Metabolite Diversity of Endophytic Fungi Associated with Medicinal Plants

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“Recite in the name of your Lord who created,
Created man from a clinging substance.
Recite, and your Lord is the most Generous,
Who taught by the pen,
Taught man what he did not know.”

(translated from the Holy Quran surah Al-‘Alaq 96: 1–5)

Dedicated to my beloved family

Wafi Nuraini

Arvind Farzani Raykharfi

Qaiser Edler Harfian

and

My Parents

Bapak Casjoyo and Ibu Wahyuning Sari

as well as

My Parents-in-laws

Bapak M. Syukri and Ibu Pariyah

CONTENTS

Abstract

The increasing demand for new antibiotics to combat the global trend of antimicrobial resistance drives scientists to engage in the discovery of new bioactive natural products. Endophytic fungi constitute a relatively unstudied and promising source of structurally diverse and/or biologically active compounds as a starting point in drug discovery. In this dissertation two plant-associated endophytic fungi were studied: *Trichoderma harzianum* and *Epicoccum nigrum.* This led to the isolation of chemically and biologically diverse secondary metabolites. These include dithiodiketopiperazines, flavipin-derived alkaloids and flavonoids, with some of them possessing antimicrobial and cytotoxic activities. Remarkably, OSMAC (One Strain MAny Compounds) and feeding experiments which were conducted with *E. nigrum* resulted in the production of kaempferol derivatives via biotransformation reactions of plant derived flavonoids.

This dissertation consists of the following three published or submitted manuscripts:

Dithiodiketopiperazine derivatives from endophytic fungi Trichoderma harzianum and Epicoccum nigrum

Investigation of *Trichoderma harzianum* and *Epicoccum nigrum*, endophytic fungi residing in leaves of *Zingiber officinale* and *Salix* sp., afforded the new epidithiodiketopiperazine (ETP) pretrichodermamide G. Moreover, three known ETPs including pretrichodermamide A, epicorazine A and ent-epicoccin G were also obtained. Pretrichodermamide A revealed anti-tubercular activity and inhibited the growth of the phytopathogenic fungus *Ustilago maydis* with minimal inhibitory concentration (MIC) values of 50 µM and 2 mM, respectively. Epicorazine A was found to induce apoptosis in lymphoma (Ramos) and leukemia (Jurkat J16) cell lines through both the intrinsic and extrinsic pathway.

Azacoccones F – H, new flavipin-derived alkaloids from an endophytic fungus Epicoccum nigrum MK214079

Fermentation of *Epicoccum nigrum* MK214079, a fungal endophyte associated with willow (*Salix* sp.) leaves**,** on solid rice medium resulted in the isolation of three new flavipin-derived alkaloids, along with several bioactive metabolites. Three known compounds, namely epicocconigrone A, epipyrone A and epicoccolide B revealed moderate antibacterial activity against *Staphylococcus aureus* ATCC 29213 and mild antinematode potential towards *Heterodera schachtii*. Furthermore, epipyrone A and epicoccamide A exhibited antifungal activity against *U. maydis* with MIC values of 1.63 and 1.80 mM, respectively. Epicorazine A showed potent cytotoxicity against the L5178Y mouse lymphoma cell line with an IC_{50} value of 1.3 µM.

Biotransformation of host plant flavonoids by the fungal endophyte Epicoccum nigrum

A new kaempferol *O*-diglycoside, together with two known kaempferol derivatives were obtained from OSMAC experiments through fermentation of *Epicoccum nigrum* isolated from leaves of *Salix* sp. on green lentil solid medium. Kaempferol *O*-glycoside was also present in green lentils as the starting point to conduct further feeding experiment. In the latter experiment, two flavonoids (kaempferol and rutin) were added as precursors to solid rice media followed by high performance liquid chromatography (HPLC) and LC-MS analyses. This study indicated that the fungal flavonoids originate through hydrolytic cleavage of kaempferol glycosides such as kaempferol *O*-rhamnoside followed by glycosylation and acetylation. Collectively, the isolated flavonoids are formed through biotransformation of plant flavonoids rather than by *de novo* biosynthesis.

CHAPTER 1
Introduction

1.1 Antimicrobial resistance and its impact on drug discovery

Antimicrobial resistance (AMR) occurs when pathogenic microorganisms, such as bacteria and fungi, no longer respond to conventional antibiotic therapies to which they were originally sensitive to (Lewis, 2013). In 2016, the World Health Organization (WHO) classified AMR as a high health priority. It requires international action, as it poses a serious threat to public health globally (Tacconelli *et al*., 2018). Accordingly, the rate of antibiotic resistance among bacteria that cause common infections (e.g., urinary tract and bloodstream infections) is high and new resistance mechanisms emerge and spread regularly. Globally, the top killer among infectious diseases that are caused by a single pathogen is tuberculosis (TB). During the 2016 WHO global TB report, six countries (China, India, Indonesia, Nigeria, Pakistan and South Africa) accounted for 60% of all newly reported TB cases, among those were approximately 580,000 cases of multidrug-resistant tuberculosis (MDR-TB) (WHO, 2017).

The number of patients suffering from drug-resistant infections increases daily and the rate of drug discovery is unable to match these demands. The strong momentum in antibiotic discovery has slowed down significantly since the its golden era (1940s−1960s), in fact the approval of new antibiotics and the discovery of novel classes has declined by up to 90% from the 1980s to the early 2000s (Luepke *et al.*, 2017). During the last two decades, merely two new antibiotic classes have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Luepke *et al*., 2017). These are the lipopeptides (daptomycin) and oxazolidinones (linezolid) which both are active against Gram-positive bacteria (Luepke *et al*., 2017). On the other side of the bacterial spectrum, fluoroquinolones, which were discovered half a century ago, were the last approved class of antibiotics with broadband activity against Gram-negative bacteria. However, to date new compounds as exemplified by the peptide darobactin are still under development as new narrow-spectrum antibiotics against Gram-negative pathogens (Imai *et al.*, 2019). Additionally, forty-four new chemical entities (NCEs) were developed until July 2018 as representatively described in Table 1.1 (Theuretzbacher *et al.*, 2019). The increasing global awareness of the current need for new antibiotics to control emerging diseases or resistant strains of microorganisms inspired a

growing number of research groups to discover and develop new drug leads to combat multidrug-resistant pathogens (Aly *et al*., 2010).

Table 1.1. Antibiotics development towards priority pathogens

Name	**Antibiotic Class**	**Indication**	**Status (07/2018)**
Delafloxacin	Fluoroquinolone	ABSSSI (CAP, Phase 3)	FDA approved
Vaborbactam + Meropenem	Boronate BLI + Carbapenem	cUTI	FDA approved
Plazomicin	Aminoglycoside	cUTI	FDA approved
Eravacycline	Tetracycline	CRE	NDA, MAA
Cefiderocol	Siderophore-cephalosporin	CRAB, CRPA, CRE	Phase III
Telacebec (Q-203)	Imidazopyridine amide	Anti-tubercular	Phase II
Zidebactam + Cefepime	DBO-BLI/ PBP2 binder + Cephalosporin	CRE	Phase I

***** ABSSSI= acute bacterial skin and skin structure infection, CAP= community-acquired pneumonia, BLI= β-lactamase inhibitor, CRAB= *Acinetobacter baumannii*, carbapenem-resistant, CRE= *Enterobacteriaceae*-, carbapenem- and third-generation cephalosporin-resistant, CRPA= *Pseudomonas aeruginosa*, carbapenem-resistant, cUTI= complicated urinary tract infection, DBO= diazabicyclooctane, MAA= marketing authorisation application, NDA= new drug application (FDA), PBP= penicillin-binding protein.
Adapted from Theuretzbacher *et al.* (2019)

1.2 Potential sources of new antibiotics or drug leads

Today medicinal plants are still globally used as one of the most valuable resources for drug discovery. However, the International Union for Conservation of Nature and the World Wildlife Foundation reported that approximately 25% of known medicinal plants are on the verge of extinction due to indiscriminate exploitation (overharvesting and habitat destruction) (Chen *et al.*, 2016). The fact that over 80% of the natural remedies available to the market originate from medicinal plants and their endophytes (Singh and Dubey, 2015) has raised the question whether the plant or rather the fungal endophytes are the real contributors of several

bioactive metabolites. These facts have shifted the focus of scientists from plants to fungal endophytes (Strobel and Daisy, 2003; Cragg and Newman, 2013) and triggered a recent strategy to screen endophytes as a major source of bioactive compounds with promising pharmaceutical application (Aly *et al.*, 2010). Interestingly, many endophytes are able to biosynthesize a number of important bioactive secondary metabolites that are characteristic of the respective host plants. One of the best-known examples of this phenomenon is the discovery of the anticancer drug paclitaxel (Taxol®) producing endophytic fungi such as *Taxomyces andreanae* from the Pacific yew tree *Taxus brevifolia* (Taxaceae) (Striele *et al.*, 1993) as well as a more recently discovered strain of *Epicoccum nigrum* (Somjaipeng *et al.* 2016). Less widely known examples include the cardiac glycoside digoxin, originally obtained from the plant *Digitalis lanata* (Plantaginaceae), which was also produced by endophytic *E. nigrum* (El-Sayed *et al.*, 2020), as well as curcumin, originally isolated from *Curcuma wenyujin* and later detected in endophytic *Gibberella fujikuroi* (Yan *et al.*, 2014[a]). These discoveries evoked the interest to examine other plants to yield endophytes not only producing pharmacologically important natural products hitherto only known from plants, but also promising novel therapeutic agents (Aly *et al*., 2010).

Generally, around ten percent of all currently known bioactive natural products are of microbial origin. The total number of known microbial natural products amounts to approximately 22,000, of which 70% are produced by actinomycetes, 20% by fungi, 7% by *Bacillus* spp. and 1–2% by other bacteria (Khalifa *et al.*, 2019). Reportedly, around 90% of all antibiotics used in clinics today are derived from microorganisms as selectively summarized in the Table 1.2 (Katz and Baltz, 2016). The untapped microbial reservoirs of natural products need to be screened and explored by scientists worldwide as they are likely candidates for the next antibiotic ‘gold mine’. The chemodiversity hidden within microorganisms such as fungi, is still being unravelled and it will be crucial for next-generation drug development. Today, fungi are still underexplored even though they have historically proved themselves to be excellent sources for biologically active compounds with therapeutic potential such as antibiotics, anticancer drugs, immunosuppressants, antiparasitics and agrochemicals (Hoeksma *et al.*, 2019).

Table 1.2. Diverse natural products of microbial origin

Natural Product	Major Use	Producer	Type of Producer
Acarbose	Antidiabetic	*Actinoplanes* sp.	Actinomycetes
Avermectin (Ivermectin)	Anthelminthic	*Streptomyces avermitilis*	
Chloramphenicol	Antibacterial	*Streptomyces venezuelae*	
Doxorubicin (Adriamycin)	Antitumor	*Streptomyces peucetius*	
Nystatin	Antifungal	*Streptomyces noursei*	
Staurosporin (aglycone)	Antitumor	*Streptomyces staurosporeus*	
Streptomycin	Anti-tubercular	*Streptomyces griseus*	
Cephalosporin	Antibacterial	*Cephalosporium acremonium*	Fungi
Cyclosporin A	Immunomodulator	*Tolypocladium inflatum*	
Ergometrine	Vasoconstrictor	*Claviceps purpurea*	
Lovastatin	Antihiperlipidemia	*Asperigillus terreus*	
Pneumocandin	Antifungal	*Glarea lozoyensis*	
Gramicidin S	Antibacterial	*Bacillus subtilis*	Bacteria
Polymyxin (Colistin)	Antibacterial	*Paenibacillus polymyxa*	
Surfactin	Surfactant	*Bacillus subtilis*	

Adapted from Katz and Baltz (2016)

1.3 Fungal secondary metabolites as pharmaceuticals

Several endophytic fungi isolated either from terrestrial or marine natural sources produce bioactive compound groups. The commercially available pharmaceuticals derived from these natural products can be divided into alkaloids, polyketides, terpenoids and miscellaneous compounds derived from other metabolic pathways.

a) *Alkaloids*

Camptothecin is a pentacyclic quinoline alkaloid that was obtained from the fungal endophytes *Entrophospora infrequens* (Puri *et al.*, 2005) and *Neurospora* sp. (Rehman *et al.*, 2008) isolated from the Indian plant *Nothapodytes foetida*. Moreover, it was also reported from *Aspergillus* sp., *Fusarium solani* and *Trichoderma atroviride* associated with the plant *Camptotheca acuminata* (Kusari *et al.*, 2009; Pu *et al.*, 2013) from which it was formerly known. Camptothecin is the parent compound of the semi-synthetic derivatives irinotecan and topotecan and acts via inhibition of the intranuclear enzyme topoisomerase-I, thus preventing DNA transcription and replication (Gutierrez *et al.*, 2012).

Phenylahistin (halimide) is a diketopiperazine metabolite originally produced by *Aspergillus* sp. that has been patented as a promising anticancer agent (Fenical *et al.*, 2000; Fukumoto *et al.*, 2002). Meanwhile, a synthetic tert-butyl analog of halimide, **plinabulin** (NPI-2358), is the first marine-derived fungal metabolite which has entered clinical trials as a selective tumor vascular disrupting agent with anti-microtubule activity (Yamazaki *et al.*, 2010; Gomes *et al.*, 2015).

Vinblastine is a Vinca alkaloid, originally isolated from the plant *Catharanthus roseus* (Apocynaceae). It is also produced by associated endophytes *Alternaria* sp. (Guo *et al*., 1998), *F. oxysporum* (Kumar *et al.*, 2013) and *Talaromyces radicus* (Palem *et al.*, 2015). **Vincristine** also known as leurocristine was also reported from *F. oxysporum* residing in leaves of *C. roseus* (Kumar *et al.*, 2013). Both antineoplastic drugs block the microtubules of the mitotic apparatus to inhibit mitosis (Wilson *et al.*, 1975), while vincristine alters the structure of chromatin by binding to chromatin and DNA, thus perturbing histone-DNA interaction (Mohammadgholi *et al.*, 2013). The chemical structures of anticancer agents including quinolone, diketopiperazine, and Vinca alkaloids are presented in Fig. 1.1.

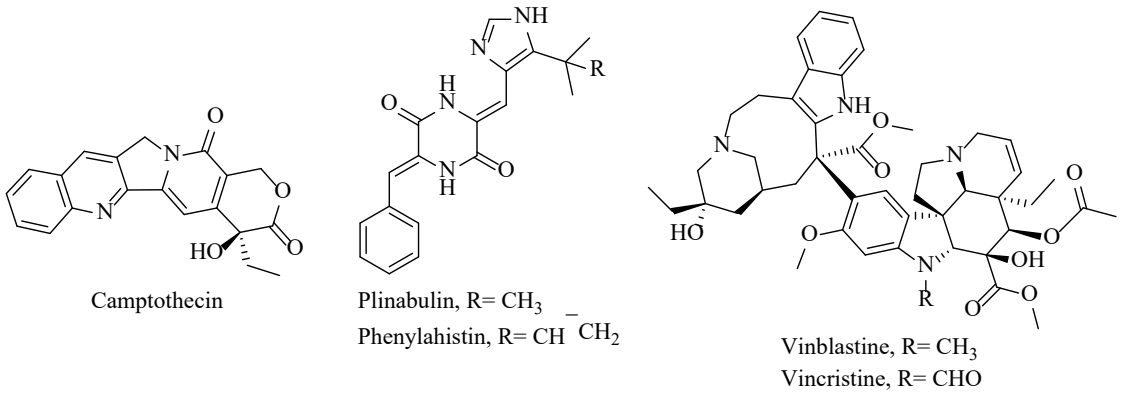

Figure 1.1. Anticancer drugs or cytotoxic alkaloids of fungal origin.

b) *Polyketides*

Griseofulvin was originally isolated from *Penicillium griseofulvum* (Oxford *et al.*, 1939). It is produced by 16 different fungi belonging to the genus *Penicillium*, as well as by *Aspergillus lanosus* (Larsen *et al.*, 2005). Griseofulvin is an antifungal agent that is used for the treatment of dermatophytosis. It acts as an antimitotic agent by inhibiting microtubule assembly through binding to a unique binding site at tubulin that is distant to the C-terminal domains of the α- and β-subunits, of which both are not affected by any conformational changes (Chaudhuri *et al.*, 2001).

