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

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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
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List of Abbreviations

Research Contributions

Declaration of Academic Honesty/Erklarung

Acknowledgments
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 IC₅₀ 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


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-
Hydroxybenzoësäure 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 Modifikationagen zien 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 beobachtet 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 IC₅₀ Wert von 1 µM und außerdem schwache
antibakterielle Aktivität gegenüber B. subtilis mit einer MIC von 53 µM.

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

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).
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).

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 mydiatic and as antidote for poisoning by organophosphate insecticides (Gryniewicz and Gadzikowska, 2008).
Introduction

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).

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).
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 approved by the FDA since 1992 for the treatment of metastatic carcinoma 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-deacetyl derivative, 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).
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).
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-positive 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).
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 B₀, 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 B₀ 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).

![Chemical structures](image)

Griseofulvin  Echinocandin B  Pneumocandin B₀

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 (Hatzioannou 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](image)

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).
Introduction

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](image)

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 Notapodytes 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 Notapodytes 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 Notapodytes 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 camptothecin 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 its derivatives act through inhibition of topoisomerase II (Guerram *et al*., 2012; Wigley, 1995).

![Podophyllotoxin, Etoposide, Teniposide](image)

**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 tricinctum 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 Thalassospia 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 *alcA* 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 *apdR* under the control of the inducible alcohol dehydrogenase promoter *alcAp*, 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 *cnsE/CSN5* demethylase subunit of the COP9 signalosome resulted in the production of a new antibiotic (Gerke *et al.*, 2012). Interestingly, the *cnsE/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.

Chapter 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 IC50 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 its 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-pentenedioic acid derivatives.
Chapter 2

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

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

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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 IC₅₀ 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*. In nature, microorganisms co-exist in complex communities, in which they interact with each other. 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. 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, thus resulting in the accumulation of new natural products. 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. A similar observation was made when co-culturing the soil-dwelling fungus *Aspergillus terreus* with either *B. subtilis* or with *B. cereus*. Besides the co-cultivation approach, treatment of fungi with epigenetic modifiers is another powerful tool for the activation of silent genes. 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.

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 and are well known to produce a wide range of natural products such as the anticancer compounds chaetocochins A - C, the antibacterial compound furano-polyene 3-epi-aureonitol, and the metallo-β-lactamases inhibitors SB236050 and SB238569. 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 acrewnsol A (3),\textsuperscript{18} 13.9 mg for SB236050 (4),\textsuperscript{16,17} 132.7 mg for SB238569 (5),\textsuperscript{16,17} and 14.6 mg for the 1:1 mixture of 3- and 4-hydroxybenzoic acid methyl esters (1 and 2, respectively)\textsuperscript{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.\textsuperscript{5,10} In addition, seven compounds, including five new natural products (8-12), as well as the known isosulochrin (6)\textsuperscript{21} and protocatechuic acid methyl ester (7)\textsuperscript{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.](image_url)
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).

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

\(^a\) 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 \(\delta_H 3.71\) (3H, s, 7-OCH\(_3\)), a methyl group at \(\delta_H 2.00\) (3H, s, H-3-2'), as well as a continuous spin system composed of two methylene groups at \(\delta_H 2.37\) (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 \(\delta_H 4.80\) (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 \(\delta_H 6.72\) (1H, dt, \(J = 3.0, 1.9\ Hz, H-2\)) (Table 2). In the HMBC spectrum of 8, the correlations observed from 7-OMe, H-2, and H-2-6 to C-7 (\(\delta_C 167.4\)) 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’ (\(\delta_C 170.8\)) 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 ($J_{3,4} = 9.0$ 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.

<table>
<thead>
<tr>
<th>Position</th>
<th>Shikimeran A (8)$^a$</th>
<th>Quinomeran (11)$^b$</th>
<th>Quinomeran (11)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta_H$</td>
<td>$\delta_C$</td>
<td>$\delta_H$</td>
</tr>
<tr>
<td>1</td>
<td>131.8, C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>7$'$-OCH$_3$</td>
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$^a$ Measured in (CD$_3$)$_2$CO at 300 ($^1$H) and 75 ($^{13}$C) MHz.

$^b$ Measured in (CD$_3$)$_2$CO at 600 ($^1$H) 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 \textit{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).

