of Science, Department of Chemistry, University of Douala, 24157 Douala, Cameroon
[c] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Health Science Center, 100191 Beijing, People's Republic of China

ARTICLE INFO

Article history:
Received 19 August 2013
Revised 25 September 2013
Accepted 1 October 2013
Available online 9 October 2013

Keywords:
Endophyte
Pestalotiopsis theae
Turraeanthus longipes
Cytosporin
Structure elucidation

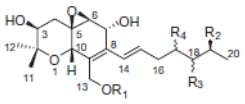
ABSTRACT

Chemical investigation of an endophytic fungus, *Pestalotiopsis theae*, isolated from the leaves of *Turraeanthus longipes* (Meliaceae) collected in Cameroon, resulted in the isolation of six new epoxyquinols, cytosporins F–K (**2**–**7**), together with the known cytosporin D (**1**). The structures of the new compounds were unambiguously determined by analysis of the 1D, 2D NMR, and HRMS spectra. Cytosporins G–K (**3**–**7**) are the first cytosporins with a hydroxyl substituted C_7 side chain, while cytosporins F–I (**2**–**5**) contain a 13-acetoxyl group that was not reported previously. A plausible biosynthetic pathway for the cytosporin derivatives is proposed.

In recent years, plant endophytic fungi have attracted considerable attention as promising sources of new bioactive compounds, which are often structurally unique and display various biological activities, such as antimicrobial activity and cytotoxicity.[1–3] In continuation of our search for new bioactive compounds from endophytes,[4,5] we isolated an endophytic strain *Pestalotiopsis theae* from leaves of the medicinal plant *Turraeanthus longipes* (Meliaceae) collected in Cameroon. *T. longipes* is a shrub commonly distributed in tropical Africa, and used in traditional medicine to treat abdominal and leg pains.[6]

The genus *Pestalotiopsis*[7,8] is reported to be a rich source of natural products with diverse chemical structures and biological activities. *P. theae* is known as a pathogenic fungus which causes tea gray blight; chemical investigation of this fungus has led to the identification of several phytotoxins,[9,10] plant growth regulators,[11] and nitrogen-containing compounds with inhibitory activities against HIV-1 replication.[12] In addition, *P. theae* was also reported as an endophytic fungus from an unidentified tree, fermentation of which on solid rice medium has afforded four pyran derivatives.[13] In the present study, we performed a chemical investigation of the titled fungus that was fermented on a solid rice medium. The fungal extract was chromatographed over silica gel and Sephadex LH-20, and compounds were purified by semi-preparative HPLC to yield the known natural product cytosporin D (**1**),[14] along with its six new derivatives (**2**–**7**) (Fig. 1). Herein, we report the isolation and structure elucidation of the new compounds.

Cytosporin F (**2**)[15] was isolated as a colorless oil. Its molecular formula was established as $C_{21}H_{32}O_6$ by HRESIMS, as a prominent peak was observed at *m/z* 398.25353 ($[M+NH_4]^+$, calcd for $C_{21}H_{36}NO_6$, 398.25371). The ^1H and ^{13}C NMR data of **2** (Tables 1 and 2) were similar to those of cytosporin D (**1**),[14] except that the oxygenated methylene protons of **2** [δ_H 4.74 (d, J = 12.0 Hz, H-13a), 4.67 (dd, J = 12.0, 1.2 Hz, H-13b)] were significantly shifted to the downfield region compared to those of **1**, and one acetoxyl



	R_1	R_2	R_3	R_4
1	H	H	H	H
2	Ac	H	H	H
3	Ac	OH	H	H
4	Ac	H	OH	H
5	Ac	H	H	OH
6	H	H	OH	H
7	H	H	H	OH

Figure 1. Structures of the isolated cytosporins (**1**–**7**).

* Corresponding authors. Tel.: +49 211 81 14187 (D.L.); tel.: +49 211 81 14163; fax: +49 211 81 11923 (P.P.).
E-mail addresses: laidaowan123@gmail.com (D. Lai), proksch@uni-duesseldorf.de (P. Proksch).

0040-4039/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tetlet.2013.10.005

Table 1
^{13}C NMR data of **2**–**7** (150 MHz, CD_3OD)

Position	**2** δ_C, m[a]	**3** δ_C, m[a]	**4** δ_C, m[a]	**5** δ_C, m[a]	**6** δ_C, m[a]	**7** δ_C, m[a]
2	77.5 s	77.5 s	77.5 s	77.5 s	77.5 s	77.5 s
3	74.0 d	74.1 d	74.0 d	74.0 d	74.1 d	74.1 d
4	36.7 t	36.7 t	36.7 t	36.7 t	36.9 t	36.9 t
5	59.9 s	59.9 s	59.9 s	59.9 s	59.9 s	59.9 s
6	63.5 d	63.5 d	63.4 d	63.4 d	63.5 d	63.5 d
7	67.2 d	67.2 d	67.2 d	67.2 d	67.2 d	67.2 d
8	138.8 s	138.6 s	138.6 s	138.6 s	135.7 s	135.5 s
9	126.7 s	126.8 s	126.9 s	127.0 s[b]	131.1 s	131.4 s[b]
10	68.1 d	68.1 d	68.1 d	68.1 d	67.6 d	67.6 d
11	16.4 q	16.4 q	16.4 q	16.4 q	16.5 q	16.5 q
12	28.1 q	28.1 q	28.1 q	28.1 q	28.1 q	28.1 q
13	63.2 t	63.1 t	63.1 t	63.1 t	60.3 t	60.3 t
14	125.7 d	126.0 d	125.8 d	127.9 d	125.9 d	128.0 d
15	138.6 d	138.4 d	138.3 d	134.8 d	137.3 d	133.8 d
16	34.6 t	34.6 t	31.0 t	42.7 t	31.0 t	42.8 t
17	30.2 t	26.6 t	37.5 t	72.0 d	37.6 t	72.1 d
18	32.7 t	39.7 t	73.3 d	40.2 t	73.4 d	40.2 t
19	23.7 t	68.6 d	31.3 t	20.0 t	31.2 t	20.1 t
20	14.5 q	23.7 q	10.5 q	14.6 q	10.5 q	14.6 q
–OAc	172.8 s, 21.0 q	172.8 s, 21.0 q	172.8 s, 21.0 q	172.8 s, 21.0 q		

[a] Multiplicities were assigned based on DEPT-135, and HSQC experiments.
[b] Signal deduced from the HMBC spectrum.

Table 2
^{1}H NMR data of **2**–**7** (600 MHz, CD_3OD)

Position	**2** δ_H, m (*J* in Hz)	**3** δ_H, m (*J* in Hz)	**4** δ_H, m (*J* in Hz)	**5** δ_H, m (*J* in Hz)	**6** δ_H, m (*J* in Hz)	**7** δ_H, m (*J* in Hz)
3	3.56 dd (12.0, 5.0)	3.56 dd (12.0, 5.0)	3.56 dd (12.0, 5.0)	3.56 dd (12.0, 5.0)	3.56 dd (12.0, 5.0)	3.56 dd (12.0, 5.0)
4 ax	2.19 dd (12.9, 11.9)	2.19 dd (12.9, 12.0)	2.19 dd (12.9, 12.0)	2.20 dd (12.9, 11.9)	2.21 dd (12.8, 12.0)	2.21 dd (12.8, 12.0)
eq	1.64 dd (12.9, 5.0)	1.64 dd (12.9, 5.0)	1.64 dd (12.9, 5.0)	1.64 dd (12.9, 5.0)	1.64 dd (12.8, 5.0)	1.64 dd (12.8, 5.0)
6	3.38 br d (2.3)	3.38 br d (2.5)	3.38 br d (2.7)	3.38 br d (2.5)	3.37 br d (2.7)	3.37 br d (2.9)
7	4.63 br s	4.63 br s	4.64 br s	4.65 br s	4.62 br s	4.64 br s
10	4.34 s	4.34 s	4.34 s	4.34 s	4.47 s	4.47 s
11	1.27 s	1.26 s	1.26 s	1.26 s	1.30 s	1.30 s
12	1.23 s	1.23 s	1.23 s	1.23 s	1.24 s	1.24 s
13	4.74 d (12.0)	4.75 d (12.0)	4.75 d (12.0)	4.76 d (12.0)	4.30 d (12.0)	4.31 d (12.0)
	4.67 dd (12.0, 1.2)	4.67 dd (12.0, 1.1)	4.68 br.d (12.0)	4.69 dd (12.0, 1.2)	4.02 dd (12.0, 1.3)	4.03 dd (12.0, 1.3)
14	6.13 d (16.0)	6.15 d (15.9)	6.17 d (15.9)	6.19 d (16.0)	6.18 d (15.9)	6.20 d (16.0)
15	5.97 dt (16.0, 6.9)	5.97 dt (15.9, 6.9)	5.99 dt (15.8, 6.9)	6.00 dt (16.0, 7.1)	5.98 dt (15.9, 7.0)	5.98 dt (16.0, 7.1)
16	2.16 q-like (7.0)	2.17 m	2.30 m	2.33 dddd	2.29 m	2.33 dddd
			2.23 m	(14.0, 7.1, 5.3, 1.2)	2.22 m	(14.0, 7.1, 5.2, 1.1)
				2.26 dddd		2.26 dddd
				(14.0, 7.2, 7.2, 1.2)		(14.0, 7.1, 7.1, 1.1)
17	1.44 pent (7.1)	1.55 m	1.58 m	3.65 m	1.58 m	3.65 m
		1.46 m	1.52 m		1.53 m	
18	1.33 m	1.46 m	3.48 m	1.47 m	3.48 m	1.48 m
				1.41 m		1.41 m
19	1.34 m	3.73 m	1.49 m	1.48 m	1.49 m	1.48 m
			1.42 m	1.37 m	1.43 m	1.38 m
20	0.91 t (7.1)	1.15 d (6.2)	0.94 t (7.4)	0.93 t (6.9)	0.94 t (7.5)	0.93 t (6.9)
–OAc	2.03 s	2.04 s	2.04 s	2.03 s		

group (δ_H 2.03 s; δ_C 172.8 s, 21.0 q) was present in the former. Taking into consideration their difference in molecular mass (42 amu),

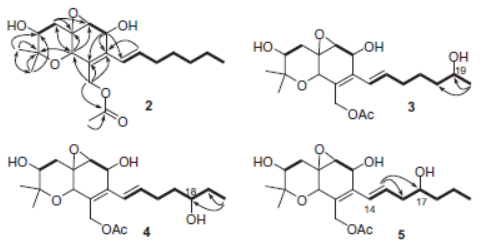


Figure 2. ^{1}H–^{1}H COSY (**bold**) and selected HMBC (H → C) correlations of **2**–**5**.

2 was deduced as a 13-acetoxy derivative of **1**, which was confirmed by the HMBC correlations observed from H_2-13 to the carbonyl carbon of the acetyl group (Fig. 2). The stereochemistry of **2** was suggested to be the same as **1**, for which the absolute configuration is known,[14] by comparison of the coupling constants, ROESY correlations, as well as the specific optical rotation value.

Cytosporin G (**3**)[16] was also isolated as a colorless oil, and exhibited a UV maximum absorption at 239.0 nm, similar to that of cytosporins D (**1**) and F (**2**). It exhibited a pseudomolecular ion peak at *m*/*z* 419.20442 $[M+Na]^+$ in the HRESIMS spectrum, indicating the molecular formula $C_{21}H_{32}O_7$, which contains one more oxygen atom compared to **2**. A comparison of the NMR data of **2** and **3** (Tables 1 and 2) reveals their great similarities, and the only noticeable difference was ascribed to the presence of an oxygenated methine group (δ_H 3.73 m; δ_C 68.6 d) of the latter instead of

a methylene group of the former, suggesting one additional hydroxyl group being present in **3**. This hydroxyl group was deduced to be located at C-19 of the side chain by analysis of the 2D NMR spectra (^{1}H–^{1}H COSY, HSQC, and HMBC). In the ^{1}H–^{1}H COSY spectrum, a diagnostic correlation was seen between the terminal methyl group (δ_H 1.15, d, J = 6.2 Hz, Me-20) and the oxymethine proton (δ_H 3.73, m, H-19) (Fig. 2). In the HMBC spectrum, cross peaks of the proton signal of this methyl group (Me-20) to the signals of C-18 (δ_C 39.7, t), and C-19 (δ_C 68.6, d) were observed (Fig. 2). Hence, compound **3** is a 19-hydroxylated derivative of **2**. To determine the absolute configuration of C-19, the modified Mosher's method was applied.[17] According to a convenient Mosher ester procedure carried out in NMR tubes,[18] the corresponding (*R*)- and (*S*)-MTPA esters (**3r** and **3s**) of **3** were prepared by treatment with (*S*)-MTPACl and (*R*)-MTPACl, respectively. After analysis of the ^{1}H NMR and ^{1}H–^{1}H COSY spectra, the chemical shift differences between the (*R*)- and (*S*)-MTPA esters ($\Delta\delta^{SR} = \delta$**3s**$-\delta$**3r**) were obtained, which suggested a 19*S* configuration (Fig. 3). In addition, analysis of the $\Delta\delta^{SR}$ values around C-7, allowed the assignment of a 7*R* configuration, which is the same as reported for cytosporin D,[14] whereas the unequivocal absolute configuration assignment for C-3 could not be done, as anomalous $\Delta\delta^{SR}$ values were observed for H-4ax (+0.101), and H-4eq (−0.014). From a biogenetic point of view, the core structure of **3** should retain the same stereochemistry as that of the co-occurring cytosporin D (**1**).

The molecular formula of cytosporin H (**4**)[19] was determined as $C_{21}H_{32}O_7$ by HRESIMS, which was the same as that of cytosporin G (**3**). The similar UV and NMR data suggested that **4** was an isomer of **3**. Comparison of their NMR data (Tables 1 and 2) revealed that **4** differed from **3** only in the side chain, as the terminal methyl group appeared as a triplet (δ_H 0.94, t, J = 7.4 Hz) in **4**, instead of a doublet in **3**, suggesting that the hydroxyl group in the side chain of **4** was not located at C-19. That the hydroxyl group was on C-18 was based on the cross peaks of Me-20 (δ_H 0.94, t)/H$_2$-19 (δ_H 1.49, 1.42 m), H$_2$-19/H-18 (δ_H 3.48, m) in the COSY spectrum, and of Me-20/C-19 (δ_C 31.3, t), Me-20/C-18 (δ_C 73.3, d) in the HMBC spectrum (Fig. 2).

The HRESIMS, ^{1}H, and ^{13}C NMR data revealed that cytosporin I (**5**)[20] was also an isomer of **3** and **4**. The only difference among these compounds is the position of the hydroxyl group in the side chain. A combined analysis of the COSY and HMBC spectra permitted us to assign the hydroxyl group to C-17, as the oxymethine proton (H-17, δ_H 3.65 m) showed COSY correlation to H$_2$-16 (δ_H 2.33, 2.26, each dddd), which in turn correlated to the olefinic proton H-15 (δ_H 6.00, dt), while H-15 showed HMBC correlations to C-16 (δ_C 42.7, t), and C-17 (δ_C 72.0, d) (Fig. 2).

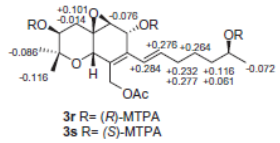
Two minor congeners, cytosporins J (**6**)[21] and K (**7**),[22] were identified as the deacetyl derivatives of cytosporins H (**4**) and I (**5**), respectively. Compounds **6** and **7** share the same molecular formula $C_{19}H_{30}O_6$ as determined by HRMS and NMR. Their molecular weights were 42 amu less than those of **4** and **5**, which were in accordance with the loss of an acetyl group. The NMR data (Tables 1 and 2) of **6** and **7** were almost identical to those of **4** and **5**, respectively, except that the signals for the acetyl group were missing. Moreover, the oxymethylene protons at C-13 of **6** and **7** were significantly shifted to upfield, as compared to those of **4** and **5**, suggesting that the acetoxyl groups at C-13 in the latters were replaced by the hydroxyl groups in the formers. The structures of **6** and **7** were further confirmed by analysis of the 2D NMR spectra, including COSY, HSQC, HMBC, and ROESY.

Cytosporins F–I (**2**–**5**) contain a 13-acetoxyl group, which is unprecedented in the cytosporin derivatives. To exclude the possibility that these acetylated compounds could be artifacts formed during extraction with EtOAc, the 'parent' compounds (i.e., **1**, **6** and **7**) were incubated in EtOAc for 72 h at room temperature; however, they remained unchanged and no formation of **2**, **4**, and **5** was detectable by HPLC and LC–MS.

Cytosporins are a type of hexahydrobenzopyran derivatives, among which cytosporins A–C, the first reported members, are known as angiotensin II binding inhibitors, which were produced by an endophytic *Cytospora* sp. isolated from living bark of *Betula alleghaniensis* (yellow birch).[23] Interestingly, two similar derivatives, named cytosporins D and E, were isolated from the mollusk (pulmonate *Onchidium* sp.) derived fungus *Eutypella scoparia*.[14] It is worth mentioning that cytosporin D has also been reported as a secondary metabolite of the endophytic fungus *Pestalotiopsis* sp. isolated from the branches of *Podocarpus macrophyllus*.[24] Notably, cytosporin D was assumed to be the precursor of pestaloquinols A and B, which contain a novel nonacyclic skeleton and showed cytotoxicity against HeLa cells.[24] Recently, the total synthesis of cytosporin D has been completed.[25]

In the current study, six new cytosporin derivatives, cytosporins F–K (**2**–**7**), were identified from the titled fungus. These compounds feature an uncommon 13-acetoxyl group and/or a hydroxylated side chain that was not reported previously. The absolute configuration of the secondary alcohol in the side chain could be determined by applying the modified Mosher's method, as in the case of **3**, however, this method is not applicable to the remaining compounds due to the limited amount. Thus, the stereochemistry of the chiral center in the side chain of **4**–**7** remains unclear.

A plausible biosynthetic pathway is proposed for the cytosporin derivatives (Fig. 4). It is possible that the biosynthesis starts from a quinol, as a similar derivative epiepoxydon has been isolated from *P. longiesta*,[9] which was reported to originate from gentisaldehyde.[9] Subsequent addition of an isoprenyl unit, followed by epoxidation of the C-2/3 double bond and hydrogenation of the keto group would give the epoxyquinol (**S1**). Similar structures, arthrobotrisins A–C, with a sesquiterpene unit attached to C-3 of the epoxyquinol ring, were isolated from a carnivorous fungus *Arthrobotrys oligospora*.[26] Then, a C$_5$ or C$_7$ aliphatic chain formed through the polyketide pathway is added to C-5 of the epoxyquinol to give the adduct (**S2**). It is interesting to note that a similar compound, ambuic acid, was isolated from the endophytic *Pestalotiopsis* spp. and *Monochaetia* sp., although the isoprenyl unit is located at C-3 of the epoxy group. The following attack from the hydroxyl group



3r R= (*R*)-MTPA
3s R= (*S*)-MTPA

Figure 3. $\Delta\delta^{SR}$ values (=$\delta_S-\delta_R$, ppm) for (*R*)- and (*S*)-MPTA tri-esters **3r** and **3s**.

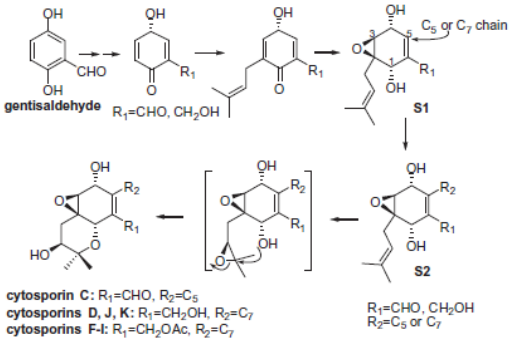


Figure 4. Proposed biosynthetic pathway for cytosporin derivatives.

(C-1) to the epoxy ring of the isoprenyl unit in the diepoxy intermediate, could give cytosporins C, D, and F–K. Cytosporin A is the dehydro product of cytosporin C, while cytosporin B is the epoxy ring-opening derivative of cytosporin C.[23]

Compounds **1**–**7** were tested for their cytotoxicity against the mouse lymphoma cell line L5178Y. However, none exhibited any significant activity (IC_{50} > 10 μg/mL). The isolated compounds were also evaluated for their antibacterial effects. The results showed that they were not active against *Staphyloccocus aureus* ATCC 25922, *Streptococcus pneumoniae* ATCC 49619, and *Escherichia coli* ATCC 25922 even at the highest test concentration of 64 μg/mL. Similarly, cytosporin D was reported to have no inhibitory activity against *S. aureus*, and *E. coli*.[14]

Acknowledgements

The financial support from BMBF granted to P.P. is gratefully acknowledged. S.H.A. wants to thank DAAD (Deutscher Akademischer Austauschdienst) for a doctoral scholarship. We are indebted to Professor W.E.G. Müller (Johannes Gutenberg University, Mainz, Germany) for cytotoxicity assays and to Professor H. Brötz-Oesterhelt (Heinrich-Heine University, Düsseldorf, Germany) for antibacterial assays.

Supplementary data

Supplementary data (experimental procedures for the isolation, identification and fermentation of the fungal strain, and the extraction and isolation of secondary metabolites from the fungal extract, as well as the biological tests) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.10.005.

References and notes

1. Aly, A. H.; Debbab, A.; Proksch, P. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1829–1845.
2. Aly, A. H.; Debbab, A.; Kjer, J.; Proksch, P. *Fungal Divers.* **2010**, *41*, 1–16.
3. Zhang, H. W.; Song, Y. C.; Tan, R. X. *Nat. Prod. Rep.* **2006**, *23*, 753–771.
4. Ebrahim, W.; Aly, A. H.; Wray, V.; Mándi, A.; Teiten, M.-H.; Gaascht, F.; Orlikova, B.; Kassack, M. U.; Lin, W.; Diederich, M.; Kurtán, T.; Debbab, A.; Proksch, P. *J. Med. Chem.* **2013**, *56*, 2991–2999.
5. Bara, R.; Zerfass, I.; Aly, A. H.; Goldbach-Gecke, H.; Raghavan, V.; Sass, P.; Mándi, A.; Wray, V.; Polavarapu, P. L.; Pretsch, A.; Lin, W.; Kurtán, T.; Debbab, A.; Brötz-Oesterhelt, H.; Proksch, P. *J. Med. Chem.* **2013**, *56*, 3257–3272.
6. Ekwalla, N.; Tongo, E. *Nos Plantes qui soignent*; Douala: Cameroon, 2003. pp 34–78.
7. Xu, J.; Ebada, S. S.; Proksch, P. *Fungal Divers.* **2010**, *44*, 15–31.
8. Yang, X.-L.; Zhang, J.-Z.; Luo, D.-Q. *Nat. Prod. Rep.* **2012**, *29*, 622–641.
9. Nagata, T.; Ando, Y.; Hirota, A. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 810–811.
10. Kimura, Y.; Kouge, A.; Nakamura, K.; Koshino, H.; Uzawa, J.; Fujioka, S.; Kawano, T. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1624–1626.
11. Shimada, A.; Takahashi, I.; Kawano, T.; Kimura, Y. Z. *Naturforsch* **2001**, *56b*, 797–803.
12. Ding, G.; Jiang, L.; Guo, L.; Chen, X.; Zhang, H.; Che, Y. *J. Nat. Prod.* **2008**, *71*, 1861–1865.
13. Li, E.; Tian, R.; Liu, S.; Chen, X.; Guo, L.; Che, Y. *J. Nat. Prod.* **2008**, *71*, 664–668.
14. Ciavatta, M. L.; Lopez-Gresa, M. P.; Gavagnin, M.; Nicoletti, R.; Manzo, E.; Mollo, E.; Guo, Y.-W.; Cimino, G. *Tetrahedron* **2008**, *64*, 5365–5369.
15. *Cytosporin F (2):* Colorless oil; $[\alpha]_D^{20}$ −5.2 (*c* 0.075, MeOH); UV (MeOH) λ_{max} (log ε) 239.5 (2.88) nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR data in CD_3OD, see Tables 1 and 2; ESIMS *m/z* 380.9 $[M+H]^+$, 782.7 $[2M+Na]^+$; HRESIMS *m/z* 398.25353 $[M+NH_4]^+$ (calcd for $C_{21}H_{36}NO_6$, 398.25371).
16. *Cytosporin G (3):* Colorless oil; $[\alpha]_D^{20}$ −2.4 (*c* 0.125, MeOH); UV (MeOH) λ_{max} (log ε) 239.0 (3.54) nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR data in CD_3OD, see Tables 1 and 2; ESIMS *m/z* 419.1 $[M+Na]^+$, 814.7 $[2M+Na]^+$; HRESIMS *m/z* 419.20442 $[M+Na]^+$ (calcd for $C_{21}H_{32}NaO_7$, 419.20402).
17. Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.
18. Su, B.-N.; Park, E. J.; Mbwambo, Z. H.; Santarsiero, B. D.; Mesecar, A. D.; Fong, H. H. S.; Pezzuto, J. M.; Kinghorn, A. D. *J. Nat. Prod.* **2002**, *65*, 1278–1282.
19. *Cytosporin H (4):* Colorless oil; $[\alpha]_D^{20}$ −2.5 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 239.0 (3.83) nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR data in CD_3OD, see Tables 1 and 2; ESIMS *m/z* 397.2 $[M+H]^+$, 793.0 $[2M+H]^+$, 815.0 $[2M+Na]^+$; HRESIMS *m/z* 397.2220 $[M+H]^+$ (calcd for $C_{21}H_{33}O_7$, 397.2221).
20. *Cytosporin I (5):* Colorless oil; $[\alpha]_D^{20}$ −9.2 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 239.7 (3.80) nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR data in CD_3OD, see Tables 1 and 2; ESIMS *m/z* 419.3 $[M+Na]^+$, 815.0 $[2M+Na]^+$; HRESIMS *m/z* 419.2041 $[M+Na]^+$ (calcd for $C_{21}H_{32}NaO_7$, 419.2040).
21. *Cytosporin J (6):* Colorless oil; $[\alpha]_D^{20}$ −9.4 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 239.9 (3.84) nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR data in CD_3OD, see Tables 1 and 2; ESIMS *m/z* 355.0 $[M+H]^+$, 709.0 $[2M+H]^+$; HRESIMS *m/z* 377.19360 $[M+Na]^+$ (calcd for $C_{19}H_{30}NaO_6$, 377.19346).
22. *Cytosporin K (7):* Colorless oil; $[\alpha]_D^{20}$ −4.5 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 239.1 (3.87) nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR data in CD_3OD, see Tables 1 and 2; ESIMS *m/z* 336.8 $[M+H-H_2O]^+$, 708.8 $[2M+H]^+$, 730.8 $[2M+Na]^+$; HRESIMS *m/z* 372.23808 $[M+NH_4]^+$ (calcd for $C_{19}H_{34}NO_6$, 372.23806).
23. Stevens-Miles, S.; Goetz, M. A.; Bills, G. F.; Giacobbe, R. A.; Tkacz, J. S.; Chang, R. S. L.; Mojena, M.; Martin, I.; Diez, M. T.; Pelae, F.; Hensens, O. D.; Jones, T.; Burg, R. W.; Kong, Y. L.; Huang, L. *J. Antibiot.* **1996**, *49*, 119–123.
24. Ding, G.; Zhang, F.; Chen, H.; Guo, L.; Zou, Z.; Che, Y. *J. Nat. Prod.* **2011**, *74*, 286–291.
25. Vannada, J.; Niehues, L.; König, B.; Mehta, G. *Tetrahedron* **2013**, *69*, 6034–6040.
26. Wei, L.-X.; Zhang, H.-X.; Tan, J.-L.; Chu, Y.-S.; Li, N.; Xue, H.-X.; Wang, Y.-L.; Niu, X.-M.; Zhang, Y.; Zhang, K.-Q. *J. Nat. Prod.* **2011**, *74*, 1526–1530.

Supplementary data

Cytosporins F–K, New Epoxyquinols from the Endophytic Fungus *Pestalotiopsis theae*

Sergi Herve Akone [a,b], Mustapha El Amrani [a], Wenhan Lin [c], Daowan Lai [a,*], Peter Proksch [a,*]

[a] Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, Geb. 26.23, 40225 Düsseldorf, Germany;

[b] Faculty of Science, Department of Chemistry, University of Douala, 24157, Douala, Cameroon

[c] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Health Science Center, 100191 Beijing, People's Republic of China;

* Author to whom correspondence should be addressed; E-Mail: laidaowan123@gmail.com (D. L.); proksch@uni-duesseldorf.de (P. P.); Tel.: +49 211 81 14187 (D. L.), +49 211 81 14163 (P. P.); fax: +49 211 81 11923 (P. P.).