Lovastatin (**monacolin K**) was obtained from the fungus *Monascus ruber* and is commercially produced by fermentation of *Aspergillus terreus* (Seenivasan *et al.*, 2008). Historically, the first reported statin is **mevastatin** purified from *Penicillium citrinum* cultures. Moreover, simvastatin (Barrios-González and Miranda, 2010) and pravastatin (McLean *et al*., 2015) are semi-synthetic statins derived from lovastatin and mevastatin, respectively. Conversion of lovastatin to simvastatin is performed via chemical reaction, while a biotransformation process is used for pravastatin synthesis (Tobert, 2003). Based on the structure and activity relationship study, the chiral lactone-derived moiety is important for inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) rather than the bicyclic moiety (Endo and Hasumi, 1993). The chemical structures of the fungal polyketides including griseofulvin and statins are presented in Fig. 1.2.

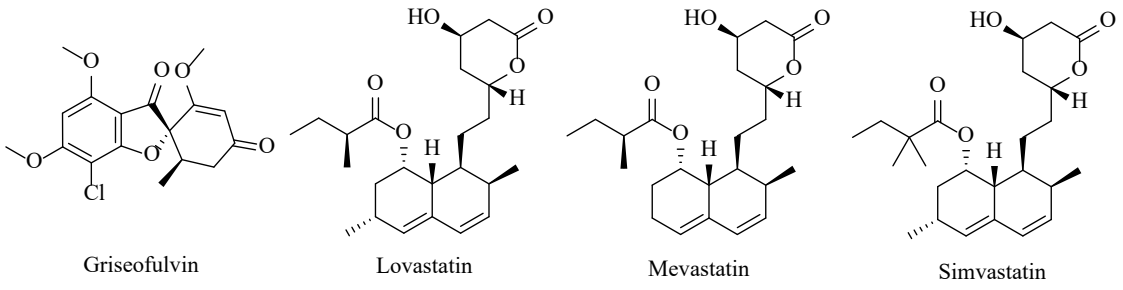

Figure 1.2. The fungal polyketides as antifungal and antihyperlipidemic agents.

c) *Terpenoids*

Fusidic acid is a fusidane-type triterpenoid originally derived from *Fusidium coccineum* (Godtfredsen *et al.*, 1962) and presently isolated from *Acremonium fusidioides* (Cao *et al.*, 2019). This oral antibiotic inhibits bacterial protein biosynthesis through interference with elongation factor G and is clinically used for the treatment of Gram-positive bacterial infections (Laurberg *et al.*, 2000).

Mycophenolic acid is the first fungal natural product discovered in 1898 from *Penicillium glaucum*, even earlier than the discovery of penicillin by Alexander Fleming in 1928. This immunosuppressant was then approved by FDA in 1995 for use in kidney transplantations. Mycophenolic acid blocks the activation of B- and T-lymphocytes by inhibiting the inosine-5'-monophosphate dehydrogenase (IMPDH) (Kim *et al.*, 2013).

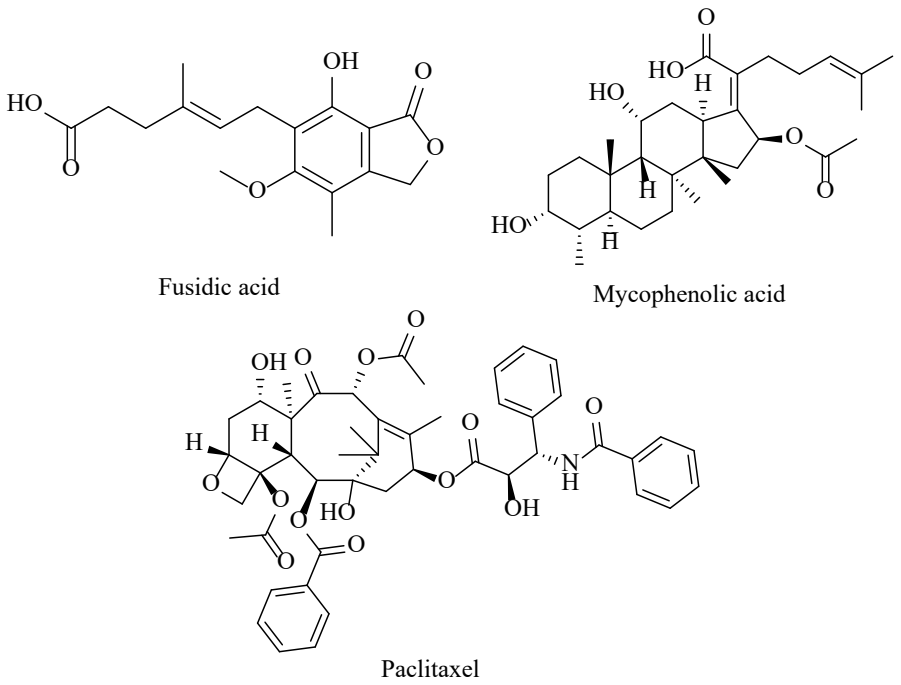

Figure 1.3. Terpenoid natural products in fungi as therapeutic agents.

Paclitaxel was firstly reported from a taxol-producing fungus *Taxomyces andreanae* in addition to the host plant *Taxus brevifolia* as an original producer (Stierle *et al.*, 1993). The other taxol-producing fungi include *Nodulisporium sylviforme* (Zhao *et al.*, 2004), *Cladosporium cladosporioides* (Zhang *et al.*, 2009) and *E. nigrum* (Somjaipeng *et al.,* 2016). Recently, semi-synthetic methods are used to produce paclitaxel through extraction of European yew *T. baccata* (Patel, 1998) and Taxus cell fermentation with the fungus *P. raistrickii* (Tabata, 2004). Paclitaxel is classified as antimitotic agent and acts by binding to the *β*-tubulin subunit, leading to a stimulated tubulin polymerization without breaking down of microtubules during cell division (Kumar, 1981). The chemical structures of fungal terpenoids are presented in Fig. 1.3.

d) *Miscellaneous*

Cyclosporine (cyclosporine A, CsA) is a lipophilic cyclic undecapeptide produced by the soil-derived fungus *Tolypocladium inflatum* (Svarstad *et al.*, 2000). Cyclosporine A is an

effective medication used for the treatment of transplant rejection and severe psoriasis (Pereira *et al.*, 2006). Additionally, it has applications for the treatment of certain viral infections, such as HIV-1 or HCV (Hatziioannou *et al.*, 2005; Paeshuyse *et al.*, 2006). This immunosuppressive drug acts via blocking the calcineurin/NFAT pathway by forming a complex with cyclophylin, thus inhibiting the phosphatase activity of calcineurin that regulates nuclear translocation followed by activation of NFAT transcription factors. Moreover, cyclosporine is a specific inhibitor of T cell activation by blocking the activation of JNK and p38 signaling pathways triggered by antigen recognition (Matsuda and Koyasu, 2000).

Ergotamine is a prenylated nonribosomal peptide originally produced by *Claviceps purpurea, C. fusiformis,* and *C. paspali*. This ergot alkaloid is clinically used as vasoconstrictor for migraine treatment or also used in combination with belladonna and phenobarbital for relief from menopausal hot flashes. Its mechanism of action is the mimicking of neurotransmitters at several receptors, such as 5-OH tryptamine (5-HT2) receptor 1B, dopamine, norepinephrine, and serotonin receptors (Bills and Gloer, 2016).

Kojic acid, a pyrone derived from glucose, was originally obtained from *A. oryzae. A. tamarii* and *A. flavus*. Kojic acid has antioxidant properties that are commercially used in cosmetic products for skin-lightening agents that work by suppressing melanogenesis, and also for treatment of abnormal hyperpigmentation via the inhibition of tyrosinase (Bills and Gloer, 2016). Structural and functional diversity of fungal metabolites are presented in Fig. 1.4.

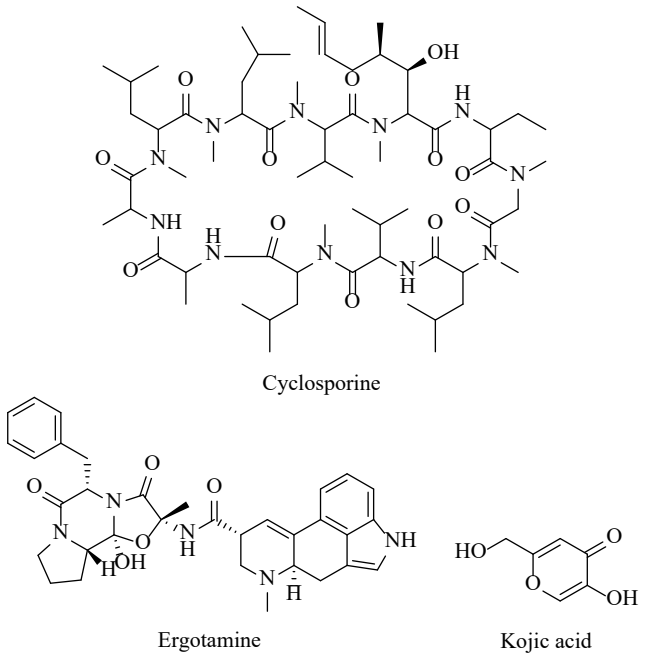

Figure 1.4. Diversity of fungal metabolites – from pharmaceuticals to cosmeceuticals.

1.4 Recent studies on plant endophytic fungi and their potential applications

Endophytes are endosymbiotic microorganisms (bacteria, fungi and actinomycetes) that colonize the inter- and/or intracellular tissue of plants, from which they can be readily isolated and cultivated on commonly used microbial or plant growth media (Jia *et al.*, 2016). To date, highly diverse natural products have been identified through investigation of various plants or their associated endophytes. Recently, the discovery of NCEs for therapeutic purposes through extensive exploration of plant sources has been shifted towards investigation of untapped resources such as endophytic microbes (Gouda *et al.*, 2016). The remarkable chemical and functional diversity of fungal metabolites makes them potential candidates for future pharmaceutical applications, as they often exhibit antimicrobial, insecticidal as well as anticancer activities or other properties. Thereby, scientists are spurred to examine the potential of fungal endophytes as alternative reservoirs of medicinally important plant metabolites or as producers of novel bioactive compounds that can contribute to a successful drug discovery (Ancheeva *et al.*, 2020).

More than eighty percent of commercially available herbal medicines are derived from medicinal plants and their associated endophytes (Singh and Dubey, 2015). On the other hand, some endophytic fungi are able to produce the same or similar bioactive metabolites as those known from their host plants, triggering a new trend towards the investigation of endophytic fungi from medicinal plants (Zhao *et al*., 2011; Venieraki *et al.*, 2017). Consequently, an increasing number of plant-derived fungal metabolites, as well as numerous publications and patents have been published and filed during the last decade (Gokhale *et al*., 2017). These positive results indicate that endophytic fungi present in the medicinal plants constitute a promising target for finding novel bioactive compounds (Gómez and Luiz, 2018). Selected examples of bioactive metabolites produced by fungal endophytes within their respective host plants are listed in Table 1.3.

Table 1.3. Bioactive metabolites of some fungal endophytes associated with plants

Host plant	Endophytic fungi	Metabolite(s) (structure)	Bioactivity	Reference(s)
Catharanthus roseus	*Fusarium oxysporum, Talaromyces radicus, Eutypella* spp.	Vinblastine, vincristine (alkaloids)	Anticancer	Palem *et al.*, 2016; Kumar *et al.*, 2013
Cinchona ledgeriana	*Phomopsis, Diaporthe, Schizophyllum, Penicillium, Fomitopsis, Arthrinium*	*Cinchona* alkaloids	Antimalarial	Maehara *et al.*, 2013
Cinnamomum spp.	*Muscodor albus*; *Annulohypoxylon squamulosum* BCRC 34022	Volatile organic compounds, Kaempferol derivatives	Antimicrobial	Strobel *et al*., 2001; Cheng *et al*., 2012
Digitalis lanata Ehrh.	*Alternaria* spp., *Penicillium* spp., *Aspergillus* spp., *Epicoccum nigrum*	Digoxin (glycoside)	Cardiotonic	Kaul *et al.*, 2013; El-Sayed *et al.*, 2020
Ginkgo biloba L.	*Fusarium oxysporum* SY0056	Ginkolide B (terpenoid lactone)	Renal, cardiovascular, and respiratory protective	Cui *et al.,* 2012
Moringa stenopetala	*Mucor irregularis*	Chlorflavonin (flavonoid)	Anti-tubercular	Rehberg *et al*., 2018
Passiflora incarnata	*Alternaria alternate, Colletotrichum capsici, Chryseobacterium taiwanense*	Chrysin (flavonoid)	Anticancer (hepatic carcinoma)	Seetharaman *et al*., 2017
Rheum palmatum L.	*Fusarium solani*	Emodin, rhein (anthraquinones)	Antibacterial, anti-inflammatory, and hepatoprotective	You *et al.*, 2013

This new resource of bioactive natural products derived from plant-associated endophytic fungi may possess great potential applications in the pharmaceutical, agricultural, and biotechnological fields (Zhao *et al*., 2011). The present study describes the investigation of the potential applications of two specific endophytic fungi, namely *Trichoderma harzanum* and *Epicoccum nigrum*.

1.4.1 *Trichoderma harzianum*

Characteristics of fungal species from the genus *Trichoderma* are rapid growth and the capability to produce abundant conidial spores as well as sclerotia. *Trichoderma* species exhibit strong mycoparasitic behavior and synergize with their host plant by antagonizing plant pathogens rather than directly promoting plant growth. Several strains of *Trichoderma* spp. are used in various industrial scales, where they are exploited for the production of antibiotics, enzymes, biofuel and other valuable substances (Błaszczyk *et al*., 2014). *Trichoderma harzianum*, a soil fungus or so-called green mold, is commercially available in form of biofertilizers and biopesticides (Vinale *et al*., 2008). Additionally, this filamentous fungus also produces diverse metabolites which can be useful for agricultural or non-agricultural applications as shown in Fig. 1.5.

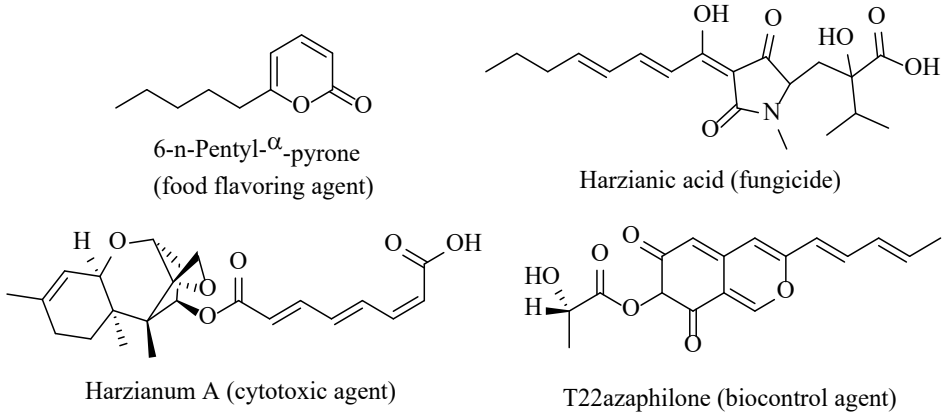

Figure 1.5. Chemical structures and potential applications of secondary metabolites from *Trichoderma harzianum*.

Strains of *T. harzianum* as well as metabolites derived from them, are successfully applied in agricultural products. The commercial biological control agents (BCA) formulated from strains of *T. harzianum* contain peptaibiotics, mainly with 11-, 14-, and 18-residue peptides, e.g., trichorzianins A–B and trichorzins HA/MA that possess antifungal and antibacterial properties (Błaszczyk *et al*., 2014; Degenkolb *et al*., 2015). The antibiotic 6-pentyl-α-pyrone (6-PP) was discovered in a solid-state fermentation of *T. harzianum* and is interestingly used as food flavoring agent (Rivera *et al*., 2014; Zeilinger *et al*., 2016). Non-volatile metabolites such as harzianopyridone and T22azaphilone, along with the volatile ones like 6-PP are factors contributing to the biocontrol activities in several crops (Vinale *et al*., 2006; Guo *et al*., 2019[b]). The siderophore harzianic acid strongly binds iron (Fe^{3+}) and was found to possess fungicidal and plant growth promoting effects (Vinale, 2013; Vinale, 2014). Unfortunately, none of the bioactive metabolites from *Trichoderma* spp., including *T.*

harzianum, was introduced into the clinical practice up to now. However, a few of these substances might have the potential to become antineoplastic agents, e.g., harzianum A and 18-deoxycytochalasin (Jin *et al*., 2007; Chen *et al*., 2015). The capability of *T. harzianum* to detoxify heavy metals, cyanides, nitrates and phenols is important for its role in bioremediation (Lynch and Moffat, 2005; Faedda *et al*., 2012). Noteworthy, biocontrol mechanisms involving cell wall degrading enzymes such as *β*-glucanases, chitinases, and proteinases are found in this fungus (Elad, 2000).