![Image](image.png)

**Figure 3.** Structures and populations of the low-energy B97D/TZVP PCM/MeCN conformers (≥2%) of (3R,4R)-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 (3R,4R)-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 ^13C 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 δH 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 δH 3.54 (6H, s, 7/7'-OMe), and two hydroxy protons at δH 8.65 (2H, br s, 5/5'-OH). In the HMBC spectrum of 2, the correlations from H-4 (/4') to C-2 (/2') (δC 134.9) and C-6 (/6') (δC 116.9), from H-3 (/3') to C-5 (/5') (δC 157.0) and C-1 (/1') (δC 132.4), as well as from H-6 (/6') to C-4 (/4') (δC 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.
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’) (δC 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>
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Table 3: NMR data of 9 measured in (CD3)2CO at 300 (1H) and 75 (13C) MHz.

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 C11H10O5. The 1H NMR spectrum of 10 revealed the presence of four coupled signals at δH 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 δH 3.90 (3H, s, 7-OCH3) and two geminal olefinic protons at δH 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-OCH3 to C-7 (δC 167.8) suggested the presence of a methyl ester group at C-1. Moreover, the HMBC correlations from H2-3’ to C-1’ (δC 165.7) and C-2’ (δC 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$_3$OD at 500 ($^1$H) and 125 ($^{13}$C) MHz.

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<td>7-OMe</td>
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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 $^1$H NMR spectrum of 11 displayed two separate aromatic spin systems; the first consisted of the signals at $\delta_H$ 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 $\delta_H$ 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 ($\delta_C$ 131.0), C-8a ($\delta_C$ 147.7), and C-4 ($\delta_C$ 138.1), from H-8 to C-6 ($\delta_C$ 129.5) and C-4a ($\delta_C$ 130.3), and from H-4 to C-5 ($\delta_C$ 128.6), C-8a, and C-2 ($\delta_C$ 153.6) (Fig. 5). The remaining signals of a methylene group resonating at $\delta_H$ 3.80 (2H, m, H$_2$-2”), an oxymethine proton at $\delta_H$ 4.83 (1H, dd, $J = 6.9$, 5.4 Hz, H-3”) and a methoxy signal at $\delta_H$ 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’ ($\delta_C$ 67.9) and C-4’ ($\delta_C$ 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’ ($\delta_C$ 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
Co-culture

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

![Compound 11](image1)

**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-pyrene derivatives. Inspection of the $^1$H NMR (Table 5) of 12 indicated the presence of two meta-coupled protons at $\delta_H$ 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 $\delta_H$ 8.28 (1H, d, $J$ = 8.8 Hz, H-1'), $\delta_H$ 7.37 (1H, dd, $J$ = 8.8, 2.7 Hz, H-2'), and $\delta_H$ 7.56 (1H, d, $J$ = 2.7 Hz, H-4'). Additional signals included those of two methoxy groups at $\delta_H$ 3.96 (3H, s, 8-OCH$_3$) and 3.90 (3H, s, 9-OCH$_3$), and a phenolic hydroxy proton at $\delta_H$ 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-pyrene 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 ($\delta_c$ 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 (1H) and 75 (13C) MHz.

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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, 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.
In a second set of experiments, the endophytic bacterium *Streptomyces lividans* 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.

Taking into consideration that posttranslational modifications of histones influence the pattern of secondary metabolites in filamentous fungi, *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, 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 cytotoxicity 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$_2$O): 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$_2$Cl$_2$/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. 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, \textit{B. subtilis} 168 trpC2, \textit{S. lividans} TK24, and \textit{M. smegamatis} (mc² 155).