General Experimental Procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. UV spectra were measured in Perkin Elmer Lambda 25 UV/VIS spectrometer. ^{1}H, ^{13}C, and 2D NMR spectra were recorded at 25 °C in CD_3OD on a Bruker ARX 600 NMR spectrometer. Chemical shifts were referenced to the solvent residual peaks, δ_H 3.31 for ^{1}H, and δc 49.15 for ^{13}C. Mass spectra (ESI) were recorded with a Finnigan LCQ Deca mass spectrometer, and HRMS (ESI) spectra were obtained with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed with a Dionex UltiMate3400 SD with a LPG-3400SD Pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. The separation column (125×4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient was used (MeOH, 0.02% H_3PO_4 in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; Pump L-7100; Eurosphere-100 C18, 300×8 mm, Knauer, Germany). Column chromatography included Diaion HP-20, LH-20 Sephadex and Merck MN Silica gel 60 M (0.04-0.063 mm) TLC plates with silica gel F254 (Merck, Darmstadt, Germany) were used to monitor fractions (CH_2Cl_2/MeOH mixtures as mobile phase); detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent**.**

Fungal material

Pestalotiopsis theae was isolated from fresh healthy leaf of *Turraeanthus longipes* (Meliaceae) collected in May 2012 in Kribi, Cameroon. The fungus was isolated under sterile conditions from the inner tissue of the leaf according to the procedure described by Kjer et al.[1] The identification was performed following a molecular biological protocol by DNA amplification and sequencing of the ITS region. A voucher strain was deposited at one of the authors' laboratory (P.P.).

Fermentation, extraction and isolation

Fermentation of the fungus was carried out in two Erlenmeyer flasks (1 L each). The fungus was grown on rice medium (to 100 g commercially available rice was added 110 mL of distilled water and kept overnight prior to autoclaving), at room temperature under static conditions for 40 days.

After incubation, each fungal culture was extracted with EtOAc (3×250 ml). The obtained EtOAc extract (933 mg) was partitioned between n-hexane and 90% MeOH. The 90% MeOH fraction (700 mg) was subjected to vacuum liquid chromatography (VLC) on silica gel employing a step gradient of hexane-ethyl acetate and dichloromethane-methanol to give twelve fractions A-L. Fraction E (65 mg) was further purified by semi-preparative HPLC to afford **1** (5.0 mg) and **2** (1.1 mg). Fraction H (200 mg) was subjected to column chromatography over Sephadex LH-20 using MeOH as eluent to give five subfractions H1-H5. Sub-fraction H5 (45 mg), was purified by semi-preparative HPLC using a gradient of MeOH-H_2O (0.1%TFA), to give **3** (2.5 mg), **6** (1.6 mg), and **7** (1.1 mg). Similarly, sub-fraction H6 was purified by semi-preparative HPLC eluting with a gradient of acetonitrile-H_2O (0.1% TFA) to yield **4** (1.1 mg), and **5** (1.1 mg).

Preparation of Mosher's esters

Aliquots of compound **3** (1 mg each) were dissolved in pyridine-d_5 (0.7 mL) and transferred to NMR tubes, prior to adding of 5 μL of (*R*)-MTPA-Cl and (*S*)-MTPA-Cl reagent (Fluka, Germany), respectively. The tubes were shaken thoroughly and allowed to stand at room temperature for 72 h. The reaction was monitored by ^{1}H NMR and ^{1}H–^{1}H COSY spectroscopy every 24 h. After treatment of **3** with (*R*)-MTPA-Cl, the (*S*)-MTPA tri-ester (**3s**) was obtained. Selected ^{1}H NMR data of **3s** (pyridine-d_5, 600 MHz): δ 5.370 (1H, dd, J = 11.0, 4.9 Hz, H-3), 2.579 (1H, dd, J = 12.8, 11.1 Hz, H-4ax), 2.293 (1H, dd, J = 12.8, 4.8 Hz, H-4eq), 4.012 (1H, d, J = 2.9 Hz, H-6), 6.610 (1H, br.s, H-7), 1.317 (3H, s, Me-11), 1.240 (3H, s, Me-12), 6.298 (1H, d, J = 16.1 Hz, H-14), 5.885 (1H, dt, J =16.1, 6.8 Hz, H-15), 2.062 (2H, m, H_2-16), 1.442 (2H, m, H_2-17), 1.632 (2H, m, H_2-18), 5.220 (1H, m, H-19), 1.203 (d, J = 6.3 Hz, Me-20). Similarly, treatment of **3** with (*S*)-MTPA-Cl afforded the (*R*)-MTPA tri-ester (**3r**). Selected ^{1}H NMR data of **3r** (pyridine-d_5, 600 MHz): δ 5.344 (1H, dd, J = 11.0, 5.0 Hz, H-3), 2.478 (1H, dd, J = 13.0, 11.1 Hz, H-4ax), 2.307 (1H, dd, J = 13.0, 4.9 Hz, H-4eq), 4.088 (1H, d, J = 2.8 Hz, H-6), 6.489 (1H, br.s, H-7), 1.403 (3H, s, Me-11), 1.356 (3H, s, Me-12), 6.014 (1H, d, J = 16.0 Hz, H-14), 5.609 (1H, dt, J = 16.0, 6.7 Hz, H-15), 1.830 (1H, m, H-16a), 1.785 (1H, m, H-16b), 1.178 (2H, m, H_2-17), 1.571 (1H, m, H-18a), 1.516 (1H, m, H-18b), 5.218 (1H, m, H-19), 1.275 (d, J = 6.2 Hz, Me-20).

Bioassay

The cytotoxic and antibacterial assays were performed as described previously[2].

References:

1. Kjer, J.; Debbab, A.; Aly, A. H.; Proksch, P. *Nat. Protoc.* **2010,** *5*, 479-490.

2. El Amrani, M.; Debbab, A.; Aly, A. H.; Wray, V.; Dobretsov, S.; Müller, W. E. G.; Lin, W.; Lai, D.; Proksch, P. *Tetrahedron Lett.* **2012,** *53*, 6721-6724.

Chapiter 4

Unguisin F, a new cyclic peptide from the endophytic fungus *Mucor irregularis*

Published in "Zeitschrift für Naturforschung C"

Impact Factor: 0.552,

The overall contribution to the paper: 80% of the first author. The first author involved to all laboratory works as well as the manuscript preparation.

DE GRUYTER Z. Naturforsch. 2016; 71(1-2)c: 15–19

Sergi H. Akone, Georgios Daletos, Wenhan Lin and Peter Proksch*

Unguisin F, a new cyclic peptide from the endophytic fungus *Mucor irregularis*

DOI 10.1515/znc-2015-0137
Received May 31, 2015; revised October 18, 2015; accepted December 15, 2015

Abstract: The new cyclic heptapeptide unguisin F (**1**) and the known congener unguisin E (**2**), were obtained from the endophytic fungus *Mucor irregularis*, isolated from the medicinal plant *Moringa stenopetala*, collected in Cameroon. The structure of the new compound was unambiguously determined on the basis of one- and two-dimensional NMR spectroscopy as well as by high-resolution mass spectrometry. The absolute configuration of the amino acid residues of **1** and **2** was determined using Marfey's analysis. Compounds **1** and **2** were evaluated for their antibacterial and antifungal potential, but failed to display significant activities.

Keywords: *Moringa stenopetala*; *Mucor irregularis*; peptide.

1 Introduction

Endophytes are microorganisms that live in the internal tissues of their host without causing any apparent disease symptoms [1]. Instead, endophytes may affect their hosts in a symbiotic way [2]. In recent years, plant endophytic fungi have attracted considerable attention as promising sources of new bioactive compounds, which are often structurally unique and display various biological activities, such as cytotoxic and antimicrobial activities [3–5]. Fungi of the genus *Mucor* occur as pathogens in a wide range of fruits, such as apple, pears and strawberries [6]. Nevertheless, they have also been described as endophytes [7] possessing heavy metal bioremediation capacities [8]. *Mucor irregularis* is known as a pathogenic fungus, which causes chronic cutaneous infection in immunocompetent humans, ultimately leading to severe morbidity if left untreated [9].

In the course of our ongoing search for new bioactive secondary metabolites [10, 11], we investigated the chemical constituents of an endophytic strain *M. irregularis*, isolated from leaves of the medicinal plant *Moringa stenopetala* (Moringaceae) collected in Cameroon. *Moringa stenopetala* is a smooth-barked deciduous tropical plant distributed in eastern and western Africa. A report on the various uses of *M. stenopetala* has shown that it is a multipurpose plant of wide use for the community [12]. It has been reported to exhibit antimicrobial activity [13], while its seeds are used as purifiers of muddy and turbid water in Africa [14, 15]. In the present study, a new γ-aminobutyric acid (GABA)-containing cyclic peptide (**1**) together with a known congener (**2**), were isolated. Details on the isolation and structure elucidation of the new compound (**1**), determination of the absolute configurations of **1** and **2** employing Marfey's method [16], as well as results of antibacterial and antifungal assays are reported.

***Corresponding author: Peter Proksch,** Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany, Phone: +49 211 81 14163, Fax: +49 211 81 11923, E-mail: proksch@uni-duesseldorf.de
Sergi H. Akone: Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany; and Faculty of Science, Department of Chemistry, University of Douala, 24157 Douala, Cameroon
Georgios Daletos: Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany
Wenhan Lin: State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Health Science Center, 100191 Beijing, People's Republic of China

2 Results and discussion

The crude EtOAc extract of *M. irregularis*, cultured on solid rice medium, was taken to dryness and partitioned between *n*-hexane and 90 % methanol. The 90 % methanol fraction was chromatographed over different stationary phases (silica gel and Sephadex LH-20). Final purification by semi-preparative HPLC yielded two peptide derivatives (**1** and **2**) (Figure 1).

Compound **1**, was isolated as a white amorphous powder. The molecular formula of **1** was established as $C_{42}H_{58}N_8O_7$ by HRESIMS, as a prominent peak was observed at *m/z* 787.4500 ($[M + H]^+$). The NMR data of **1** were very similar to those recorded for unguisin E (**2**) [17], which

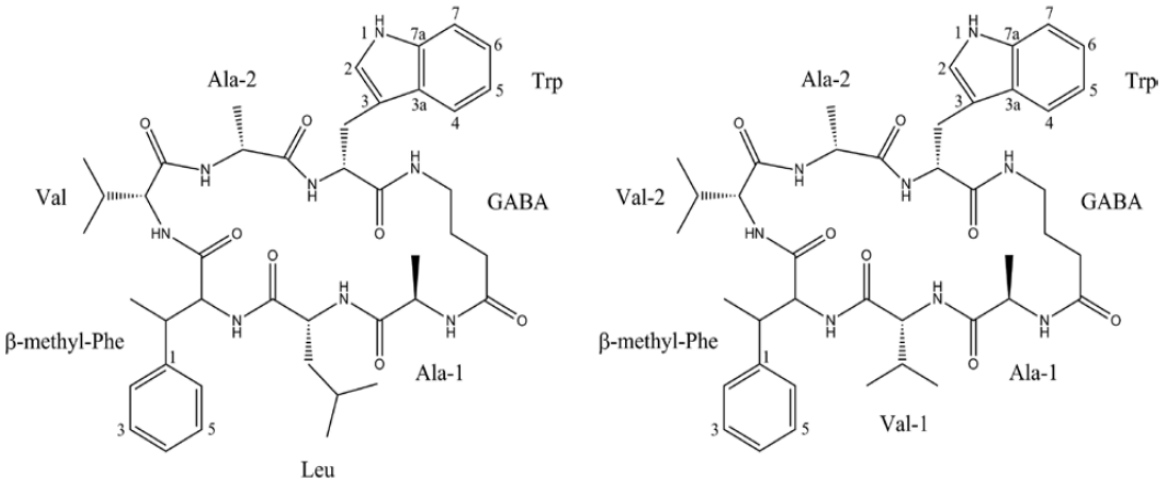


Figure 1: Structures of compounds **1** and **2**.

suggested a close structural relationship between the two compounds. Evidence for the peptide nature of this compound was provided by analysis of the ^{1}H NMR data, which revealed the presence of seven amide NH signals in the region between 7.64 and 8.36 ppm (Table 1). Detailed analysis of the ^{1}H, COSY, TOCSY and HMBC spectra established the individual structures of five α-amino acids including Ala (2 eq.), Val (1 eq.), Leu (1 eq.) and Trp (1 eq.), in addition to γ-aminobutyric acid (GABA) (1 eq.) and the unusual amino acid β-methyl-Phe (1 eq.) (Figure 1). The indole ring system of tryptophan was assigned based on the ^{1}H NMR signals including an ABCD spin system at δ_H 7.52 (H-4, d, J = 7.9 Hz), 6.97 (H-5, t, J = 7.9 Hz), 7.06 (H-6, t, J = 7.9 Hz), and 7.33 (H-7, d, J = 7.9 Hz), an exchangeable proton at δ_H 10.82 (NH-1), and an aromatic proton at δ_H 7.09 (H-2, d, J = 2.3 Hz). This was further corroborated by analysis of the HMBC spectrum, in which the correlations from H-4 to C-7a (δ_C 135.5) and C-6 (δ_C 120.8); H-5 to C-7 (δ_C 111.2) and C-3a (δ_C 127.1); H-6 to C-7a; and H-7 to C-5 (δ_C 118.1) were discerned (Figure 2). Moreover, the presence of a β-methyl substituted phenylalanine unit was evidenced through the COSY correlation between the β-proton at δ_H 3.49 (H-β, m) and the methyl group at δ_H 1.16 (H_3-γ, d, J = 7.2 Hz), in addition to the observed HMBC correlation of the latter (H_3-γ) to the aromatic carbon C-1 (δ_C 142.7).

The connectivity of the respective moieties was accomplished by analysis of the ROESY spectrum of **1**. Accordingly, ROESY correlations (Figure 2) between Trp-NH (δ_H 8.02, d, J = 6.5 Hz)/Ala(-2)-Hα (δ_H 3.90, m) and Ala(-2)-NH (δ_H 8.36, d, J = 6.1 Hz)/Val-Hα (δ_H 4.04, m) disclosed the peptide fragment Trp–Ala(-2)–Val. Likewise, the ROESY correlations between Val-NH (δ_H 7.77, d, J = 9.3 Hz)/β-methyl-Phe-Hα (δ_H 4.38, m); β-methyl-Phe-NH (δ_H 8.30, d, J = 8.8 Hz)/Leu-Hα (δ_H 4.02, m); and Leu-NH (δ_H 7.85, d, J = 5.8 Hz)/Ala(-1)-Hα (δ_H 4.11, m) extended this fragment leading to the partial sequence Trp–Ala(-2)–Val–β-methyl-Phe–Leu–Ala(-1). Finally, the cyclic planar structure of **1** was established based on the cross-peak between GABA-NH (δ_H 7.64, t, J = 5.2 Hz) and Trp-Hα (δ_H 4.09, m) (Figure 2), which is consistent with the eighteen elements of unsaturation required by the molecular formula. These data are similar to those of **2**, except for the replacement of valine unit in **2** by leucine in **1**, which accounts for the 14 amu molecular weight difference between the two compounds. Thus, **1** was identified as a new natural product for which the name unguisin F is proposed.

Compound **2** was identified by analysis of the NMR and MS data and by comparison with the literature as unguisin E, which has been previously reported from a soil-derived fungus *Aspergillus* sp. [17]. It should be noted, however, that only the planar structure of **2** has been reported so far, which prompted us to investigate further the stereochemistry of the respective amino acids.

The absolute configurations of the individual amino acid constituents of **1** and **2** were determined employing Marfey's method [16]. LC-MS analysis of **1** and **2** revealed D-configurations for all amino acids, except for β-methyl-Phe for which no standard was available. The absolute configuration of tryptophan, in the case of **1**, could not be unambiguously determined and further attempts to detect the N*α*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA)-derivatized ion peak in the MS spectrum were

Table 1: ^{1}H NMR (600 MHz, in DMSO-d_6) and ^{13}C NMR (150 MHz, in DMSO-d_6) of **1**.

Position	δ_H (mult., J in Hz)	δ_C[a]	ROESY[b]
Trp			
C=O		[c]	
NH	8.02 (d, J = 6.5)		H-α, H-β (Ala-2)
α	4.09 (m)	52.0	
β	3.21 (2H, m)	24.8	
NH-1	10.82 (d, J = 1.9)		
2	7.09 (d, J = 2.3)	123.4	
3		110.5	
4	7.52 (d, J = 7.9)	118.2	
5	6.97 (t, J = 7.9)	118.1	
6	7.06 (t, J = 7.9)	120.8	
7	7.33 (d, J = 7.9)	111.2	
3a		127.1	
7a		135.5	
GABA			
NH	7.64 (t, J = 5.2)		H-α, H-β (Trp)
α	3.02 (m), 3.06 (m)	38.6	
β	1.61 (m), 1.69 (m)	25.3	
γ	1.96 (m), 2.12 (m)	32.7	
C=O (γ)		171.7	
Ala-1			
C=O		172.6	
NH	7.82 (d, J = 5.7)		H-γ (GABA)
α	4.11 (m)	48.0	
β	1.14 (d, J = 7.0)	17.4	
Leu			
C=O		[c]	
NH	7.85 (d, J = 5.8)		H-α, H-β (Ala-1)
α	4.02 (m)	54.9	
β	1.23 (m)	39.4	
γ	0.96 (m)	23.3	
δ	0.66 (d, J = 6.5)	22.4	
δ	0.80 (d, J = 6.5)	22.1	
β–Methyl-Phe			
C=O		170.3	
NH	8.30 (d, J = 8.8)		H-α, H-β (Leu)
α	4.38 (m)	58.5	
β	3.49 (m)	38.9	
γ	1.16 (d, J = 7.2)	14.0	
1		142.7	
2	7.21 m	127.4	
3	7.22 m	127.6	
4	7.14 m	125.8	
5	7.22 m	127.6	
6	7.21 m	127.4	
Val			
C=O		172.0	
NH	7.77 (d, J = 9.3)		H-α, H-β (β –Methyl-Phe)
α	4.04 (m)	58.0	
β	2.01 (m)	29.4	
γ	0.63 (d, J = 6.6)	18.2	
γ	0.68 (d, J = 6.6)	18.5	
Ala-2			
C=O		172.6	
NH	8.36 (d, J = 6.1)		H-α (Val)
α	3.90 (m)	49.9	
β	1.18 (3H, d, J = 6.7)	16.7	

[a]Data extracted from HSQC and HMBC spectra. [b]Sequential ROEs. [c]Not observed.

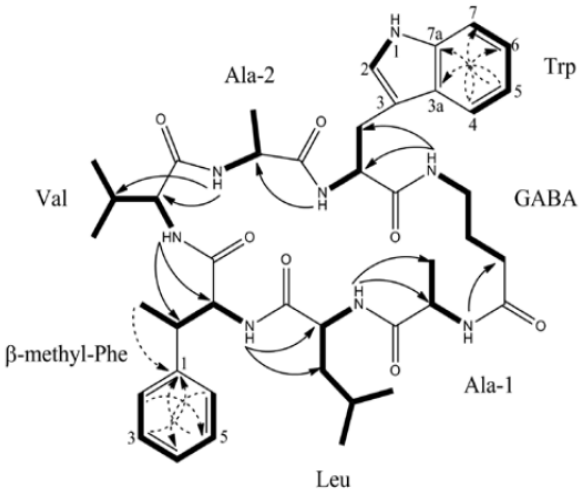


Figure 2: COSY (bold), selected HMBC (dashed) and ROESY (plain) correlations of **1**.

impeded due to the limited amount of sample. However, based on the similar NMR data and optical rotations for both compounds, as well as on their close biogenetic relationship; it could be assumed that the tryptophan unit in **1** possesses the D-configuration as assigned for **2**. The proposed absolute configurations of the amino acid residues of **1** and **2** are in agreement with those of reported unguisin derivatives [18], thus indicating a high specificity of the non-ribosomal peptide synthetases involved in the synthesis of these peptides.

Interestingly, the first unguisin substances, *viz.* unguisin A, B, and C, were obtained from the fungus *Emericella unguis*, isolated from the jellyfish *Stomolophus meleagris*, collected in Venuzuelan waters [18, 19]. Subsequently, the analogue unguisin D was detected upon feeding the culture medium of *E. unguis* with L-leucine [19]. These peptides are unusual in containing a GABA moiety in their macrocycle, as well as a high proportion of hydrophobic amino acids possessing D configuration. GABA-containing natural products are rarely encountered in nature and include to date imacidins A-E from *Streptomyces olivaceus* [20] and N-acylasparagylpolyamine derivatives from *Nephilengys borbonica* [21]. The incorporation of a GABA moiety in unguisins is suggested to enhance their conformational mobility [18].

In an attempt to explore the biological potential of this interesting class of peptides, compounds **1** and **2** were evaluated for their antibacterial activities, but failed to show activity against *Staphyloccocus aureus* ATCC 25922, *Streptococcus pneumonia* ATCC 49619 and *Escherichia coli* ATCC 25922 at a concentration of 64 µg/mL. Both compounds were further tested for their antifungal

activity against *Cladosporium cladosporioides* at a dose of 50 µg/disk. However, none exhibited significant activity.

3 Experimental section

3.1 General experimental procedure

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ^{1}H, ^{13}C and 2D NMR spectra were recorded at 25 °C in DMSO-d_6 on a Bruker ARX 600 NMR spectrometer. Chemicals shifts were referenced to the solvent residual peaks, δ_H 2.50 for ^{1}H and δ_C 39.5 for ^{13}C NMR. Mass spectra (ESI) were recorded with a Finnigan LCQ Deca mass spectrometer, and HRMS (ESI) spectra were obtained with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed with a Dionex UltiMate3400 SD with a LPG-3400SD Pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient was used (MeOH, 0.1 % HCOOH in H_2O): 0 min (10 % MeOH), 5 min (10 % MeOH), 35 min (100 % MeOH), 45 min (100 % MeOH). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; Pump L-7100; Eurosphere-100 C18, 300 mm × 8 mm, Knauer, Berlin, Germany). Column chromatography included LH-20 Sephadex and Merck MN Silica gel 60 M (0.04–0.063 mm). TLC plates with silica gel F254 (Merck, Darmstadt, Germany) were used to monitor fractions (CH_2Cl_2/MeOH mixtures as mobile phase); detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent.

3.2 Fungal material

Mucor irregularis was isolated from fresh healthy leaves of *M. stenopetala* (Moringaceae) collected in Koukoue, Littoral, Cameroon in July 2013. The fungus was isolated under sterile conditions from the inner tissue of the leaf according to the procedure described by Kjer et al. [22]. The identification was performed following a molecular biological protocol by DNA amplification and sequencing of the ITS region. The sequence data have been submitted to GenBank, accession number KP067786.

3.3 Fermentation, extraction and isolation

Fermentation of the fungus was carried out in two flasks (1 L each). The fungus was grown on rice medium (to 100 g commercially available rice was added 110 mL of distilled water and kept overnight prior to autoclaving), at room temperature under static conditions for 40 days.

After incubation, each fungal culture was extracted with EtOAc (3 × 250 mL). The obtained EtOAc extract (856 mg) was partitioned between *n*-hexane and 90 % MeOH. The 90 % MeOH fraction (598.4 mg) was subjected to vacuum liquid chromatography (VLC) on silica gel employing a step gradient of hexane-ethyl acetate and dichloromethane-methanol to give six fractions A–F. Fraction E (20 % dichloromethane in methanol, 210.8 mg) was subjected to column chromatography over Sephadex LH-20 using MeOH as eluent to give five subfractions E1–E5. Sub-fraction E5 (45 mg), was further purified by semi-preparative HPLC using a gradient of MeOH-H_2O (0.1 % TFA), to afford **1** (1.3 mg), **2** (3.5 mg).

Compound **1**. White amorphous powder. $[\alpha]_D^{20}$ + 34.7 (*c* 0.05, MeOH); UV (MeOH) λ_{max} 217.4, 281.1 nm; ^{1}H (600 MHz), and ^{13}C (150 MHz) NMR in DMSO-d_6, see Table 1; ESIMS *m/z* 787.4500 $[M + H]^+$ (calcd for $C_{42}H_{59}N_8O_7$, 787.4501).

3.4 Advanced Marfey's analysis of 1 and 2

Marfey's method [16] was used to determine the absolute configuration of the **1** and **2**. Compounds **1** and **2** (0.5 mg) were separately dissolved in 1 mL of 6 N HCl and heated at 110 °C for 24 h. This solution was evaporated, and traces of HCl were removed by repeatedly drying the compounds under vacuum after addition of distilled water. To the hydrolysate (0.5 mg), 20 µL of 1 N $NaHCO_3$ and 100 µL of 1 % L-FDAA in acetone were added. The mixture was stirred at 40 °C for 1 h. After the reaction had been quenched by addition of 10 µL of 2 N HCl, the mixture was analysed by ESI-LC/MS to assign the chirality of the amino acids. Derivatization of each commercially available standard amino acid (D- or L-form) being of interest was achieved in the same manner as applied to the isolated peptides. The retention times of the derivatized standard amino acids and of the derivatized amino acids obtained following hydrolysis of the peptide were compared to distinguish D- and L-amino acids.

3.5 Bioassays

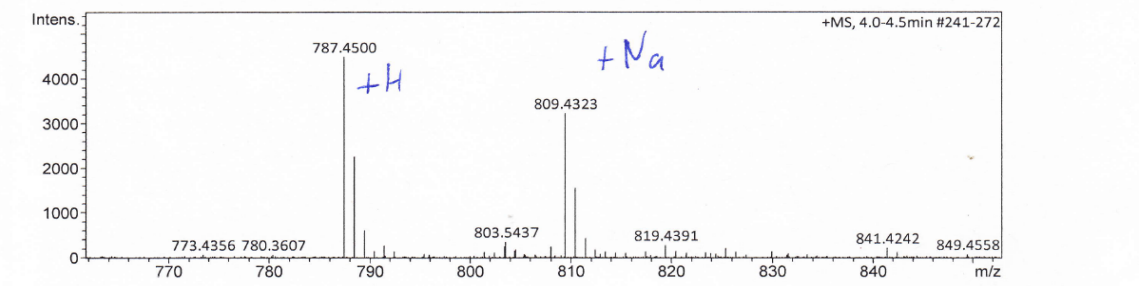
Compounds **1** and **2** were evaluated for their antibacterial activities against *S. aureus* ATCC 25922, *S. pneumonia* ATCC 49619 and *E. coli* ATCC 25922, following the broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [23]. For preparation of the inoculum, the direct colony suspension method was used. Both compounds were further tested for their antifungal activity against *C. cladosporioides* using the agar disc diffusion method.

Acknowledgments: The financial support by the German Federal Ministry of Education and Support (BMBF; Bundesministerium für Bildung und Forschung) granted to P.P. is gratefully acknowledged. S. A. wishes to thank the German Academic Exchange Service (DAAD; Deutscher Akademischer Austauschdienst) for a doctoral scholarship. We are indebted to Prof. H. Brötz-Oesterhelt (Heinrich-Heine University, Duesseldorf, Germany) for antibacterial assays and to Mrs. Rini Muharini (Heinrich-Heine University, Duesseldorf, Germany) for antifungal tests.

References

1. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003;67: 491–502.
2. Rodriguez RJ, White JF Jr, Arnold AE, Redman RS. Fungal endophytes: diversity and functional roles. New Phytol 2009;182:314–30.
3. Aly AH, Debbab A, Proksch P. Fungal endophytes: unique plant inhabitants with great promises. Appl Microbiol Biotechnol 2011;90:1829–45.
4. Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers 2010;41: 1–16.
5. Zhang HW, Song YC, Tan RX. Biology and chemistry of endophytes. Nat Prod Rep 2006;23:753–71.
6. Michailides TJ. Characterization and comparative studies of *Mucor* isolates from stone fruits from California and Chile. Plant Dis 1991;75:373–80.
7. Selim KA, El-Beih AA, AbdEl-Rahman TM, El-Diwany AI. Biodiversity and antimicrobial activity of endophytes associated with Egyptian medicinal plants. Mycosphere 2011;2:669–78.
8. Deng Z, Cao L, Huang H, Jiang X, Wang W, Shi Y, et al. Characterization of Cd- and Pb-resistant fungal endophyte *Mucor* sp. CBRF59 isolated from rapes (*Brassica chinensis*) in a metal-contaminated soil. J Hazard Mater 2011;185:717–24.
9. Lu XL, Najafzadeh MJ, Dolatabadi S, Ran YP, Gerrits van den Ende AH, Shen YN, et al. Taxonomy and epidemiology of *Mucor irregularis*, agent of chronic cutaneous mucormycosis. Persoonia 2013;30:48–56.
10. Akone SH, Amrani ME, Lin W, Lai D, Proksch P. Cytosporins F–K, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*. Tetrahedron Lett 2013;54:6751–4.
11. Akone SH, Rahn S, Henrich B, Daletos G, Vardamides JC, Nkengfack AE, et al. 2-Pentenedioc acid derivatives from a soil-derived fungus *Gongronella butleri*. Phytochem Lett 2014;10:184–8.
12. Mekonnen Y, Gessesse A. Documentation on the uses of *Moringa stenopetala* and its possible antileishmanial and antifertility effects. SINET: Ethiop J Sci 1998;21:287–95.
13. Eilert U, Wolters B, Nahrstedt A. The antibiotic principle of seeds of *Moringa oleifera and Moringa stenopetala*. Planta Med 1981;42:55–61.
14. Berger MR, Habs M, Jahn SA, Schmahl D. Toxicological assessment of seeds from *Moringa oleifera* and *Moringa stenopetala*, two highly efficient primary coagulants for domestic water treatment of tropical raw waters. East Afr Med J 1984;61:712–6.
15. Jahn SA. African plants used for the improvement of drinking water. Curare 1979;2:183–99.
16. Marfey P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Res Commun 1984;49:591–6.
17. Liu S, Shen Y. A new cyclic peptide from the marine fungal strain *Aspergillus* sp. AF119. Chem Nat Compd 2011;47:786–8.
18. Malmstrøm J. Unguisins A and B: new cyclic peptides from the marine-derived fungus *Emericella unguis*. J Nat Prod 1999;62:787–9.
19. Malmstrøm J, Ryager A, Anthoni U, Nielsen PH. Unguisin C, a GABA-containing cyclic peptide from the fungus *Emericella unguis*. Phytochemistry 2002;60:869–72.
20. Brecht-Fischer A, Zähner H, Laatsch H. Metabolic products of microorganisms 183. Imacidin, a new peptid antibiotic from *Streptomyces olivaceus*. Arch Microbiol 1979;122:219–29.
21. Nakajima T, Fujita G, Naoki H, Matahiro I. Extraction of N-acylasparagylpolyamine derivatives from *Nephilengys borbonica* venom for treatment of cerebral diseases. Jpn. Kokai Tokkyo Koho. Japanese Patent JP 07304718 A2 19951121 Heisei. Chem Abstr 1995;124:212043.
22. Kjer J, Debbab A, Aly AH, Proksch P. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nat Protoc 2010;5:479–90.
23. Cockerill FR, Wikler MA, Alder J, Dudley MN, Eliopoulos GM, Ferraro MJ, et al. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed. Wayne, PA: CLSI document M07-A9: Clinical and Laboratory Standards Institute, 2012.