1.4.2 *Epicoccum nigrum*

Epicoccum nigrum is one of the best investigated species among the genus of *Epicoccum*. This fungicolous fungus can be found in various plant tissues, in association with insects and it is also often isolated from marine sources. Similar to *T. harzianum*, this mycoparasitic fungus is widely used as BCA against phytopathogens. Therefore, *E. nigrum* plays a pivotal role in agriculture to prevent fungal infections of several crops such as cotton, potato, sorghum, sunflower, waxflower and many others. In addition, this fungus has been found to be capable of increasing potato yield, decreasing the stem disease severity index, improving the root system biomass and even of influencing membrane permeability or cell wall synthesis (Braga *et al*., 2018).

Several bioactive metabolites from *E. nigrum* are also applicable as pharmaceuticals. Cytotoxic compounds produced by *E. nigrum* include paclitaxel, together with epicorazines A/B, epicocconigrone A and epicoccolide B (Braga *et al*., 2018). Interestingly, paclitaxel production could be enhanced by serine feeding and growing the fungus under a specific condition (Somjaipeng *et al*., 2016). The latter two compounds are promising antitumor agents which work as inhibitors of histone deacetylases (HDACs) and protein kinases (El Amrani *et al*., 2014), while an isopimarane diterpene has therapeutic potential as α-glucosidase inhibitor in Diabetes Mellitus (Xia *et al*., 2015). Moreover, the cardiac glycoside digoxin (El-Sayed *et al*., 2020) and the potential antiviral agent orevactaene (Shu *et al*., 1997) were reported from this fungal species.

The biotechnological applications demonstrated for *E. nigrum*, include biotransformation of chemicals, e.g., diclofenac, ionones and ketones; degradation of biogenic amines in wine; biosynthesis of gold and silver nanoparticles; and synthesis of lipids for biodiesel production (Braga *et al*., 2018). Remarkably, a commercial fluorophore epicocconone, used for cell staining and protein detection in electrophoresis gels, is originally

derived from this fungus (Bell and Karuso, 2003; Choi *et al*., 2006). The other pigments obtained from *E. nigrum* such as carotenoids, epirodin, orevactaene, prodiginine and quinizarin have potential use as colorants in the food or textile industries (Braga *et al*., 2018). The bioactive compounds from this fungus and their potential applications are shown in Fig. 1.6.

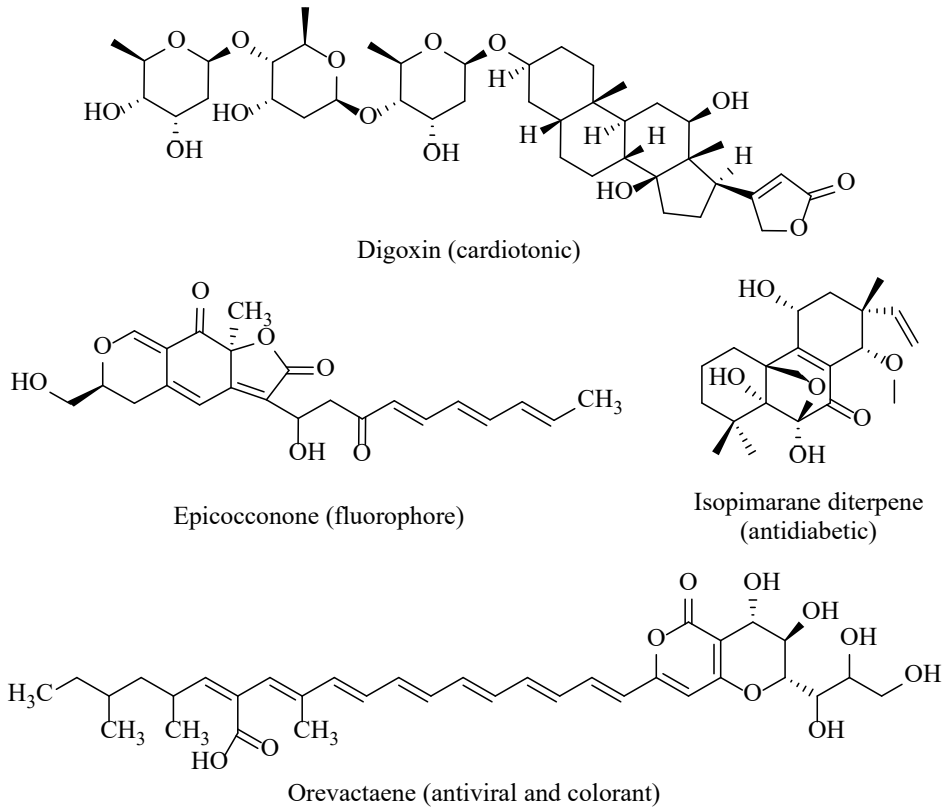

Figure 1.6. Chemical structures and potential applications of secondary metabolites from *Epicoccum nigrum*.

1.5 Strategies to diversify the fungal secondary metabolites

The strategic action plans to enhance the rate of discovery of novel lead compounds from endophytic fungi can be achieved through new culturing techniques that aim to activate silent gene clusters, leading to the production of distinct and unique metabolites that are not expressed under standard laboratory culturing conditions (Ancheeva *et al*., 2018). These strategies include the OSMAC (One Strain Many Compounds) approach, stress-inducing culturing methods and co-cultivation with either other fungal strains or with bacteria (Romano *et al*., 2018; Pan *et al*., 2019). Other feasible approaches include the feeding of precursors, adding of biotic or abiotic elicitors, the addition of inhibitors, the use of special enzymes or other substances (Pan *et al*., 2019). All of these methods are dependent on investigation of the

metabolic profile of the fungus during fermentation and may be experimetally adopted to optimize the production of novel bioactive metabolites.

The term OSMAC basically describes the modulating effects of changing certain fermentation parameters such as media type and composition, temperature, osmolarity, salinity, pH value or other cultural conditions (Romano *et al*., 2018). The OSMAC principle attempts to extend the diversity of fungal metabolites by stimulating silent biogenetic gene clusters, thereby inducing new natural compounds, which are undetected under conventional culturing conditions or enhancing the accumulation of constitutively present compounds (Daletos *et al*., 2017). Recent examples include media modification as in the case of a strain of *A. ochraceus* that was capable of producing a new waspergillamide B when cultured on white bean medium (Frank *et al*., 2019), as well as the fermentation of *F. tricinctum* on fruit and vegetable juice-supplemented solid rice media that resulted in the production of new fusarielins K and L (Hemphill *et al*., 2017). The culture broths of *Trichoderma* sp. TPU199, consisting of freshwater media supplemented with 1.5% NaCl, 3.0% NaBr and 3.0% NaI to set different salinity, afforded two rare epidithiodiketopiperazines (ETPs), gliovirin and pretrichodermamide A, along with two new bromo and iodo ETP derivatives (Yamazaki *et al*., 2015). Additionally, a recent study revealed that cultivation of *Trichocladium* sp. on rice medium supplemented with 2% tryptophan yielded a new bismacrolactone 13-*N*-(2-carboxyphenyl)colletoketol with cytotoxic potential (Tran-Cong *et al*., 2019).

Today, the rate of discovery of new drugs or drug leads is getting lower, while the possibility of rediscovery of known compounds is higher than before. In the production of microbial secondary metabolites, this challenge could be alleviated by employing an effective and efficient strategy such as OSMAC, or one of the alternative approaches such as co-cultivation, addition of epigenetic modifiers (e.g., suberoylanilide hydroxamic acid/SAHA) and of biosynthetic precursor(s). Nowadays, new additional opportunities arise, such as the investigation of the uncultivable microorganisms, as well as metabolomic tools and gene activation experiments (Romano *et al*., 2018). Examples of the application of new methods in the discovery of novel bioactive metabolites include transcriptomics and metabolomics-based studies (Covington *et al*., 2017), MS/MS-based molecular networking analysis (Wang *et al*., 2016), as well as microbe genome mining (Hug *et al*., 2018). Hence, the scientific community is expected to open up an exciting area of research for the discovery of lead compounds circumventing time-consuming methods (Ancheeva *et al*., 2018).

1.6 Aims and significance of the study

Natural products take a leading place in drug discovery of antimicrobial agents as an almost inexhaustible, reproducible and cost-effective supply of bioactive compounds. Microorganisms, which are used as a source of bioactive secondary metabolites, include different classes of fungi, bacteria, actinomycetes and lichens. Among them, plant endophytic fungi are of considerable importance as promising sources of bioactive natural products featuring novel and unique carbon frameworks. Most of these secondary metabolites show biological activities in pharmaceutical bioassay systems and hence represent lead structures with a wide range of potentials in medicinal, pharmaceutical and agricultural.

Thus, this present study was directed at the investigation of new promising natural products derived from endophytic fungi associated with medicinal plants. Furthermore, the isolated pure substances were submitted to various bioassays such as investigation of cytotoxicity against mouse or human carcinoma cell lines, antibacterial activities against *Mycobacterium tuberculosis* and a panel of Gram-positive and Gram-negative bacteria, as well as antifungal activity towards the plant pathogenic fungus *Ustilago maydis* and antinematode activity against *Heterodera schachtii* (chapters 2–5). The results of these studies are described in this dissertation.

CHAPTER 2

Dithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and *Epicoccum nigrum*

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Dithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and *Epicoccum nigrum*

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ABSTRACT

A new epidithiodiketopiperazine (ETP), pretrichodermamide G (**1**), along with three known (epi)dithiodiketopiparazines (**2-4**) were isolated from cultures of *Trichoderma harzianum* and *Epicoccum nigrum*, endophytic fungi associated with medicinal plants *Zingiber officinale* and *Salix* sp., respectively. The structure of the new compound (**1**) was established on the basis of spectroscopic data, including 1D/2D NMR and HRESIMS. The isolated compounds were investigated for their antifungal, antibacterial and cytotoxic potential against a panel of microorganisms and cell lines. Pretrichodermamide A (**2**) displayed antimicrobial activity towards the plant pathogenic fungus *Ustilago maydis* and the human pathogenic bacterium *Mycobacterium tuberculosis* with MIC values of 1 mg/mL (2 mM) and 25 μg/mL (50 μM), respectively. Meanwhile, epicorazine A (**3**) exhibited strong to moderate cytotoxicity against L5178Y, Ramos, and Jurkat J16 cell lines with IC_{50} values ranging from 1.3 to 28 μM. Further mechanistic studies indicated that **3** induces apoptotic cell death.

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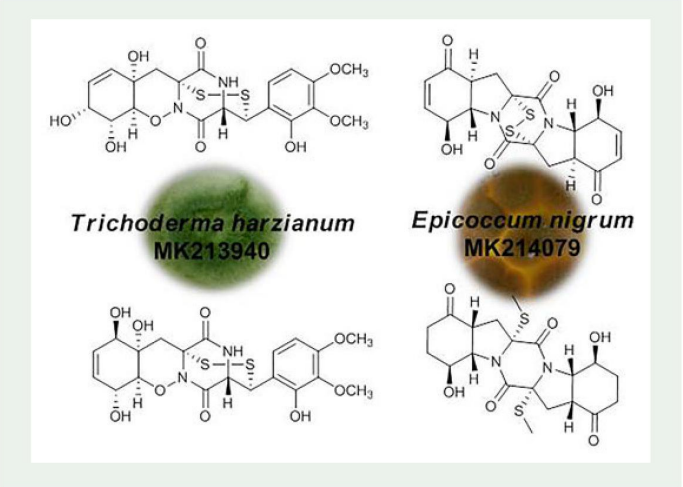

1. Introduction

Microorganisms residing in plant tissues without causing any apparent symptoms, namely endophytes, are important resources for the discovery of biologically active compounds with promising agricultural and pharmaceutical applications (Aly et al. 2011). Moreover, endophytes possess the advantage of large-scale production of diverse bioactive metabolites and potential drug leads (Aly et al. 2010) which is not always possible in plants. *Trichoderma* species are recognized as mycoparasites and are widely used in agriculture as biofungicides and bioremediation agents (Błaszczyk et al. 2014; Zeilinger et al. 2016; Ghorbanpour et al. 2018). Similarly, *Epicoccum nigrum* (synonym *E. purpurascens*), which can parasitize the mycelium of fungal pathogens of crops, is used as a biocontrol agent (Madrigal et al. 1991; Koutb and Ali 2010). Diverse bioactive compounds including diketopiperazine alkaloids and various terpenoids were currently reported from these two biocontrol fungi (Wang et al. 2010; Yamazaki et al. 2015a, 2015b; Perveen et al. 2017; Fang et al. 2018; Han et al. 2018; Liang et al. 2019). Moreover, *T. harzianum* was shown to be tolerant towards the heavy metals cadmium and mercury, thus underlying its potential role in the detoxification of environmental pollutants (Faedda et al. 2012).

In the course of our screening for new and bioactive compounds from endophytes associated with medicinal plants, we investigated two endophytic fungal strains, *T. harzianum* MK213940 and *E. nigrum* MK214079 which were isolated from healthy leaves of *Zingiber officinale* (Zingiberaceae) and *Salix* sp. (Salicaceae), respectively. Ginger rhizome is traditionally used as antiemetic and its crude extract and various constituents were shown to exhibit antitumor, immunomodulatory, antiviral, and antimicrobial effects (Chrubasik et al. 2005). Interestingly, the ginger endophyte *T. harzianum* was found to be highly active against common fungal and bacterial pathogens associated with Zingiberaceae crops (Rajan et al. 2002). The second fungus investigated in this study, *E. nigrum* was isolated from willow leaves collected in Russia. *Salix*

sp. (Salicaceae) have been profoundly associated with the discovery of salicylic acid and derivatives exhibiting antiinflammatory, antirheumatic, antipyretic, analgesic, and antiseptic effects (Barnes et al. 2002).

In our previous reports we described a new cytochalasin with potent cytotoxicity, isolated from *T. harzianum* that was obtained from *Cola nitida* leaves (Chen et al. 2015). In addition, *E. nigrum* isolated from leaves of *Mentha suaveolens* Ehr. produced new polyketides that showed *in vitro* inhibitory activity against a panel of protein kinases and histone deacetylases (El Amrani et al. 2014). Herein, we investigated the chemical profiles of new isolates of these fungi. HPLC-DAD and LC-MS analyses of their crude extracts showed that both fungi are able to accumulate a rare fungal secondary metabolites class, epidithiodiketopiperazines (ETPs). These natural products are characterized by an internal disulfide linkage across the diketopiperazine ring (Guimarães et al. 2010). Examples of diketopiperazines (DKPs) that have been previously isolated from fungi belonging to the genus *Trichoderma* or *Epicoccum*, include gliovirin, pretrichodermamide A, trichodermamides A and B, five halogenated ETPs, epicoccins E-T, and epicorazines A and B displaying a wide range of biological activities (Deffieux et al., 1978a, 1978b, Garo et al. 2003; Seephonkai et al. 2006; Guo et al. 2009; Wang et al. 2010; Yamazaki et al. 2015a, 2015b). To date, only eighteen DKP derivatives have been previously reported possessing a rare 1,2-oxazadecaline core (Yokose et al. 1984; Orfali et al. 2015; Yamazaki et al. 2015a, 2015b; Kajula et al. 2016; Yurchenko et al. 2016). In this study, chemical investigation of *T. harzianum* and *E. nigrum* resulted in the isolation of a new ETP (**1**), along with three known derivatives (**2**-**4**). The structure of the new compound **1** was unequivocally deduced by NMR spectroscopic analysis and mass spectrometry, as well as by comparison with the literature. Antifungal, antibacterial and cytotoxic assays results of **1**-**4** are likewise reported.

2. Results and discussion

Four dithiodiketopiperazines from two endophytes were isolated and identified in this study. Among them, a new ETP derivative (**1**) together with pretrichodermamide A (**2**) were isolated from the endophytic fungus *T. harzianum* and two known bioactive secondary metabolites, epicorazine A (**3**) and ent-epicoccin G (**4**) were obtained from the culture of *E. nigrum* (Deffieux et al. 1978a; Seephonkai et al. 2006; Wang et al. 2010). Structures of these compounds are shown in Figure 1. To obtain these compounds, the EtOAc extracts of the aforementioned fungi were partitioned against *n*-hexane and 10% MeOH and the MeOH phases were further separated by VLC, following by size-exclusion chromatography over Sephadex LH-20. As a final purification step, semi-preparative HPLC was applied to afford compounds **1**-**4**.