### 4.3. Co-cultivation experiment of \textit{Chaetomium} sp. with \textit{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 \textit{Chaetomium} sp., five for co-cultures of \textit{Chaetomium} sp. and \textit{B. subtilis}, five for \textit{Chaetomium} sp. treated with autoclaved \textit{B. subtilis}, five for axenic \textit{B. subtilis} and five for autoclaved \textit{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 \textit{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 (OD₆₀₀) 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 \textit{Chaetomium} sp. grown on malt agar (three pieces, 1 cm-1 cm) to the rice medium containing viable \textit{B. subtilis}. On the other hand, after 4 days incubation, ten flasks cultured with \textit{B. subtilis} were autoclaved followed by addition of the fungus to five of them, in the same manner like for viable cultures of \textit{B. subtilis}. Both microorganisms were also grown axenically on rice medium. Co-cultures and axenic cultures of \textit{Chaetomium} sp. and \textit{B. subtilis} were kept under static conditions at 23°C until they reached their stationary phase of growth (3 weeks for \textit{Chaetomium} sp. and \textit{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 \textit{Chaetomium} sp. with \textit{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 (mc² 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 methyltransferase 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 CH₂Cl₂-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 B₁-B₉. Subfraction B₂, eluted with *n*-hexane-EtOAc (9:1, v/v) was further purified using Sephadex LH-20 followed by semipreparative HPLC with MeOH-H₂O (0.1%TFA) to afford the mixture 1+2 (50 mg). Subfraction B₃ eluted with *n*-hexane-EtOAc (8.5:1.5, v/v) was purified by semipreparative HPLC with MeOH-H₂O (0.1%TFA), to yield 11 (1.1 mg) and 7 (1.2 mg). Subfraction B₄, eluted with *n*-hexane-EtOAc (8:2, v/v) was further purified using Sephadex LH-20 followed by semipreparative HPLC with MeOH-H₂O to yield 8 (11 mg), 12 (6 mg) and 6 (4 mg). Subfraction B₅ eluted with *n*-hexane-EtOAc (7.5:2.5, v/v) was treated in the same manner as B₄ 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 CH₂Cl₂-MeOH to give six fractions A-F. Fraction E eluted with EtOAc was purified by semipreparative HPLC (MeOH-H₂O) to yield 4 (1.9 mg) and 5 (3.1 mg). Compounds 1₁⁹, 2ₐ, 3₁₈, 4₁₆, 5₁₆, 6₂₁, 7₂₂ were identified by comparing their ¹H 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; [α]^{20}_D -58 (c 1.01, MeOH); UV (MeOH) λ_{max}: 227 nm; ECD (MeCN,λ [nm] (Δε), c = 3.50×10^{-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; [α]^{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 \textit{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. The direct colony suspension method was used with an inoculum of $5 \times 10^5$ 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 $1 \times 10^5$ 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-2H-tetrazolium bromide) assay and compared to that of untreated controls, as described previously. 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
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References


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**.

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 (3R,4R)-8.

Figure 4. Experimental ECD spectrum of 8 in MeCN compared with the Boltzmann-weighted B3LYP/TZVP PCM/MeCN ECD spectrum of (3R,4R)-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

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Chapter 3

Cytosporins F–K, new epoxyquinols from the endophytic fungus

_Pestalotiopsis theae_

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

Sergi Herve Aloue, Mustapha El Amrani, Wenhan Lin, Daowan Lai, Peter Proksch

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. In continuation of our search for new bioactive compounds from endophytes, we isolated an endophytic strain Pestalotiopsis theae from leaves of the medicinal plant Turraeaanthus 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 pain.

The genus Pestalotiopsis 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, plant growth regulators, and nitrogen-containing compounds with inhibitory activities against HIV-1 replication. 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. 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), 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) was isolated as a colorless oil. Its molecular formula was established as C_{21}H_{20}O_6 by HRESIMS, as a prominent peak was observed at m/z 398.2535 ([M+Na]^+) for C_{21}H_{20}O_6Na_2. The ^1H and ^13C NMR data of 2 (Tables 1 and 2) were similar to those of cytosporin D (1), except that the oxygenated methylene protons of 2 (H_4, 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 acetyl group was present. The stereochemistry of 2 was determined by NOE experiments. The coupling constants indicate that the configuration at C-9 is trans. The structures of the isolated cytosporins (1–7) were confirmed by spectroscopic analysis.
Cytosporins from *Pestalotiopsis theae*

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* Multiplicities were assigned based on DEPT-135, and HSQC experiments.
* Signal deduced from the HMBC spectrum.

Table 2

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* δ_H (H in Hz) for 2-7: 600 MHz, CDCl₃/DMSO-

A group (δ_C 203.3 s; δ_C 172.8 s, 21.0 q) was present in the former. Taking into consideration their difference in molecular mass (42 amu), 2 was deduced as a 13-acetoxy derivative of 1, which was confirmed by the HMBC correlations observed from H₂-13 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 con-

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Figure 2. 1H-1H COSY (bold) and selected HMBC (H → C) correlations of 2-5.
Cytosporins from *Pestalotiopsis theae*