Supporting informations



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
787.4500	1	C42H59N8O7	787.4501	0.2	6.9	1	100.00	17.5	even	ok
	2	C43H55N12O3	787.4515	1.9	8.4	2	47.53	22.5	even	ok
	3	C39H51N18O	787.4488	-1.5	11.1	3	53.06	23.5	even	ok
	4	C41H63N4O11	787.4488	-1.5	17.5	4	47.06	12.5	even	ok
	5	C54H55N6	787.4483	-2.2	59.9	5	10.68	30.5	even	ok
	6	C58H59O2	787.4510	1.2	74.1	6	10.33	29.5	even	ok
809.4323	1	C41H62N4NaO11	809.4307	-1.9	16.4	1	48.31	12.5	even	ok
	2	C42H58N8NaO7	809.4321	-0.2	16.5	2	100.00	17.5	even	ok
	3	C39H50N18NaO	809.4307	-1.9	20.6	3	43.89	23.5	even	ok
	4	C43H54N12NaO3	809.4334	1.4	23.7	4	52.55	22.5	even	ok
	5	C54H54N6Na	809.4302	-2.5	70.6	5	7.11	30.5	even	ok
	6	C58H58NaO2	809.4329	0.8	83.9	6	10.17	29.5	even	ok

Figure 1: HRESIMS spectrum of Ungusin F.

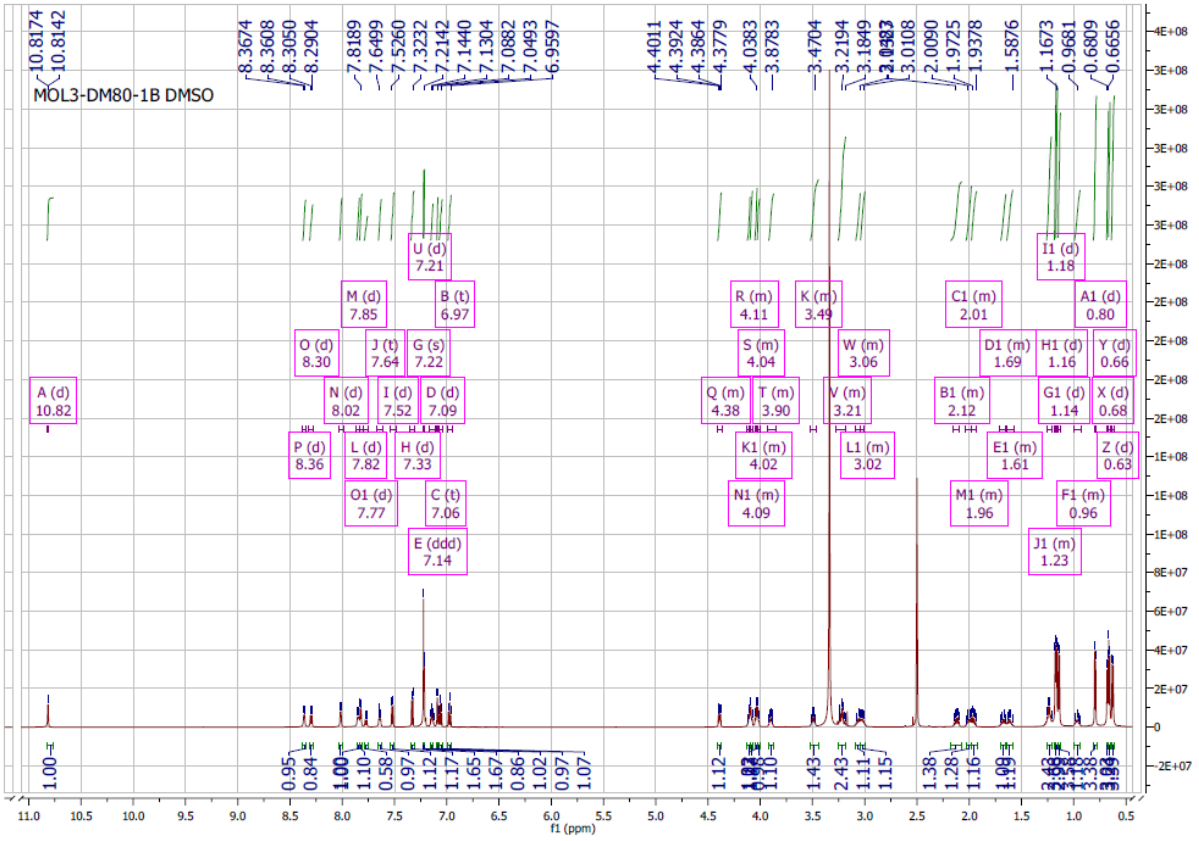


Figure 2: ^{1}H NMR of Ungusin F in DMSO (600 MHz).

Chapiter 5

2-Pentenedioic acid derivatives from a soil-derived fungus *Gongronella butleri*

Published in "Phytochemistry letters"

Impact Factor: 1.450,

The overall contribution to the paper: 80% of the first author. The first author involved to all laboratory works as well as the manuscript preparation.

Phytochemistry Letters 10 (2014) 184–188

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

2-Pentenedioic acid derivatives from a soil-derived fungus *Gongronella butleri*

Sergi Herve Akone [a,b], Sebastian Rahn [c,d], Birgit Henrich [d], Georgios Daletos [a], Juliette Catherine Vardamides [b], Augustin Ephrem Nkengfack [e], Wenhan Lin [f], Daowan Lai [a,*], Peter Proksch [a,**]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, 40225 Duesseldorf, Germany
[b] Faculty of Science, Department of Chemistry, University of Douala, 24157 Douala, Cameroon
[c] Department of Hematology, Oncology and Clinical Immunology, University Clinic of the Heinrich-Heine University of Duesseldorf, 40225 Duesseldorf, Germany
[d] Institute of Medical Microbiology and Hospital Hygiene, University Clinic of the Heinrich-Heine University, 40225 Duesseldorf, Germany
[e] Faculty of Science, Department of Organic Chemistry, University of Yaoundé I, 812 Yaounde, Cameroon
[f] State Key Laboratory of Natural and Biomimetic Drugs, Health Science Center, Peking University, 100191 Beijing, PR China

ARTICLE INFO

Article history:
Received 25 May 2014
Received in revised form 5 September 2014
Accepted 9 September 2014
Available online 19 September 2014

Keywords:
2-Pentenedioic acid
Gongronella butleri
Isolation
Structure elucidation

ABSTRACT

Eight new 2-pentenedioic acid derivatives (**1**–**8**), and one known congener, 2-decanyl-2-pentenedioic acid (**9**), were isolated from a soil-derived fungus *Gongronella butleri* collected in Cameroon. The structures of the new compounds were established by spectroscopic methods including 1D, 2D NMR, and MS. The isolated compounds feature a 2-pentenedioic acid core structure substituted by a 2-alkyl chain that has even number of carbon atoms (C_6, C_8, and C_{10}) with or without an oxygenated substituent. These compounds were screened for their cytotoxic and antibacterial potentials; however, the tested compounds displayed no significant activities.

1. Introduction

Soil harbors the largest population of microbes of any habitat. Soil microorganisms especially actinomycetes, are well known antibiotic producers (Baltz, 2008; Sanglier et al., 1993). Soil-derived fungi were also reported to be prolific sources of biologically active metabolites, as exemplified by the discovery of cytotoxic peniciketals A–C from *Penicillium raistrichii* (Liu et al., 2013), waikialoid A, isolated from *Aspergillus* sp., which was found to inhibit biofilm formation by *Candida albicans* (Wang et al., 2012), and antimicrobial xanthepinone from an unidentified fungus (Liermann et al., 2009).

As a continuation of our search for bioactive substances from fungi (Akone et al., 2013; El Amrani et al., 2014; Lai et al., 2013), we investigated a soil sample collected in Cameroon, which resulted in the isolation of several fungal strains. A small-scale fermentation of these fungi was performed, and the corresponding HPLC chromatogram was obtained. Then, a preliminary de-replication was done by searching the UV spectrum in a lab-built library that included hundreds of fungal secondary metabolites isolated in the past. Some candidate extracts with peaks of interest were further analysis by HPLC–MS to do a further de-replication. Among those, one fungus *Gongronella butleri* (strain no. ESS2) was selected for further chemical investigation based on the HPLC profile of its fungal extract. As we observed several peaks with a maximum absorption at 214 nm in the HPLC chromatogram, the de-replication indicated that these compounds were not previously obtained. The following large-scale cultivation of this fungus on the solid rice media permitted the isolation of these interesting metabolites in sufficient quantities for structure elucidation. The isolated compounds (**1**–**9**) were characterized as 2-pentenedioic acids with a 2-alkyl chain that varied in carbon numbers and oxygenated function group. Herein, we reported the isolation, structure elucidation, and biological evaluation of the isolated compounds.

* Corresponding author. Present address: Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University, 100193 Beijing, PR China. Tel.: +49 211 81 14163; fax: +49 211 81 11923.
** Corresponding author. Tel.: +49 211 81 14163; fax: +49 211 81 11923.
E-mail addresses: laidaowan123@gmail.com (D. Lai), proksch@uni-duesseldorf.de (P. Proksch).

http://dx.doi.org/10.1016/j.phytol.2014.09.001
1874-3900/

2. Results and discussion

The fungal extract was chromatographed over silica gel and Sephadex LH-20, followed by purification by semi-preparative HPLC to yield the known natural product 2-decanylpent-2-enedioic acid (**9**) (Endo et al., 1985), along with eight new congeners (**1**–**8**) (Fig. 1).

Compound **1** was isolated as a colorless oil. Its molecular formula was determined as $C_{11}H_{18}O_5$, as a prominent protonated ion peak observed at *m/z* 231.1228 ([M+H]$^+$) in the HRESIMS spectrum, bearing three degrees of unsaturation. The NMR data of **1** (Tables 1 and 2) indicated the presence of two carboxylic groups (δ_C 171.0, 175.0), one trisubstituted double bond (δ_C 135.6 CH, 136.2 C; δ_H 6.94, t), one oxymethine group (δ_C 68.5, CH; δ_H 3.71, m) and one methyl group (δ_C 23.5 CH_3; δ_H 1.14, d), together with five aliphatic methylene groups. These functional groups accounted for three degrees of unsaturation, thus indicating a linear structure of **1**. Through inspection of the ^{1}H–^{1}H COSY spectrum, we easily established a long spin system that started from H_2-1′ (δ_H 2.32, t), via the oxymethine (H-5′, δ_H 3.71, m), which terminated at the methyl group (Me-6′, δ_H 1.14, d) (Fig. 2). The unassigned methylene group (H_2-4, δ_H 3.23, d) was found to couple with the olefinic proton (H-3, δ_H 6.94, t) as observed in the COSY spectrum. In the HMBC spectrum, the allylic protons (H_2-4) showed correlations to the double bond (C-2/C-3), as well as to one carboxylic group (C-5, δ_C 175.0), indicating that this group was attached to the allylic carbon (C-4) (Fig. 2). The HMBC correlations from the olefinic proton (H-3) to the second carboxylic group (C-1, δ_C 171.0) and the methylene group (C-1′, δ_C 27.8), as well as from H_2-1′ to C-1, C-2, and C-3, suggested that the C1′–C6′ chain and the second carboxylic group were all connected to C-2. Thus, the molecule has a conjugated system, i.e., an αβ-unsaturated carboxylic group, which is consistent with the UV spectrum of **1** showing maximum absorption at λ_{max} 213.6 nm. (Mistry, 2009) The geometry of the double bond was determined to be *Z*, as a correlation between H-3 and H_2-1′ was seen in the ROESY spectrum of **1**. The optical rotation of **1** was close to zero, suggesting it to be a racemic mixture. Therefore, compound **1** was established as (*Z*)-2-(5-hydroxyhexyl)pent-2-enedioic acid.

Compound **2** exhibited a UV maximum absorption at 213.1 nm similar to that of **1**, indicating that they shared a similar chromophore. It exhibited a pseudomolecular ion peak at *m/z* 237.1097 [M+Na]$^+$ in the HRESIMS spectrum, indicating its molecular formula as $C_{11}H_{18}O_4$, which contained one less oxygen atom compared to **1**. Comparison of the NMR data (Tables 1 and 2) revealed pronounced similarities, and the only difference was

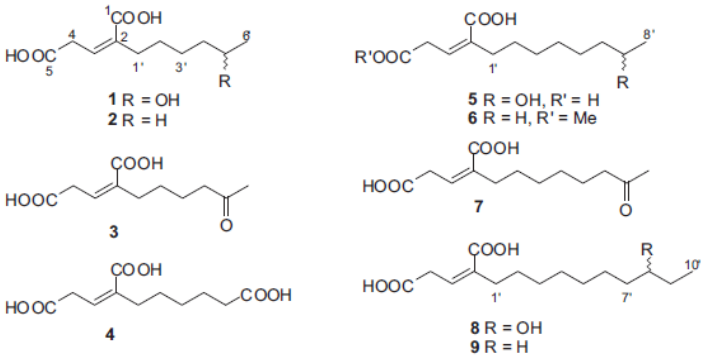


Fig. 1. Structures of the isolated 2-pentenedioic acids (**1**–**9**).

Table 1
^{13}C NMR data of **1**–**8** (150 MHz, CD_3OD).[a]

Position	1	2	3	4	5	6	7	8
1	171.0 C	170.8 C	170.8 C	170.7 C[b]	170.8 C	170.6 C	171.0 C	170.6 C
2	136.2 C	136.4 C	136.1 C	136.2 C	136.3 C	136.9 C	136.2 C	136.2 C
3	135.6 CH	135.1 CH	135.8 CH	135.6 CH	135.2 CH	134.3 CH	135.4 CH	135.1 CH
4	34.5 CH_2	34.7 CH_2	34.8 CH_2	34.4 CH_2	34.6 CH_2	34.5 CH_2	34.6 CH_2	34.2 CH_2
5	175.0 C	174.2 C	174.7 C	174.4 C[b]	174.3 C	172.6 C	175.1 C	174.8 C
1′	27.8 CH_2	27.8 CH_2	27.5 CH_2	27.6 CH_2	27.8 CH_2	27.8 CH_2	27.7 CH_2	27.7 CH_2
2′	30.2 CH_2	30.1 CH_2[c]	29.8 CH_2	29.8 CH_2	30.1 CH_2	30.1 CH_2	29.8 CH_2	30.0 CH_2
3′	26.8 CH_2	30.2 CH_2[c]	24.6 CH_2	30.0 CH_2	30.5 CH_2[d]	30.6 CH_2[e]	29.95 CH_2[f]	30.4 CH_2
4′	40.0 CH_2	32.8 CH_2	44.0 CH_2	25.9 CH_2	30.6 CH_2[d]	30.5 CH_2[e]	30.00 CH_2[f]	30.4 CH_2
5′	68.5 CH	23.6 CH_2	212.0 C	34.9 CH_2	26.8 CH_2	30.4 CH_2[e]	24.8 CH_2	30.4 CH_2
6′	23.5 CH_3	14.4 CH_3	29.6 CH_3	177.7 C[b]	40.2 CH_2	33.0 CH_2	44.3 CH_2	26.9 CH_2
7′					68.6 CH	23.7 CH_2	212.1 C	39.8 CH_2
8′					23.5 CH_3	14.4 CH_3	29.6 CH_3	73.6 CH
9′								30.9 CH_2
10′								10.1 CH_3
–OMe						52.5 CH_3		

[a] Multiplicities were assigned based on DEPT-135 and HSQC experiments.
[b] Signal deduced from HMBC spectrum.
[c] Signals may be interchanged within the same column.
[d] Signals may be interchanged within the same column.
[e] Signals may be interchanged within the same column.
[f] Signals may be interchanged within the same column.

Table 2
^{1}H NMR data of **1–8** (600 MHz, CD_3OD).

Position	1	2	3	4	5	6	7	8
	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)	δ_H, mult., (*J*, Hz)
3	6.94 t (7.4)	6.91 t (7.4)	6.94 t (7.4)	6.93 t (7.5)	6.92 t (7.4)	6.88 t (7.4)	6.93 t (7.4)	6.92 t (7.4)
4	3.23 d (7.4)	3.24 d (7.4)	3.23 d (7.4)	3.24 d (7.5)	3.24 d (7.4)	3.29 d (7.4)	3.23 d (7.4)	3.23 d (7.4)
1′	2.32 t (6.7)	2.30 t (7.6)	2.32 t (7.7)	2.32 m	2.31 t (7.6)	2.30 t (7.6)	2.31 t (7.6)	2.31 t (6.6)
2′	1.36–1.50 m	1.40 m	1.41 m	1.44 m	1.42 m	1.40 m	1.42 m	1.41 m
3′	1.36–1.50 m	1.28–1.37 m	1.58 m	1.37 m	1.28–1.37 m	1.25–1.36 m	1.33 m	1.28–1.37 m
4′	1.36–1.50 m	1.28–1.37 m	2.50 t (7.3)	1.62 m	1.28–1.37 m	1.25–1.36 m	1.33 m	1.28–1.37 m
5′	3.71 m	1.28–1.37 m		2.29 m	1.40 m 1.33 m	1.25–1.36 m	1.55 m	1.28–1.37 m
6′	1.14 d (6.2)	0.90 t (6.8)	2.12 s		1.44 m 1.39 m	1.25–1.36 m	2.47 t (7.4)	1.28–1.37 m
7′					3.71 m	1.25–1.36 m		1.41 m
8′					1.14 d (6.2)	0.90 t (7.0)	2.13 s	3.43 m
9′								1.45 m
10′								0.93 t (7.4)
–OMe						3.71 s		

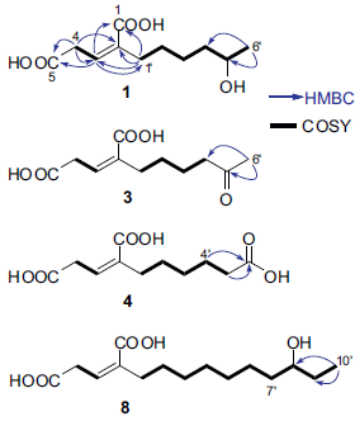


Fig. 2. ^{1}H–^{1}H COSY (bold), and selected HMBC (H → C) correlations of **1**, **3**, **4** and **8**.

ascribed to the side chain, in which the hydroxyl group in **1** was replaced by a hydrogen atom in **2**. Thus, **2** was deduced as a deoxy derivative of **1**, and named (*Z*)-2-hexylpent-2-enedioic acid.

Compound **3** was also identified as a congener of **1**, as their UV, and NMR data were quite similar to each other. The HRESIMS measurement established the molecular formula of **3** as $C_{11}H_{16}O_5$, which was two protons less than that of **1**. The ^{13}C NMR data of **3** showed a signal for a ketone group at 212.0 ppm (C-5′), instead of a hydroxyl group as found in **1**. The methyl group appeared as a singlet (Me-6′, δ_H 2.12, s) in the ^{1}H NMR spectrum of **3**, suggesting that the ketone group was located at C-5′ as expected. This was corroborated by HMBC, as correlations were found from Me-6′ to C-5′, and C-4′ (δ_C 44.0) (Fig. 2). Therefore, **3** was assigned as 5′-dehydro derivative of **1**, and named (*Z*)-2-(5-oxohexyl)pent-2-enedioic acid.

The molecular formula of **4** was deduced as $C_{11}H_{16}O_6$ by HRESIMS analysis. The NMR data of **4** were similar to those of **2**, as both shared the same pent-2-enedioic moiety. The structural differences lie in the side chain. The methyl group in **2** was replaced by a carboxylic group (δ_C 177.7) in **4**, as a long spin system spanned from C-1′ to C-5′ in **4** as evident by analysis of the COSY spectrum. Moreover, HMBC correlations were observed from both H_2-4′ (δ_H 1.62, m) and H_2-5′ (δ_H 2.29, m) to the carboxylic group (C-6′) (Fig. 2). Thus, compound **4** was elucidated as (*Z*)-oct-2-ene-1,3,8-tricarboxylic acid.

Compound **5** was isolated and identified as a close congener of **1**, as they had similar NMR data (Tables 1 and 2). The differences were attributed to the chain length, in which two more methylene groups were present in **5** comparing to **1**, which was in agreement with the fact that the molecular mass of **5** was 28 amu higher than that of **1**. The terminal methyl group (Me-8′, δ_H 1.14) appeared as a doublet in the ^{1}H NMR, and showed COSY correlation to the neighboring oxymethine proton (H-7′, δ_H 3.71), suggesting that the hydroxyl group was located at C-7′ of the octyl side chain of **5**. Compound **5** was also a racemic mixture as its specific optical rotation was close to zero. Thus, **5** was determined as (*Z*)-2-(7-hydroxyoctyl)pent-2-enedioic acid.

Compound **6** showed a prominent peak at *m*/*z* 257.1749 $[M+H]^+$in the HRESIMS spectrum, corresponding to the molecular formula $C_{14}H_{24}O_4$. The NMR data of **6** were similar to those of **2**. However, the length of the alkyl chain differed between both compounds. An octyl chain instead of a hexyl chain was substituted at C-2 of **6**, as deduced from the NMR data. In addition, one methoxyl group (δ_H 3.71, s; δ_C 52.5, CH_3) was present in **6**, which showed HMBC correlation to the carbonyl group at C-5 (δ_C 172.6). Thus, the structure of **6** was determined as (Z)-2-(3-methoxy-3-oxopropylidene)decanoic acid.

Compound **7** was characterized as a dehydro derivative of **5**. The molecular formula of **7** was established as $C_{13}H_{20}O_5$, which was two protons less than that of **5**. Inspection of their NMR data revealed close similarities between both compounds except that the signals for the oxymethine group were missing in **7**, while an isolated methyl group (δ_H 2.13, s; δ_C 29.6, CH_3) and a ketone group (δ_C 212.1, C-7′) appeared. This suggested a 7′-keto group instead of 7′-OH group in **7**, which was corroborated by the HMBC experiment where a methyl group showed correlation to C-7′ and C-6′ (δ_C 44.3). Thus, **7** was determined as (*Z*)-2-(7-oxooctyl)-pent-2-enedioic acid, a homologous structure of **3**.

Compound **8** was isolated likewise as a colorless oil. It had the molecular formula $C_{15}H_{26}O_5$, which contained two more CH_2 units than that of **5**, indicating that a longer chain was present. Interestingly, the hydroxyl group was not located next to the terminal methyl group as seen in **5**; rather, it was situated at C-8′ as the methyl group appeared as a triplet in the ^{1}H NMR spectrum, and showed HMBC correlations to the adjacent methylene group (C-9′, δ_C 30.9) and the carbon (C-8′, δ_C 73.6) bearing the hydroxyl group (Fig. 2). Thus, compound **8** was established as (*Z*)-2-(8-hydroxydecyl)pent-2-enedioic acid. We tried to determine the absolute configuration of this secondary alcohol by applying the

modified Mosher's method (Ohtani et al., 1991; Su et al., 2002), however, this reaction failed to give the desired Mosher's esters. Hence, the absolute configuration of **8** remained to be resolved. A Scifinder search indicated that a compound (CAS registry number: 1174387-28-0) has a same planar structure as **8**, but no reference regarding this structure was found, nor were any spectral data available. Thus, **8** was a new natural product.

Compound **9** was identified as (*Z*)-2-decylpent-2-enedioic acid by analysis of the NMR and MS data. It is a known natural product, which had been previously reported from a soil-derived fungus *G. butleri* (Endo et al., 1985).

In the current study, eight new 2-pentenedioic acid derivatives (**1**–**8**) and one known congener (**9**) were identified from the titled fungus. A previous chemical investigation of *G. butleri* had led to the isolation of three 2-pentenedioic acids and one pentanedioic acid, which were inhibitors of acetyl CoA carboxylase (Endo et al., 1985). To the best of our knowledge, this is the second report of the natural 2-pentenedioic acids produced by fungi.

All isolated compounds were tested for their cytotoxicity against the mouse lymphoma cell line L5178Y. However, none exhibited any significant activity ($IC_{50} > 10$ μg/mL). The antibacterial potentials of these compounds were evaluated against *Staphyloccocus aureus* ATCC 25922, *Streptococcus pneumonia* ATCC 49619, and *Escherichia coli* ATCC 25922, but also in this bioassay they were inactive even at the highest concentration tested (64 μg/mL).

Interestingly, compound **9** was previously reported to inhibit rat liver acetyl CoA carboxylase (IC_{50} 55 μg/mL) (Endo et al., 1985). A 3-carboxylated analog of **9**, oreganic acid, isolated from an endophytic fungus of *Berberis oregano*, was found to be a potent (IC_{50}, 14 nM) and specific inhibitor of farnesyl-protein transferase (Jayasuriya et al., 1996). In the current study, however, the isolated pentenedioic acids were inactive in the assay employed. Thus, to explore the bioactivities of these compounds in other bioassays could be promising.

3. Materials and methods

3.1. General experimental procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ^1H, ^{13}C, and 2D NMR spectra were recorded at 25 °C in CD_3OD on a Bruker ARX 600 NMR spectrometer. Chemical shifts were referenced to the solvent residual peaks, δ_H 3.31 for ^1H, and δ_C 49.0 for ^{13}C. Mass spectra (ESI) were recorded with a Finnigan LCQ Deca mass spectrometer, and HRMS (ESI) spectra were obtained with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed with a Dionex UltiMate3400 SD with a LPG-3400SD Pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient was used (MeOH, 0.02% H_3PO_4 in H_2O): 0 min (10% MeOH), 5 min (10% MeOH), 35 min (100% MeOH), 45 min (100% MeOH). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; Pump L-7100; Eurosphere-100 C18, 300 mm × 8 mm, Knauer, Germany). Column chromatography included LH-20 Sephadex and Merck MN Silica gel 60 M (0.04–0.063 mm) TLC plates with silica gel F254 (Merck, Darmstadt, Germany) were used to monitor fractions (CH_2Cl_2/MeOH mixtures as mobile phase); detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent.

3.2. Isolation and identification of the fungus

The fungus was isolated from a soil sample collected in May 2012 in forest at Koukoue, village located at 10 km from Edea in the littoral region of Cameroon. This fungus was identified by using a molecular biological protocol by DNA amplification and sequencing of the ITS region (Kjer et al., 2010). The identification of the fungus was done by sequencing the 18S rDNA region and the region comprising ITS1–ITS2, followed by a BlastN search in the database. A phylogenetic tree that covers the highest number of homologous sequences in the database was generated. To give final evidence that this fungus belongs to *Gongronella*, we analyzed 1.2 kb of the 18S rDNA gene sequence and found 99% homology to *Gongronella* (e.g. accession numbers EU253966, DQ191324, AF157137 and FJ866597) which clustered in the same branch of a phylogenetic tree framed by *Absidia*, *Cunninghamella* and *Mucorales* (see Fig. S1 in the Supporting Information). BlastN analysis of a 680 bp ITS1–ITS2 region showed homologies of 88–90% homology to *G. butleri* (accession numbers JN206607, KJ439178, AF157191, HM849698 and JN939198) building a distinct branch in the ITS1–ITS2 based phylogenetic tree (see Fig. S2 in the Supporting Information). Thus, we named this fungal isolate as *G. butleri* subspecies AHS. A voucher strain was deposited at one of the authors' laboratory (P.P.).

3.3. Cultivation of the fungus

Scale-up fermentation of the fungus for the isolation and identification of secondary metabolites was carried out in Erlenmeyer flasks (1 L each). The fungus was grown on solid rice medium (to six flasks of 100 g commercially available rice was added 110 mL of distilled water and kept overnight prior to autoclaving) at room temperature under static conditions for 40 days.