Compound **1** was isolated as yellow solid. Its molecular formula was established by HRESIMS as $C_{20}H_{22}N_2O_9S_2$ (11 degrees of unsaturation) on the basis of the protonated molecular ion peak at *m/z* 499.0839 $[M+H]^+$. The ^1H and ^{13}C NMR data of **1** (Table S1) were similar to those of pretrichodermamides D or E, secondary metabolites biosynthesized by the marine algae-derived fungus *Penicillium* sp. (Yurchenko et al. 2016). Accordingly, the ^1H and ^{13}C NMR spectra of **1** showed the signals of two methoxy groups (δ_H 3.78/3.67; δ_C 55.5/60.0), one phenolic hydroxy proton (δ_H 9.47), and two

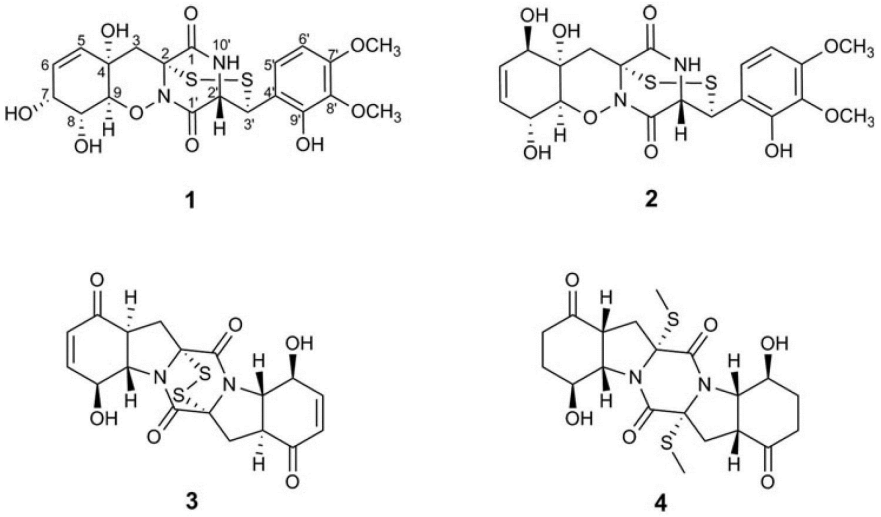

Figure 1. Dithiodiketopiperazines produced by the endophytic fungi *Trichoderma harzianum* (**1-2**) and *Epicoccum nigrum* (**3-4**).

ortho coupled aromatic methines (δ_H 7.43/6.55, $J = 8.8$ Hz; δ_C 122.6/103.1) (Yurchenko et al. 2016). Observed HMBC correlations arising from the aromatic proton H-5′ to C-7′ (δ_C 153.1) and C-9′ (δ_C 147.6) and from H-6′ to C-4′ (δ_C 116.2) and C-8′ (δ_C 136.0) suggested the presence of a tetrasubstituted benzene ring, in which 7′-OMe and 8′-OMe groups are attached to the respective carbons. Further correlations from 9′-OH to C-4′, C-8′ and C-9′ confirmed a 2,3-dimethoxyphenol unit in **1**.

A series of ^{1}H-^{1}H COSY cross-peaks established a spin system starting from H-5 (δ_H 5.53) and extending till H-9 (δ_H 4.13). Moreover, HMBC correlations from H-5 and H-9 to the oxygenated sp^3 carbon C-4 (δ_C 66.8) indicated the presence of a cyclohexene ring in the structure of **1**, bearing a double bond at C-5/C-6 and four oxygenated carbons, as in the corresponding ring of the known pretrichodermamide D. These signals were indicative of an oxazine–cyclohexene moiety, identical to that observed for **2** and pretrichodermamide D (Yurchenko et al. 2016), as confirmed by the HMBC correlations from H_2-3 (δ_H 2.10, 2.36) to C-4 (δ_C 70.8), C-5 (δ_C 133.4), and C-9 (δ_C 82.2), from 4-OH (δ_H 5.27) to C-3 (δ_C 37.9), C-4, and C-9, from H-5 to C-7 (δ_C 65.8) and C-9, from H-6 to C-4 and C-8 (δ_C 66.3) (Figure S1). The ^{1}H-^{1}H COSY correlation between H-2′ (δ_H 4.37) and NH-10′ amino group (δ_H 8.97) in addition to HMBC correlations from NH-10′ to C-1′ (δ_C 164.8) and C-2 (δ_C 68.0), and the remaining two sulfur atoms in the molecular formula of **1**, suggested the presence of a 6-6-6-7 epidithiodiketopiperazine skeleton similar to that of its derivatives, pretrichodermamides A-F (Orfali et al. 2015; Yurchenko et al. 2016).

The relative configuration of stereogenic centers in **1** was deduced to be the same as in the closely related natural product, pretrichodermamide D based on the identical values of observed chemical shifts at positions CH5 – CH9 ($\Delta\delta_H < 0.05$ ppm; $\Delta\delta_c < 0.5$ ppm), and ^{1}H – ^{1}H coupling constants patterns of $H_{7\text{-}8}$ (4.8 Hz) and $H_{8\text{-}9}$ (8.8 Hz) and $H_{2'\text{-}3'}$ (2.1 Hz) (Seephonkai et al. 2006; Orfali et al. 2015; Yurchenko et al. 2016).

Moreover, the ROESY correlations of H-9 with 4-OH, 7-OH, and 8-OH (Figure S1) supported this structural assignment, as observed for **2** and pretrichodermamide D. Considering the common biosynthetic origin of **1** and other pretrichodermamides and its negative optical rotation value, the absolute configuration of **1** is suggested to be the same as for pretrichodermamide D (Orfali et al. 2015; Yurchenko et al. 2016). On the basis of the above data, **1** was determined as a new 1,2-oxazadecaline epidithiodiketopiperazine, N-demethyl derivative of pretrichodermamide D, and the trivial name pretrichodermamide G is proposed for this natural product (Orfali et al. 2015; Yurchenko et al. 2016).

The structures of pretrichodermamide A (**2**), epicorazine A (**3**) and ent-epicoccin G (**4**) were identified by NMR and ESI-MS data and by comparison with the literature (Deffieux et al. 1978a; Seephonkai et al. 2006; Wang et al. 2010). Compounds **1** and **2** are rare derivatives of ETPs as the disulfide bond is connected between the α- and β-positions of two amino acid residues, similar to gliovirin (Yamazaki et al. 2015a, 2015b). Interestingly, compound **2** was previously isolated from the same genus, *Trichoderma* sp., associated with bamboo leaves and red algae collected in Northeast Thailand and Palau, respectively (Seephonkai et al. 2006; Yamazaki et al. 2015a, 2015b). Compounds **3** and **4** feature symmetrical DKP scaffolds, but only compound **4** possesses free thiomethyl groups among these derivatives.

DKPs **1**-**4** were subjected to antifungal and antibacterial assays against the phytopathogenic fungus *Ustilago maydis* and a panel of human pathogenic bacteria. Remarkably, pretrichodermamide A (**2**) selectively inhibited the growth of *U. maydis* with an MIC value of 1 mg/mL compared with that of the positive controls nystatin (MIC = 0.02 mg/mL) and nourseothricin (MIC = 2 mg/mL), as shown in Table S2. The latter compound is extensively used as a selection marker to generate transgenic fungal strains (Brachmann et al. 2004). *U. maydis* has been used as a model phytopathogenic fungus, which can infect only *Zea mays* and its progenitor plant (teosinte) causes corn smut, resulting in stunted growth and crop yield losses (Vollmeister et al. 2012; Mueller et al. 2016). Only pretrichodermamide A (**2**) and epicorazine A (**3**) displayed potential inhibitory effects against *U. maydis*, showing zones of inhibition of 15 and 10 mm at 100 μg/disk, respectively. Meanwhile, nystatin 10 mg/mL and nourseothricin 20 mg/mL displayed inhibition diameters of 29 and 14 mm, respectively (Figure S9). To the best of our knowledge, the growth inhibitory activity of pretrichodermamide A (**2**) and epicorazine A (**3**) against *U. maydis* are reported in this study for the first time. In addition, pretrichodermamide A (**2**) exhibited moderate antitubercular (MIC = 25 μg/mL) and weak cytotoxic activities, consistent with earlier reports (Seephonkai et al. 2006; Zhou et al. 2013). A further dithiodiketopiperazine related to pretrichodermamide A (**2**), peniciadametizine B, isolated from *Penicillium adametzioides* was reported to display antifungal activity against *Alternaria brassicae* (Liu et al. 2015). Several strains of *T. harzianum* are well-known producers for antifungal metabolites such as isoharzianic acid, harzianopyridone, and trichoharzianol (Vinale et al. 2014; Ahluwalia et al. 2015; Jeerapong et al. 2015).

The initial screening for cytotoxicity of the isolated compounds (**1-4**) indicated that only epicorazine A (**3**) had potent cytotoxic activity against the mouse lymphoma cell line (L5178Y) with IC_{50} value of 1.3 μM, as well as moderate cytotoxicity against human

lymphoma (Ramos) and leukemia (Jurkat J16) cell lines with IC_{50} values of 28 and 20 μM, respectively (Table S3). On the other hand, the new compound **1** and its derivatives pretrichodermamide A (**2**) and ent-epicoccin G (**4**) showed no cytotoxic activity when assayed at doses of 20-30 μM. Similarly, previous cytotoxicity assays for related compounds, i.e. pretrichodermamides A-F and epicoccins A-T indicated weak cytotoxicities (Guo et al. 2009; Wang et al. 2010; Orfali et al. 2015; Yurchenko et al. 2016). The presence of a chlorine atom in several DKP related natural products such as N-methylpretrichodermamide B, trichodermamide B, chlorotrithiobrevamide, and chloro-ETP (DC1149B) was found to play a vital role for their cytotoxicity (Garo et al. 2003; Orfali et al. 2015; Yamazaki et al. 2015a, 2015b; Jans et al. 2017). Hence, the chlorohydrin moiety might be precursor to a reactive epoxide in the active form of those derivatives (Garo et al. 2003). In consequence, the replacement of this reactive moiety in case of pretrichodermamides A-F or trichodermamides A and C by a trans vicinal diol eliminates their cytotoxic activity (Orfali et al. 2015; Yurchenko et al. 2016; Jans et al. 2017).

The strong cytotoxic properties of epicorazine A (**3**) have been previously described in the literature, however none of the former studies provided insides into the mechanism of action of this dithiodiketopiperazine (Kleinwächter et al. 2001; Kong et al. 2014; Bingui et al. 2015). In this study, we investigated the influence of **3** on apoptosis, the programmed cell death which is generally characterized by membrane blebbing and DNA fragmentation leading to cell death. Cysteine-dependent aspartate-directed proteases (caspases) are the key regulators for inducing apoptosis through intrinsic (mitochondrial) or extrinsic (death receptor) pathways (Taylor et al. 2008). Accordingly, in order to estimate the potential contribution of pro-apoptotic mechanisms for the cytotoxicity of epicorazine A (**3**), we analyzed activation of caspase-3 through two different methods: first by immunoblotting for the cleavage of the caspase-3 substrate poly (ADP-ribose) polymerase-1 (PARP-1) and second by measuring the fluorescence of the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC.

Western blot experiments revealed cleavage of the caspase-3-substrate PARP after treatment with epicorazine A (**3**) in both Ramos and Jurkat J16 cell lines, indicating activation of caspase-3 and thus induction of apoptosis. Furthermore, we performed co-incubation with the pan-caspase inhibitor N-(2-quinolyl)-L-valyl-L-aspartyl-(2,6-difluorophenoxy) methylketone (QVD) to prove caspase dependency of the observed cleavage of PARP (Figure S10). QVD completely blocked cleavage of PARP after treatment with epicorazine A (**3**) at concentrations up to 30 μM, demonstrating a causative role of caspases and thereby occurrence of apoptosis. Briefly, epicorazine A (**3**) has the potential to induce apoptosis, even though high doses and long incubation periods are needed to kill the tested cancer cells. Thereby, epicorazine A (**3**) and its congeners (epicorazines B-C) have potential therapeutic value for targeting cancer cell (Kleinwächter et al. 2001; Vigushin et al. 2004).

3. Conclusion

Chemical investigation of two endophytic fungi *T. harzianum* and *E. nigrum* resulted in the isolation and characterization of the new pretrichodermamide G (**1**) along with

three known derivatives (**2-4**). Among these dithiodiketopiperazines, **2** exhibited selective antifungal activity against *U. maydis*, whereas **3** revealed potent cytotoxicity through apoptotic cell death. Structure of cyclohexene unit in **2** (substitution pattern and/or location of double bond) is important for the selective antifungal activity of this metabolite (**2** vs **1**), while the presence of α,β-unsaturated carbonyl moiety in **3** is likely to be responsible for its cytotoxicity as the contribution of this functionality to the cytotoxic properties of structurally distinct compounds is well described in the literature (Amslinger 2010; Nakayachi et al. 2004).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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SUPPLEMENTARY MATERIAL

Dithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and *Epicoccum nigrum*

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Abstract

A new epidithiodiketopiperazine (ETP), pretrichodermamide G (**1**), along with three known (epi)dithiodiketopiparazines (**2-4**) were isolated from cultures of *Trichoderma harzianum* and *Epicoccum nigrum*, endophytic fungi associated with medicinal plants *Zingiber officinale* and *Salix* sp., respectively. The structure of the new compound (**1**) was established on the basis of spectroscopic data, including 1D/2D NMR and HRESIMS. The isolated compounds were investigated for their antifungal, antibacterial and cytotoxic potential against a panel of microorganisms and cell lines. Pretrichodermamide A (**2**) displayed antimicrobial activity towards the plant pathogenic fungus *Ustilago maydis* and the human pathogenic bacterium *Mycobacterium tuberculosis* with MIC values

of 1 mg/mL (2 mM) and 25 μg/mL (50 μM), respectively. Meanwhile, epicorazine A (**3**) exhibited strong to moderate cytotoxicity against L5178Y, Ramos, and Jurkat J16 cell lines with IC_{50} values ranging from 1.3 to 28 μM. Further mechanistic studies indicated that **3** induces apoptotic cell death.

Keywords: *Trichoderma harzianum*; *Epicoccum nigrum*; epidithiodiketopiperazine; endophytic fungi; antimicrobial activity; cytotoxicity; apoptosis.

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Experimental section

General experimental procedures

HPLC analysis was performed with a Dionex p580 DAD3000RS (Dionex Softron, Munich, Germany) with a LPG-3400SD pump coupled with a photodiode array detector (UVD340S), using routine detection channels at 235, 254, 280, and 340 nm wavelengths. ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz), and 2D NMR spectra were recorded at 297.9°K in DMSO-d_6 Bruker Avance III 600 MHz NMR spectrometers (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts are in ppm referring to the deuterated solvent peaks at δ_H 2.50 and δ_C 39.5 (DMSO-d_6) for ^{1}H and ^{13}C, respectively. Mass spectra were measured by a HP110 Agilent Finnigan LCQ Deca XP Thermoquest mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), while high resolution mass spectra (HRESIMS) were recorded on a UHR-TOF maxis 4G mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A Jasco P-2000 digital polarimeter (Jasco International, Tokyo, Japan) was used for determination of specific optical rotation. All solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements.

The separation column (125×4 mm, L×ID) was prefilled with Eurosphere-10C_{18} (Knauer, Germany), and the following gradient was used (MeOH, 0.1% formic acid in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semi-preparative RP-HPLC was performed using a Merck Hitachi system (UV detector L-7400; pump L-7100; Eurosphere-100C_{18}, 300×8 mm, Merck KGaA, Darmstadt, Germany). Column chromatography included LH-20 Sephadex, and Merck silica gel 60 M (0.04 - 0.063 mm). TLC plates pre-coated with silica gel 60 F_{254} (Macherey-Nagel, Dueren, Germany) were used to monitor fractions, followed by detection under UV 254 and 366 nm or after spraying with anisaldehyde-sulfuric acid reagent.

Fungal material

The fungus *Trichoderma harzianum* was isolated from fresh and healthy leaves of *Zingiber officinale*, collected in Banyumas, Central Java, Indonesia, in May 2016. *Epicoccum nigrum* was isolated from fresh and healthy leaves of *Salix* sp., collected in Lago Naki, the Republic of Adygea (North Caucasus), Russia, in May 2017. Isolation of fungi was achieved by the dilution plate method employing isolation medium (15 g/L bacto agar, 15 g/L malt extract in distilled water, at pH 7.4–7.8) supplied by chloramphenicol (0.20 g/L) and streptomycin sulfate (0.25 g/L) in order to inhibit the growth of bacteria and actinomycetes.

Fungal identification was performed according to a molecular biology protocol by DNA amplification and sequencing of the ITS region with GenBank accession No. MK213940 and MK214079 for *T. harzianum* and *E. nigrum*, respectively (Kjer et al. 2010). Voucher strains were deposited at the corresponding authors' laboratory (P.P.).