Cytosporins from *Pestalotiopsis theae* are a group of compounds that have been identified in the study. One particular compound, cytosporin A, was found to 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 detected 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 *Cyclospora* isolated from a species of *Pestalotiopsis*. The stereochemistry of cytosporin A was confirmed by the synthesis of its derivative (2). In the current study, six new cytosporin derivatives, cytosporins F–K (2–7), were identified from the same fungus. These compounds contain an uncommon 13-acetoxyl group and/or a hydroxylated side chain that was not reported previously. The absolute configuration of the alcohol side chain could be determined by applying the modified Mosher's method, as the case of 2, 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 epiperoxyn has been isolated from *P. longiseta* (2), which was reported to originate from gentisaldehyde. Subsequent addition of an isoprenyl unit, followed by epoxidation of the C-2/C3 double bond and hydrogenation of the keto group would give the epoxiquinol (S1). Similar structures, arbutrinins A–C, with a sesquiterpene unit attached to C-3 of the epoxiquinol ring, were isolated from a carnivorous fungus *Arthrobotrys oligospora*. Then, a C6 or C9 aliphatic chain formed through the polyketide pathway is added to C-5 of the epoxiquinol to give the adduct (S2). It is interesting to note that a similar compound, ambic Acid, was isolated from the endophytic *Pestalotiopsis* sp. and *Monochete* sp., although the isoprenyl unit is located at C-5 of the epoxiquinol group. The following attack from the hydroxyl group

![Figure 3. Proposed biosynthetic pathway for cytosporin derivatives.](image-url)
Cytosporins from *Pestalotiopsis theae*

**References and notes**


Supplementary data

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

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Cytosporins from *Pestalotiopsis theae*

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**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$_3$OD 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$_3$PO$_4$ in H$_2$O): 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$_2$Cl$_2$/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. 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-H2O (0.1%TFA), to give 3 (2.5 mg), 6 (1.6 mg), and 7 (1.1 mg). Similarly, subfraction H6 was purified by semi-preparative HPLC eluting with a gradient of acetonitrile-H2O (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): $\delta$  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): $\delta$  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:


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

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

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

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**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.

**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 $C_{31}H_{63}N_5O_8$ 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
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 $\alpha$-amino acids including Ala (2 eq.), Val (1 eq.), Leu (1 eq.) and Trp (1 eq.), in addition to $\gamma$-aminobutyric acid (GABA) (1 eq.) and the unusual amino acid $\beta$-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 $\delta_H$ 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 $\delta_H 10.82$ (NH-1), and an aromatic proton at $\delta_H 7.09$ (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 C7a ($\delta_C 135.5$) and C-6 ($\delta_C 120.8$); H-5 to C7 ($\delta_C 111.2$) and C-3a ($\delta_C 127.1$); H-6 to C7a; and H-7 to C5 ($\delta_C 118.1$) were discerned (Figure 2). Moreover, the presence of a $\beta$-methyl substituted phenylalanine unit was evidenced through the COSY correlation between the $\beta$-proton at $\delta_H 3.49$ (H-\(\beta\), m) and the methyl group at $\delta_C 1.16$ (H$_2$-\(\gamma\), d, $J = 7.2$ Hz), in addition to the observed HMBC correlation of the latter (H$_2$-\(\gamma\)) to the aromatic carbon C-1 ($\delta_C 142.7$).

The connectivity of the respective moieties was accomplished by analysis of the ROESY spectrum of 1. Accordingly, ROESY correlations (Figure 2) between Trp-NH ($\delta_H 8.02$, d, $J = 6.5$ Hz)/Ala(2)-Hx ($\delta_H 3.90$, m) and Ala(2)-NH ($\delta_H 8.36$, d, $J = 6.1$ Hz)/Val-Hx ($\delta_H 4.04$, m) disclosed the peptide fragment Trp–Ala(2)–Val. Likewise, the ROESY correlations between Val-NH ($\delta_H 7.77$, d, $J = 9.3$ Hz)/$\beta$-methyl-Phe-Hx ($\delta_H 4.38$, m); $\beta$-methyl-Phe-NH ($\delta_H 8.30$, d, $J = 8.8$ Hz)/Leu-Hx ($\delta_H 4.02$, m); and Leu-NH ($\delta_H 7.85$, d, $J = 5.8$ Hz)/Ala(1)-Hx ($\delta_H 4.11$, m) extended this fragment leading to the partial sequence Trp–Ala(2)–Val–$\beta$-methyl-Phe–Leu–Ala(1). Finally, the cyclic planar structure of 1 was established based on the cross-peak between GABA-NH ($\delta_H 7.64$, t, $J = 5.2$ Hz) and Trp-Hx ($\delta_H 4.09$, 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 unguisfin F is proposed.

Compound 2 was identified by analysis of the NMR and MS data and by comparison with the literature as unguisfin 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 $\beta$-configurations for all amino acids, except for $\beta$-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 Na(2,4-dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA)-derivatized ion peak in the MS spectrum were
**DE GRUYTER**

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

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$^a$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 $\alpha$-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 unguisins derivatives [18], thus indicating a high specificity of the non-ribosomal peptide synthetases involved in the synthesis of these peptides.