3.4. Extraction and isolation

After incubation, 250 mL EtOAc was added to each flask and left overnight, followed by filtration. The extraction was done in three times. The EtOAc extract was combined and taken to dryness. The dry residue was partitioned between *n*-hexane and 90% MeOH. The 90% MeOH fraction (1.54 g) was subjected to vacuum liquid chromatography (VLC) on silica gel employing a step gradient of hexane/ethyl actetate and dichloromethane/methanol to give twelve fractions A–L. Fraction C (160 mg) was subjected to size exclusion column chromatography over Sephadex LH-20 eluting with MeOH to give four subfractions C_1–C_4. Subfraction C_2 (30 mg) and C_3 (46 mg) were purified by semi-preparative HPLC using MeOH-H_2O (0.1% TFA) as eluant to afford compounds **2** (5.0 mg) and **6** (2.6 mg) from the former subfraction, and **9** (1.6 mg) from the latter. Fraction E (129 mg) was also processed in a same manner as that of fraction C by chromatographed over Sephadex LH-20 to give rise to three subfractions (E1–E3). Subfraction E1 (55.8 mg) was further purified by semi-preparative HPLC to obtain compounds **3** (1.4 mg), **4** (1.6 mg), **7** (2.3 mg), and **8** (2.0 mg). Fraction F (85 mg) was also chromatographed over Sephadex LH-20 to give two subfractions F1 and F2. Subfraction F1 (55 mg), after semi-preparative HPLC using MeOH-H_2O (0.1% TFA), gave compounds **5** (2.1 mg), and **1** (1.0 mg). The HPLC chromatogram of the crude extract and the isolated compounds were shown in Figs. **S3**–**S12** in the Supporting Information.

Compound **1**: colorless oil; $[\alpha]_D^{20} = +0.1$ (*c* 0.23, CH_3OH); UV (MeOH) λ_{max} 213.6 nm; ^1H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 230.8 $[M+H]^+$, 253.0 $[M+Na]^+$; HRESIMS *m/z* 231.1228 $[M+H]^+$ (calcd for $C_{11}H_{19}O_5$, 231.1227).

Compound **2**: colorless oil; UV (MeOH) λ_{max} 213.1 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 214.7 $[M+H]^+$; HRESIMS *m/z* 237.1097 $[M+Na]^+$ (calcd for $C_{11}H_{18}NaO_4$, 237.1097).

Compound **3**: colorless oil; UV (MeOH) λ_{max} 213.9 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 228.8 $[M+H]^+$, 251.0 $[M+Na]^+$; HRESIMS *m/z* 229.1072 $[M+H]^+$ (calcd for $C_{11}H_{17}O_5$, 229.1071).

Compound **4**: colorless oil; UV (MeOH) λ_{max} 214.1 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 244.8 $[M+H]^+$; HRESIMS *m/z* 245.1023 $[M+H]^+$ (calcd for $C_{11}H_{17}O_6$, 245.1020).

Compound **5**: colorless oil; $[\alpha]_D^{20} = +0.1$ (*c* 0.08, CH_3OH); UV (MeOH) λ_{max} 214.4 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 258.9 $[M+H]^+$, 281.0 $[M+Na]^+$; HRESIMS *m/z* 259.1536 $[M+H]^+$ (calcd for $C_{13}H_{23}O_5$, 259.1540).

Compound **6**: colorless oil; UV (MeOH) λ_{max} 213.3 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 256.7 $[M+H]^+$, 279.0 $[M+Na]^+$; HRESIMS *m/z* 257.1749 $[M+H]^+$ (calcd for $C_{14}H_{25}O_4$, 257.1747).

Compound **7**: colorless oil; UV (MeOH) λ_{max} 214.2 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 256.8 $[M+H]^+$; HRESIMS *m/z* 257.1383 $[M+H]^+$ (calcd for $C_{13}H_{21}O_5$, 257.1384).

Compound **8**: colorless oil; $[\alpha]_D^{20} = +4.8$ (*c* 0.34, CH_3OH); UV (MeOH) λ_{max} 213.6 nm; ^{1}H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data, see Tables 1 and 2; ESIMS *m/z* 286.8 $[M+H]^+$, 309.1 $[M+Na]^+$; HRESIMS *m/z* 287.1854 $[M+H]^+$ (calcd for $C_{15}H_{27}O_5$, 287.1853).

4. Bioassay

The cytotoxic and antibacterial assays were performed as described previously (El Amrani et al., 2012).

Acknowledgements

The authors are grateful to BMBF for the financial support (grant No. 0315772). S.H.A. wants to thank DAAD (Deutscher Akademischer Austauschdienst) for a doctoral scholarship. We are indebted to Professor W.E.G. Müller (Johannes Gutenberg University, Mainz, Germany) for cytotoxicity assays and to Professor H. Brötz-Oesterhelt (Heinrich-Heine University, Düsseldorf, Germany) for antibacterial assays.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2014.09.001.

References

Akone, S.H., El Amrani, M., Lin, W., Lai, D., Proksch, P., 2013. Cytosporins F–K, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*. Tetrahedron Lett. 54, 6751–6754.

Baltz, R.H., 2008. Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. 8, 557–563.

El Amrani, M., Debbab, A., Aly, A.H., Wray, V., Dobretsov, S., Müller, W.E.G., Lin, W., Lai, D., Proksch, P., 2012. Farinomalein derivatives from an unidentified endophytic fungus isolated from the mangrove plant *Avicennia marina*. Tetrahedron Lett. 53, 6721–6724.

El Amrani, M., Lai, D., Debbab, A., Aly, A.H., Siems, K., Seidel, C., Schnekenburger, M., Gaigneaux, A., Diederich, M., Feger, D., Lin, W., Proksch, P., 2014. Protein kinase and HDAC inhibitors from the endophytic fungus *Epicoccum nigrum*. J. Nat. Prod. 77, 49–56.

Endo, A., Takeshima, H., Kuwabara, K., 1985. Acetyl CoA carboxylase inhibitors from the fungus *Gongronella butleri*. J. Antibiot. 38, 599–604.

Jayasuriya, H., Bills, G.F., Cascales, C., Zink, D.L., Goetz, M.A., Jenkins, R.G., Silverman, K.C., Lingham, R.B., Singh, S.B., 1996. Organic acid: a potent novel inhibitor of ras farnesyl-protein transferase from an endophytic fungus. Bioorg. Med. Chem. Lett. 6, 2081–2084.

Kjer, J., Debbab, A., Aly, A.H., Proksch, P., 2010. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nat. Protoc. 5, 479–490.

Lai, D., Brötz-Oesterhelt, H., Müller, W.E.G., Wray, V., Proksch, P., 2013. Bioactive polyketides and alkaloids from *Penicillium citrinum*, a fungal endophyte isolated from *Ocimum tenuiflorum*. Fitoterapia 91, 100–106.

Liermann, J.C., Kolshorn, H., Opatz, T., Thines, E., Anke, H., 2009. Xanthepinone, an antimicrobial polyketide from a soil fungus closely related to *Phoma medicaginis*. J. Nat. Prod. 72, 1905–1907.

Liu, W.-Z., Ma, L.-Y., Liu, D.-S., Huang, Y.-L., Wang, C.-H., Shi, S.-S., Pan, X.-H., Song, X.-D., Zhu, R.-X., 2013. Peniciketals A–C, new spiroketals from saline soil derived *Penicillium raistrichii*. Org. Lett. 16, 90–93.

Mistry, B.D., 2009. A Handbook of Spectroscopic Data Chemistry: UV, IR, PMR, ^{13}C NMR and Mass Spectroscopy. Oxford Book Company, Jaipur, India.

Ohtani, I., Kusumi, T., Kashman, Y., Kakisawa, H., 1991. High-field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. J. Am. Chem. Soc. 113, 4092–4096.

Sanglier, J.J., Haag, H., Huck, T.A., Fehr, T., 1993. Novel bioactive compounds from actinomycetes: a short review (1988–1992). Res. Microbiol. 144, 633–642.

Su, B.-N., Park, E.J., Mbwambo, Z.H., Santarsiero, B.D., Mesecar, A.D., Fong, H.H.S., Pezzuto, J.M., Kinghorn, A.D., 2002. New chemical constituents of *Euphorbia quinquecostata* and absolute configuration assignment by a convenient Mosher ester procedure carried out in NMR tubes. J. Nat. Prod. 65, 1278–1282.

Wang, X., You, J., King, J.B., Powell, D.R., Cichewicz, R.H., 2012. Waikialoid A suppresses hyphal morphogenesis and inhibits biofilm development in pathogenic *Candida albicans*. J. Nat. Prod. 75, 707–715.

Supporting Information

2-Pentenedioic acid derivatives from a soil-derived fungus *Gongronella butleri*

Sergi Herve Akone[a,b], Sebastian Rahn[c,d], Birgit Henrich[d], Georgios Daletos[a], Juliette Catherine Vardamides[b], Augustin Ephrem Nkengfack[e] , Wenhan Lin[f] , Daowan Lai[a,1,*], Peter Proksch[a,*]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, 40225, Duesseldorf, Germany

[b] Faculty of Science, Department of Chemistry, University of Douala, 24157, Douala, Cameroon

[c] Department of Hematology, Oncology and Clinical Immunology, University Clinic of the Heinrich-Heine University of Duesseldorf, 40225, Duesseldorf, Germany

[d] Institute of Medical Microbiology and Hospital Hygiene, University Clinic of the Heinrich-Heine University, 40225, Duesseldorf, Germany

[e] Faculty of Science, Department of Organic Chemistry, University of Yaoundé I, 812, Yaounde, Cameroon

[f] State key Laboratory of Natural and Biomimetic Drugs, Health Science Center, Peking University, 100191, Beijing, PR China

[1] Present address: Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University, 100193, Beijing, PR China

*Author for correspondence

E-mail: laidaowan123@gmail.com; proksch@uni-duesseldorf.de

Tel.: +49 211 81 14163; fax: +49 211 81 11923.

Sequenced 18S rDNA region of the unknown fungus

CTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTAAAAGCAATTTTTACAGCGAAACTGC
GAATGGCTCATTAAATCAGTTATGATCTACATGGCAATGTATTTTTTTACTATTGGATAAC
CGTGGTAATTCTAGAGCTAATACATGCAAAAAAGGGGTGCTTTCGGGCACTCTGCACTTA
TTAGATCAAAGCCAACAGCCTGGAAACAGGTTTCTTTTGGTGAATCATAATAATTAAGCG
GATCGCATGGCCTTGTGCCGGCGACGGTTCATTCGATTTTCTGCCCTATCAGGCTTTTGAT
GGTAAGGTAGTGGCTTACCATGGCTATCACGGGTGACGGGGAATAAGGGTTCGATTCCGG
AGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACC
CAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATGCAGAGCCTTTCAGGTTTTGC
AATTGGAATGAGTACAATTTAAATCTCTTAACGAGAACCAATTGGAGGGCAAGTCTGGTG
CCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAACG
TCCGTAGTCGAATTTTGGTTTGGGCTTGTGCCTGGCGTCCTTCATTGGGCGTTTGGGTTGC
ATGCCTTGCCATGTTGTAGGGGTGCGGTCCTTCGGGGCCGATGATCTCTGCGTATTACCAT
GAGCAAATCAGAGTGCTCAAAGCAGGCTTTATAGCTTGAATGTTTTAGCATGGAATAATG
AAATAAGCCTTAGGTCTTGTTTCGTTGGTTTACTTGACACTGGGGAATGATGAATAGGAA
CGGTTGGGGGCATTTGTATTTGGCCGCTAGAGGTGAAATTCTTGGATTGGCCGAAGACAA
ACTACTGCGAAAGCATTTGACCCAGGACGTTTTCATTGATCAGGGACTAAAGTTGAGGGA
TCGAAGACGATTAGATACCGTCGTAGTCTTAACCACAAACTATGCCGACTAGCGATCGCA
TGGATACTTTTTTTGTTCCATGCGGCAGCTTAGCGAAAGTAAAGTCTTTGGGTTCTGGGGG
GAGTATGGGACGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTG
GAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACATAAGAAG
GATTGACAGATTGAAAGCTCTTTCTAGATTTTATGGGTGGTGGTGCATGGCCGTTCTTAGT
TCGTGGAGTGATTTGTCT

Of these 1281 nucleotides which were used for BlastN analysis, region of nt 47-1281 showed the highest number of homologous sequences and was thus used for the generation of a phylogenetic tree with the highest homologue sequences of the databases that cover the same sequence region (Species Abbr. and Acc. numbers are indicated). The 18S rDNA region of the titled fungus clusters in the *Gongronella* branch nearest to *G. butleri*.

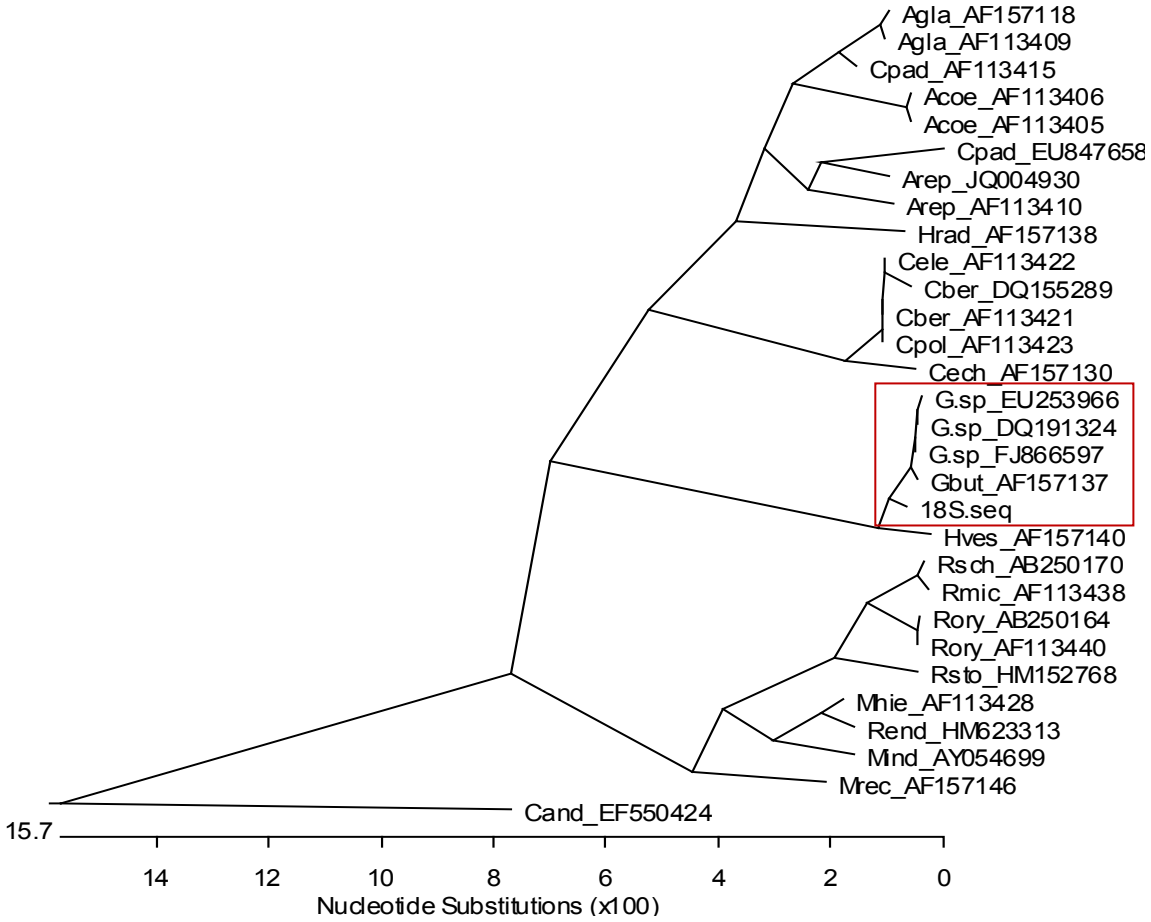


Fig. S1 Phylogenetic tree based on part of the18S rDNA

Sequenced region of the unknown fungus comprising ITS1-ITS2

TTTTGATGGGTGGTATAAAGCCTTGTGCTTTCCGTCCGCCTATTCCGACTACTGAATTCAA
TTCAGGGGAAGTATGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGC
GCGCTACACTGACAAAGGCAACGAGTTTTTTCCTTGGCCGGAAGGTCTGGGTAAACTTTT
GAAACTTTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGAATTCCT
AGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT
CGCTACTACCGATTGAATGGTCATAGTGAGCATGTGGGATTGACGTCGTCACAGCGGCAA
CGCTGAGATGATGTTGAGAACTATGGCAAACTAGGCTATTTAGAGGAAGTAAAAGTCGT
AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATAATGAGCTTTTTCTAGTTG
TAAAAGATTAGAAAAAGTCCTTTTAACTTTTTTTCCTGCTCTCGCAAGAGTGCTAGGRAAA
AAATCCACTGTGATCTGTTTTCGAGTGGGGTCTTGGGTTAACCTAGGCTTCACSCAAGCCT
CTTCTGGTACTTTTTTGAGAAATCATAAAGTGCTTGTCGAGGTGTTTATTACACTTTATTA
AATTACTTTTTGAAATACAAATCAAAAAAACAACTTTCAACAATGGATCTCTCGGCTTAC
GCATCGATGAAGAACGCAGCGAATCGCGATATRTAATGTGATCTGCATTTAGTGAATCAT
CGAGTTTTTGAACGCATCTTGCGCCCAAGGGTAATCCTTTGGGCACGCCTGTTTCAGTGTT
ATTTTCAACCCCTCTCCTAGATTGGGTGATGAAACGATGGGTTTGCAATCTGCAAAGATT

GTCCTACTCTGAAGAAATCGAGCTGGATTTTCTAATAAATCAGGCTTTGGTAATTACCAA
AAGTCGCGTTTATTTTGAAAACCAAATACCAGTGACCTCTTTCAAAATTTAACCTGAAATC
AGGCGGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAAATAACAA
TGATTCCCCTAGTAACGGCGAGTGAAGAGGGAAAAGCTCAAGTTTGGAAGCTTCGTGGC
ATAGCTACGACGCGTTGTAAACTGAAGTTTTTTGGGTGATTGGGCGACCGGATTAAAAAG
TCCTTTGGAAAGGGGCAACATAGAGGGTGACATTCCCGTCTTTGGTCCGAGTCAGTTCAG
TCATTGCTCAGGAAACGACGAGTCAGGTTGTTTGGGAATGCAGCCTAAAATGGGAGGTA
AATCTCTCCTAAAGCTAAATATTGGCGTAAGACCGATGCGAACAAGTACCGTGAGGGAA
AGATGAAAAGGACTTTGAAAAGAGGGTCAAACAGTACGTGAAATTGTTGAAAGGGAACC
GTATGAAAGTAGACCGGTTTGGTAGTGATCAATGTGTGTTTCGGCATGCATGCACTCATT
GCCAATTCCAGCCAGCGGCAGTTTTTGTTGAAGGAAGAAAATCTTGGGAATGTGGCTTCG
GCTTCGGCGGAAGTGTTATAGCCCTTGATAAAATTACTTTGGCGGGGACTGAGGCTTGCA
GCGGGTTTTTGGGAGCGGCGCGCGCTTGCTTGTTGCAGTGTAAATTTCCCTCGGGTGTTTG
CATTGTAAGGGGTAACTCGTGTGTTGTCTTACCGTCTCGCTTAGGTCGCTGGCGTAATGCT
TTTATATGACCCGTCTTGA

Of these 1763 nucleotides which were used for BlastN analysis, region of nt 1064-1750 (ITS1-ITS2.seq) showed the highest number of homologous sequences and was thus used for the generation of a phylogenetic tree with the highest homologue sequences of the databases (Species abbr. and Acc. numbers are indicated). The ITS1-ITS2 region of the titled fungus clusters in the *Gongronella butleri* branch.

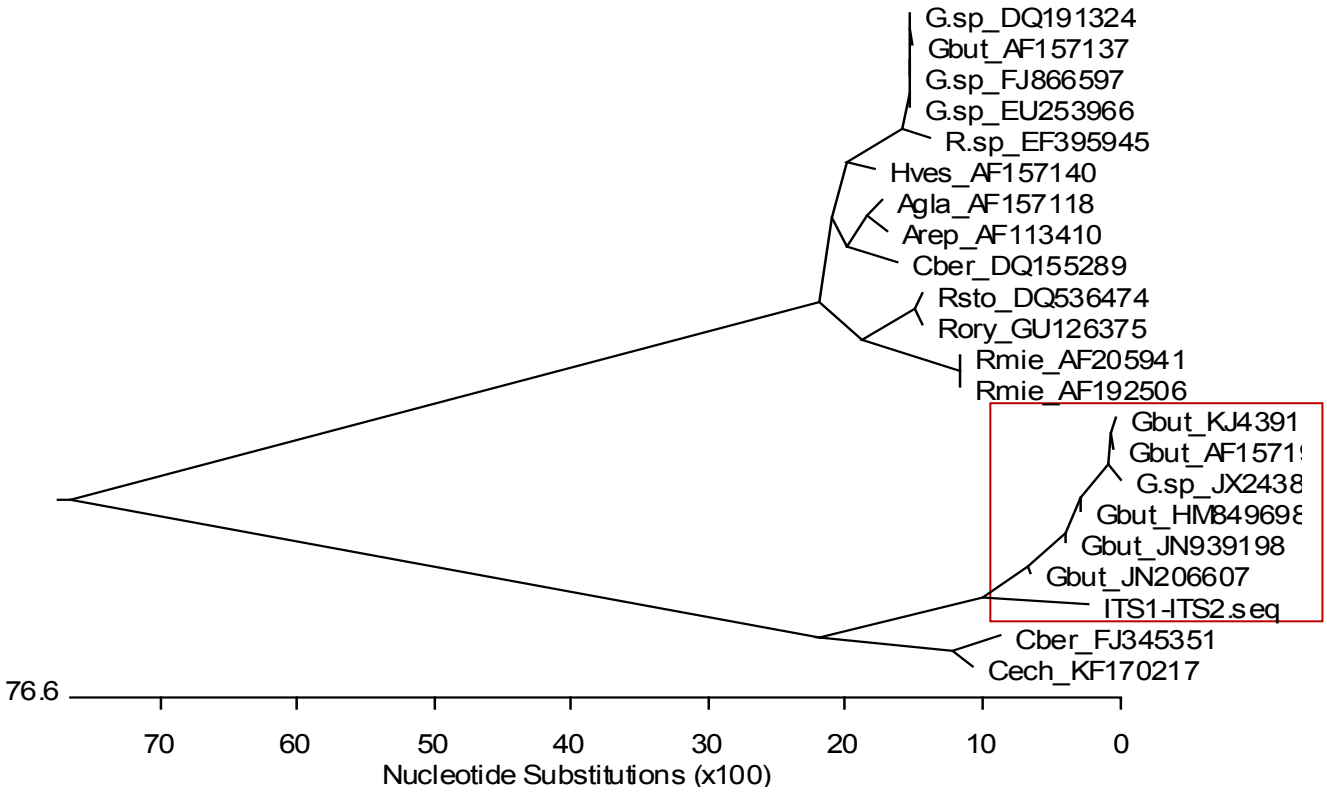


Fig. S2 Phylogenetic tree based on part of the ITS1-ITS2 region

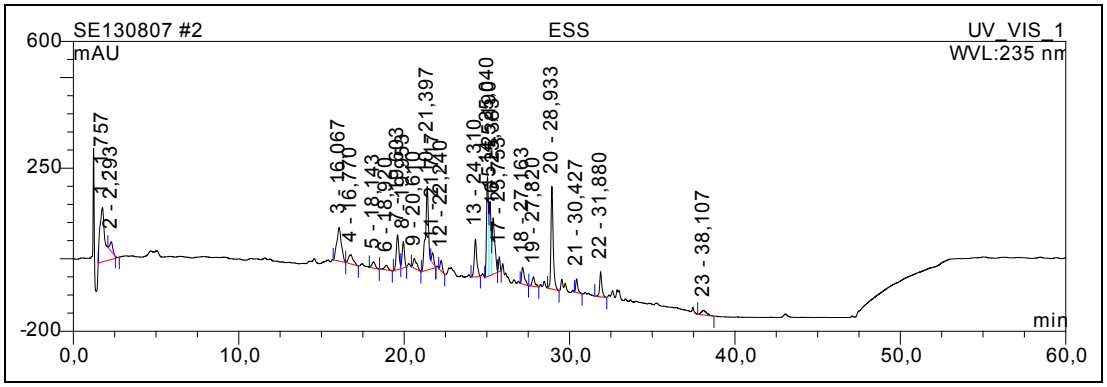


Fig. S3 HPLC chromatogram of the crude extract (detected at UV 235 nm)

Note: A Eurosphere-10 C_{18} (Knauer, Germany) column (i.d. 125×4 mm) was used. The following gradient was employed (mobile phase: MeOH, 0.02% H_3PO_4 in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Flow rate: 1 mL/min. The same condition was used for the detection of the isolated compounds.

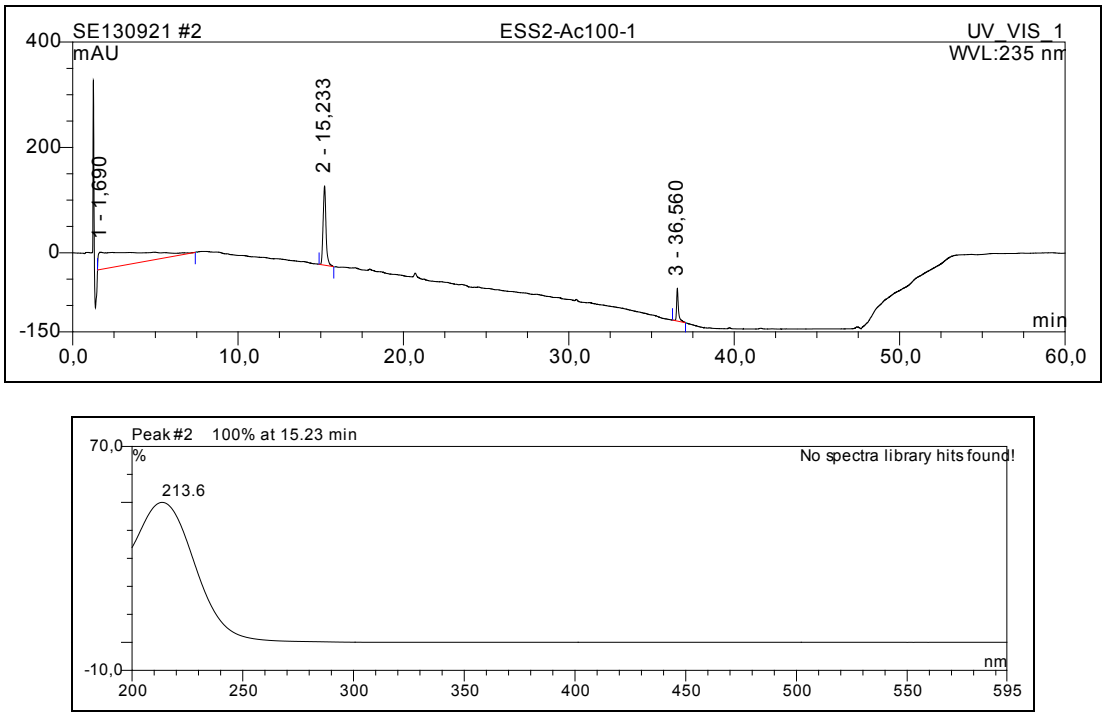


Fig. S4 HPLC chromatogram of **1** (detected at UV 235 nm) and its on-line UV spectrum

Note: The peak at around 36.6 min (the mobile phase during 35-45 min was 100% MeOH) represented an impurity from the column. This peak appeared at all the HPLC chromatograms of the isolated compounds.