Fermentation and isolation

The fungal strains were cultivated on solid rice medium, which was prepared by autoclaving 100 g of rice and 110 mL of water in a 1 L Erlenmeyer flask, except for compound **4** isolated from *E. nigrum* cultivated on green lentil medium which was prepared in the same manner. Large scale fermentation of these two fungi was performed in five flasks for each fungus for 14 days at room temperature under static condition. The cultures were diced and extracted with EtOAc (5 L). The crude extract of *T. harzianum* (11.34 g) was partitioned by liquid-liquid extraction using 90%MeOH-H_2O and *n*-hexane to yield the MeOH fraction (4.40 g), which was then subjected to vacuum liquid chromatography (VLC) using a step gradient of *n*-hexane/EtOAc, followed by DCM/MeOH, to yield 15 fractions (F1-F15). Fractions 7 and 8 (F78, 0.49 g), eluted with DCM/MeOH (95/90:5/10), were subjected to size exclusion chromatography using a Sephadex LH-20 column (100×2.5 cm) with 50%DCM-MeOH as eluting solvent. The subfractions HTH-V78-SD1-2-3 containing ETPs (TLC monitoring) were selected for further purification via semi-preparative RP-HPLC using gradient elution of water and methanol yielding compounds **1** (8.84 mg) and **2** (22.69 mg). The crude extract of *E. nigrum* (4.5 g) was subjected to VLC using n-hexane/EtOAc (10/90) to yield fraction F5, then F5 subjected to a size exclusion chromatography using a Sephadex LH-20 column with 100% methanol as eluting solvent to yield six subfractions. Two subfractions (HEN-V5-D4/5) were further purified by semi-preparative RP-HPLC to yield compounds **3** (0.50 mg) and **4** (2.0 mg).

*Pretrichodermamide G (**1**)*

yellow solid; $[\alpha]^{20}_{D}$: − 103.7 (*c* 0.17, MeOH); UV λ_{max} (MeOH) 203, 283 nm; ^{1}H and ^{13}C NMR spectral data in DMSO-d_6, see Table S1; HRESIMS (+) *m/z* 499.0828 $[M + H]^+$ (calcld for $C_{20}H_{22}N_2O_9S_2$, 499.0839, Δ 1.1 mmu).

Biological assay

Antibacterial assay

The antibacterial assay was carried out using the broth microdilution method following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012).

A panel of Gram-positive and Gram-negative bacterial strains, including *Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli* as well as *Mycobacterium tuberculosis* $H_{37}Rv$ were selected for this evaluation.

Antifungal assay

The antifungal assay was performed using the agar diffusion (Kirby-Bauer) method. *Ustilago maydis* AB33 (Brachmann et al. 2001) and *Saccharomyces cerevisiae* ESM356-1 (Pereira et al. 2001) were grown in CM medium supplemented with 10 g/L glucose (Holliday, 1974; Banuett and Herskowitz, 1989) and YPD respectively. Sterile filter paper disks of 5 mm diameter were placed on agar plates previously inoculated with *U. maydis* or *S. cerevisiae*. The disks were then impregnated with 100 µg of the compounds, which were dissolved in DMSO. Double distilled water (ddH_2O) and DMSO were used as negative controls, while nystatin (100 µg) and nourseothricin (200 µg) were used as positive controls. The treated agar plates were incubated at 28°C for 48 h, afterwards antifungal activity was recorded as growth inhibition zones (in mm) of inhibition surrounding the disk. Antifungal assay against *Candida albicans* was performed by microdilution method with the same protocol as antibacterial assay. The test compound was considered active when the growth of inhibition zone was greater than 8 mm, while the lowest concentration at which no observed growth inhibition zone was taken to be the minimum inhibitory concentration (MIC). Natural products and controls were tested in triplicate apart from epicorazine A which was tested once due to the limited amount of this compound.

Cytotoxicity and apoptosis assays

Cytotoxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay employing mouse lymphoma cells (L5178Y), lymphoblastic leukemia T cells (Jurkat J16, no. ACC-282), Burkitt's lymphoma B cells (Ramos, no. ACC-603), and human ovarian cancer cells (A2780 sens). Kahalalide F and staurosporine (STS) were used as positive controls and media with 0.1% DMSO as negative control. All experiments were carried out in triplicate and performed as described earlier (Liu et al. 2017). With regard to the observed cytotoxicity of compound **3**, we subsequently evaluated the potential contribution of pro-apoptotic mechanisms through two different methods: (1) western blotting of the caspase-3 substrate PARP and (2) measuring the increase in fluorescence of the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC. Caspase activity

was determined as the slope of the resulting linear regressions. Data points shown are the mean of triplicates, error bars = SD. Values are normalized to DMSO (0.1% v/v) treated cells (=100 %).

Table S1. NMR spectroscopic data of **1** recorded at 600 (^{1}H) and 150 (^{13}C) MHz (DMSO-d_6, δ in ppm).

Position	**δ_C, type***	**δ_H, mult (*J* in Hz)**	**COSY**	**ROESY**	**HMBC**
1	166.7, C	-			
2	68.0, C	-			
3a	37.9, CH_2	2.10, d (15.0)	3b	5, 4-OH	1, 2, 4, 5, 9
3b		2.36, d (15.0)	3a	5, 6	1, 2, 4, 5
4	70.8, C	-			
5	133.4, CH	5.53, d (10.0)	6	3ab, 4-OH, 7	7, 9
6	127.7, CH	5.60, dd (10.0; 4.2)	5, 7		4
7	65.8, CH	4.04, q (4.8)	6, 7-OH, 8	5	
8	66.3, CH	3.78, ddd (8.8, 6.5, 4.8)	7, 8-OH, 9	3b	
9	82.2, CH	4.13, d (8.8)	8	4-OH	
1'	164.8, C	-			
2'	59.0, CH	4.37, dd (4.7, 2.1)	10'-NH	3', 5'	1', 3'
3'	44.9, CH	4.49, d (2.1)		2', 9-OH, 10'-NH	1', 2', 4', 5', 9'
4'	116.2, C	-			
5'	122.6, CH	7.43, d (8.8)	6'	2'	3', 7', 9'
6'	103.1, CH	6.55, d (8.8)	5'	7'-OCH_3	4', 7', 8'
7'	153.1, C	-			
8'	136.0, C	-			
9'	147.6, C	-			
10'-NH	-	8.97, d (4.7)	2'	3'	1', 2
7'-OCH_3	CH_3	3.78, s		6'	7'
8'-OCH_3	CH_3	3.67, s		9'-OH	8'
4-OH	-	5.27, s		3a, 5, 9	3, 4, 9
7-OH	-	4.95, d (5.5)	7		6
8-OH	-	4.48, d (6.5)	8		
9'-OH	-	9.47, s		3', 8'-OCH_3	9, 4', 8'

* Data extracted from HSQC and HMBC spectra

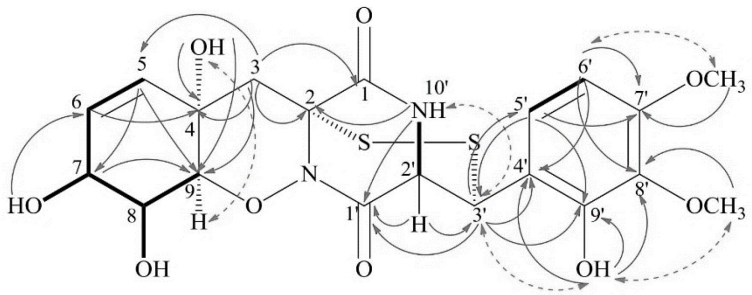

Figure S1. COSY (bold), HMBC (blue line) and key ROESY (red dashed) correlations of compound **1**.

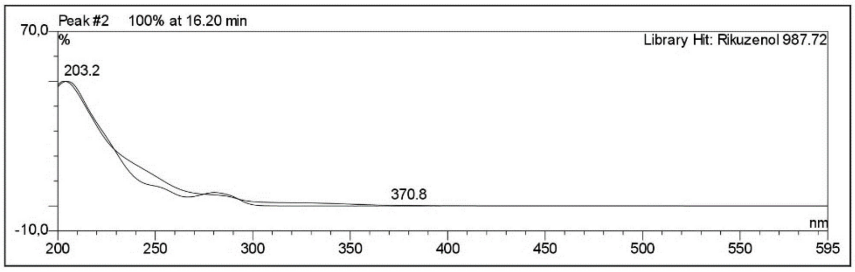

Figure S2. UV spectrum of compound **1**.

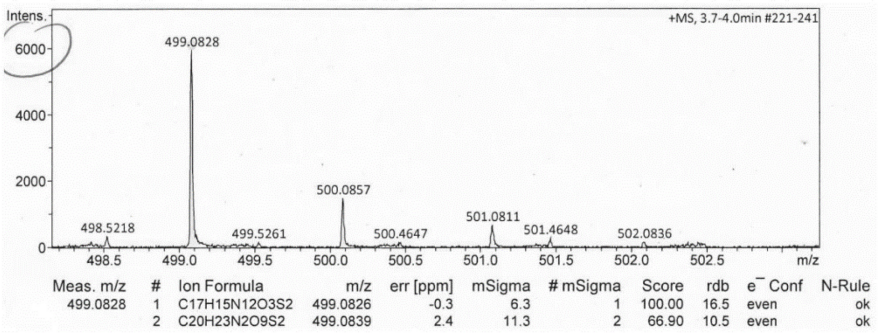

Figure S3. HRESIMS spectrum of compound **1**.

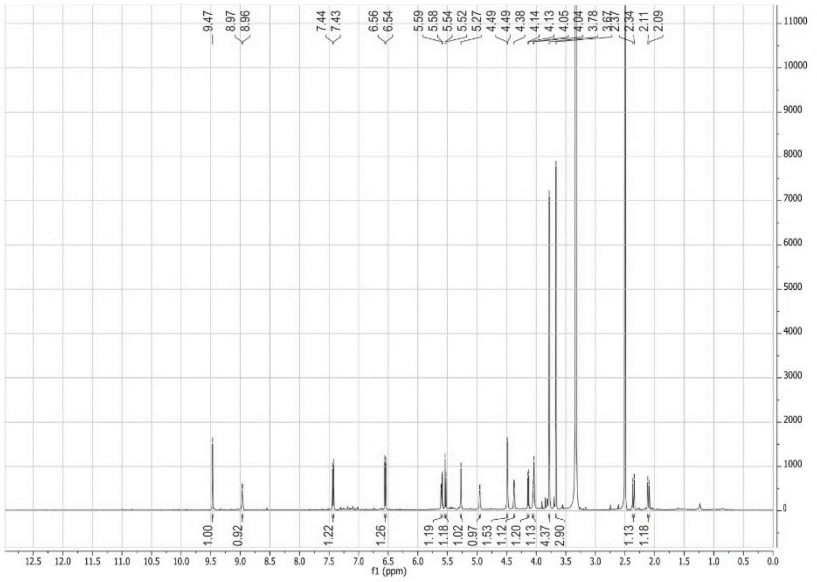

Figure S4. ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **1**.

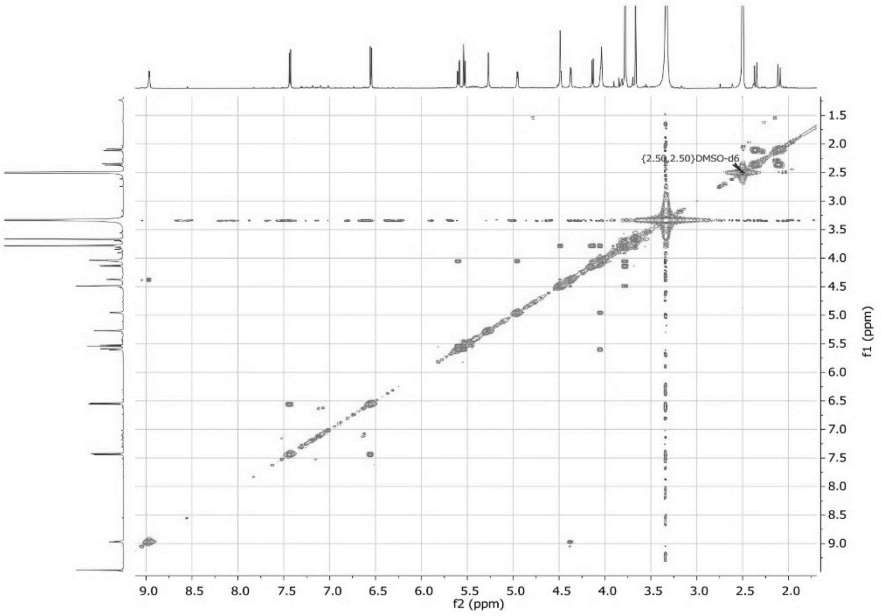

Figure S5. ^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) spectrum of compound **1**.

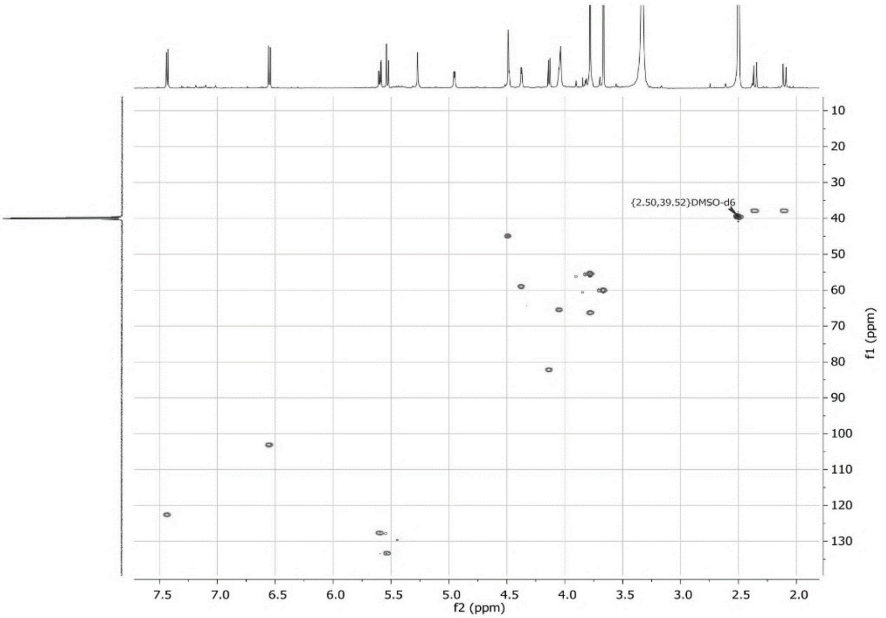

Figure S6. HSQC (600 and 150 MHz, DMSO-d_6) spectrum of compound **1**.

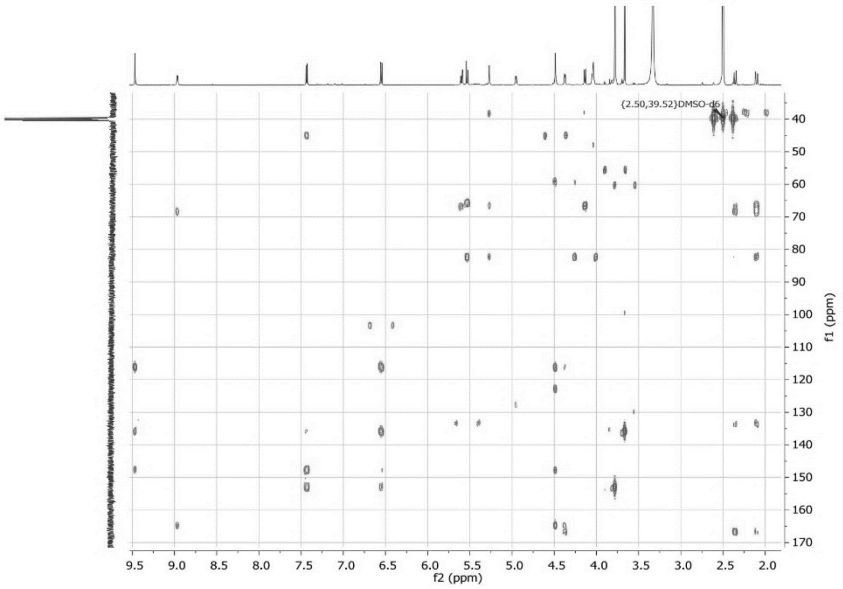

Figure S7. HMBC (600 and 150 MHz, DMSO-d_6) spectrum of compound **1**.