Interestingly, the first unguisins substances, viz. unguisin A, B, and C, were obtained from the fungus *Emericella unguis*, isolated from the jellyfish *Stomolophus meleagris*, collected in Venezuelan 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 imacindins A–E from *Streptomyces olivaceus* [20] and N-acylasparaglypolyamine derivatives from *Nephielengys 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 *Staphylococcus aureus* ATCC 25922, *Streptococcus pneumonia* ATCC 49619 and *Escherichia coli* ATCC 25922 at a concentration of 64 $\mu$g/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. "H, "C and 2D NMR spectra were recorded at 25 °C in DMSO-\textit{d}_6 on a Bruker ARX 600 NMR spectrometer. Chemical shifts were referenced to the solvent residual peaks, δ_\text{H} 2.50 for "H and δ_\text{C} 39.5 for "C NMR. Mass spectra (ESI) were recorded with a Finnigan LCQ Deca mass spectrometer, and HRMS (ESI) spectra were obtained with a 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 UltiMate 3400 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 prewelled with Eurosphere-10 C18 (Knauf, Germany), and the following gradient was used (MeOH, 0.1% HCOOH in H\textsubscript{2}O): 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 L7400; Pump L7100; Eurosphere-100 C18, 300 mm × 8 mm, Knauf, Berlin, Germany). Chromatography included H\textsubscript{2}O: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\textsubscript{3}Cl/MeOH mixtures as mobile phase); detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent.

3.4 Advanced Marfev's analysis of 1 and 2

Marfev'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\textsubscript{3} and 100 μL of 1% L-FDA 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-QL/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 29222, S. pneumoniae ATCC 49619 and E. coli ATCC 2922, 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

Figure 1: HRESIMS spectrum of Ungusin F.

Figure 2: $^1$H NMR of Ungusin F in DMSO (600 MHz).
Chapter 5

2-Pentenedioic acid derivatives from a soil-derived fungus

*Gongronella butleri*

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Impact Factor: 1.450,

The overall contribution to the paper: 80% of the first author. The first author involved in all laboratory works as well as the manuscript preparation.
2-Pentenedioic acid derivatives from *Gongronella butleri*

Sergi Herve Akone, Sebastian Rahn, Birgit Henrich, Georgios Daletos, Juliette Catherine Vardamides, Augustin Ephrem Nkengfack, Wenhan Lin, Daowan Lai, Peter Proksch

**Abstract**

Eight new 2-pentenedioic acid derivatives (1–8) and one known congener, 2-decyl-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<sub>6</sub>, C<sub>8</sub>, and C<sub>10</sub>) with or without an oxygenated substituent. These compounds were screened for their cytotoxic and antibacterial potentials; however, the tested compounds displayed no significant activities.

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1. Introduction

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

As a continuation of our search for bioactive substances from fungi (Akone et al., 2013; ElAmrani 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-bred library that included hundreds of fungal secondary metabolites isolated in the past. Some candidate extracts with peaks of interest were further examined 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.
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-decanoyl pent-2-enolic 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_{14}O_4, 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, 136.2, 136.2), one oxymethine group (δ_C 68.5, CH, δ_H 3.71, m) and one methyl group (δ_C 23.5, δ_H 1.4, 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 1H-1H COSY spectrum, we easily established a long spin system that started from H-2 (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-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-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’ 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 α,β-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’ 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-hydroxylellyl)pent-2-enolic 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_{14}O_5, 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

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Multiplicities were assigned based on DEPT-135 and HSQC experiments.

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3 Signals may be interchanged within the same column.
2-Pentenedioic acid derivatives from *Gongronella butleri*

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</table>

Fig. 2. 1H-1H COSY (bold), and selected HMBC (H → C) correlations of 1, 3, 4 and 8.

The molecular formula of 4 was deduced as C21H24O8 by HRESIMS analysis. The NMR data of 4 were similar to those of 2, as both shared the same pent-2-enoic moiety. The structural differences lie in the side chain. The methyl group in 2 was replaced by a carbonyl group (δC 127.7) and a long spin system spanning 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' (δH 1.62, m) and H-5' (δH 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.

Compounds 5 was isolated and identified as a close congeners of 1, as they had similar NMR data (Tables 1 and 2). The differences were attributed to the chain length in which two more methine 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', δH 1.14) appeared as a doublet in the 1H NMR, and showed COSY correlation to the neighboring oxymethylene proton (H-7', δH 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-enoic acid.