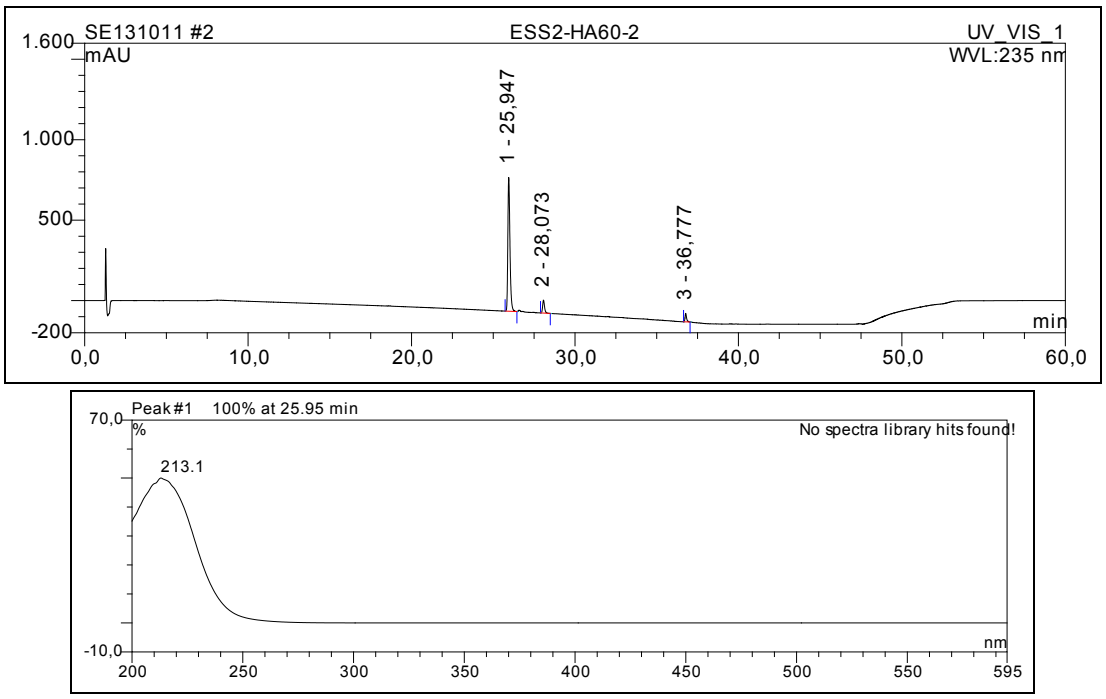


Fig. S5 HPLC chromatogram of **2** (detected at UV 235 nm) and its on-line UV spectrum

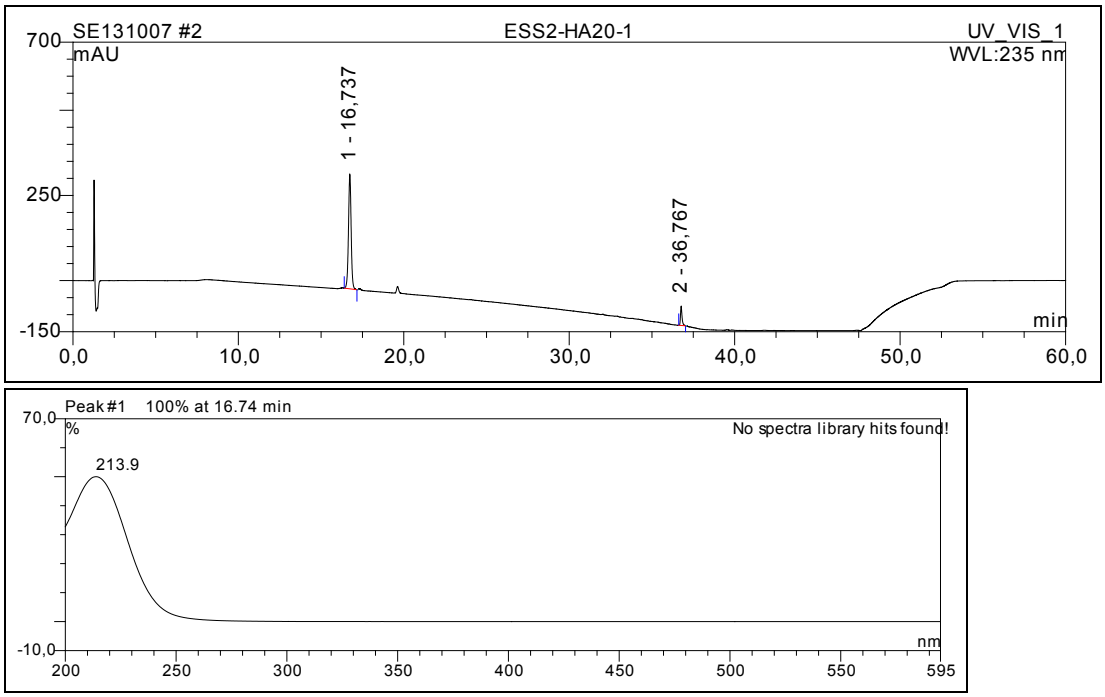


Fig. S6 HPLC chromatogram of **3** (detected at UV 235 nm) and its on-line UV spectrum

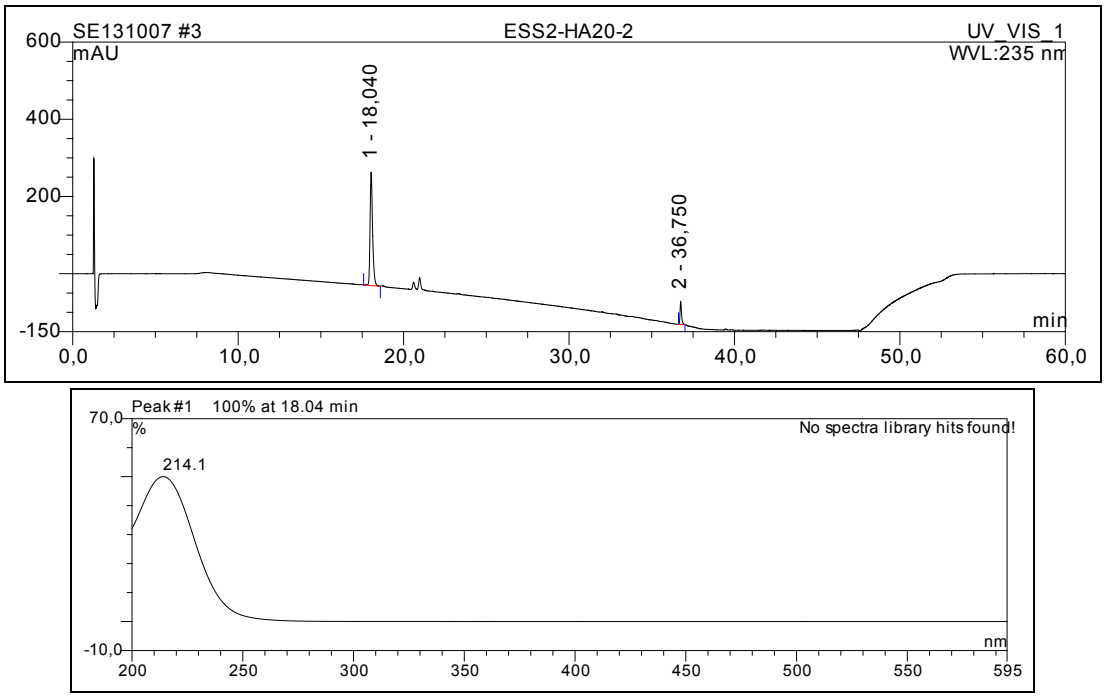


Fig. S7 HPLC chromatogram of **4** (detected at UV 235 nm) and its on-line UV spectrum

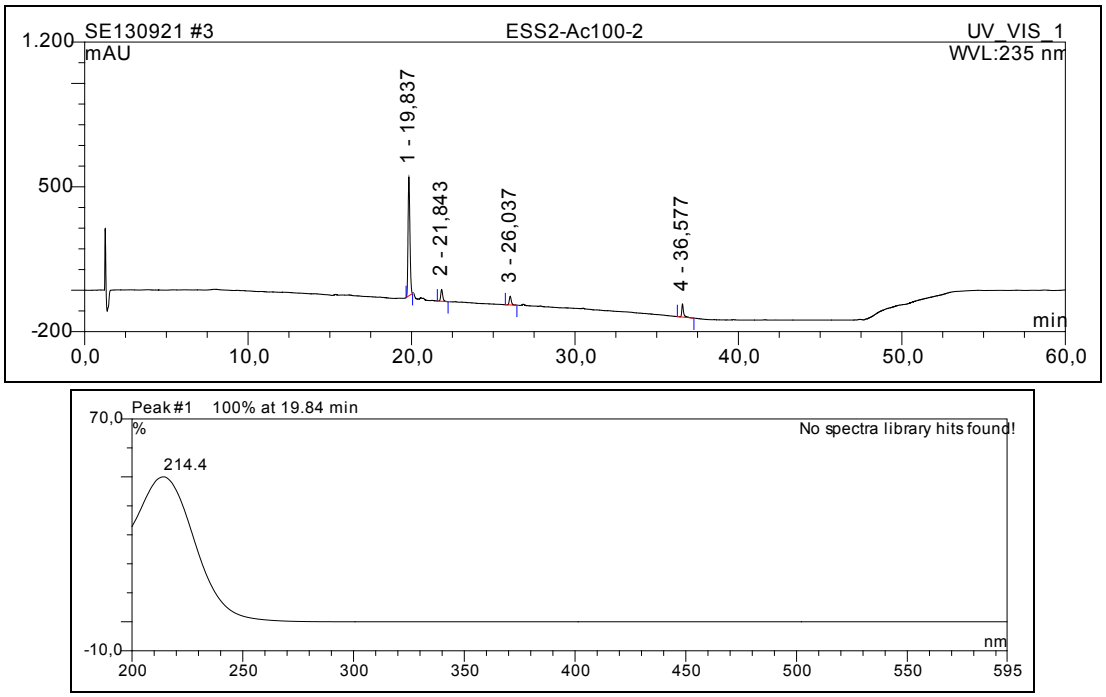


Fig. S8 HPLC chromatogram of **5** (detected at UV 235 nm) and its on-line UV spectrum

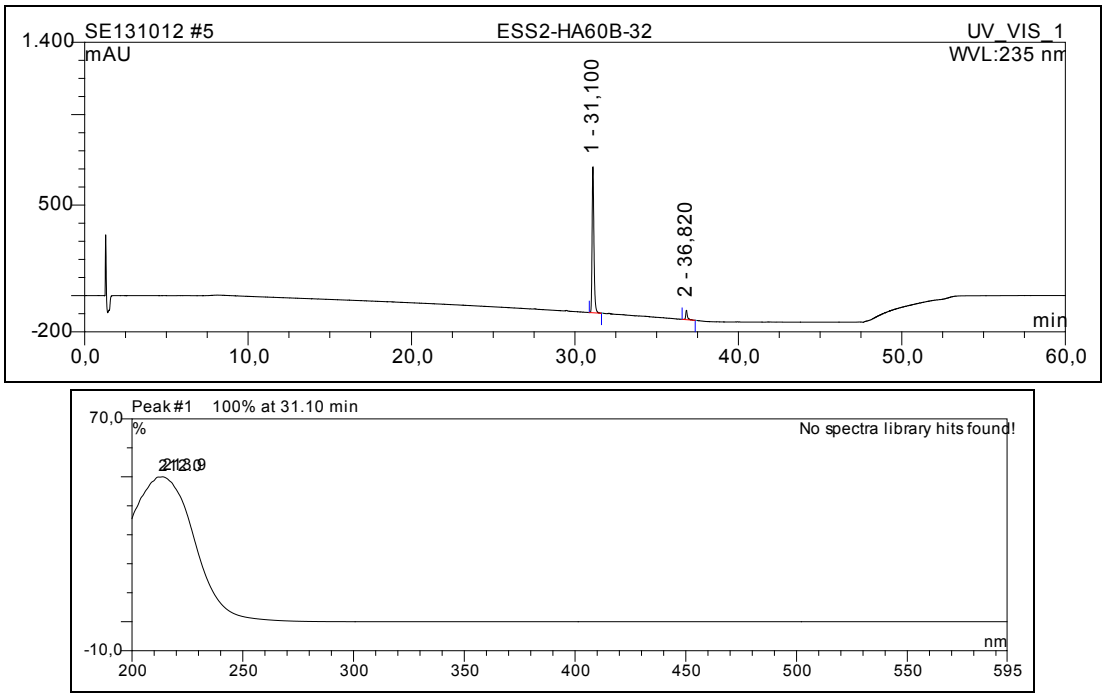


Fig. S9 HPLC chromatogram of **6** (detected at UV 235 nm) and its on-line UV spectrum

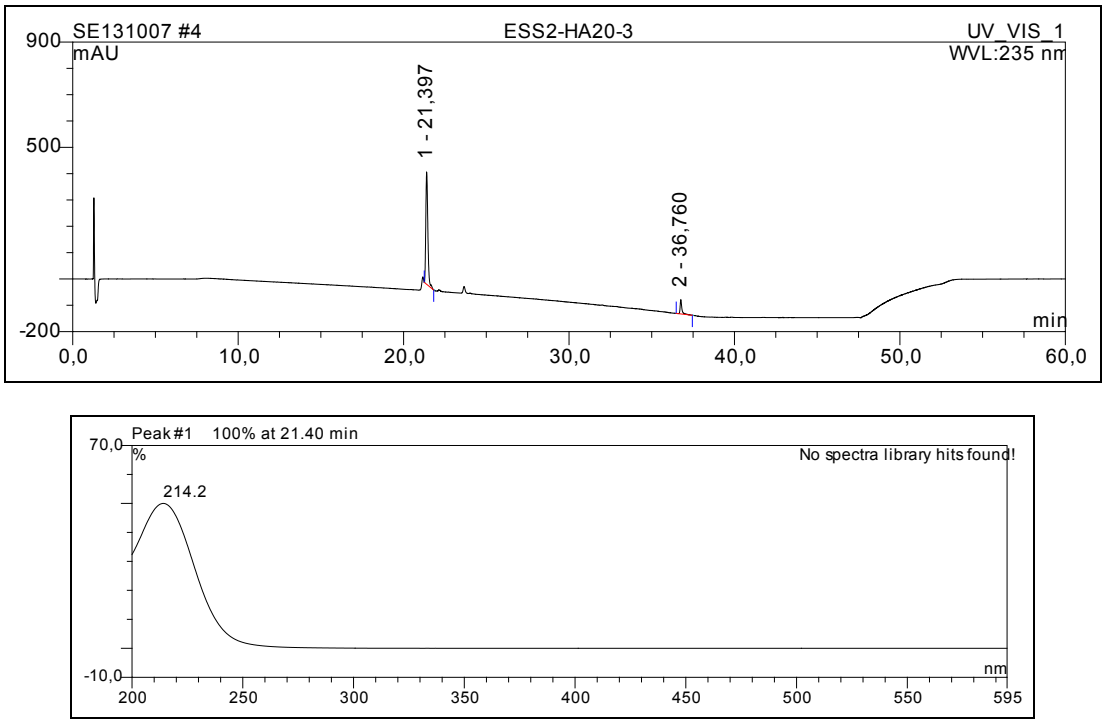


Fig. S10 HPLC chromatogram of **7** (detected at UV 235 nm) and its on-line UV spectrum

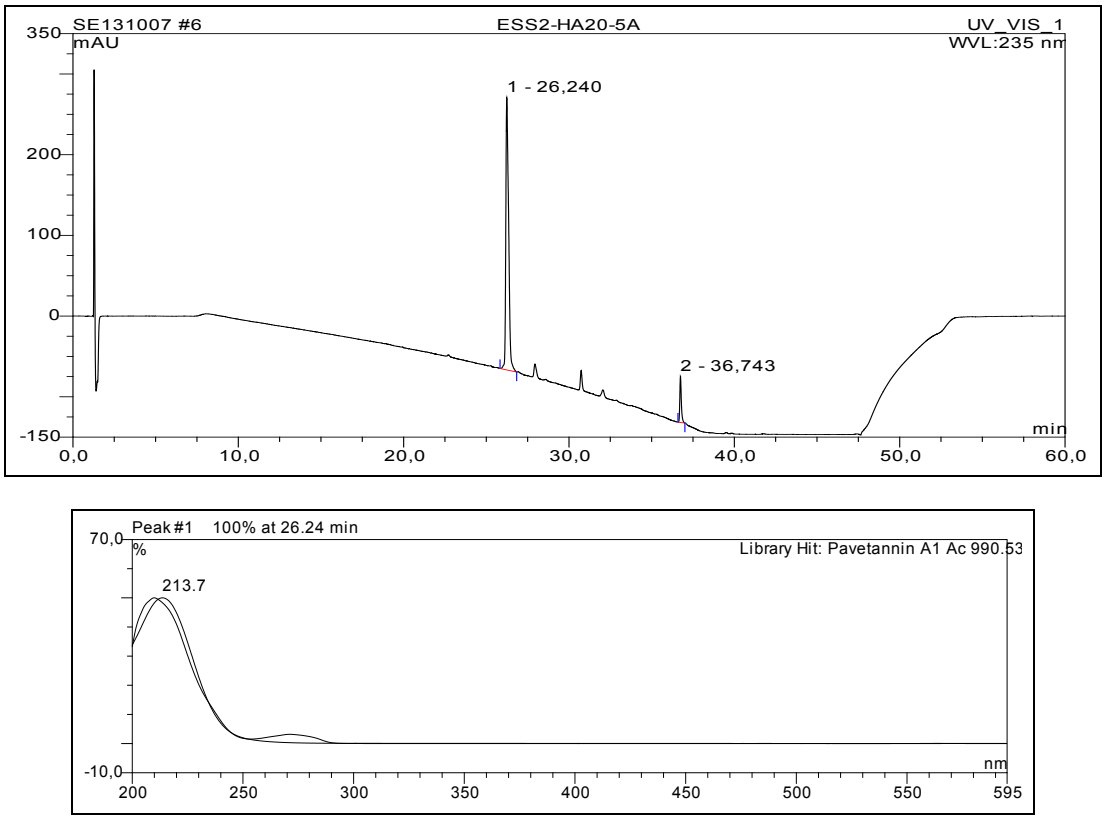


Fig. S11 HPLC chromatogram of **8** (detected at UV 235 nm) and its on-line UV spectrum

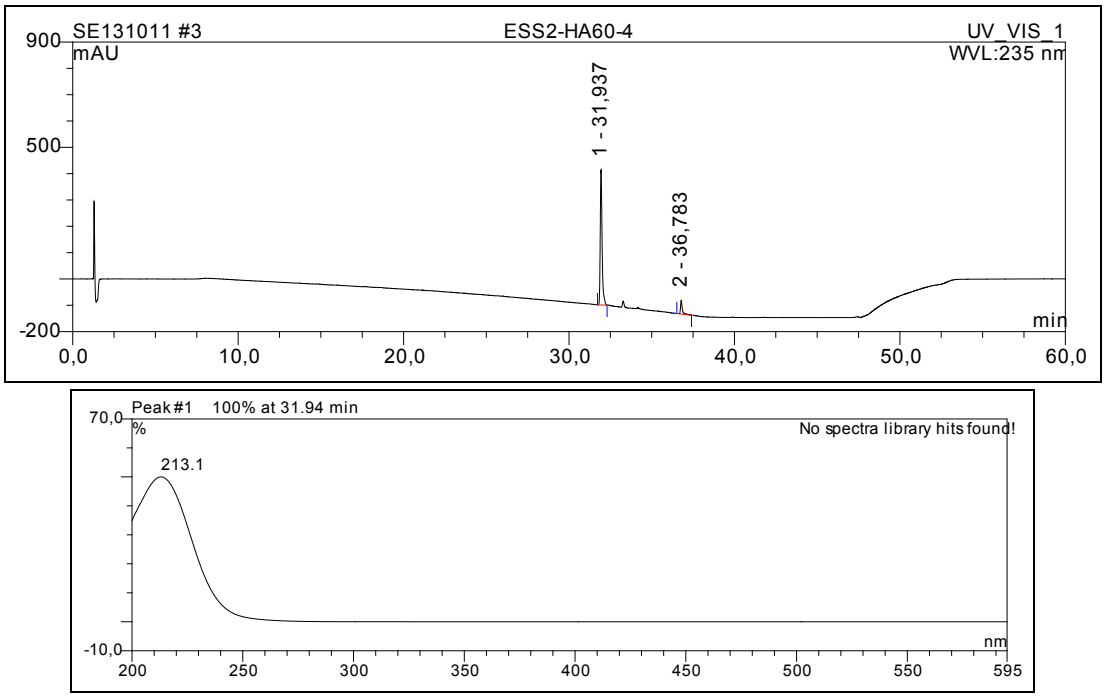


Fig. S12 HPLC chromatogram of **9** (detected at UV 235 nm) and its on-line UV spectrum

Chapter 6

Discussion

6.1 Induction of Silent Biosynthetic Pathways of the Endophytic Fungus *Chaetomium* sp. through Co-Cultivation Experiments

Microorganisms, including fungi, are a vast and largely untapped resource of novel and structurally diverse metabolites. Many of these metabolites have been used in drug discovery (Pettit, 2011). Re-isolation of known secondary metabolites has significantly increased in the past few years (Laatsch, 2011; Hong *et al.*, 2009), which poses serious problems for terrestrial bioprospecting from microorganisms. However, modern molecular methods have demonstrated that the genetic potential of microorganisms, in terms of producing new natural compounds, has been underestimated (Knight *et al.*, 2003; Brakhage *et al.*, 2008). In fact, it was shown that many biosynthetic genes of microorganisms are remaining silent under laboratory conditions. One strategy that has successfully been applied to solve the above highlighted difficulties in drug discovery is the application of mixed fermentation or co-cultivation. In nature, microorganisms such as bacteria and fungi live in complex communities, involving several signaling-based interactions, such as modulation of the physiochemical environment, protein secretion, and gene transfer (Frey-Klett *et al.*, 2011). Another important interaction between microorganisms is the competition for limited nutrients, known as trophic competition, which plays an eminent role in the production of natural products in prokaryotes and eukaryotes alike (Scherlach and Hertweck, 2009).

Co-cultivation of different microorganisms instead of maintaining axenic cultures, as usually practiced under standard laboratory conditions, forces direct interactions that lead to an enhancement in the accumulation of constitutively present metabolites (Oh *et al.*, 2007; Schroeckh *et al.*, 2009; Nützmann *et al.*, 2011) or may trigger the expression of silent biosynthetic pathways, affording new natural products (Oh *et al.*, 2005; Cueto *et al.*, 2001). Several co-cultivation experiments, highlighting the importance of this ecological powerful tool in the discovery of new natural products, have been reported in the literature. Co-cultivation of a marine-derived fungus *Pestalotia* sp. with a marine α-proteobacterium (strain

CNJ-328) resulted in the production of a new antibiotic, called pestalone (Cueto *et al.*, 2001). Subsequent co-culture of the same bacterium with a marine derived fungus *Libertella* sp. resulted in the discovery of four potent cytotoxic pimarane diterpenoids, libertellenones A-D (Oh *et al.*, 2005). Recently, co-cultivation of the endophytic fungus *Fusarium striatum* with *Bacillus subtilis* led to the production of three new polyketides (Ola *et al.*, 2013). Likewise, co-cultivation of the soil-derived fungus *Aspergillus terreus* with *Bacillus subtilis* afforded two new butyrolactone derivatives (Chen *et al.*, 2015).

In a current study, the endophytic fungus *Chaetomium* sp. was co-cultivated with different bacteria (Manuscript submitted). During co-cultivation of *Chaetomium* sp. with *B. subtilis*, there was a significant change in the optical appearance of the co-culture flasks, as a yellow pigmentation was observed, instead of the green pigmentation discerned in the axenic fungal controls. It has also been reported that the induction of secondary metabolites during co-culture may occur either by secretion of chemical signals or by physical contact of the respective organisms (Scherlach and Hertweck, 2009). In order to get a further insight into the possible mechanism of fungal metabolite induction by *B. subtilis*, the fungus was treated with cell-free medium of viable or autoclaved *B. subtilis*. However, none of these treatments was effective in inducing the production of fungal cryptic metabolites or in changing the optical appearance of the fungus. Moreover, to exclude the possibility that the induction was due to metabolites produced by the bacterium to the medium, we treated the fungus with culture filtrates of the bacterium previously grown on rice for four days. Likewise, no induction of fungal cryptic metabolites was observed, suggesting that the induction of cryptic fungal metabolites was probably due to fungus-bacterium physical contact.

When *Chaetomium* sp. was co-cultured with *Streptomyces lividans* or *Mycobacterium tuberculosis* no induction of fungal metabolites was observed (Manuscript submitted).

The treatment of *Chaetomium* sp. with the epigenetic modifier suberoylanilide hydroxamic acid or 5-azacytidine resulted in an enhanced accumulation of isosulochrin, which was likewise detected when co-culturing *Chaetomium* sp. with *Bacillus subtilis*, but not in axenic fungal controls. Thus, the induction of new secondary metabolites during co-culture may occur through histone modifications (Manuscript submitted), as recently reported during co-cultivation of *Aspergillus nidulans* with *Streptomyces rapamycinicus* (Nützman *et al.*, 2011). Accordingly, *S. rapamycinicus* triggered histone modifications in *A. nidulans* through the Saga/Ada complex resulting in the induction of the orselinic acid by the latter (Nützman *et al.*, 2011).

6.2 Induction of Silent Biosynthetic Pathways of the Endophytic Fungus Chaetomium sp. through Epigenetic Remodelling

An approach to induce the production of cryptic secondary metabolites from fungi might be the application of epigenetic modifiers. Many fungal genomes have been identified and it was demonstrated that genes dedicated for secondary metabolite production are encoded in clusters existing in the distal regions of chromosomes (Shwab *et al.*, 2007). These gene clusters are known to exist in a heterochromatin state, whose constitutive genes are usually controlled by epigenetic regulations, such as histone deacetylation and DNA methylation. Histones are proteins associated to DNA units, called nucleosomes that form chromatin (Ramakrishnan, 1997). It is suggested that histone modification patterns control the interaction of histone with transcriptional activators and repressors (Jenuwein and Allis, 2001). Histone acetylations are the most understood histone modifications and are controlled by the opposite action of histones acetyltransferases and histone deacetylases (HDACs). As a general rule, hypoacetylation of histone tends to be associated with heterochromatin and silencing genes, while hyperacetylation is usually associated with euchromatin and gene activation (Shwab *et al.*, 2007).

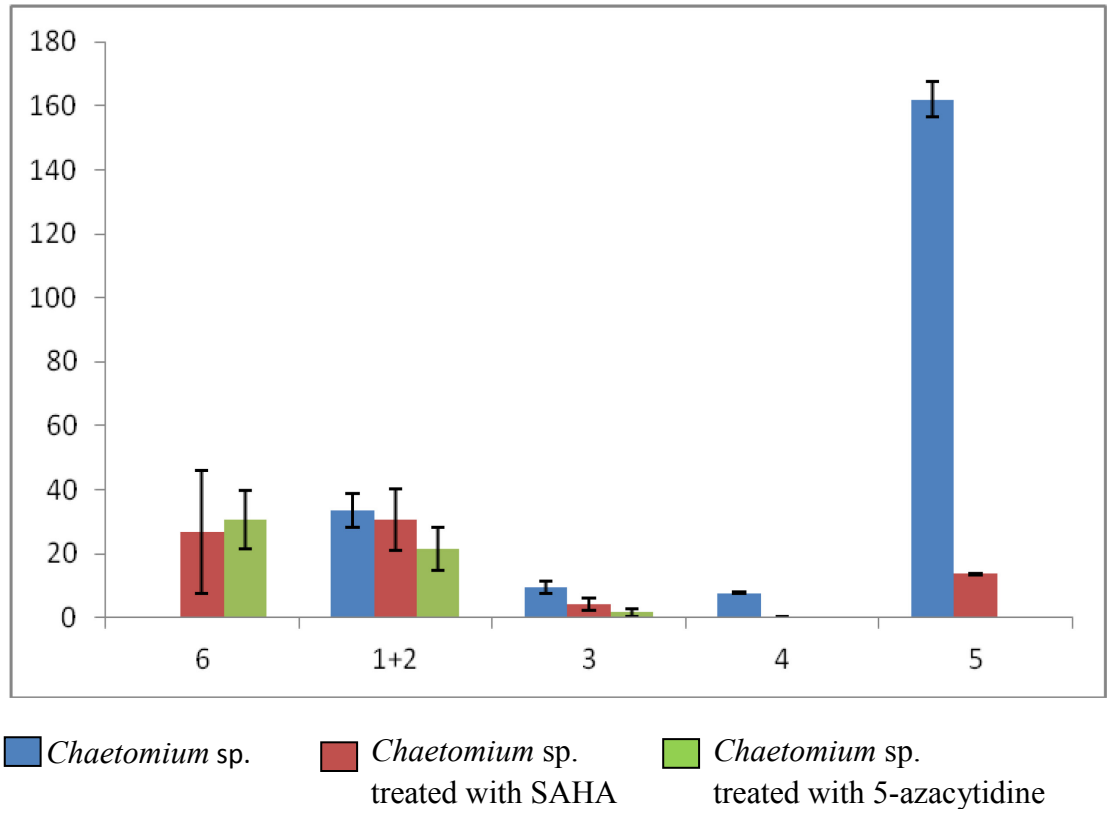
Disruption of the histone deacetylase activity in *Aspergillus nidulans* resulted in the transcriptional activation of gene clusters responsible for the production of sterigmatocystin and penicillin (Shwab *et al.*, 2007). Furthermore, in the same study, treatment of other fungal genera with HDACs resulted in the production of unidentified natural products. In another study, addition of the DNA methyltransferase inhibitor 5-azacytidine to the culture of the endophytic fungus *Pestalotiopsis crassiuscula* afforded one new coumarin (Yang *et al.*, 2014). These observations showed that application of small-molecule epigenetic modifiers, such as inhibitors of histone acetyltransferases (HAT), histone deacetylases (HDACs) or DNA methyltransferases (DMATs) could be a rational approach for the activation of silent biosynthetic pathways, and thus for the induction of fungal metabolite production.