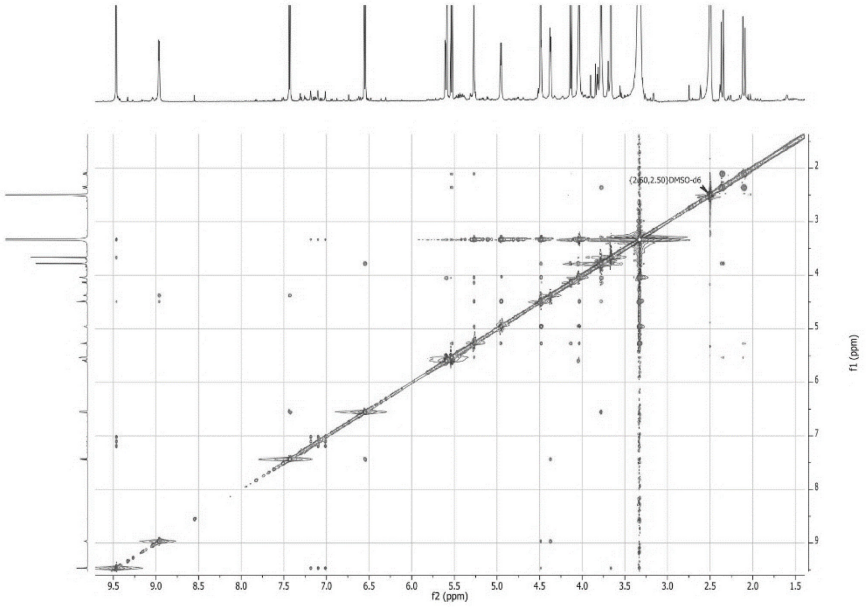

Figure S8. ROESY (600 MHz, DMSO-d_6) spectrum of compound **1**.

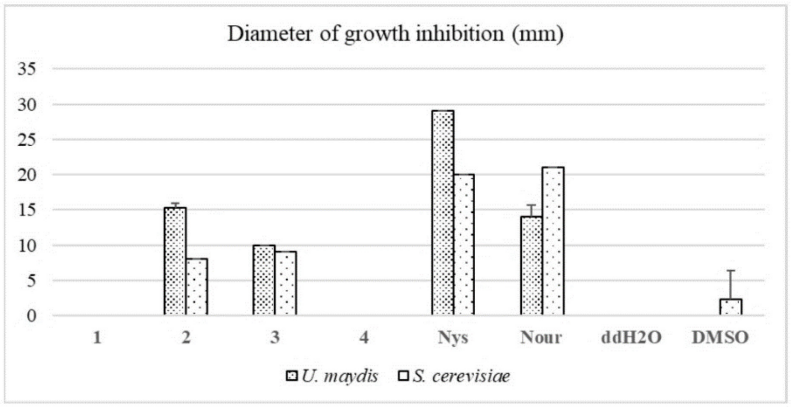

Figure S9. Diameter of growth inhibition (mm) towards *Ustilago maydis* and *Saccharomyces cerevisiae* of pretrichodermamide G (**1**), pretrichodermamide A (**2**), epicorazine A (**3**), and ent-epicoccin G (**4**), along with nystatin (Nys) and nourseothricin (Nour) as well as double distilled water (ddH_2O) and DMSO.

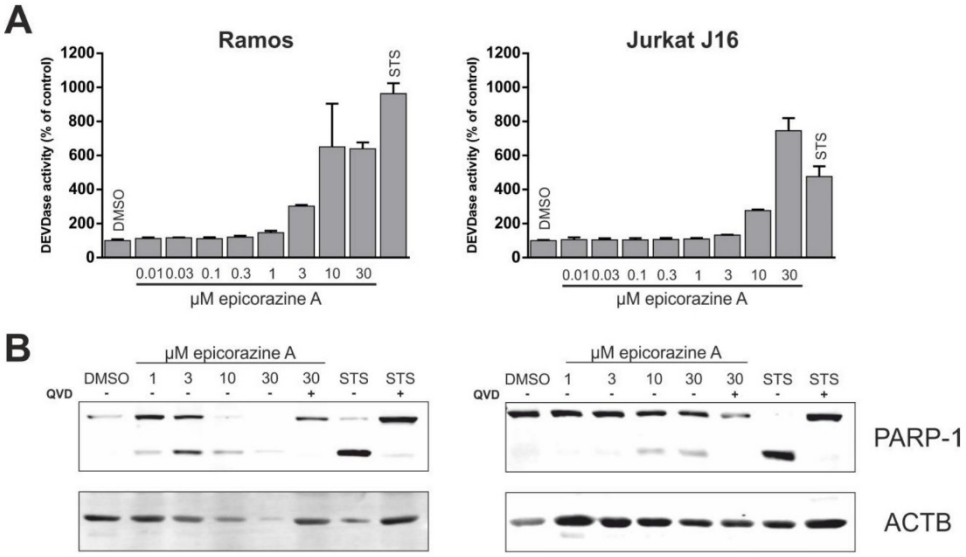

Figure S10. Epicorazine A (**3**) induces apoptosis.

(A) Ramos cells (Burkitt's lymphoma B lymphocytes) and Jurkat J16 cells (acute T cell leukemia cells) were treated with the indicated concentrations of epicorazine A (**3**) or the positive control staurosporine (2.5 µM) for 24 h. Afterwards the cells were lysed and loaded with the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC and fluorescence of AMC was measured over the course of 2 h in a microplate reader. Increase in fluorescence (DEVDase activity) is considered as activation of pro-caspase-3. (B) Ramos and Jurkat J16 cells were treated with the indicated concentrations of epicorazine A (**3**) in the absence or presence of the caspase inhibitor Q-VD-OPh (10 µM) for 24 h. Cells treated with staurosporine (STS, 2.5 µM) were used as positive control for caspase-dependent cleavage of Poly (ADP-ribose) polymerase-1 (PARP). After incubation period, cleavage of PARP was determined by Western blot analysis. The expression of Beta-actin was determined as protein loading control. Figure S10B shows the result of a representative blot.

Table S2. Minimum inhibitory concentrations (µg/mL) of the isolated compounds **1-4**

Compound	*M. tuberculosis*	*Candida albicans*	*Ustilago maydis*
1	> 50	> 50	n.a
2	25	> 50	1,000
3	> 42	-	n.t.
4	> 45	> 45	n.a
Nystatin	-	-	20
Nourseothricin	-	-	2,000

* n.a : not active, n.t. – not tested

Table S3. Cytotoxicity of the isolated compounds **1-4** measured after 24 h of incubation and reported as IC_{50} (µM)

Compound	L5178Y	Ramos	Jurkat J16
1	n.a	n.a	n.a
2	n.a	n.a	n.a
3	1.3	28	20
4	n.a	n.a	n.a
Kahalalide F	4.3	-	-
Staurosporine	-	1.1	-

* n.a : not active

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CHAPTER 3

Azacoccones F – H, new flavipin-derived alkaloids from an endophytic fungus *Epicoccum nigrum* MK214079

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Azacoccones F – H, new flavipin-derived alkaloids from an endophytic fungus *Epicoccum nigrum* MK214079

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Abstract

Three new flavipin-derived alkaloids (**1**−**3**), along with six known compounds were isolated from the endophytic fungus *Epicoccum nigrum* MK214079 associated with leaves of *Salix* sp. The structures of the new compounds were established by analysis of their 1D/2D NMR and HRESIMS data. The known compounds epicocconigrone A (**4**), epipyrone A (**5**) and epicoccolide B (**6**) revealed moderate antibacterial activity against *Staphylococcus aureus* ATCC 29213 with MIC values ranging from 25 to 50 μM. Furthermore, epipyrone A (**5**) and epicoccamide A (**7**) displayed mild antifungal activity against *Ustilago maydis* with MIC values of 1.63 and 1.80 mM, respectively. Epicorazine A (**8**) exhibited pronounced cytotoxicity against the L5178Y mouse lymphoma cell line. Furthermore, electronic circular dichroism (ECD) methods are being performed to determine the absolute configuration of the new compounds.

Keywords: *Epicoccum nigrum*, flavipin, alkaloids, Pictet-Spengler reaction

1. Introduction

The fungal aldehyde flavipin, a well known polyketide, was first isolated from *Aspergillus* spp. [1], but was later also found in *Epicoccum* spp. [2] and *Chaetomium globosum* [3] exhibiting a wide range of biological activities such as antioxidant [3], antinematodal [4] and antifungal [5] activities. Bioactive fungal metabolites from the genus *Epicoccum* such as epicoccones A−B, epicoccolides A−B and epicocconigrones A−B are biosynthetically related to flavipin [6]. To date, natural products which presumably originate via an unusual Pictet-Spengler (PS) reaction starting from the precursor flavipin are rare in fungi. The PS reaction which is featured by a condensation of *β*-arylethylamine with an aldehyde to form a piperidine ring, is known to generate diverse plant alkaloids including morphine, camptothecin, and reserpine [7]. Interestingly, the first fungal metabolites derived from this PS reaction are the indole alkaloids chaetoglines A−H produced by *C. globosum* [7], followed by polyketides azacoccones A−E found in *A. flavipes* [8].

Epicoccum nigrum Link has been mainly studied for its potential biotechnological and pharmaceutical applications, as exemplified by the commercial fluorophore epicocconone and the well known anticancer drug taxol which is better known from plants but was likewise obtained from this fungus [6]. During our previous studies on *E. nigrum* we have isolated polyketides [9], dithiodiketopiperazine alkaloids [10], and flavonoids [11] with anticancer and

antifungal potential from this fungal species residing in medicinal plants. We have now studied *E. nigrum* MK214079 isolated from *Salix* sp. leaves. Chemical investigation of this fungal strain resulted in the isolation of three new natural products (**1**–**3**), together with six known metabolites. The known compounds can be classified into five classes including polyketides (**4** and **6**), pyronepolyenes (**5**), tetramic acid derivatives (**7**), alkaloids (**8**) and chromanones (**9**). Consecutively, they were identified as epicocconigrone A (**4**) [9], epipyrone A (**5**) [12], epicoccolide B (**6**) [13], epicoccamide A (**7**) [14], epicorazine A (**8**) [10] and a chromanone derivative (**9**) [15] as shown in Fig. 1. Furthermore, all isolated compounds were assessed for their biological activities toward pathogenic microorganisms and a cancer cell line.

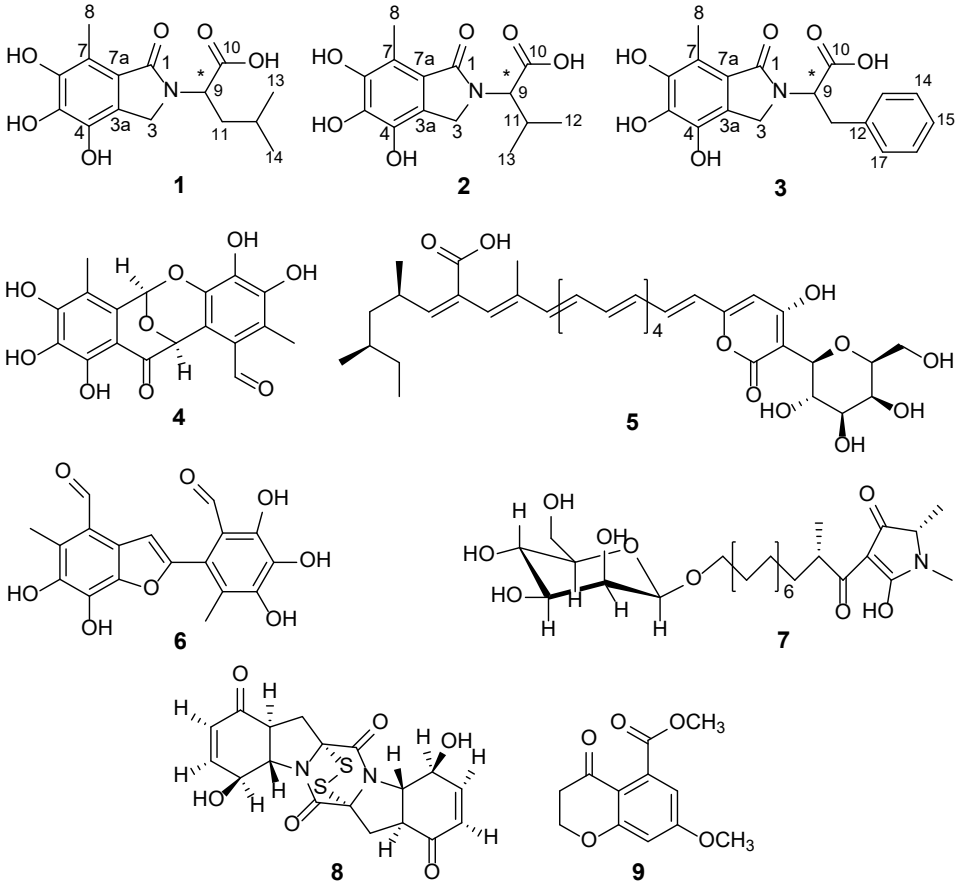

Fig. 1. Structures of compounds **1**−**3**, epicocconigrone A (**4**), epipyrone A (**5**), epicoccolide B (**6**), epicoccamide A (**7**), epicorazine A (**8**) and chromanone (**9**).

2. Results and Discussion

Compound **1** was isolated as a green solid with the molecular formula $C_{15}H_{19}NO_6$ (seven degrees of unsaturation) as established by the pseudomolecular ion peak at *m/z* 308.1140 $[M - H]^-$ in the negative-ion HRESIMS. The UV absorptions maxima at 214 and 268 nm were similar

to those of azacoccones C or E, suggesting that **1** is an aza-epicoccone derivative [8]. The ^{1}H and ^{13}C NMR data of **1** (Table 1) resembled the basic skeleton of azacoccone E including a methyl group at δ_H 2.46 (s)/δ_C 9.6, a methylene function at δ_H 4.39 (d, 16.4); 4.19 (d, 16.4)/δ_C 44.5, a methine proton at δ_H 4.98 (dd, 11.7, 4.3)/δ_C 52.9, and two carbonyl groups at δ_C 172.8 and 174.9. The typical signals of Me-8 (δ_H 2.46/δ_C 9.6) along with those of six aromatic carbons at δ_C 116.9, 120.4, 122.5, along with three oxygenated carbons resonating at downfield shifts (δ_C 138.7, 138.8, and 145.2) suggested the presence of a hexasubstituted benzene moiety with a 1,2,3-trihydroxy pattern. Further HMBC correlations of an aromatic methyl proton H_3-8 (δ_H 2.46) to C-6 (δ_C 145.2), C-7a (δ_C 120.4) and C-7 (δ_C 116.9), in addition to its long range HMBC correlations to meta positioned C-3a (δ_C 122.5) and C-5 (δ_C 138.8), as well as to the adjacent exocyclic carbonyl C-1 (δ_C 172.8) confirmed this unit in **1** (Fig. 2). Moreover, signals observed in the HMBC spectrum from methylene H_2-3 to carbonyl C-1, C-7a and C-4 (δ_C 138.4) confirmed that they are connected in a five membered ring bearing one heteroatom. Collectively, the presence of a 4,5,6-trihydroxy-7-methylisoindolin-1-one unit was confirmed by detailed analysis of 2D NMR spectra. This basic scaffold is similar to epicoccone, a polyketide previously isolated from *Epicoccum* spp. [9,15,16], however the chemical shift of the methylene carbon at C-3 (δ_C 44.5) is strongly shielded relative to epicoccone (δ_C 68.0) [15,16], indicating that the oxygen binding site is replaced by nitrogen in **1** which is consistent with its molecular formula.

The remaining NMR signals included two methyl groups at δ_H 0.98 (d, 4.3)/δ_C 21.2 and δ_H 0.98 (d, 4.3)/δ_C 23.2, one methylene at 1.94 (ddd, 14.3, 11.6, 4.2); 1.84 (ddd, 14.2, 10.0, 4.3)/δ_C 39.2, as well as two aliphatic methine protons at δ_H 4.98 (dd, 11.7, 4.3)/δ_C 52.9 and δ_H 1.48 (m)/δ_C 26.0. Detailed analysis of the COSY spectrum revealed that all of them are connected in a single-spin system coinciding with a leucine unit, confirmed by the HMBC correlation of H-11 (δ_H 1.94) to carboxyl C-10 (δ_C 174.9) (Fig. 2). The leucine side chain of **1** was determined to be connected to the aromatic part through the amino function by formation of an isoindolin-1-one system. This attachment was established by the HMBC correlations of the α-amino proton H-9 (δ_H 4.98) to carbonyl C-1 (δ_C 172.8) and methylene C-3 (δ_C 44.5), as well as of the methylene proton H_2-3 to C-9 (δ_C 52.9). The structural assignment was further supported by ROESY correlation between H-3 and H-9/H-11. Hence, the planar structure of **1** was elucidated.

Table 1. ^{13}C and ^{1}H NMR Data of **1**−**3**.