Compound 6 showed a prominent peak at m/z 257.1749 [M+H]⁺ in the HRESIMS spectrum, corresponding to the molecular formula C16H16O5. The NMR data of 6 were similar to those of 2. However, the length of the allyl 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 (δH 3.71; δC 52.5, CH₃O) was present in 6, which showed HMBC correlation to the carbonyl group at C-5 (δC 172.6). Thus, the structure of 6 was determined as (Z)-2-(3-methoxy-3-oxopropyl)decanolic acid.

Compound 7 was characterized as a dehydro derivative of 6. The molecular formula of 7 was established as C21H22O8, 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 oxymethylene group were missing in 7, while an isolated methyl group (δH 2.13; δC 29.6, CH₃) and a ketone group (δC 121.2-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' (δC 44.3). Thus, 7 was determined as (Z)-2-(7-oxo-octyl)pent-2-enoic acid, a homologous structure of 3.

Compound 8 was isolated likewise as a colorless oil. It had the molecular formula C21H22O8, which contained two more CH₂ 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 1H NMR spectrum, and showed HMBC correlations to the adjacent methylene group (C-8'; δC 30.5) and the carbon (C-8'; δC 73.6) bearing the hydroxyl group (Fig. 2). Thus, compound 8 was established as (Z)-2-(8-hydroxyoctyl)pent-2-enoic acid. We tried to determine the absolute configuration of this secondary alcohol by applying the
2-Pentendioic acid derivatives from *Gongronella butleri*

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: 1174367-28-0) has a similar 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-pentendioic acid derivatives (1-8) and one known congeners (9) were identified from the title fungus. A previous chemical investigation of *G. butleri* had led to the isolation of three 2-pentendioic acids and one pentendioic 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-pentendioic 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 (Minimal Inhibitory Concentration) and antifungal (Minimal inhibitory concentration) activities of these compounds were evaluated against *Staphylococcus aureus* ATCC 25922, *Streptococcus pneumoniae* ATCC 49619, and *Escherichia coli* ATCC 25922, but also in this bioassy 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, organic acid, isolated from an endophytic fungus of *Berberis oregano*, was found to be a potent (IC_{50} 14 mM) and specific inhibitor of farnesyl-protein transferase (Jayasurya et al., 1996). In the current study, however, the isolated pentendioic 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. H, 13C, and 2D NMR spectra were recorded at 25 °C in CD3OD on a Bruker AV 600 NMR spectrometer. The shifts were referenced to the solvent residual peaks, δH 3.31 for H, and δC 49.0 for 13C. Mass spectra (ESI) were recorded with a Finnigan LCQ Deca mass spectrometer, and HRMS (ESI) spectra were obtained with a FHRMS/Ms-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 UltiMate 3000 SD with a UPLC-3600D Pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. The separation column (125 mm x 4 mm) was pre-filled with Eurosphere-10 C18 (Knaur, Germany) and the following gradient was used: MeOH, 0.02% H3PO4 in H2O: 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-10 C18, 300 mm x 8 mm, Knaur, 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 (CHCl3/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 Koudou village located at 10 km from Edéa 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 98% homology to *Gongronella* (e.g. accession numbers EU253396, DQ191324, AF157137 and FJ865979) which clustered in the same branch of a phylogenetic tree framed by *Absidia*, *Cunninghamamia* and *Mucorales* (see Fig. S1 in the Supporting Information). BlastN analysis of a 680 bp ITS1–ITS2 sequence (GenBank) showed 90% homology to *G. butleri* (accession numbers JN266607, JF491978, AF157191, HM849698 and JN291188) 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 of EOA (acetone) was added to each flask and left overnight, followed by filtration. The extraction was done in three times. The EOA 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 acetate 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 C1-C4. Subfraction C2 (30 mg) and C4 (46 mg) were purified by semi-preparative HPLC using MeOH-H2O (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 the 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 2 (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-H2O (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; [a]_D^25 = +0.1 (c 0.23, CHCl3); UV (MeOH): 213.6 nm, (H: 200 MHz) and 1H: 150 MHz (CD3OD) 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]^+ (calcld for C_{11}H_{10}O_{3}, 231.1227).
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Compound 2: colorless oil; UV (MeOH) λ\text{max} 213.1 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) NMR data, see Tables 1 and 2; ESIMS m/z 244.8 [M+H]+; HRESIMS m/z 245.1023 [M+H]+ (calcd for C14H14O3, 245.1020).