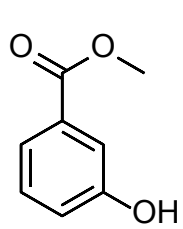
In this study, we applied the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the DMAT inhibitor 5-azacytidine to the endophytic fungus *Chaetomium* sp. on rice medium in a single treatment (Manuscript submitted). Each epigenetic modifier was used at a concentration of 6 mM. We noticed a restriction of the fungal growth the first week of the fermentation compared to the non-treated fungus, whereas the appearance of the yellow pigmentation turned to green. Therefore, a combination of both epigenetic modifiers was not undertaken that could lead to a significant growth restriction and/or generation of metabolite

profiles dominated by the effects of a single component in the mixture. The cultures exhibited convergent responses to both treatments, leading to the induction of the polyketide isosulochrin (**6**) that was not detected in the fungal control (Manuscript submitted). Interestingly, isosulochrin has not yet been reported from *Chaetomium* species, but only from *Pestalotiopsis theae* (Shimada *et al.*, 2001) and *Aspergillus wentii* (Hamasaki and Kimura, 1983). This result constitutes a strong evidence for the role of epigenetic modifiers in the activation of silent genes. Both epigenetic modifiers triggered the same gene clusters, even though the associated mechanisms are different. Importantly, isosulochrin (**6**) was also a co-cultivation product of *Chaetomium* sp. with the bacterium *Bacillus subtilis*, suggesting that the bacterium may trigger partial histone modifications to activate the expression of silent genes for the production of isosulochrin (**6**) (Manuscript submitted). This is in aggreement with findings during co-cultivation of *Aspergillus nidulans* with *Streptomyces rapamycinicus*, in which the latter triggered histone modifications to produce orsellinic acid through the complex Saga/Ada containing the HAT GncE and the AdaB protein in *A. nidulans* (Nützman *et al*., 2011). Interestingly, isosulochrin (**6**) was only observed in the culture of *Chaetomium* sp. treated with *B. subtilis*, confirming the applicability of the use of small epigenetic molecules for the elicitation of new secondary metabolites.

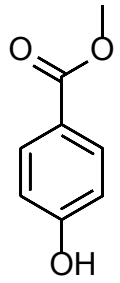
However, application of each epigenetic modifier to the endophytic fungus *Chaetomium* sp. also resulted in the down-regulation of some fungal constitutively secondary metabolites, with a pronounced effect for 5-azacytidine. This observation suggests that epigenetic modifiers are also involved in silencing or repressing fungal gene cluster expression.

Single treatment of SAHA or 5-azacytidine on *Chaetomium* sp.

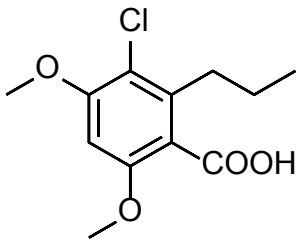




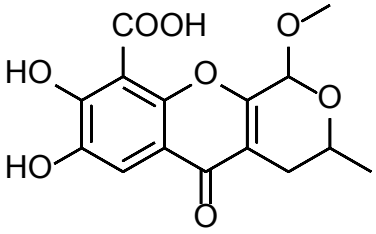
1. Methyl 3-hydroxy-benzoate



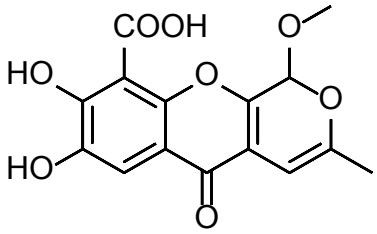
2. Methyl 4-hydroxy-benzoate



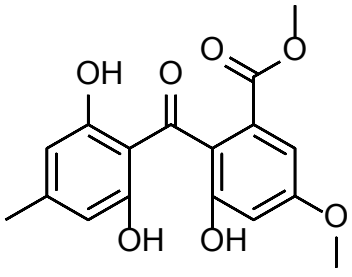
3. Acremonisol



4. SB236050



5. SB238569



6. Isosulochrin

The findings of the current study support the role of epigenetic regulation in fungal secondary metabolite production. This approach is based on the use of epigenetic small molecules, which provide a rapid access of cryptic fungal natural products and can be easily

implemented in most laboratories. However, it depends on finding the right concentration of epigenetic modifiers, under which they trigger histone modifications and lead to the induction of new natural products. Nevertheless, epigenetic modification is a promising tool for natural scientists to further explore fungal diversity (Scherlach and Hertweck, 2009).

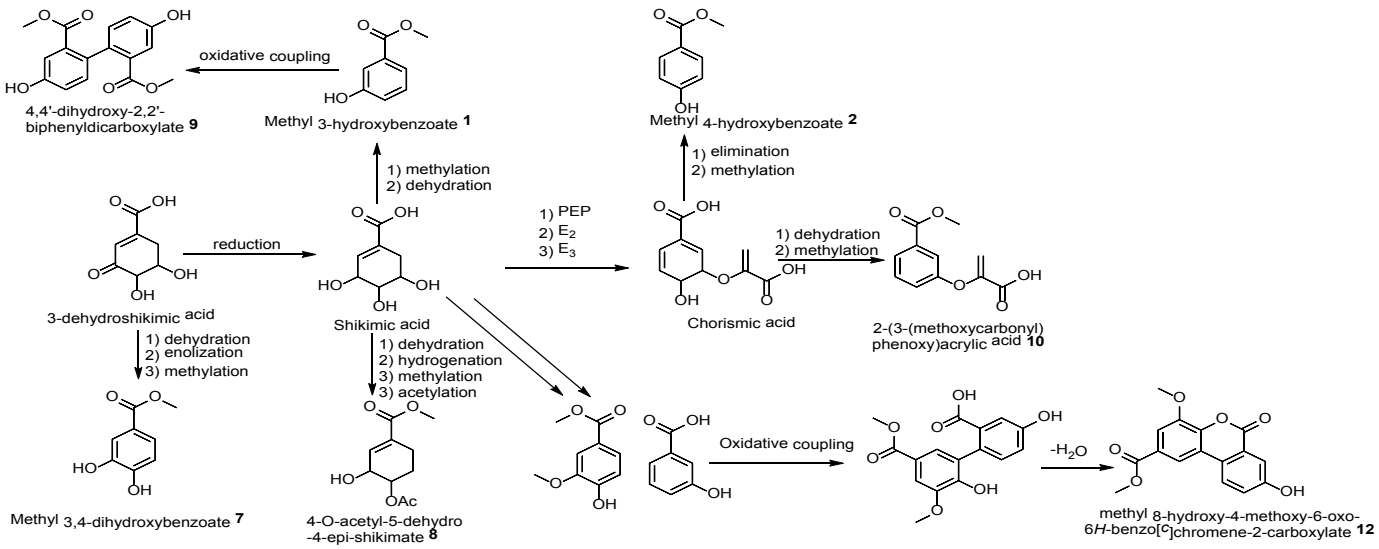
6.3 Proposed Biosynthesis of the Isolated Compounds during Co-Cultivation Experiments of *Chaetomium* sp. vs. *B. subtilis*

During the co-cultivation experiments, *Chaetomium* sp. implemented three different pathways for the production of secondary metabolites.

Shikimic acid pathway

We propose that compounds **1**,**2**,**7,8,9,10** and **12** could originate from the shikimate pathway. Shikimic acid may undergo a series of reactions, including dehydration, hydrogenation, methylation, and acetylation to afford compound **8**. Moreover, methylation followed by dehydration of shikimic acid could lead to compound **1**, which would then undergo oxidative coupling to yield the new compound **9**. Compound **10** could be produced by dehydration of chorismic acid through the enzyme chorismate dehydratase (Mahanta *et al.*, 2013), followed by methylation. Compound **2** is a well known product of shikimic acid, which is formed by elimination and subsequent methylation of chorismic acid (Dewick, 2009). Shikimic acid could also undergo a cascade of dehydration and methylation reactions to afford two benzoic acid derivatives followed by oxidative coupling and hydrolysis to afford the biphenyl compound **12**. Likewise, the well known 3-dehydroshikimic acid could afford compound **7** after a series of dehydration, enolisation, and methylation reactions (Dewick, 2009).

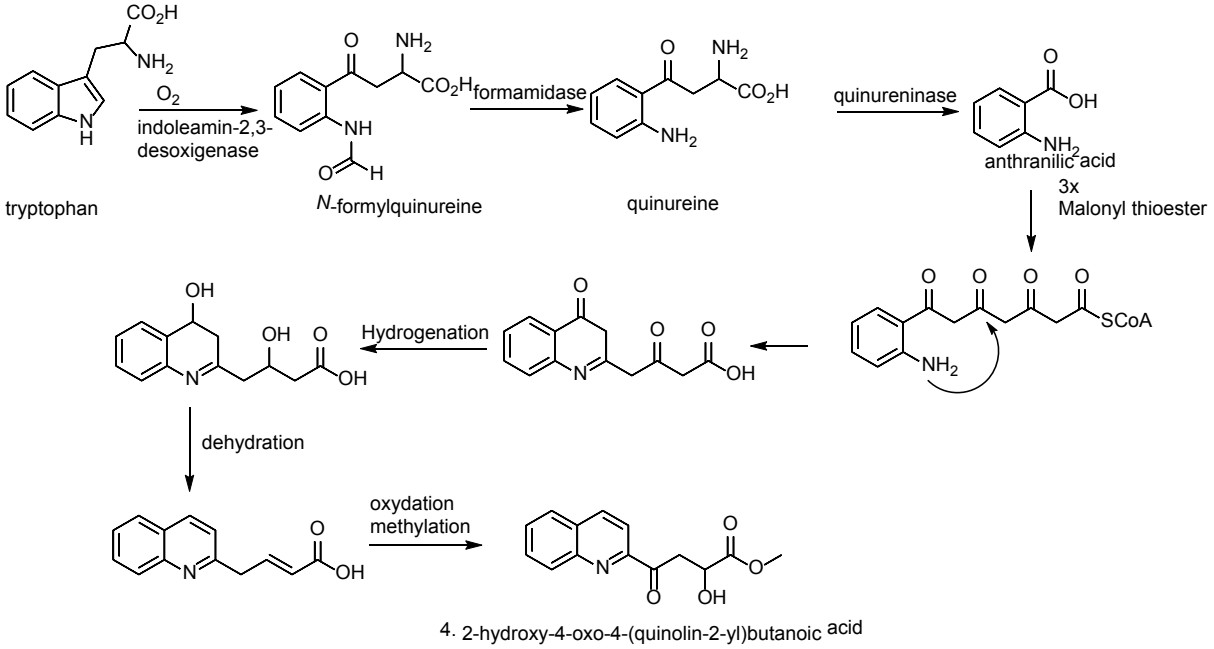
Scheme depicting the proposed biosynthesis for compounds 1,2,7,8,9,10 and 12



Alkaloid

Compound **11**, which is a quinoline derivative, was probably produced by *Chaetomium* sp., as quinolines have already been reported from fungi (Teichert *et al.*, 2008; Abraham and Spassov, 1991). Quinoline biosynthesis has recently been proposed (Diaz *et al.*, 2015). Biosynthetically, compound **11** probably originates from tryptophan, which through a series of enzymatic reactions could afford anthranilic acid. The latter is condensed with three units of malonyl CoA, followed by Mannich condensation and a cascade of hydrogenation, dehydration, oxidation, and methylation reactions to yield **11**.

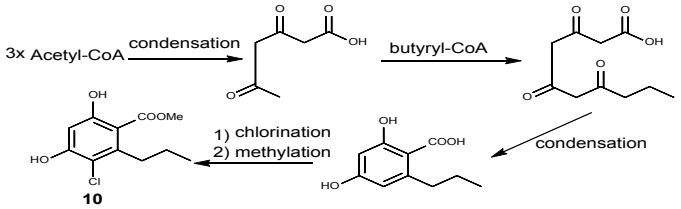
Scheme depecting the proposed biosynthesis of compound 4



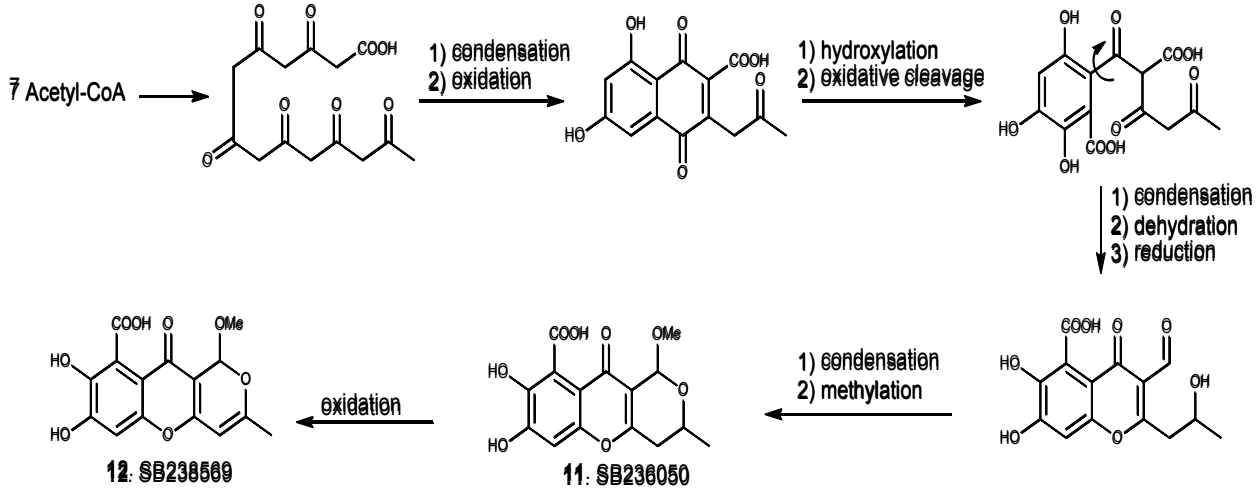
Polyketides

Acremonisol A (**3**) has been reported from the marine-derived fungus *Acremonium* sp. (Pontius *et al.*, 2008). It is possible that its biosynthesis starts from condensation of one butyryl-CoA and three acetyl-CoA units. The product of this reaction could then undergo internal condensation, followed by chlorination and methylation to yield acremonisol A (**3**).

Scheme depecting the proposed biosynthesis acremonisol A (3)

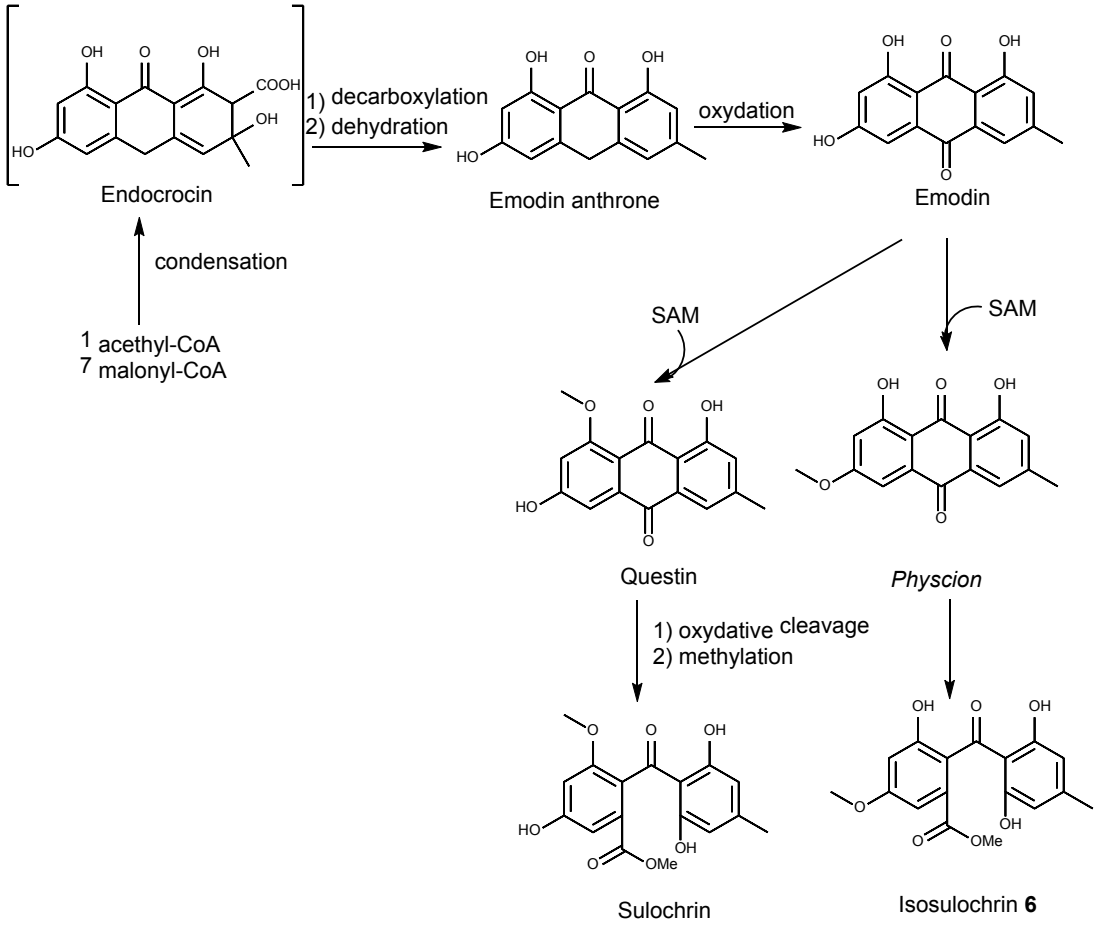


Compounds **4** and **5** are formed through condensation of seven acetate units, followed by a series of reactions, including rotation, condensation, methylation and oxidation reactions (Lu *et al.*, 2013). The proposed pathway is in agreement with the literature, in which the biosynthesis of related compounds known as chaetocyclinones, that differ from **5** by the position of a hydroxy group in the aromatic ring and the presence of an acetyl ester function instead of a carboxylic acid, has been proved by ^{13}C-labelled acetate feeding experiments (Lösgen *et al.,* 2007).



Proposed biosynthesis of compounds **4** and **5** (Lösgen *et al*., 2007; Lu *et al*., 2013)

Another compound involved in the polyketide pathway is isosulochrin (**6**). The biosynthesis of its congener sulochrin has been reported in the literature and since both compounds differ only in the position of the methoxy group, it can be assumed that they share the same biosynthesis. The first step in sulochrin biosynthesis consists of condensation of one acetyl-CoA primer and seven malonyl-CoA extenders, thus leading to the formation of the intermediate endocrocin. Decarboxylation and dehydration is then required to form emodin anthrone, which in turn is oxidized to emodin. Subsequent methylation of emodin through S-adenosylmethionine (SAM) could lead to questin or physcion and S-adenosylhomocysteine (SAH) formation. Finally, oxidation and methylation of questin or its metha isomer physcion (Wijesekara et al., 2014) could yield sulochrin or isosulochrin (Couch, and Gaucher, 2004).



Proposed biosynthesis leading to Isosulochrin (**6**) (Couch and Gaucher, 2004)

6.4 Unguisin Derivatives from the Endophytic Fungus *Mucor irregularis*

6.4.1 Non-ribosomal peptide synthesis

Microorganisms can synthesize peptides either through the classical pathway of ribosomal biosynthesis or through non-ribosomal peptide biosynthesis, where the process is carried out by specific enzymes, called non-ribosomal peptide synthetases (NRPSs) (Colegate *et al.*, 2007). NRPSs are large multimodular biocatalysts that utilize complex regiospecific reactions to assemble structurally and functionally diverse peptides (Strieker *et al.*, 2010). Non-ribosomal peptides (NRPs) often contain non-proteogenic amino acid constituents, as well as fatty acid, polyketide, carbohydrate or isoprenoid moieties (Evans *et al.*, 2011). Notable examples of NRPs are the antibiotics (Fischbach and Walsh, 2006; Saleem *et al.*, 2010) bacitracin, rifamycin, and daptomycin (Kaya *et al.*, 2013), the anticancer agent bleomycin, as well as the immunosuppressant drug cyclosporine A (Fischbach and Walsh, 2006; Walsh, 2003).

NRPSs are multimodular enzymes that act in an assembly line manner, with each module adding a single amino acid during the chain elongation. Accordingly, each amino acid is activated, as an aminoacyl-AMP, in the adenylation domain of the module. Then, the amino acid is transferred to the PCP domain followed by amide bond formation with the next PCP-bound amino acid. During this step, modification of the respective amino acid may take place, e.g. from L- to D-form by an epimerization domain. Finally, the peptide chain is transferred to the thioesterase (TE) domain, in which it is then released either by hydrolysis or macro-cyclization forming a linear or a cyclic peptide, respectively (Strieker *et al.*, 2010).

6.4.2 Unguisin F, a GABA-containing cyclic peptide

Unguisins F, isolated in this study (publication 2), may biogenetically arise from non-ribosomal peptide synthesis, as almost all constituent amino acids were of the D-form after Marfey's analysis, which is in agreement with the literature (Malmstrøm 1999). Moreover, this peptide is unusual in containing a *gamma*-aminobutyric acid (GABA) unit, which is a four carbon non-proteinogenic amino acid. GABA bears an amino group at the gamma-carbon rather than at the alpha-carbon, as in the common α-amino acids. It mainly exists in an unbound form, adopting numerous conformations in solution (Shelp *et al.*, 1999). Likewise, incorporation of a GABA unit in unguisin F peptides is suggested to enhance its conformational mobility (publication 2).

In nature, GABA is widely distributed among microorganisms, plants, and animals (Ueno, 2000). In microorganisms for instance, GABA is functionally involved in spore germination, as reported for *Neurospora crassa* (Kubicek *et al.*, 1979) and *Bacillus megaterium* (Foerster and Foerster, 1979). It is also known to confer resistance in bacteria, such as *Escherichia coli* (Castanie-Cornet *et al.*, 1999).

GABA is the principal inhibitory neurotransmitter in the mammalian central nervous system (Dhakal *et al.*, 2012). In the pharmaceutical industry, several modulators of GABA receptors have been developed, such as benzodiazepines and volatile anaesthetics. Moreover, GABA is an active component in foods (e.g. cheese) and beverages (e.g. Gabaron tea) (Dhakal *et al.*, 2012; Nomura *et al.*, 1998; Sawai *et al.*, 2001). Interestingly, GABA has been documented as having hypotensive, tranquilising, diuretic and antidiabetic effects (Adeghate *et al.*, 2002; Capitani *et al.*, 2003).

Despite the fact that unguisin F showed no significant biological activity in the bioassays employed (publication 2), synthetic cyclic peptides containing GABA have been reported as

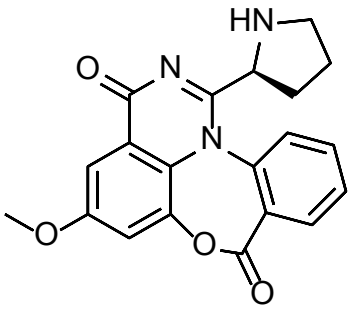
potential drug carriers (Sheh *et al.*, 1995; Chen *et al.*, 1998) and potent antitumor agents (Sheh *et al.*, 1990).

6.5 Induction of Secondary Metabolites in the Soil-derived Fungus *Gongronella butleri* through Co-Culture with *Fusarium striatum*

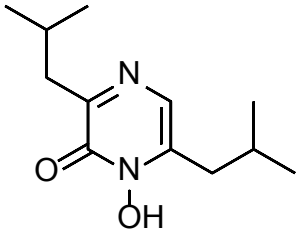
It is suggested that microorganisms, including fungi, live in mixed communities where they interact with each other. These interactions include symbiosis or competition that may lead to the production of a wide range of antifungal metabolites. Therefore, mimicking this natural ecological situation on a laboratory scale may lead to the discovery of new metabolites. Fungal-fungal co-cultivation has been applied in many studies for the production of specific enzymes. For example, co-cultivation of *Aspergillus niger* with *Trichoderma reesei* led to the production of cellulose (Maheshwari *et al.*, 1994). Likewise, co-cultivation of *Aspergillus fumigatus* with *Aspergillus ellipticus* produced cellulase and β-glucosidase (Gupte and Madamwar, 1997). Fungal-fungal co-cultivation has also been used for pigment production. Indeed, when the fungus *Monascus* was co-cultured with *Aspergillus oryzae*, there was a significant increase in the pigment production, more than 30-fold, compared to monocultures of *Monascus* (Shin *et al.*, 1998). Moreover, co-cultivation of fungi has been applied for the production of bioactive compounds in drug discovery. When *Phomopsis* sp. K38 was co-cultured with *Alternaria* sp. E33, three new secondary metabolites were isolated including a xanthone derivative, which displayed antifungal, antibacterial, and cytotoxic activities (Huang *et al.*, 2014). When two different fungi of the genus *Aspergillus* were co-cultured, a new alkaloid aspergicin, as well as the known compounds neoaspergillic acid and ergosterol were produced. The new compound aspergicin showed antibacterial activity against *Bacillus subtilis* (Zhu *et al.*, 2011). In a further study, co-cultivation of two unidentified mangrove-derived endophytic fungi led to the production of two new alkaloids, marinamide and marinamide methylether which showed pronounced cytotoxicity against several cancer cell lines (Zhu *et al.*, 2006 and 2013). Recently, co-cultivation of two *Penicillium* strains (IO1 and IO2), isolated from the Mediterranean sponge *Ircinia oros*, led to the known norlichexanthone and monocerin that were not detected in either of the two axenic fungal controls (Chen, *et al.*, 2015). Monocerin exhibited strong cytotoxicity against the murine lymphoma (L5178Y) cell line with an IC50 value of 8.4 μM. All these experiments showed that the mixed fermentation of two fungi is a powerful tool for the activation of silent genes that could lead to the production of new secondary metabolites.

The soil-derived fungus *Gongrenella butleri*, analysed in this study, yielded eight new 2-pentenedioc acid derivatives (publication 3). We challenged this fungus with the fungus *Fusarium striatum* through a mixed fermentation, in order to activate its silent genes, aiming at the production of new bioactive metabolites. However, no induction of new secondary metabolites was observed, whereas the major compound detected in the HPLC chromatogram appeared to originate from *F. striatum* by comparison with the monoculture. Interestingly, even though both fungi were inoculated at the same time, *F. striatum* took over the surface of the co-culture flask after one week. There are different antagonistic mechanisms between fungi by which mycelia interact with each other. These include hyphal interference, competition for nutrients, and combative interactions (Wicklow *et al.*, 1992). Hyphal interference is very common in fungi-fungi interactions and has been described as leading to the death of hyphae from one species promoted by physical contact with other species (Ikediugwu and Webster, 1970a). Ikediugwu and Webster suggested that hyphal interference is a mean for fungi to win competition when they encounter other microorganisms, especially slow growing ones, and access the nutrients released from dying hyphae (Ikediugwu and Webster, 1970b). It is also assumed that death occurring during the hyphal interference may be caused by toxic substances released only after direct contact (Silar, 2012). Another antagonistic mechanism that might be possible when two fungi are present is via combative interactions. This mechanism differs from hyphal interference by the fact that it does not require contact between the mycelia, but includes antagonism at distance through diffusible substances (known as antibiosis) (Boddy, 2000).

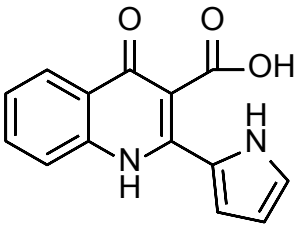
Accordingly, our results suggested that simultaneously inoculation of *G. butleri* and *F. striatum* may favor one of them, which could dominate the competitor by hyphal interference and/or combative interactions. Thus, it is possible that the absence of the induction of new secondary metabolites could be due to the rapid domination of *F. striatum* over *G. butleri*. As the rate of growth of both fungi is different, the success of fungi co-cultivation for the induction of new secondary metabolite may rely on finding the right conditions under which the slowest fungus should be inoculated in a manner that both fungi could coexist for an appreciable time.



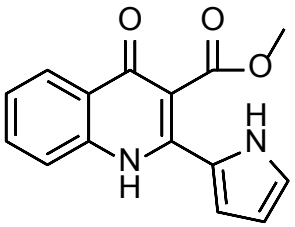
aspergicin



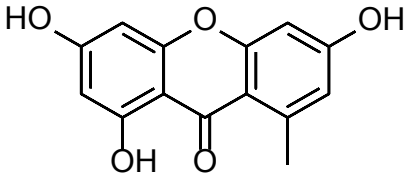
neoaspergillic acid



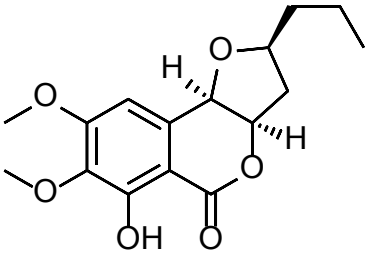
marinamide



marinamide methylether



norlichexanthone



monocerin

REFERENCES

Abraham, W.-R., and Spassov, G. **1991**. '4-Hydroxymethyl-quinoline from *Polyporus* species. *Phytochemistry,* 30: 371-372.

Adeghate, E., and Ponery, A. **2002**. 'GABA in the endocrine pancreas: cellular localization and function in normal and diabetic rats'. *Tissue and Cell,* 34: 1-6.

Alexopoulos, C., J., Mims, C., N., and Blackwell, M. **1996**. "Introductory Mycology". John Wiley and Sons Inc', *New York, USA*.