Position	**1**[a]		**2**[b]		**3**[b]	
	δ_C*	δ_H mult (*J* in Hz)	δ_C*	δ_H mult (*J* in Hz)	δ_C*	δ_H mult (*J* in Hz)
1	172.8		173.4		172.5	
3	44.5	4.39 d (16.4) 4.19 d (16.4)	45.2	4.49 d (16.8) 4.27 d (16.8)	45.3	4.40 d (16.5) 4.18 d (16.5)
3a	122.5		122.4		122.4	
4	138.7		138.4		138.5	
5	138.8		n.d.[c]		139.0	
6	145.2		145.3		145.2[c]	
7	116.9		117.2		116.5	-
7a	120.4		119.9		120.7	
8	9.6	2.46 s	9.5	2.46 s	9.5	2.39 s
9	52.9	4.98 dd (11.7, 4.3)	61.3	4.57 d (10.1)	57.3	5.12 dd (11.5, 4.7)
10	174.9		173.6		174.3	
11	39.2	1.94 ddd (14.3, 11.6, 4.2) 1.84 ddd (14.2, 10.0, 4.3)	29.6	2.33 q (7.8, 6.9)	36.7	3.51 dd (14.8, 4.7) 3.16 dd (14.8, 11.5)
12	26.0	1.48 m	20.6	1.09 d (6.6)	139.2	
13	21.2	0.98 d (4.3)	19.7	0.91 d (6.7)	129.4	7.25 d (7.0)
14	23.2	0.98 d (4.3)			129.4	7.21 t (7.6)
15					127.3	7.13 t (7.2)
16					129.4	7.21 t (7.6)
17					129.4	7.25 d (7.0)

[a] Recorded at 700 MHz for ^{1}H and 176 MHz for ^{13}C in CD_3OD.

[b] Recorded at 750 MHz for ^{1}H and 189 MHz for ^{13}C in CD_3OD.

[c] Not clearly detected.

* Data extracted from HSQC and HMBC spectra.

Compound **2**, a yellowish solid, had the molecular formula $C_{14}H_{17}NO_6$ on the basis of the pseudomolecular ion peak at *m/z* 296.1125 $[M + H]^+$ established by HRESIMS which is 14 amu less than that of **1**. Comparison of the NMR data of **2** with those of **1** (Table 1) displayed that both compounds are similar with the exception of one methylene unit missing in the side chain of **2**. Detailed analysis of 2D NMR spectra revealed **2** to be the valine analogue of **1** (Fig. 2).

Compound **3** was obtained as a yellowish solid with the molecular formula $C_{18}H_{17}NO_6$ (11 degrees of unsaturation) as determined from the pseudomolecular ion peak at *m/z* 342.0980 $[M - H]^-$ in the negative-ion HRESIMS which was 48 mass unit larger than that of **2**. The NMR spectra of **3** were again similar to those of **1** and **2** in their basic scaffolds whereas the major difference consisted of the signals of five aromatic protons (δ_H 7.13–7.25) which formed a separate spin system in the COSY spectrum of **3** (Fig. 2), indicating the presence of a monosubstituted benzene unit. This benzene is adjacent to a deshielded methylene unit at δ_H 3.51 (dd, 14.8, 4.7)/ 3.16 (dd, 14.8, 11.5) in the side chain, as confirmed by ROESY correlations between methylene H_2-11 (δ_H 3.51/3.16) and aromatic protons H-13/H-17 (δ_H 7.25), as well as by the HMBC correlations from H-11 to C-13/C-17 (δ_C 129.4) and from H-13/H-17 to C-11 (δ_C 36.7) (Fig. 2). Detailed analysis of 2D NMR spectra revealed **3** to be the phenylalanine analogue of **1** and **2**. Hence, the structures of the new compounds **1**−**3** represented the same epicoccone core structure, but differed in their amino acid moieties. The trivial names azacoccones F – H (**1**−**3**) are proposed for these new metabolites, respectively.

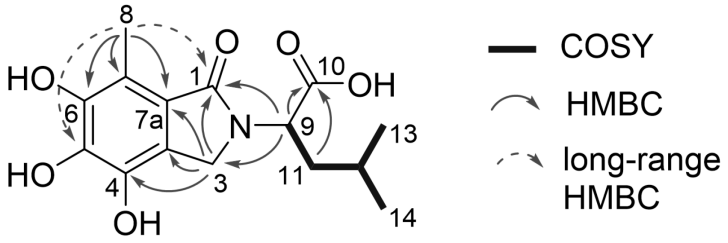

Fig. 2. COSY and key HMBC correlations of **1**.

The new compounds **1**−**3** are biosynthetically likely derived from flavipin with the replacement of an oxygen atom by a nitrogen atom. Biogenetically, compounds **1**−**3** are probably constructed via an unusual PS-reaction as reported for the structurally related compounds, azacoccones and chaetoglines [7,8]. Flavipin is also the precursor of epicoccone [13]. The two known polyketides (**4** and **6**) are presumably formed through condensation of two molecules of flavipin [9,13].

Compounds **1**−**3** feature a single chiral center at C-9 as also reported for azacoccone E and chaetoglines C/D [7,8]. In contrast, their specific optical rotation values were found to be opposite of those of (*S*)-azacoccone E [8], however, the values were found to be similar to those of chaetoglines C and D [7] which are L-tryptophane analogues, which had been independently established by X-ray analysis and ECD calculation. Comparison of the ECD spectrum of **1** with those of chaetoglines C and D which are characterized by negative Cotton effects at 230 and

270 nm as well as a positive Cotton effect at 250 nm (Fig. 3) indicated a good agreement. However, further ECD calculations are being performed for compounds **1** and **3** to assign their absolute configuration.

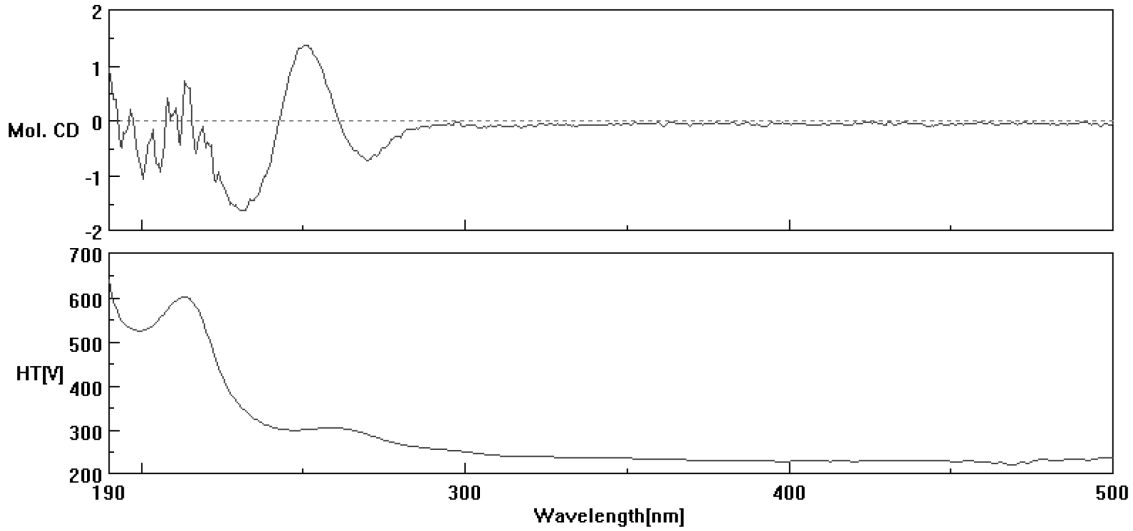

Fig. 3. The ECD and UV spectra of compound **1**

All isolated metabolites were evaluated for their bioactivities including antibacterial, antifungal and cytotoxic activities. Epicocconigrone A (**4**), epipyrone A (**5**) and epicoccolide B (**6**) displayed moderate antibacterial activity against *Staphylococcus aureus* ATCC 29213 with MIC values of 25−50 μM (Table 2). Moreover, epipyrone A (**5**) showed mild antifungal potential towards *Ustilago maydis* with an MIC value of 1.63 mM, whereas epicoccamide A (**7**) selectively inhibited the growth of this phytopathogenic fungus with a MIC value of 1.80 mM which the latter was previously undescribed. When tested for cytotoxicity, the isolated compounds were inactive with the exception of epicorazine A (**8**) that revealed potent cytotoxicity against the L5178Y mouse lymphoma with an IC_{50} value of 1.3 μM as reported earlier [10]. All bioactive compounds obtained in this study (Table 2) possess a *β*-hydroxy-*α,β*-unsaturated carbonyl moiety which is absent in all inactive compounds (i.e., **1**−**3** and **9**), suggesting that this moiety is likely to be responsible for the bioactivities [9,10].

In summary, three new flavipin-derived alkaloids namely, azacoccones F−H (**1**−**3**), were isolated from an endophytic fungus *E. nigrum* MK214079 residing in the leaves of *Salix* sp., together with six known bioactive compounds including four non-cytotoxic metabolites (**4**−**7**) with some of them exhibiting antimicrobial or cytotoxic activities. This study highlights a rare biosynthetic pathway in the fungal kingdom which is characterized by the Pictet-Spengler reaction starting from the precursor flavipin to produce unprecedented natural products [7,8].

Table 2. Bioactive compounds isolated from *E. nigrum* MK214079

Compound	MIC		IC_{50}
	S. aureus (μM)	*U. maydis* (mM)	L5178Y (μM)
Epicocconigrone A (**4**)	25	n.a.	n.a.
Epipyrone A (**5**)	25	1.63	n.a.
Epicoccolide B (**6**)	50	n.a.	n.a.
Epicoccamide A (**7**)	> 100	1.80	n.a.
Epicorazine A (**8**)	> 100	n.d.	1.33[#]
Moxifloxacin[a]	3.89	-	-
Nourseothricin sulfate/clonNAT[b]	-	3.33	-
Nystatin[b]	-	0.02	-
Kahalalide F[c]	-	-	4.30

***** n.a. : not active, n.t. – not tested, n.d. : not determined,

[#] This value was cited from our previous report [10]

a–c were used as the positive controls in antibacterial (a), antifungal (b), and cytotoxicity (c) assays.

3. Experimental

3.1. General experimental procedures

HPLC analysis was performed on a Dionex UltiMate3400 SD with an LPG-3400SD pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. ^{1}H, ^{13}C, and 2D NMR spectra were recorded at 297.9°K in CD_3OD on Bruker Avance III 600 or AV III HD 750 NMR spectrometers (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts are in ppm referring to the deuterated solvent peaks at δ_H 3.31 and δ_C 49.0 (MeOH-d_4) for ^{1}H and ^{13}C, respectively. Low-resolution mass spectra (ESI) were measured on a Finnigan LCQ Deca mass spectrometer, and HRMS (ESI) spectra were recorded with an FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Optical rotations were measured on a Jasco P-2000 digital polarimeter (Jasco International, Tokyo, Japan). ECD spectra were recorded on a J-810 spectropolarimeter in acetonitrile. The separation column (125×4 mm, L×ID) was prefilled with Eurosphere-10C_{18} (Knauer, Germany), and the following gradient was used (MeOH, 0.1% HCOOH in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semi-preparative RP-HPLC was performed using a Merck Hitachi system (UV detector L-7400; pump L-7100; Eurosphere-100C_{18}, 300×8

mm, Merck KGaA, Darmstadt, Germany). Column chromatography included LH-20 Sephadex, and Merck silica gel 60 M (0.04 - 0.063 mm). TLC plates pre-coated with silica gel 60 F_{254} (Macherey-Nagel, Dueren, Germany) were used to monitor fractions, followed by detection under UV 254 and 366 nm or after spraying with anisaldehyde-sulfuric acid reagent.

3.2. Fungal material and fermentation

The fungus *Epicoccum nigrum* was isolated from fresh and healthy leaves of *Salix* sp. (Salicaceae), collected in the Caucasus mountain, Lago-Naki, Russia, in May 2017. Leaves washed with sterilized water, surface sterilized with 70% ethanol for 1 min, and cut into small pieces (around 1 × 1 × 1 cm) using a flame sterilized blade. The pieces were put on malt agar plates (15 g/L malt extract, 15 g/L bacto agar, as well as 0.20 g/L chloramphenicol and 0.25 g/L streptomycin sulfate in distilled water, pH 7.4–7.8 with sodium hydroxide or hydrochloric acid), and then incubated at room temperature for several days. The fungal strain was identified as *Epicoccum nigrum* by amplification and sequencing of the ITS-region including the 5.8S ribosomal DNA and subsequent BLAST search as previously described [17], then submitted to GenBank with the accession number MK214079 [10]. Voucher strain was deposited at the corresponding authors' laboratory (P.P.).

3.3. Extraction and isolation

The fungal strain was cultivated on solid rice medium, which was prepared by autoclaving 100 g of rice and 110 mL of water in a 1 L Erlenmeyer flask. Large scale fermentation of this fungus was performed in 7 flasks for 14 days at room temperature under static condition. The culture was diced and extracted with EtOAc (4 L). The crude extract of *E. nigrum* (15.0 g) was subjected to vacuum liquid chromatography (VLC) using a step gradient of n-hexane/EtOAc, followed by DCM/MeOH, to yield 14 fractions (F1-F14). Four interesting fractions based on HPLC-DAD profiles were subjected to size exclusion chromatography using a Sephadex LH-20 column (100×2.5 cm) with 100% MeOH as eluting solvent. Three subfractions SD3−5 were selected for further purification via semi-preparative RP-HPLC using gradient elution of water and methanol yielding compounds **5** (6.36 mg), **8** (0.45 mg) and **9** (0.50 mg). In addition, subfraction SD6 was purified by semi-preparative RP-HPLC to afford compound **6** (1.80 mg). Fraction 5 (eluted by 20% n-hexane/80% EtOAc) was subjected to a Sephadex LH-20 column eluted with 100% MeOH, followed by purification via semi-preparative RP-HPLC to yield **4** (1.28 mg) and **6** (2.17 mg). Fraction 9 (eluted by 85% CH_2Cl_2/15% MeOH) was subjected to a C-18 reversed phase column using a step gradient of MeOH/H_2O, then subfraction F9-R1 was purified by semi-preparative RP-HPLC to afford **2** (0.45 mg). Four-mixed fractions 10-13

(eluted by CH_2Cl_2/MeOH from 80/20 to 20/80) containing the flavipin-derived alkaloids were subjected to a Sephadex LH-20 column and eluted with 100% MeOH, followed by purification via semi-preparative RP-HPLC to afford **1** (1.45 mg), **3** (0.43 mg), **5** (2.72 mg) and **7** (0.83 mg).

*3.3.1. Azacoccone F (**1**)*

Greenish solid; $[\alpha]^{20}_D$: −98.1° (*c* 0.10, MeOH); UV λ_{max} (MeOH) 268 and 214 nm; ECD (0.12 mM, MeCN) λ_{max} ($\Delta\varepsilon$): 307sh (−0.06), 270 (−0.62), 251 (+1.37), 230 (−1.52); ^{1}H and ^{13}C NMR spectral data in MeOH-d_4 see Table 1; HRESIMS *m*/*z* 308.1140 $[M - H]^-$ (calcd for $C_{15}H_{18}NO_6$, 308.1140).

*3.3.2. Azacoccone G (**2**)*

Yellowish solid; $[\alpha]^{20}_D$: −12.0° (*c* 0.14, MeOH); UV λ_{max} (MeOH) 270 and 214 nm; ^{1}H and ^{13}C NMR spectral data in MeOH-d_4 see Table 1; HRESIMS *m*/*z* 296.1125 $[M + H]^+$ (calcd for $C_{14}H_{18}NO_6$, 296.1125).

*3.3.3. Azacoccone H (**3**)*

Yellowish solid; $[\alpha]^{20}_D$: −26.7° (*c* 0.15, MeOH); UV λ_{max} (MeOH) 269 and 214 nm; ^{1}H and ^{13}C NMR spectral data in MeOH-d_4 see Table 1; HRESIMS *m*/*z* 342.0980 $[M - H]^-$ (calcd for $C_{18}H_{16}NO_6$, 342.0980).

3.4. Antibacterial assay

The antibacterial screening was carried out using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. A panel of Gram-positive and Gram-negative bacterial strains, including *S. aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, as well as *Mycobacterium tuberculosis* H_{37}Rv were selected for this evaluation. After incubation at 37 °C for 24 h, the 96-well plates were checked by visual inspection. Moxifloxacin and rifampicin were used as the positive controls for the Gram-positive and Gram-negative strains, respectively. Pure compounds were added from stock solution (10 mg/mL in DMSO), resulting in a final DMSO amount of 0.64% at the highest antibiotic concentration tested (64 μg/mL). Serial 2-fold dilutions of antibiotics were prepared with DMSO being diluted along with the compounds.