Compound 3: colorless oil; UV (MeOH) λ\text{max} 213.9 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) NMR data, see Tables 1 and 2; ESIMS m/z 239.0 [M+Na]+; HRESIMS m/z 239.1072 [M+Na]+ (calcd for C13H13NaO3, 239.1071).

Compound 4: colorless oil; UV (MeOH) λ\text{max} 214.1 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) 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 C12H12O3, 229.1071).

Compound 5: colorless oil; UV (MeOH) λ\text{max} 214.4 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) 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 C15H15O3, 259.1540).

Compound 6: colorless oil; UV (MeOH) λ\text{max} 213.3 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) 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 C14H13O3, 257.1747).

Compound 7: colorless oil; UV (MeOH) λ\text{max} 214.2 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) NMR data, see Tables 1 and 2; ESIMS m/z 256.8 [M+H]+; HRESIMS m/z 257.1383 [M+H]+ (calcd for C14H13O3, 257.1384).

Compound 8: colorless oil; UV (MeOH) λ\text{max} 213.6 nm; H\text{2} (600 MHz, CD\text{3}OD) and 13C (150 MHz, CD\text{3}OD) 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 C15H15O3, 287.1852).

4. Bioassay

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

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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.phytochem.2014.09.001.

References


Supporting Information

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

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Sequenced 18S rDNA region of the unknown fungus

CTTGTCCTCAAGAGATTTACAGCATGCTAGTCTAAGTAAAGCAATTTTTACAGCAGGACTGCG
GAATGGGCTCATATATATCGTATGATCTACATGGCAGATGATTTTTTACTATTGGAATAAC
CGTGTTAATTTCGACTAATACATGCAAAAAAGGGGTCTCGGACACTGGGAACCTGGGGAAT
TTAGATCAAAGCCAACAGCCTGGAAACAGGTTTCTTTTGGATATCAATAAATAAATGACAGC
GATCCGATGGCCTGGCCGAGCAGGTTCCATCGGTTTCTGCCTCCTCAGCTTTTGCT
GGATATGGGTAGATGCTACTCCCTATACAGGTTGACGGGGAATAGGTCTCGGAACCT
GAGGGGAGCCTGAGAAACCGGCTACCACCATCCAAAGGAAGCAGGAGCAGGAGCGCAAATTACC
CAATCCCGACACCGGGAGGAGTTGCTAAGCACAATAAAATAAACAATGCAAGAGGCTTTTTGC
AATTGGAAATGATGATCAATATCTACTTCAACGGAACCAATTTGGAAGGCGAAGTCTGGTG
CCAGCAGCCGGTAAATTCAGTCGAATCGTTAACTAAAGTTGGAGTACGTTAAAACG
TCCGTAGTCGAAGTTGTTGGCTTTGTCGGTACGCTTCATCTGGAGGGGGCTGGTGAC
ATGCTTGGCAGCTGTTAGGTTGCTGGAGCGCTCTGGTTGTGCTGTCTGGAGTTCT
GACAAATCAGAGCTTCTCAAGACAGGCTTTTATAGCTCTGATGTTTTAGCTGGAATAATG
AAATAAGCCTTAGTGCTTGTGTTGTTTACTTTGACACTGGGGAATGATGAATAGGGAA
CGGTTGGGGGCTATTTGATACTTGGCAGGAAATTCTTGGATTTGAGCGGAACAA
ACTACTGCAAAAGCATTGACCAGGGCTAAAATTTGATCACAGGACTAAAGTTGGAGGA
TCGAAAGCATTAGATACCAGTCGTAGTCTTAAACACACAAACTATGCGAGCTAAGCGA
TGATACCTTTTTTGTTCCATAGCGCCAGCTTAGCGGAAATAGTTCTTGGGTGAGGG
GAAGGACGACGCGATGAACTCTAAGGAATTTGGACCGGAGGCGAACAGGAGGCTG
GAGCCGCTGCTTAAACTTTAGCTAAGACCAGGGAAACTCAACAGGCTCCAGACATAAGAG
GATGGACAGATTGAAACGCTTTTCTAGATTTTTATGGTGGTGGTGCATGCGCCGTCTTAGT
TCTGGGAGTGATTTGCT

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.
2-Pentenedioic acid derivatives from *Gongronella butleri*