Allen, D., E, and Hatfield, G. **2004**. 'Medicinal plants in folk tradition: an ethnobotany of Britain & Ireland'.

Allison, A., C. **2000**. 'Immunosuppressive drugs: the first 50 years and a glance forward'. *Immunopharmacology*, 47: 63-83.

Aly, A., H., Debbab, A.., and Proksch, P. **2011**. 'Fungal endophytes: unique plant inhabitants with great promises'. *Applied Microbiology and Biotechnology*, 90: 1829-45.

Arnold, A., E., Henk, D. A., Eells, R., L., Lutzoni, F., and Vilgalys, R. **2007**. 'Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR'. *Mycologia*, 99: 185-206.

Asano, K., Lee, J-B, Yamamura, Y., and Kurosaki, F. **2013**. 'Enhanced accumulation of atropine in *Atropa belladonna* transformed by Rac GTPase gene isolated from *Scoparia dulcis*'. *Transgenic Research*, 22: 1249-55.

Bacon, C., W, and White, J. **2000**. *Microbial endophytes* (CRC Press).

Barrios-González, J., and Miranda, R., U. **2010**. 'Biotechnological production and applications of statins'. *Applied Microbiology and Biotechnology*, 85: 869-83.

Bary, A. **1866**. 'Morphologie und physiologie der pilze, flechten und myxomyceten′ (W. Engelmann).

Beith, M. **1995**. 'Healing threads. Traditional medicines of the highlands and islands'. *Edinburgh: Polygon:* 294. *ISBN*, 74866199.

Bell, A., Wernli, B., and Franklin, R., M. **1994**. 'Roles of peptidyl-prolyl cis-trans isomerase and calcineurin in the mechanisms of antimalarial action of cyclosporin A, FK506, and rapamycin'. *Biochemical Pharmacology*, 48: 495-503.

Bent, K., J, and Moore, R., H. **1966**. "The mode of action of griseofulvin." In *Symp. Soc. Gen. Microbiol*, 16:1.

Bergmann, S., Schümann, J., Scherlach, K., Lange, C., Brakhage, A., A. and Hertweck, C. **2007**. 'Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*'. *Nature Chemical Biology*, 3: 213-17.

Boddy, L. **2000**. 'Interspecific combative interactions between wood-decaying basidiomycetes'. *FEMS Microbiology Ecology, 31*, 185-194.

Bode, Björn, H., Bethe, B. Höfs, R., and Zeeck, A. **2002**. 'Big effects from small changes: possible ways to explore nature's chemical diversity'. *European Journal of Chemical Biology*, 3: 619-27.

Borel, J., F., Feurer, C., Gubler, H., U. and Stähelin, H. **1994**. 'Biological effects of cyclosporin A: a new antilymphocytic agent'. *Agents and Actions*, 43: 179-86.

Bosman, A., and Mendis, K., N.. **2007**. 'A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies'. *The American Journal of Tropical Medicine and Hygiene*, 77: 193-97.

Brahmachari, G. **2011**. 'Natural Products in Drug Discovery: Impacts and', *Bioactive Natural Products: Opportunities and Challenges in Medicinal Chemistry*: 1.

Brakhage, Axel A. **1998**. 'Molecular regulation of β-lactam biosynthesis in filamentous fungi'. *Microbiology and Molecular Biology Reviews*, 62: 547-85.

Brakhage, A., A, Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., and Hertweck, C. **2008**. 'Activation of fungal silent gene clusters: a new avenue to drug discovery.' *Progress in Drug Research*. 66: 3-12.

Buckland, B., Gbewonyo, K., Hallada, T., Kaplan, L., and Masurekar, P. **1989**. 'Production of lovastatin, an inhibitor of cholesterol accumulation in humans', Novel microbial products for medicine and agriculture. *Elsevier, Amsterdam*: 161-69.

Butler, M., S. **2004**. 'The role of natural product chemistry in drug discovery'. *Journal of Natural Products*, 67: 2141-53.

Cameron, J. **1900**. 'The Gaelic Names of Plants:(Scottish, Irish, and Manx)'. John Mackay.

Canel, C., Moraes, R., M., Dayan, F., E. and Ferreira, D. **2000**. 'Podophyllotoxin'. *Phytochemistry*, 54: 115-20.

Capitani, G., De Biase, D., Aurizi, C., Gut, H., Bossa, F., and Grütter, M. G. **2003**. 'Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase'. *The EMBO Journal,* 22: 4027-4037.

Carroll, G. **1988**. 'Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont'. *Ecology*, 69: 2-9.

Castanie-Cornet, M.-P., Penfound, T. A., Smith, D., Elliott, J. F., and Foster, J. W. **1999**. 'Control of acid resistance in *Escherichia coli* '. *Journal of Bacteriology,* 181, 3525-3535.

Chai, Y.-J, Cui, C.-B, Li, C.-W., Wu, C.-J., Tian, C.-K., and Hua, W. **2012**. 'Activation of the dormant secondary metabolite production by introducing gentamicin-resistance in a marine-derived *Penicillium purpurogenum* G59'. *Marine Drugs*, 10: 559-82.

Chang, H.-M., But, P., P., H., and Yao, S.-C. **1986**. *Pharmacology and applications of Chinese materia medica* (World Scientific).

Chaudhuri, A., R., and Ludueña, R., F. **2001**. 'Griseofulvin: Interaction with normal and subtilisin-treated tubulin'. *Drug Development Research*, 53: 44-49.

Chen, H., Aktas, N., Konuklugil, B., Mandi, A., Daletos, G., Lin, W., Dai, H., Kurtan, T., Proksch, P. **2015**. 'A new fusarielin analogue from *Penicillium* sp. isolated from the Mediterranean sponge *Ircinia oros'. Tetrahedron Letters, 56*, 5317-5320.

Chen, H., Daletos, G., Abdel-Aziz, M. S., Thomy, D., Dai, H., Brötz-Oesterhelt, H., Lin, W., Proksch, P. **2015**. 'Inducing secondary metabolite production by the soil-dwelling fungus *Aspergillus terreus* through bacterial co-culture'. *Phytochemistry Letters, 12*, 35-41.

Chen, S.-L., Wong, R. N. S., Lo, V., Chang, S., Chiang, C., Sheh, L. **1998**. 'Synthesis, DNA cleavage and cytotoxicity of some novel cyclic peptide-2, 6-dimethoxy-hydroquinone-3-mercaptoacetic acid conjugates containing D-amino acids'. *Anti-Cancer Drug Design,* 13, 501-518.

Chiang, Y.-M., Chang, S.-L., Oakley, B. R., and Wang, 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: 137-143.

Chiang, Y.-M., Szewczyk, E., Davidson, A., D., Keller, N., Oakley, B., R., and Wang, C., C. **2009**. 'A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, asperfuranone, in *Aspergillus nidulans*'. *Journal of the American Chemical Society*, 131: 2965-70.

Christian, O., E., Compton, J., Christian, K., R., Mooberry, S., L., Valeriote, F., A., and Crews, P. **2005**. 'Using jasplakinolide to turn on pathways that enable the isolation of new chaetoglobosins from *Phomospis asparagi*'. *Journal of Natural Products*, 68: 1592-97.

Cichewicz, R. **2012**. 'Epigenetic regulation of secondary metabolite biosynthetic genes in fungi.' in, *Biocommunication of Fungi* (Springer): 57-69.

Clay, K. **1988**. 'Fungal endophytes of grasses: a defensive mutualism between plants and fungi'. *Ecology*, 69: 10-16.

Colegate, S., M., and Molyneux, R., J. **2007**. '*Bioactive natural products: detection, isolation, and structural determination'*: CRC press.

Couch, R., D., and Gaucher, G., M. **2004**. 'Rational elimination of *Aspergillus terreus* sulochrin production'. *Journal of Biotechnology,* 108: 171-177.

Crabbé, R., Vuagniaux, G., Dumont, J.-M., Nicolas-Métral,V., Marfurt, J., and Novaroli, L. **2009**. 'An evaluation of the cyclophilin inhibitor Debio 025 and its potential as a treatment for chronic hepatitis C', *Expert Opinion on Investigational Drugs*, 18: 211-20.

Crabtree, G., R. **1989**. 'Contingent genetic regulatory events in T lymphocyte activation'. *Science*, 243: 355-61.

Cragg, G., M. **1998**. 'Paclitaxel (Taxol®): a success story with valuable lessons for natural product drug discovery and development'. *Medicinal Research Reviews*, 18: 315-31.

Cragg, G., M, and Newman, D., J. **2001**. 'Natural product drug discovery in the next millennium'. *Pharmaceutical Biology*, 39: 8-17.

Cragg, G., M., and Newman, D., J. **2005**. 'Biodiversity: A continuing source of novel drug leads'. *Pure and Applied Chemistry*, 77: 7-24.

Cragg, G., M., and Newman, D., J. **2013**. 'Natural products: a continuing source of novel drug leads', *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1830: 3670-95.

Cueto, M., Jensen, P., R., Kauffman, C., Fenical, W., Lobkovsky, E., and Clardy, J. **2001**. 'Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge'. *Journal of Natural Products, 64*(11), 1444-1446.

Cui, L., and Su, X.-Z. **2009**. 'Discovery, mechanisms of action and combination therapy of artemisinin'. *Expert Review of Anti-infective Therapy*, 7: 999-1013.

Denis, J., N., Greene, A., E., Guenard, D., Gueritte-Voegelein, F., Mangatal, L., and Potier, P. **1988**. 'Highly efficient, practical approach to natural taxol'. *Journal of the American Chemical Society*, 110: 5917-19.

Denning, D.,W. 2003. 'Echinocandin antifungal drugs', *The Lancet*, 362: 1142-51.

DerMarderosian, A., and Beutler, J., A. **2002**. *The review of natural products: the most complete source of natural product information* (Facts and Comparisons).

Dev, S. **1999**. 'Ancient-modern concordance in Ayurvedic plants: some examples'. *Environmental Health Perspectives*, 107: 783-89.

Dewick, P. M. **2002**. "*Medicinal natural products: a biosynthetic approach"*. John Wiley & Sons.

Dhakal, R., Bajpai, V., K., and Baek, K.-H. **2012**. 'Production of GABA (γ-aminobutyric acid) by microorganisms: a review'. *Brazilian Journal of Microbiology, 43*, 1230-1241.

Dias, D., A, Urban, S., and Roessner, U. **2012**. 'A historical overview of natural products in drug discovery'. *Metabolites*, 2: 303-36.

Diaz, G., Miranda, I. L., & Diaz, M. A. N. **2015**. "Quinolines, Isoquinolines, Angustureine, and Congeneric Alkaloids—Occurrence, Chemistry, and Biological Activity" In Phytochemicals-Isolation, characterization and role in human health. *Agricultural and Biological Sciences*. 141-162.

Dillenius, J., J., and Ray, J. **1724.** "Synopsis Methodica Stirpium Britannicarum." In.: G. and J. Innys: London, UK.

Duke, J., A. **2008**. "*Duke's handbook of medicinal plants of Latin America*." CRC Press.

Evans, B., S., Chen, Y., Metcalf, W., W., Zhao, H., and Kelleher, N., L. **2011**. 'Directed evolution of the nonribosomal peptide synthetase AdmK generates new andrimid derivatives in vivo'. *Chemistry & Biology,* 18, 601-607.

Eyberger, A., L., Dondapati, R.,, and Porter, J., R. **2006**. 'Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin'. *Journal of Natural Products*, 69: 1121-24.

Fabbretti, A., Gualerzi, C., O., and Brandi, L. **2011**. 'How to cope with the quest for new antibiotics'. *FEBS Letters*, 585: 1673-81.

Fenical, W., and Jensen, P., R. **2006**. 'Developing a new resource for drug discovery: marine actinomycete bacteria'. *Nature Chemical Biology*, 2: 666-73.

Fenical, W., Jensen, P., R., Palladino, M., A., Lam, K., S., Lloyd, G., K., and Potts, B., C. **2009**. Discovery and development of the anticancer agent salinosporamide A (NPI-0052). *Bioorganic & Medicinal Chemistry,* 17: 2175-2180.

Figueiredo, E., L., and Machado, F., P. **2010**. 'Digoxin's roles in heart failure patients: An overview'. *Insuficiencia Cardíaca,* 5: 59-64.

Fischbach, M., A., and Walsh, C., T. **2006**. 'Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms'. *Chemical Reviews, 106*, 3468-3496.

Fitch, C., D. **2004**. 'Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs'. *Life Sciences*, 74: 1957-72.

Florey, H., W., Abraham, E., P., Newton, G., G., F., Burton, H., S., Kelly, B., K., Hale, C., W., and Miller, G., A. **1956**. 'Improvements relating to the production of an antibiotic substance by a *Cephalosporium* species'. *British Patent*, 745208.

Foerster, C., W., and Foerster, H., F. **1973**. 'Glutamic acid decarboxylase in spores of *Bacillus megaterium* and its possible involvement in spore germination'. *Journal of Bacteriology,* 114, 1090-1098.

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M.,and Sarniguet, A. **2011.** 'Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists'. *Microbiology and Molecular Biology Reviews,* 75, 583-609.

Fröhlich, J., and Hyde, K., D. **1999.** 'Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic?', *Biodiversity & Conservation*, 8: 977-1004.

Gerke, J., Bayram, Ö., Feussner, K., Landesfeind, M., Shelest, E., Feussner, I., and Braus, G. H. **2012**. 'Breaking the silence: protein stabilization uncovers silenced biosynthetic gene clusters in the fungus *Aspergillus nidulans*'. *Applied and Environmental Microbiology,* 78: 8234-8244.

Gonzalez, A., Jimenez, A., Vazquez, D., Davies, J., and Schindler, D. **1978**. 'Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes'. *Biochimica et Biophysica Acta (BBA)-Nucleic Acids and Protein Synthesis,* 521: 459-469.

Grisham, L., M., Wilson, L., and Bensch, K., G. **1973**. 'Antimitotic action of griseofulvin does not involve disruption of microtubules': 294-296.

Grove, J., F., and McGowan, J., C. **1947**. 'Identity of griseofulvin and "curling factor"', *Nature*, 160: 1947.

Grynkiewicz, G., and Gadzikowska, M. **2008**. 'Tropane alkaloids as medicinally useful natural products and their synthetic derivatives as new drugs'. *Pharmacological Reports,* 60: 439-463.

Guéritte, F., and Fahy, J. **2005.** 'The vinca alkaloids', *Anticancer agents from natural products*, 10: 123-35.

Guerram, M., Jiang, Z.-Z., and Zhang, L.-Y. **2012**. 'Podophyllotoxin, a medicinal agent of plant origin: past, present and future', *Chinese Journal of Natural Medicines*, 10: 161-69.

Gull, K., and Trinci, A., P., J. **1973**. 'Griseofulvin inhibits fungal mitosis'. 292-294.

Gulyamova, T., G., Nasmetova, S., M., Ruzieva, D., M., Ziyavitdinov, J., F., Sattarova, R., S. and Rasulova, G., A. **2014.** 'Composition of statins produced by indigenous strain of

Aspergillus terreus'. *International Journal of Engineering, Science and Technology*, 6: 71-76.

Guo, B., H., Kai, G., Y., Jin, H., B., and Tang, K., X. **2006**. 'Review-Taxol synthesis'. African *Journal of Biotechnology*, 5: 15-20

Guo, B., Li, H., and Zhang, L. **1997**. 'Isolation of an fungus producting vinbrastine'. *Journal of Yunnan University (Natural Sciences)*, 20: 214-15.

Gupte, A., and Madamwar, D. **1997.** 'Solid state fermentation of lignocellulosic waste for elclulase and β-glucosidase production by co-cultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus*. *Biotechnology Progress,* 13, 166-169.

Gurib-Fakim, A. **2006**. 'Medicinal plants: traditions of yesterday and drugs of tomorrow'. *Molecular Aspects of Medicine*, 27: 1-93.

Gurnani, N., Mehta, D., Gupta, M., and Mehta, B., K. **2014**. 'Natural Products: Source of Potential Drugs'. *African Journal of Basic & Applied Sciences*, 6: 171-86.

Hamasaki, T., and Kimura, Y. **1983**. 'Isolation and structures of four new metabolites from *Aspergillus wentii'*. *Agricultural and Biological Chemistry,* 47, 163-165.

Harborne, J. B. **1984**. "Methods of plant analysis *Phytochemical methods"*, Springer: 1-36.

Hartley, S., E., and Gange, A., C **2009**. 'Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context'. *Annual Review of Entomology*, 54: 323-42.

Harvey, A.,L. **2008**. 'Natural products in drug discovery'. *Drug Discovery Today*, 13: 894-901.

Hatfield, G. **1994**. *Country remedies: Traditional East Anglian plant remedies in the twentieth century*: Boydell.

Hatziioannou, T., Perez-Caballero, D., Cowan, S., and Bieniasz, P., D. **2005.** 'Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells'. *Journal of Virology*, 79: 176-83.

Hay, S., I., Guerra, C., A., Tatem, A., J., Noor, A., M., and Snow, R., W. **2004**. 'The global distribution and population at risk of malaria: past, present, and future'. *The Lancet Infectious Diseases*, 4: 327-36.

Hinshaw, C., Feldman, W., H., and Pfuetze, K., H. **1946.** 'Treatment of tuberculosis with streptomycin: a summary of observations on one hundred cases'. *Journal of the American Medical Association*, 132: 778-82.

Holton, R., A, Kim, H., B., Somoza, C., Liang, F., Biediger, R., J., Boatman, P., D., Shindo, M., Smith, C., C., and Kim, S. **1994.** 'First total synthesis of taxol. 2. Completion of the C and D rings'. *Journal of the American Chemical Society*, 116: 1599-600.

Holwell, S., E., Cooper, P., A., Grosios, K., Lippert, J., W., Pettit, G., R., Shnyder, S., D., and Bibby, M., C. **2001.** 'Combretastatin A-1 phosphate a novel tubulin-binding agent with in vivo anti vascular effects in experimental tumours'. *Anticancer Research*, 22: 707-11.

Hong, K., Gao, A.-H., Xie, Q.-Y., Gao, H., G., Zhuang, L., Lin, H.-P., Yu, H.-P., Li, J., Yao, X.-S, Goodfellow, M. **2009**. 'Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China'. *Marine Drugs,* 7: 24-44.

Huang, S., Ding, W., Li, C., and Cox, D. G. **2014**. 'Two new cyclopeptides from the co-culture broth of two marine mangrove fungi and their antifungal activity'. *Pharmacognosy Magazine, 10*, 410.

Hubler, F., Rückle, T., Patiny, L., Muamba, T., Guichou, J.-F., Mutter, M., and Wenger, R.. **2000.** 'Synthetic routes to NEtXaa 4-cyclosporin A derivatives as potential anti-HIV I drugs', *Tetrahedron Letters*, 41: 7193-96.

Hyde, K., D., and Soytong, K. 2008. 'The fungal endophyte dilemma', *Fungal Diversity*, 33: 2.

Ikediugwu, F., and Webster, J. **1970** (a). 'Hyphal interference in a range of coprophilous fungi'. *Transactions of the British Mycological Society, 54*, 205-IN206.

Ikediugwu, F., E., O., and Webster, J. **1970** (b). Antagonism between *Coprinus heptemerus* and other coprophilous fungi'. *Transactions of the British Mycological Society, 54*, 181IN182-204IN184.

Jenuwein, T., and Allis, C., D. **2001.** 'Translating the histone code'. *Science, 293*, 1074-1080.

Jonsson, E., Dhar, S., Jonsson, B., Nygren, P., Graf, W., and Larsson, R. **2000**. 'Differential activity of topotecan, irinotecan and SN-38 in fresh human tumour cells but not in cell lines'. *European Journal of Cancer,* 36: 2120-2127.

Joyce, Christopher. 1993. 'Taxol: search for a cancer drug'. *Bioscience*, 43: 133-36.

Kannan, R., Sahal, D., and Chauhan, V., S. **2002**. 'Heme-artemisinin adducts are crucial mediators of the ability of artemisinin to inhibit heme polymerization'. *Chemistry & Biology*, 9: 321-32.

Kaya, S., Yilmaz, G., Kalkan, A., Ertunç, B., and Köksal, İ. **2013.** 'Treatment of Gram-positive left-sided infective endocarditis with daptomycin'. *Journal of Infection and Chemotherapy, 19*, 698-702.

Keates, R., A., B. **1981**. 'Griseofulvin at low concentration inhibits the rate of microtubule polymerization in vitro', *Biochemical and Biophysical Research Communications*, 102: 746-52.

Kedei, N., Lundberg, D., J., Toth, A., Welburn, P., Garfield, S., H., and Blumberg, P., M. **2004.** 'Characterization of the interaction of ingenol 3-angelate with protein kinase C', *Cancer Research*, 64: 3243-55.

Keglevich, P., Hazai, L., Kalaus, G., and Szántay, C. **2012.** 'Modifications on the basic skeletons of vinblastine and vincristine', *Molecules*, 17: 5893-914.

Khosroushahi, A. Y., Valizadeh, M., Ghasempour, A., Khosrowshahli, M., Naghdibadi, H., Dadpour, M., and Omidi, Y. **2006**.'Improved Taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata'*. *Cell Biology International,* 30: 262-269.

Kinghorn, A., D., Pan, L., Fletcher, J., N., and Chai, H. **2011**. The relevance of higher plants in lead compound discovery programs. *Journal of Natural Products,* 74: 1539-1555.

Kingston, D., G. **2009**. 'Tubulin-interactive natural products as anticancer agents (1)', *Journal of Natural Products*, 72: 507-15.

Kingston, D. **2011**. 'Taxol and Its Analogs 6', *Anticancer agents from natural products*: 123.

Kjeldsen, E., Svejstrup, J., Q., Gromova, I., I., Alsner,J., and Westergaard, O. **1992.** 'Camptothecin inhibits both the cleavage and religation reactions of eukaryotic DNA topoisomerase I', *Journal of Molecular Biology*, 228: 1025-30.

Klonis, N., Dilanian, R., Hanssen, E., Darmanin, C., Streltsov, V., Deed, S., Quiney, H., and Tilley, L. **2010**. 'Hematin− hematin self-association states involved in the formation and reactivity of the malaria parasite pigment, hemozoin'. *Biochemistry*, 49: 6804-11.

Knight, V., Sanglier, J.-J., DiTullio, D., Braccili, S., Bonner, P., Waters, J., Hughes, D., Zhang, L. **2003**. 'Diversifying microbial natural products for drug discovery'. *Applied Microbiology and Biotechnology,* 62, 446-458.

Kocken, C., H., M., van der Wel, A., Rosenwirth, B., and Thomas, A., W. **1996**. 'Plasmodium vivax: In VitroAntiparasitic Effect of Cyclosporins'. *Experimental Parasitology*, 84: 439-43.

Kovács, P., Csaba, G., Pállinger, É, and Czaker, R. **2007.** 'Effects of taxol treatment on the microtubular system and mitochondria of Tetrahymena'. *Cell Biology International*, 31: 724-32.

Kubicek, C., Hampel, W., and Röhr, M. **1979**. 'Manganese deficiency leads to elevated amino acid pools in citric acid accumulating *Aspergillus niger*'. *Archives of Microbiology, 123*, 73-79.

Kuhn, Y., Rohrbach, P., and Lanzer, M. **2007**. 'Quantitative pH measurements in *Plasmodium falciparum*-infected erythrocytes using pHluorin'. *Cellular Microbiology*, 9: 1004-13.

Kumar, A., Patil, D., Rajamohanan, P., R., and Ahmad, A. **2013**. 'Isolation, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*'. *PloS one*, 8: e71805.

Kurtz, M., B., and Rex, J., H. **2001**. 'Glucan synthase inhibitors as antifungal agents'. *Advances in Protein Chemistry*, 56: 423-75.

Kusari, S., Zühlke, S., and Spiteller, M. **2009**. 'An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues'. *Journal of Natural Products*, 72: 2-7.

Laatsch, H. **2011**. *Antibase 2012: The natural compound identifier*: Wiley-Vch.

Larsen, T., O., Smedsgaard, J., Nielsen, K., F., Hansen, M., E., and Frisvad, J., C. **2005**. 'Phenotypic taxonomy and metabolite profiling in microbial drug discovery'. *Natural Product Reports*, 22: 672-95.

Lösgen, S., Schlörke, O., Meindl, K., Herbst-Irmer, R., and Zeeck, A. **2007**. 'Structure and biosynthesis of chaetocyclinones, new polyketides produced by an endosymbiotic fungus'. *European Journal of Organic Chemistry,* 2007: 2191-2196.

Liu, Y., Gong, G., Xie, L., Yuan, N., Zhu, C., Zhu, B., and Hu, Y. **2010.** 'Improvement of cephalosporin C production by recombinant DNA integration in *Acremonium chrysogenum*'. *Molecular Biotechnology*, 44: 101-09.

Lu, K., Zhang, Y., Li, L., Wang, X., and Ding, G. **2013**. 'Chaetochromones A and B, two new polyketides from the fungus *Chaetomium indicum* (CBS. 860.68)'. *Molecules,* 18: 10944-10952.

Lucas, R., De Sante, K., Hatcher, B., Heminway, J., Lachno, R., and Brooks, S. **1996**. "LY303366 single dose pharmacokinetics and safety in healthy volunteers". In *Program and abstracts of the 36th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy. New Orleans*.

Ma, L.-J., and Fedorova, N., D. **2010**. 'A practical guide to fungal genome projects: strategy, technology, cost and completion'. *Mycology*, 1: 9-24.

MacFarlane, A. **1924**. *Gaelic names of plants: Study of their uses and lore*.

Madej, C. W., and Clay, K. **1991**. 'Avian seed preference and weight loss experiments: the effect of fungal endophyte-infected tall fescue seeds'. *Oecologia*, 88: 296-302.

Mahanta, N., Fedoseyenko, D., Dairi, T., and Begley, T., P. **2013**. 'Menaquinone biosynthesis: formation of aminofutalosine requires a unique radical SAM enzyme'. *Journal of the American Chemical Society,* 135: 15318-15321.

Maheshwari, D., Gohade, S., Paul, J., and Varma, A. **1994.** 'Paper mill sludge as a potential source for cellulase production by *Trichoderma reesei* QM 9123 and *Aspergillus niger* using mixed cultivation'. *Carbohydrate Polymers,* 23, 161-163.

Malik, S., Cusidó, R., M., Mirjalili, M., H., Moyano, E., Palazón, J., and Bonfill, M. **2011**. 'Production of the anticancer drug taxol in *Taxus baccata* suspension cultures: a review'. *Process Biochemistry*, 46: 23-34.

Malmstrøm, J. **1999**. 'Unguisins A and B: new cyclic peptides from the marine-derived fungus *Emericella unguis'*. *Journal of Natural Products,* 62, 787-789.

Mann, John. **1994**. "Magic, Murder and Medicine." In.: Oxford University Press, New York.

Marmann, A., Aly, A. H., Lin, W., Wang, B., and Proksch, P. **2014**. 'Co-cultivation—A powerful emerging tool for enhancing the chemical diversity of microorganisms'. *Marine Drugs*, 12: 1043-65.

Martin, M. **1934**. "*A Description of the Western Isles of Scotland*". 4th ed.; Macleod, D.J., Ed.; Stirling: Eneas Mackay: Cornhill, UK.

Masurekar, P., S., Fountoulakis, J., M., Hallada, T., C., Sosa, M., S., and Kaplan, L. **1992**. 'Pneumocandins from Zalerion arboricola. II. Modification of product spectrum by mutation and medium manipulation'. *The Journal of Antibiotics,* 45: 1867-1874.

Mathijssen, R., H., Loos, W. J., Verweij, J., and Sparreboom, A. **2002**. 'Pharmacology of topoisomerase I inhibitors irinotecan (CPT-11) and topotecan'. *Current Cancer Drug Targets,* 2: 103-123.

Matsuda, S., and Koyasu, S. **2000**. 'Mechanisms of action of cyclosporine'. *Immunopharmacology*, 47: 119-25.

Matsuda, S., Moriguchi, T., Koyasu, S., and Nishida, E. **1998**. 'T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A'. *Journal of Biological Chemistry*, 273: 12378-82.

McMurry, J., **2010**. 'Organic Chemistry with biological approach, In Secondary metabolites: an introduction to natural products chemistry, Cengage Learning, *Science* : 1016

Mohammadgholi, A., Rabbani-Chadegani, A., and Fallah, S. **2013**. 'Mechanism of the interaction of plant alkaloid vincristine with DNA and chromatin: spectroscopic study', *DNA and cell biology*, 32: 228-35.

Müller, I., B., and Hyde, J., E. **2010**. 'Antimalarial drugs: modes of action and mechanisms of parasite resistance'. *Future Microbiology*, 5: 1857-73.

Musmade, P., B., Tumkur, A., Trilok, M., and Bairy, K., L. **2013**. Fusidic acid-topical antimicrobial in the mamagement of *Staphylococcus aureaus*. ' *International Journal of Pharmacy and Pharmaceutical Sciences*, 5: 381-90.

Nair, D., N., and S., Padmavathy. **2014.** 'Impact of endophytic microorganisms on plants, environment and humans'. *The Scientific World Journal*, 2014: 1-11.