Fig. S1 Phylogenetic tree based on part of the 18S rDNA

**Sequenced region of the unknown fungus comprising ITS1-ITS2**

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TTTTGTATGGGTATAAAGCCCTTGTCCTTCGTCGCCCTATCCGACTACGATTTCA
TTTCAAGGGAAAGTATGGCATAAACAGGTCTCTGATGCGCTTTAGATGTTCGCTGGCCACGC
GCGTACACTGACAAAGGCAACGAGAGTTTTTTTCCTTGCGCCGGAAGGCTGGGTAAACTTTT
GAAACTTTTGCTCTGGGATTGAGCATTGCAATTATTGCTCTTCAACGAGGAATTTCTAG
AGTAAGCGCAAGTCATCAGCTTTGCCTTGATTACGTCCCTGCCCTTTGTACACACACCCGT
CGCTACTACCGATTTGATGCTAGTGAAGCATGTGGGATTGACGTCGTCACAGCGGCAA
CGCTGAGATGATGTTGAGAATCTATGGCAAACTAGGCTATTTAGAGGAAGTAAAAGTCGT
AACAAGTTAAAAAGCTCCTTTTACCTTTTTTTCGCTCTCTCGCAAGGGTAATCCTTTGGGCACGC
CTGTTTCAGTGTTTTTCAACCCCTCCTAGATTGGGTGATGAAACGATGGGTTTGCAATCGCAAAGATT
```
2-Pentenedioic acid derivatives from *Gongronella butleri*

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.
2-Pentenedioc acid derivatives from *Gongronella butleri*

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 C18 (Knauer, Germany) column (i.d. 125×4 mm) was used. The following gradient was employed (mobile phase: MeOH, 0.02% H3PO4 in H2O): 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.
2-Pentenedioc acid derivatives from *Gongronella butleri*

**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
2-Pentenedioc acid derivatives from *Gongronella butleri*

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
2-Pentenedioic acid derivatives from *Gongronella butleri*

**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
2-Pentenediolic acid derivatives from *Gongronella butleri*

**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; Schroechk 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

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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 cocultivation 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 agreement 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.

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.
Discussion

Scheme depicting the proposed biosynthesis of compound 4

\[
\text{tryptophan} \xrightarrow{\text{indoleamin-2,3-desoxigenase}} \text{N-formylquinureine} \xrightarrow{\text{formamidase}} \text{quinureine} \xrightarrow{\text{quinureninase}} \text{anthranilic acid}
\]

\[
\text{3x Malonyl thioester} \xrightarrow{\text{H}_{2}\text{O}} \text{anthranilic acid} \xrightarrow{\text{3x Malonyl thioester}} \text{anthranilic acid}
\]

\[
\text{Hydrogenation} \xrightarrow{\text{dehydration}} \text{H}_{2}\text{O} \xrightarrow{\text{oxydation}} \text{H}_{2}\text{O} \xrightarrow{\text{methylaion}} \text{H}_{2}\text{O}
\]

\[
4. \text{2-hydroxy-4-oxo-4-(quinolin-2-yl)butanoic acid}
\]

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 depicting 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).
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 adenyltransfer 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 macrocyclization 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
Discussion

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-pentenediolic 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.
Discussion

Aspergicin

Marinamide

Neoaspergilllic acid

Marinamide methylether

Norlichexanthone

Monocerin
REFERENCES


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


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


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


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


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


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.


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.


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.


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.

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.


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


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


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

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>[α]D</td>
<td>specific rotation at the sodium D-line</td>
</tr>
<tr>
<td>ax</td>
<td>axial</td>
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<tr>
<td>br</td>
<td>broad signal</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
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<tr>
<td>CH₂Cl₂</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>CD₃OD</td>
<td>deuterated methanol</td>
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<tr>
<td>c</td>
<td>concentration</td>
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<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
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<td>d</td>
<td>doublet</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<tr>
<td>dd</td>
<td>doublet of doublet</td>
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<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eq</td>
<td>equatorial</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>et al.</td>
<td>et altera (and others)</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond connectivity</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
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<tr>
<td>H₂O</td>
<td>water</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>HRESIMS</td>
<td>high resolution electrospray ionisation mass spectrometry</td>
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<td>high resolution mass spectrometry</td>
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<tr>
<td>Hz</td>
<td>Herz</td>
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<td>ITS</td>
<td>Internal transcriber spacers</td>
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<td>L</td>
<td>liter</td>
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<tr>
<td>LC</td>
<td>liquid chromatography</td>
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</table>
LC/MS  liquid chromatography-mass spectrometry
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
<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VLC</td>
<td>Vacuum liquid chromatography</td>
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Research Contributions

Publications


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Sergi Herve Akone.
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