Negishi, S., Cai-Huang, Z., Hasumi, K., Murakawa, S., and Endo, A. **1986** (in Japanese). *Hakko Kogaku Kaishi* 64: 509-512

Netzker, T., Fischer, J., Weber, J., Mattern, D., J., König, C., C., Valiante, V., Schroeckh, V., and Brakhage, A., A. **2015.** 'Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters'. *Frontiers in Microbiology*, 6: 1-13.

Newman, D., J., and Cragg, G., M. **2010**. 'Natural products as drugs and leads to drugs: the historical perspective'. *Natural Product Chemistry for Drug Discovery, Cambridge, UK: RSC Publishing*, 18: 23-24.

Newton, G., G., F., and Abraham, E., P. **1956**. 'Isolation of cephalosporin C, a penicillin-like antibiotic containing D-α-aminoadipic acid'. *Biochemical Journal*, 62: 651.

Nicolaou, K., C., Yang, Z., Liu, J., J., Ueno, H., Nantermet, P., G., Guy, R., K., Claiborne, C., F., Renaud, J., Couladouros, E., A., and Paulvannan, K. **1994**. 'Total synthesis of taxol'. *Nature*, 367: 630-34.

Nomura, M., Kimoto, H., Someya, Y., Furukawa, S., and Suzuki, I. **1998**. 'Production of γ-aminobutyric acid by cheese starters during cheese ripening'. *Journal of Dairy Science,* 81: 1486-1491.

Nützmann, H.-W., Reyes-Dominguez, Y., Scherlach, K., Schroeckh, V., Horn, F., Gacek, A., Schümann, J., Hertweck, C., Strauss, J, and Brakhage, A. A. **2011**. 'Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation'. *Proceedings of the National Academy of Sciences,* 108: 14282-14287.

Nyfeler, R., and Keller-Schierlein, W. **1974**. 'Echinocandin B, A novel polypeptide-antibiotic from *Aspergillus nidulans* var. echinulatus: isolation and structural components'. *Helvetica Chimica Acta* 57: 2459-77.

Ochi, K., and Hosaka, T. **2013**. 'New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters'. *Applied Microbiology and Biotechnology*, 97: 87-98.

Ochi, K., Okamoto, S., Tozawa, Y., Inaoka, T., Hosaka, T., Xu, J., and Kurosawa, K. **2004**. 'Ribosome engineering and secondary metabolite production'. *Advances in Applied Microbiology*, 56: 155-84.

Oda, T. **2006.** 'Effects of 2′-demethoxy-2′-propoxygriseofulvin on microtubule distribution in Chinese hamster V79 cells'. *The Journal of antibiotics*, 59: 114-16.

Ogbourne, S., M., Suhrbier, A., Jones, B., Cozzi, S.-J., Boyle, G., M., Morris, M., McAlpine, D., Johns, J., Scott, T., M., and Sutherland, K. P. **2004**. 'Antitumor activity of 3-ingenyl angelate plasma membrane and mitochondrial disruption and necrotic cell death'. *Cancer Research*, 64: 2833-39.

Oh, D.-C., Jensen, P., R., Kauffman, C., A., and Fenical, W. **2005**. 'Libertellenones A–D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition'. *Bioorganic & Medicinal Chemistry*, 13: 5267-73.

Oh, D.-C., Kauffman, C., A., Jensen, P., R., and Fenical, W. **2007**. 'Induced production of emericellamides A and B from the marine-derived fungus *Emericella* sp. in competing co-culture'. *Journal of Natural Products,* 70, 515-520.

Ola, A., R., Thomy, D., Lai, D., Brötz-Oesterhelt, H., and Proksch, P. **2013**. 'Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through co-culture with *Bacillus subtilis'*. *Journal of Natural Products,* 76, 2094-2099.

Osment, L., S. **1969**. 'The many effects of griseofulvin'. *The Alabama Journal of Medical Sciences*, 6: 392.

Overy, D., P., Smedsgaard, J., Frisvad, J., C., Phipps, R., K., and Thrane, U. L., F. **2006**. 'Host-derived media used as a predictor for low abundant, in planta metabolite production from necrotrophic fungi'. *Journal of Applied Microbiology*, 101: 1292-300.

Oxford, A., E., Raistrick, H. and Simonart, P. **1939**. 'Studies in the biochemistry of micro-organisms: Griseofulvin, C17H17O6Cl, a metabolic product of *Penicillium griseo-fulvum* Dierckx'. *Biochemical Journal*, 33: 240.

Paeshuyse, J., Kaul, A., De Clercq, E., Rosenwirth, B., Dumont, J.-M., Scalfaro, P., Bartenschlager, R., and Neyts, J. **2006.** 'The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro'. *Hepatology*, 43: 761-70.

Papp-Wallace, K., M., Endimiani, A., Taracila, M., A., and Bonomo, R., A. **2011**. 'Carbapenems: past, present, and future'. *Antimicrobial Agents and Chemotherapy*, 55: 4943-60.

Park, H.,B., Kwon, H., C., Lee, C.-H., and Yang, H., O. **2009**. 'Glionitrin A, an antibiotic−antitumor metabolite derived from competitive interaction between abandoned mine microbes'. *Journal of Natural Products*, 72: 248-52.

Pereira, T.,M., Vieira, A., P., Fernandes, J., C., and Sousa-Basto, A. **2006.** 'Cyclosporin A treatment in severe childhood psoriasis'. *Journal of the European Academy of Dermatology and Venereology*, 20: 651-56.

Petrini, O. **1991**. 'Fungal endophytes of tree leaves. In *Microbial ecology of leaves*.(Springer) : 179-197.

Pettit, R. K. **2011**. 'Culturability and secondary metabolite diversity of extreme microbes: Expanding contribution of deep sea and deep-sea vent microbes to natural product discovery'. *Marine Biotechnology,* 13, 1-11.

Pontius, A., Mohamed, I., Krick, A., Kehraus, S., and König, G. M. **2008**. 'Aromatic polyketides from marine algicolous fungi'. *Journal of Natural Products,* 71: 272-274.

Pu, X., Qu, X., Chen, F., Bao, J., Zhang, G., and Luo, Y. **2013**. 'Camptothecin-producing endophytic fungus *Trichoderma atroviride* LY357: isolation, identification, and fermentation conditions optimization for camptothecin production'. *Applied Microbiology and Biotechnology*, 97: 9365-75.

Puri, S., C., Verma, V., Amna, T., Qazi, G., N., and Spiteller, M. **2005**. 'An endophytic fungus from *Nothapodytes f oetida* that produces Camptothecin. *Journal of Natural Products,* 68: 1717-1719.

Purvis, W. **2000**. "*Lichens. London: Natural History Museum"*. London, Washington DC: Smithsonian Books.

Ramakrishnan, V. **1997**. 'Histone structure and the organization of the nucleosome'. *Annual Review of Biophysics and Biomolecular Structure, 26*: 83-112.

Ramawat, K., G., and Mérillon, J-M. **2008**. "*Bioactive molecules and medicinal plants"* (Springer).

Rammelkamp, C., H., and Maxon, T. **1942**. 'Resistance of *Staphylococcus aureus* to the Action of Penicillin'. *Experimental Biology and Medicine*, 51: 386-89.

Rao, S.,K., Prasad, T., Mohanta, G., P., and Manna, P., K. **2011.** 'An overview of statins as hypolipidemic drugs'. *International Journal of Pharmaceutical Science and Drug Research*, 3: 178-83.

Rebacz, B., Larsen, T., O., Clausen, M., H., Rønnest, M., H., Löffler, H., Ho, A., D., and Krämer, A. **2007**. 'Identification of griseofulvin as an inhibitor of centrosomal clustering in a phenotype-based screen'. *Cancer Research*, 67: 6342-50.

Reen, F., J., Romano, S., Dobson, A., D., W., and O'Gara, F. **2015**. 'The Sound of silence: activating silent biosynthetic gene clusters in marine microorganisms'. *Marine Drugs*, 13: 4754-83.

Rehman, S., Shawl, A., S., Kour, A., Andrabi, R., Sudan, P., Sultan, P., Verma, V., and Qazi, G. N. **2008**. 'An endophytic *Neurospora* sp. from *Nothapodytes foetida* producing camptothecin'. *Applied Biochemistry and Microbiology*, 44: 203-09.

Rodriguez, R., and Redman, R. **2008**. 'More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis'. *Journal of Experimental Botany*, 59: 1109-14.

Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T., and Jones, E. **2008**. 'Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*'. *Thailand. Fungal Diversity,* **33**: 139-161.

Saleem, M., Nazir, M., Ali, M., S., Hussain, H., Lee, Y., S., Riaz, N., and Jabbar, A. **2010.** 'Antimicrobial natural products: an update on future antibiotic drug candidates'. *Natural Product Reports,* 27, 238-254.

Samuelsson G. **2004**. "*Drugs of Natural Origin: A Textbook of Pharmacognosy*, 5th ed', *Swedish Pharmaceutical Press*, Stockholm.

Sawada, S., Yokokura, T., and Miyasaka, T. **1995**. 'Synthesis and antitumor activity of A-ring or E-lactone modified water-soluble prodrugs of 20 (S)-camptothecin, including development of irinotecan hydrochloride trihydrate (CPT-11)'. *Current Pharmaceutical Design*, 1: 113-32.

Sawai, Y., Yamaguchi, Y., Miyama, D., and Yoshitomi, H. **2001**. 'Cycling treatment of anaerobic and aerobic incubation increases the content of γ-aminobutyric acid in tea shoots'. *Amino Acids,* 20: 331-334.

Scherlach, K., Nützmann, H.-W., Schroeckh, V., Dahse, H.-M., Brakhage, A., A., and Hertweck, C. **2011**. 'Cytotoxic pheofungins from an engineered fungus impaired in posttranslational protein modification'. *Angewandte Chemie International Edition*, 50: 9843-47.

Scherlach, K., and Hertweck, C. **2009**. 'Triggering cryptic natural product biosynthesis in microorganisms'. *Organic & Biomolecular Chemistry,* 7, 1753-1760.

Schiff, P., B., Fant, J., and Horwitz, S., B. **1979**. 'Promotion of microtubule assembly in vitro by taxol'. *Nature*. , 277: 665-7.

Schulz, B., Wanke, U., Draeger, S., and Aust, H.-J. **1993**. 'Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods'. *Mycological Research*, 97: 1447-50.

Schroeckh, V., Scherlach, K., Nützmann, H.-W., Shelest, E., Schmidt-Heck, W., Schuemann, J., Martin, K., Hertweck, C., Brakhage, A. A. **2009**. 'Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences,* 106: 14558-14563.

Schroeckh, V., Nützmann, H., W., and Brakhage, A., A. **2014**. 'Fungal-actinomycete interactions–wakening of silent fungal secondary metabolism gene clusters via interorganismic interactions. *Natural Products: Discourse, Diversity, and Design*, 147-158.

Seenivasan, A., Subhagar, S., Aravindan, R., and Viruthagiri, T. **2008**. 'Microbial production and biomedical applications of lovastatin'. *Indian Journal of Pharmaceutical Sciences*, 70: 701.

Sengupta, S., and Chattopadhyay, M. H.-P. **2013**. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology,* 4: 1-13.

Sheh, L., Chang, H., Ong, C., Chen, S., Bailly, C., Linssen, R., and Waring, M. **1995**. 'Synthesis, DNA binding, and sequence specificity of DNA alkylation by some novel cyclic peptide-chlorambucil conjugates'. *Anti-Cancer Drug Design,* 10: 373-388.

Sheh, L., Chen, B., L., and Chen, C., F. **1990**. 'Synthesis of cyclic peptide homologs of glutathione as potential antitumor agents'. *International Journal of Peptide and Protein Research,* 35: 55-62.

Shelp, B., J., Bown, A., W., and McLean, M., D. **1999.** 'Metabolism and functions of gamma-aminobutyric acid'. *Trends in Plant Science,* 4, 446-452.

Shimada, A., Takahashi, I., Kawano, T., and Kimurab, Y. **2001.** 'Chloroisosulochrin, chloroisosulochrin dehydrate, and pestheic acid, plant growth regulators, produced by *Pestalotiopsis theae'*. *Zeitschrift für Naturforschung B,* 56: 797-803.

Shin, C., S., Kim, H., J., Kim, M., J., and Ju, J., Y. **1998**. 'Morphological change and enhanced pigment production of *Monascus* when co-cultured with *Saccharomyces cerevisiae* or *Aspergillus oryzae'*. *Biotechnology and Bioengineering,* 59, 576-581.

Shu, Y.-Z. **1998**. 'Recent natural products based drug development: a pharmaceutical industry perspective'. *Journal of Natural Products*, 61: 1053-71.

Shwab, E., K., Bok, J., W., Tribus, M., Galehr, J., Graessle, S., and Keller, N., P. **2007.** 'Histone deacetylase activity regulates chemical diversity in *Aspergillus*'. *Eukaryotic Cell*, 6: 1656-64.

Sieber, T., N. **2007**. 'Endophytic fungi in forest trees: are they mutualists?'. *Fungal Biology Reviews*, 21: 75-89.

Siegel, M., R., and Bush, L., P. **1997**. "Toxin production in grass/endophyte associations." In, *Plant Relationships* (Springer): 185-207.

Silar, P. **2012**. "Hyphal interference: self versus non-self fungal recognition and hyphal death" *Biocommunication of Fungi* (Springer) : 155-170.

Stierle, A., Strobel, G., and Stierle, D. **1993**. 'Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew'. *Science*, 260: 214-16.

Strahl, B. D., and Allis, C. D. **2000**. 'The language of covalent histone modifications'. *Nature,* 403: 41-45.

Strieker, M., Tanović, A., and Marahiel, M., A. **2010**. 'Nonribosomal peptide synthetases: structures and dynamics'. *Current Opinion in Structural Biology,* 20, 234-240.

Strobel, G., and Daisy, B. **2003**. 'Bioprospecting for microbial endophytes and their natural products'. *Microbiology and Molecular Biology Reviews*, 67: 491-502.

Strobel, G., Daisy, B., Castillo, U., and Harper, J. **2004**. 'Natural products from endophytic microorganisms'. *Journal of Natural Products*, 67: 257-68.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. **1994**. 'JNK is involved in signal integration during costimulation of T lymphocytes'. *Cell*, 77: 727-36.

Swanton, E., W. **1916**. 'Economic and folk lore notes'. *Transactions of the British Mycological Society*, 5: 408-09.

Szewczyk, E., Chiang, Y.-M., Oakley, C. E., Davidson, A. D., Wang, C. C., and Oakley, B. R. **2008**. 'Identification and characterization of the asperthecin gene cluster of *Aspergillus nidulans*'. *Applied and Environmental Microbiology,* 74: 7607-7612.

Tafreshi, S., Hosseini, A., Shariati, M., Mofid, M., R., and Nekui, M., K. **2011**. 'Rapid germination and development of *Taxus baccata* L. by in vitro embryo culture and hydroponic growth of seedlings'. *In Vitro Cellular & Developmental Biology-Plant*, 47: 561-68.

Teichert, A., Schmidt, J., Porzel, A., Arnold, N., and Wessjohann, L. **2008**. '(Iso)-quinoline alkaloids from fungal fruiting bodies of Cortinarius subtortus'. *Journal of Natural Products,* 71: 1092-1094.

Thorn, C., F, Klein, T., E., and Altman, R., B. **2009.** 'Codeine and morphine pathway', *Pharmacogenetics and Genomics*, 19: 556-58.

Ueno, H. **2000**. 'Enzymatic and structural aspects on glutamate decarboxylase'. *Journal of Molecular Catalysis B: Enzymatic,* 10: 67-79.

Verbist, L. **1990**. 'The antimicrobial activity of fusidic acid'. *Journal of Antimicrobial Chemotherapy*, 25: 1-5.

Vidensek, N., Lim, P., Campbell, A., and Carlson, C. **1990**. 'Taxol content in bark, wood, root, leaf, twig, and seedling from several *Taxus* species'. *Journal of Natural Products*, 53: 1609-10.

Wainwright, M. **1990**. "*Miracle cure: The story of penicillin and the golden age of antibiotics"* (Blackwell).

Wall, M., E., Wani, M., C., Cook, C., E., Palmer, A., T., and McPhail, and Sim, G., A. **1966.** 'Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*1, 2'. *Journal of the American Chemical Society*, 88: 3888-90.

Walsh, C. **2003**. 'Antibiotics: actions, origins, resistance'. (*American Society for Microbiology Press*, Washington, DC) .

Wang, T.-H., Wang, H.-S., and Soong, Y.-K. **2000**. 'Paclitaxel-induced cell death'. *Cancer*, 88: 2619-28.

Wang, W.-J., Li, D.-Y., Li, Y.-C., Hua, H.-M., Ma, E.-L., and Li, Z.-L. **2014**. 'Caryophyllene sesquiterpenes from the marine-derived fungus *Ascotricha* sp. ZJ-M-5 by the One Strain–Many Compounds strategy'. *Journal of Natural Products*, 77: 1367-71.

Wicklow, D. T. **1992**. "Interference competition. *The fungal community: its organization and role in the ecosystem, 2nd Edn". Carroll, GC and Wicklow, DT, Eds.:* 265-274.

Wigley, D., B. **1995**. 'Structure and mechanism of DNA topoisomerases'. *Annual Review of Biophysics and Biomolecular Structure*, 24: 185-208.

Wijesekara, I., Zhang, C., Van Ta, Q., Vo, T.-S., Li, Y.-X., and Kim, S.-K. **2014**. 'Physcion from marine-derived fungus *Microsporum* sp. induces apoptosis in human cervical carcinoma HeLa cells'. *Microbiological Research,* 169, 255-261.

Williams, D., I., Marten, R., H., and Sarkany, I. **1958**. 'Oral treatment of ringworm with griseofulvin'. *The Lancet*, 272: 1212-13.

Williams, R., B., Henrikson, J., C., Hoover, A., R., Lee, A., E., and Cichewicz, R., H. **2008**. 'Epigenetic remodeling of the fungal secondary metabolome'. *Organic & Biomolecular Chemistry*, 6: 1895-97.

Wilson, L., Creswell, K., M., and Chin, D. **1975**. 'Mechanism of action of vinblastine. binding of [acetyl-3H]-vinblastine to embryonic chick brain tubulin and tubulin from sea urchin sperm tail outer doublet microtubules'. *Biochemistry*, 14: 5586-92.

Yang, X.-L., Huang, L., and Ruan, X.-L. **2014**. 'Epigenetic modifiers alter the secondary metabolite composition of a plant endophytic fungus, *Pestalotiopsis crassiuscula* obtained from the leaves of *Fragaria chiloensis'*. *Journal of Asian Natural Products Research,* 16: 412-417.

Yeung, T., K, Germond, C., Chen, X., and Wang, Z. **1999**. 'The mode of action of taxol: apoptosis at low concentration and necrosis at high concentration'. *Biochemical and Biophysical Research Communications*, 263: 398-404.

Yukimune, Y., Tabata, H., Higashi, Y., and Hara, Y. **1996**. 'Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in Taxus cell suspension cultures'. *Nature Biotechnology*, 14: 1129-32.

Zervosen, A., Sauvage, E., Frère, J.-M., Charlier, P., and Luxen, A. **2012**. 'Development of new drugs for an old target—the penicillin binding proteins'. *Molecules*, 17: 12478-505.

Zhang, P., Zhou, P.-P., and Yu, L.-J. **2009**. 'An endophytic taxol-producing fungus from *Taxus* media, *Cladosporium cladosporioides* MD2'. *Current Microbiology*, 59: 227-32.

Zhao, J., Zhou, L., Wang, J., Shan, T., Zhong, L., Liu, X., and Gao, X. **2010**. 'Endophytic fungi for producing bioactive compounds originally from their host plants'. *Current Researcgh, Technology and Education Topic in Appled Microbiology and Microbial Biotechnology*, 1: 567-76.

Zhong, J.-J. **2002**. 'Plant cell culture for production of paclitaxel and other taxanes'. *Journal of Bioscience and Bioengineering*, 94: 591-99.

Zhu, F., Chen, G., Chen, X., Huang, M., and Wan, X. **2011**. 'Aspergicin, a new antibacterial alkaloid produced by mixed fermentation of two marine-derived mangrove epiphytic fungi'. *Chemistry of Natural Compounds,* 47, 767-769.

Zhu, F., and 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'. *Chinese Science Bulletin,* 51, 1426-1430.

Zhu, F., Chen, G., Wu, J., and Pan, J. **2013**. 'Structure revision and cytotoxic activity of marinamide and its methyl ester, novel alkaloids produced by co-cultures of two

marine-derived mangrove endophytic fungi'. *Natural Product Research,* 27, 1960-1964.

Zhu, Y., Zeng, H., Xie, J., Ba, L., Gao, X., and Lu, Z. **2004**. 'Atomic force microscopy studies on DNA structural changes induced by vincristine sulfate and aspirin'. *Microscopy and Microanalysis,* 10: 286-290.

List of Abreviations

$[\alpha]_D$	specific rotation at the sodium D-line
ax	axial
br	broad signal
CD	Circular Dichroism
CH_2Cl_2	dichloromethane
CD_3OD	deuterated methanol
c	concentration
COSY	correlation spectroscopy
d	doublet
DCM	dichloromethane
dd	doublet of doublet
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eq	equatorial
ESI	electrospray ionization
et al.	et altera (and others)
EtOAc	ethyl acetate
g	gram
HMBC	heteronuclear multiple bond connectivity
HSQC	heteronuclear single quantum coherence
H_2 O	water
HPLC	high performance liquid chromatography
HRESIMS	high resolution electrospray ionisation mass spectrometry
HR-MS	high resolution mass spectrometry
Hz	Herz
ITS	Internal transcriber spacers
L	liter
LC	liquid chromatography

LC/MS	liquid chromatography-mass spectrometery
m	multiplet
M	molar
MeOH	methanol
mg	milligram
MHz	mega Herz
min	minute
mL	milliliter
mm	millimeter
MS	mass spectrometry
MTPA	α-methoxy-α-trifluoromethylphenylacetic acid
MTT	microculture tetrazolium assay
m/z	mass per charge
µg	microgram
µL	microliter
µM	micromol
NaCl	sodium chloride
nm	nanometer
nM	nanomolar
NMR	nuclear magnetic resonance
ppm	parts per million
q	quartet
ROESY	rotating frame overhauser enhancement spectroscopy
RP 18	reversed phase C 18
s	singlet
sp.	species (singular)
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy

UV	Ultra-violet
VLC	Vacuum liquid chromatography

Research Contributions

Publications

Sergi Herve Akone, Georgios Daletos, Tibor Kurtán, Rudolf Hartmann, Wenhan Lin, and Peter Proksch. Inducing secondary metabolites production by the endophytic fungus *Chaetonium* sp. through bacterial co-culture and epigenetic modification. *Tetrahedron* (**2016**, submitted).

Sergi Herve Akone, Mustapha El Amrani, Wenhan Lin, Daowan Lai, Peter Proksch. Cytosporins F–K, new epoxyquinols from the endophytic fungus *Pestalotiopsis theae*. *Tetrahedron letters*, 54, **2013**, 6751-6754.

Sergi Herve Akone, Georgios Daletos, Wenhan Lin and Peter Proksch. Unguisin F, a new cyclic peptide from the endophytic fungus *Mucor irregularis*. *Z. Natuforsch*. **2016**, 71(1-2)c: 15-19.

Sergi Herve Akone, Sebastian Rahn, Birgit Henrich, Georgios Daletos, Juliette Catherine Vardamides, Augustin Ephrem Nkengfack, Wenhan Lin, Daowan Lai, Peter Proksch. 2-Pentenedioic acid derivatives from a soil-derived fungus *Gongronella butleri*. *Phytochemistry Letters* 10, **2014**, 184-188.

Declaration of Academic Honesty/Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel „Sekundärstoffe Produziert bei Pilzen - Ansätze zur Aktivierung stiller Biosynthesewege, Strukturaufklärung und Bioaktivität“ selbst angefertigt habe. Außer den angegebenen Quellen und Hilfsmitteln wurden keine weiteren verwendet. Diese Dissertation wurde weder in gleicher noch in abgewandelter Form in einem anderen Prüfungsverfahren vorgelegt.

Düsseldorf, den 01.07.2016

Sergi Herve Akone.

Aknowledgments

I wish to express my gratitude and cordial thanks to Prof. Dr. rer. Nat. Peter Proksch for giving me this opportunity to start and achieve my doctoral research here at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität, Düsseldorf. I will never forget his fruitful discussions, his support at all circumstances, his valuable suggestions, his permanent availability, the excellent work facilities always available at the Institute.

My appreciation goes to Prof. Dr. Rainer Kalscheuer for his collaboration on the project regarding the development of a new lead drug in the quest for TB, his support for co-cultivation study, antibacterial tests and for officiating as a second referee.

I would like to especially thank, Dr. Georgios Daletos for his assistance, his guidance, his patience, his constructive discussions, his help on the elucidation of the structures, preparation of the manuscripts during my entire Ph.D.

I also appreciate the kind support from Mrs. Claudia Eckelskemper and all the technicians Mrs. Waltraud Schlag, Mrs. Simone Miljanovic, Mrs. Katja Friedrich, Mrs. Heike Goldbach-Gecke, Mr. Dieter Jansen and Mrs Eva Müller.

I am deeply indepted to Prof. Dr. Augustin Nkengfack, Faculty of Science, University of Yaounde 1, for his guidance and support for getting my scholarship.

This research was done in colaboration with other research groups. In this regards, I would like to thank:
Prof. Dr. Wenhan Lin (National Research Laboratories of Natural and Biomimetic Drugs, Peking University, Health Science Center) for his kind suggestions on structure elucidation
Prof. Dr. rer. nat. Werner E. G. Müller and Renate Steffen, Institut für Physiologische Chemie und Pathobiochemie, University of Mainz, and Prof. Dr. rer. nat. Matthias Kassack, Institut für Pharmazeutische und Medizinische Chemie, Heinrich Heine University Duesseldorf, for carrying out the cytotoxicity tests.

Dr. Tibor Kurtán, Department of Organic Chemistry, University of Debrecen, Hungary for CD measurement.

Dr. Rudolf Hartmann, Institute of Complex Systems: Strukturbiochemie (ICS-6), Forschungszentrum Julich, for 700 MHz NMR measurements.
Prof. Dr. Horst Weber for his professional discussion regarding the synthesis.

I am grateful to the past Dean, Prof. Dr. Paul Bilong, and the present Prof. Adolphe Mukengue Imano of the Faculty of Science, University of Douala, for allowing to come in Germany.

My deep appreciation to Prof. Dr. Luc Léonard Mbaze Meva'a, Department of Chemistry, Faculty of Sciences, University of Douala, who always support me and signed all the documents needed from the University of Douala.

Further thanks to the past postdocs Dr. Daowan Lai who guided me at the early beginning of my study here and shared with me his experience on structure elucidation and manuscripts preparation of manuscripts. I also appreciate the support of Dr. Amal Hassan and Dr. Abdessamad Debbab for sharing their experience with me.

My great appreciation to DAAD (German Academic Exchange Service) for the financial support during my stay in Germany.

During my PhD study, I received help, support, and happiness from time to time, so I would
like to take this opportunity to express my profound gratitude to all of these group members:

- The current postdoctors, Dr. Zhen Liu for fruitful NMR discussion and suggestions regarding the synthesis. Dr. Weaam Ebrahim for his kind advices. Dr. Elena Ancheeva for her nice collaboration. Dr. Ramsay Kamdem, for the nice time enjoyed together in the same laboratory.
- The former colleagues Dr. Andreas Marmann for the guidances regarding the work on fungi, Dr. A. R. B. Ola for the initiation on co-cultivation experiments, Dr. Lena Hammerschmidt and Dr. Sherif Elsayed, Dr. Cong-Dat Pham for their technical support.
- Dr. Hendrick Niemann, Jens Hagenow, for their support on the synthesis.
- Ms. Yang Liu, Mrs. Rini Muharini, Ms. Imke Form, Ms. Arta Kuci, Ms. Catalina Perez Hemphill, Ms. Mi-Young Chung, Mr. Marian Frank (thanks for translation of abstract

part in the thesis, and enjoying), Mr. Liu Shuai, Haiqian Yu for the nice conversations, sharing experience during the time we spent together.

- Mr. Hao Wang for the special support and advices regarding the structure elucidation.
- Special thank to my best friend Mr. Amin Mokhlesi for his support in all circumstances, his advices, suggestions, and discussions.
- Ms. Chen Huiqin for her support in the co-cultivation experiments, construction of the graphes
- Ms. Nidja Rehberg for bacterial strain preparation, antibacterial activities and all the experiments regarding chlorflavonin.
- My former colleagues Mr. Mohamed Elnaggar, Mrs. Catherine Bogner, Dr. Mousa Al Tarabeen, Ms. Lisa Kuppers and Mr. Ferhat Oezkaya, Blessing Umeokoli, Peter Eze, Dr. Odontuya, Pamela Nangmo Kemda, Rudiyansyah (Thanks for the suggestions on the proposed biosynthesis), Dr. Festus Okoye for all their kind support.
- My brother and friend, M. Minoue Ndjee Adonis fot his constant support.

At the end, my deepest gratitude to my family in Cameroon and here in Germany for their permanent support for the termination of my study in Germany.

To all of you, thank you very much!