3.5. Antifungal assay

The antifungal assay was performed using the agar diffusion (Kirby-Bauer) method adopted from the same protocol as described earlier [10]. *Ustilago maydis* AB33 [19] and *Saccharomyces cerevisiae* ESM356-1 [20] were pre-cultured in Complete Minimal (CM) medium supplemented with 10 g/L glucose [21–22] and Yeast Extract–Peptone–Dextrose

(YPD), respectively. The overnight cultures (500 µL) were diluted to OD_{600} of 0.5 and then inoculated on agar plates. Sterile filter paper disks of 5 mm diameter were placed on the agar plates. Each of the tested compounds (100 µg) was dissolved in DMSO to impregnate the disks. Nystatin (100 µg) and nourseothricin sulfate/clonNAT (200 µg) were used as positive controls, while double distilled water (*dd*H_2O) and DMSO were used as negative controls. The isolated compounds and controls were tested in triplicate. In order to observe antifungal activity, the treated agar plates were incubated at 28°C for 48 h. Growth inhibition zone (in mm) surrounding the disk was measured and the lowest concentration of the tested compounds, which did not exhibit growth inhibition, was considered as the minimum inhibitory concentration (MIC).

3.6. Cytotoxicity assay

Cytotoxicity was employed against the L5178Y mouse lymphoma cells using an MTT assay [10]. Experiments were repeated three times and conducted in triplicate. Kahalalide F and 0.1% ethylene glycol monomethyl ether (EGMME)–DMSO were used as a positive and negative controls, respectively.

3.7. Computational methods

Mixed torsional/low-mode conformational searches were carried out by means of the Macromodel 10.8.011 software using the MMFF with an implicit solvent model for $CHCl_3$ applying a 21 kJ mol^{-1} energy window [23]. Geometry reoptimizations of the resultant conformers [ωB97X/TZVP with PCM solvent model for MeCN and MeOH, and SOGGA11-X/TZVP SMD/MeCN], TDDFT ECD and OR calculations were performed with Gaussian 09 using various functionals (B3LYP, BH&HLYP, CAM-B3LYP, PBE0) and the TZVP basis set with the same solvent model as in the preceding DFT optimization step [24]. ECD spectra were generated as the sum of Gaussians with 1800 cm^{-1} half-height widths, using dipole-velocity-computed rotational strengths [25]. Boltzmann distributions were estimated from the ωB97X and SOGGA11-X energies. The MOLEKEL program was used for visualization of the results [26].

Acknowledgements

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Strategieprojekt BioSC (No. 313/323-400-002 13) to M.F. T.K. was supported by the National Research, Development and Innovation Office (K120181) and A.M. by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. The Governmental Information-Technology Development Agency (KIFÜ) is acknowledged for CPU time.

Appendix A. Supplementary data

Supplementary data (HPLC chromatograms, UV, HRESIMS, and NMR spectra of **1**, **2**, and **3**) associated with this article can be found online at: http://doi.org/xxxxx

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Supporting Information

Azacoccones F–H, new flavipin-derived alkaloids from an endophytic fungus *Epicoccum nigrum* MK214079

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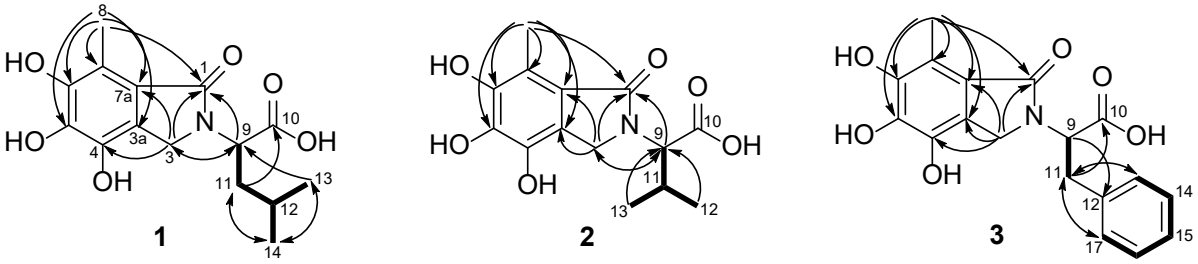

Figure S1. Key HMBC (arrow) and ^{1}H-^{1}H COSY (bold) correlations of compounds **1**–**3**.

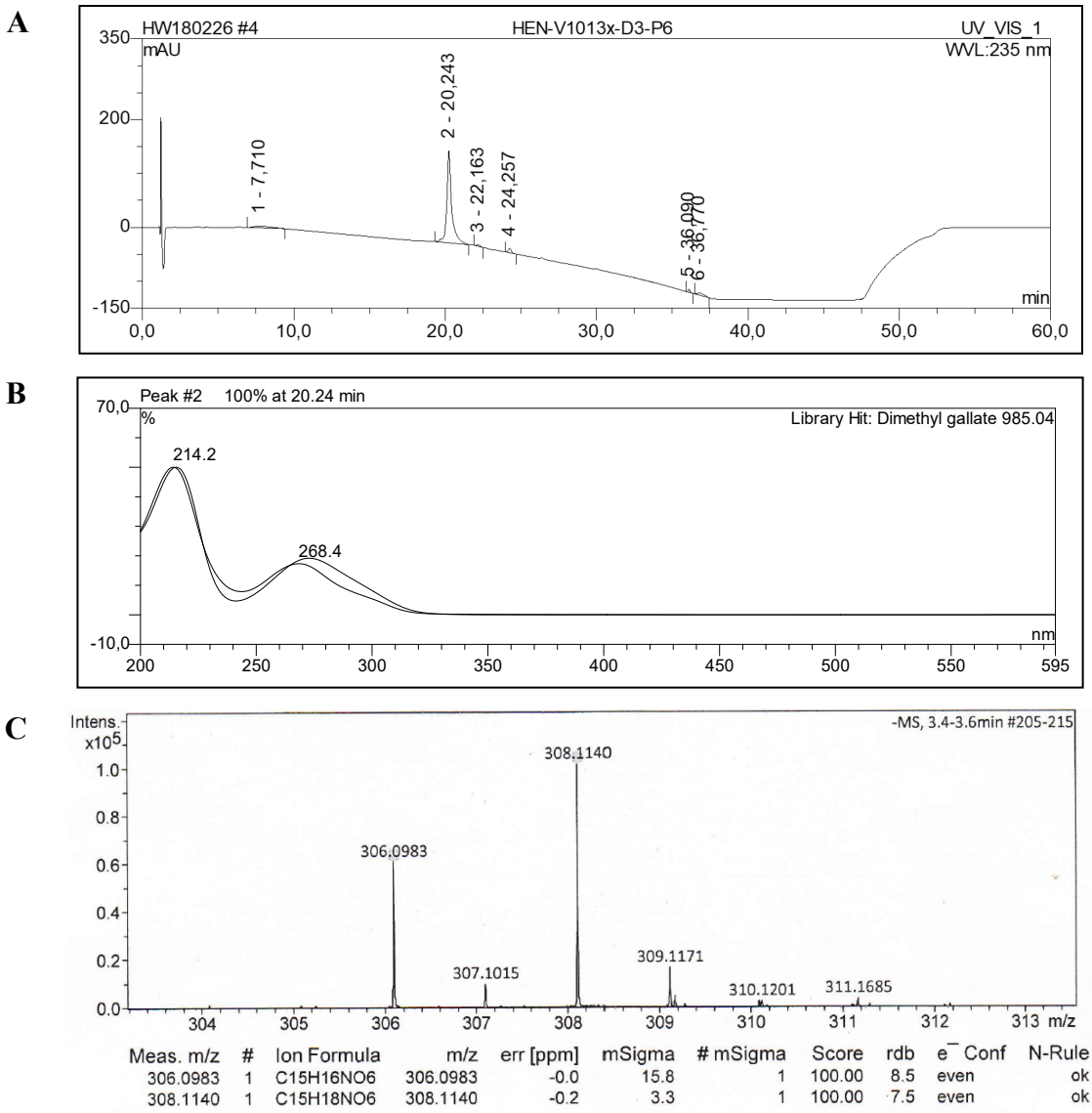

Figure S2. HPLC chromatogram (**A**), UV (**B**), and HRESIMS spectra (**C**) of **1**.

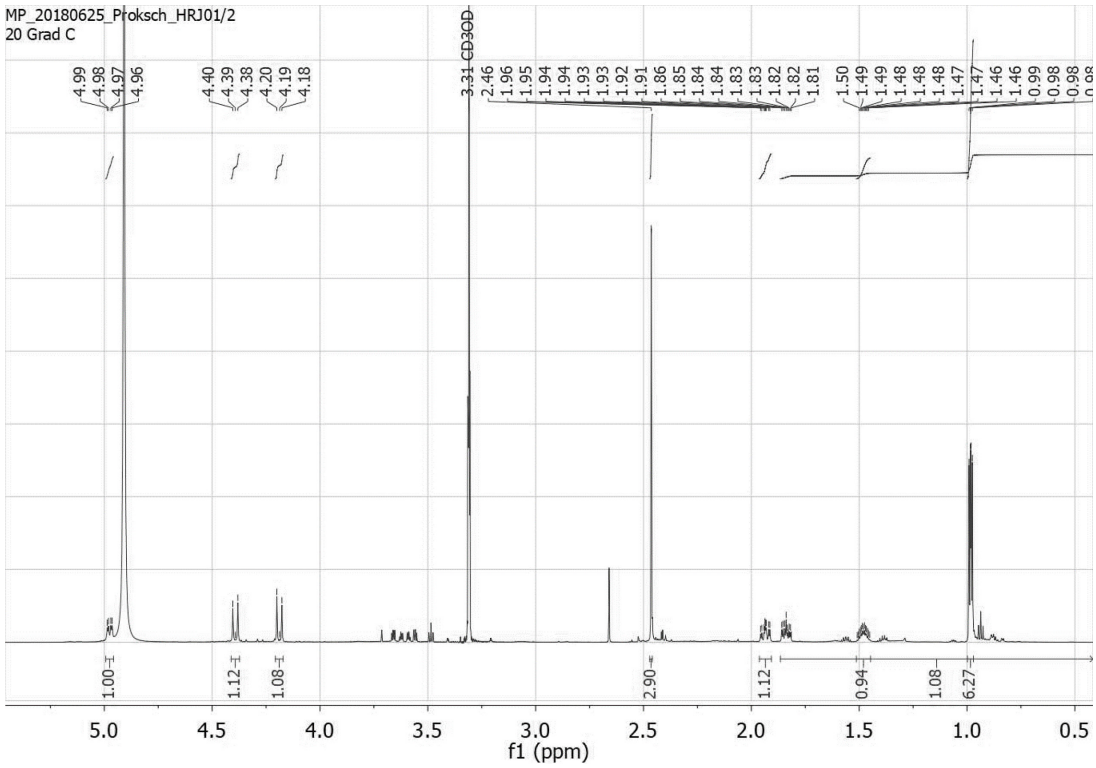

Figure S3. ^{1}H NMR spectrum of **1** in MeOH-d_4 (700 MHz; T=293 K).

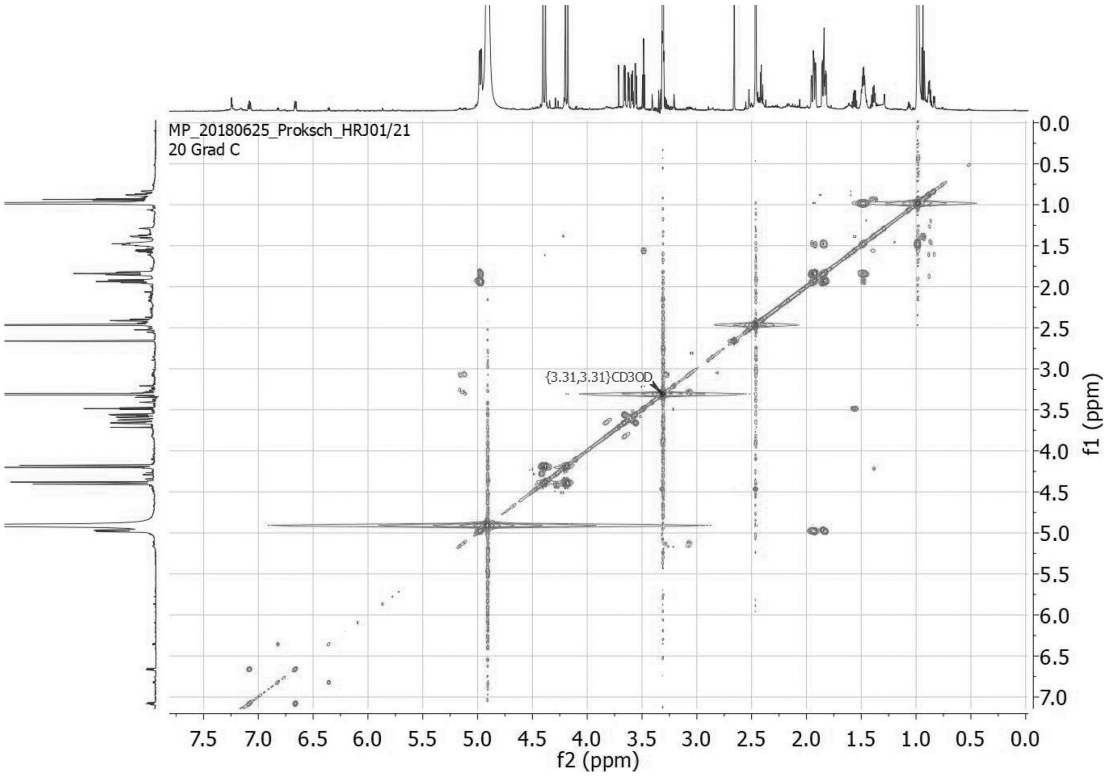

Figure S4. ^{1}H-^{1}H COSY spectrum of **1** in MeOH-d_4 (700 MHz; T=293 K).

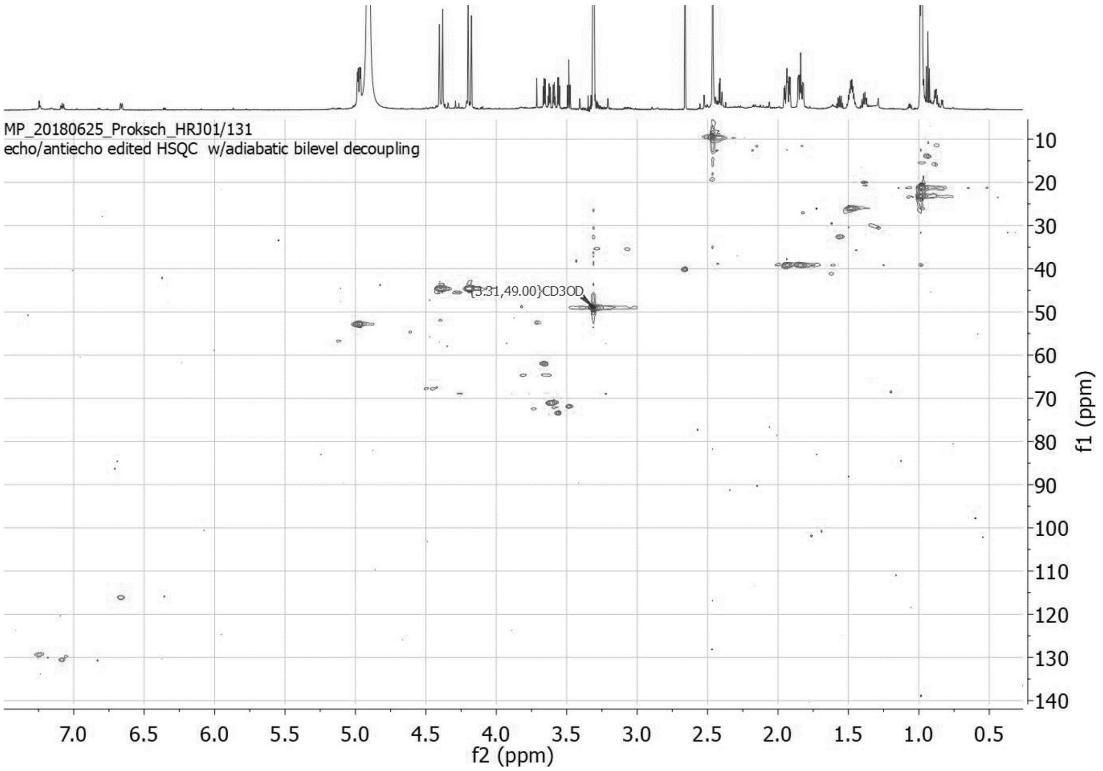

Figure S5. HSQC spectrum of **1** in MeOH-d_4 (700 and 176 MHz; T=293 K).

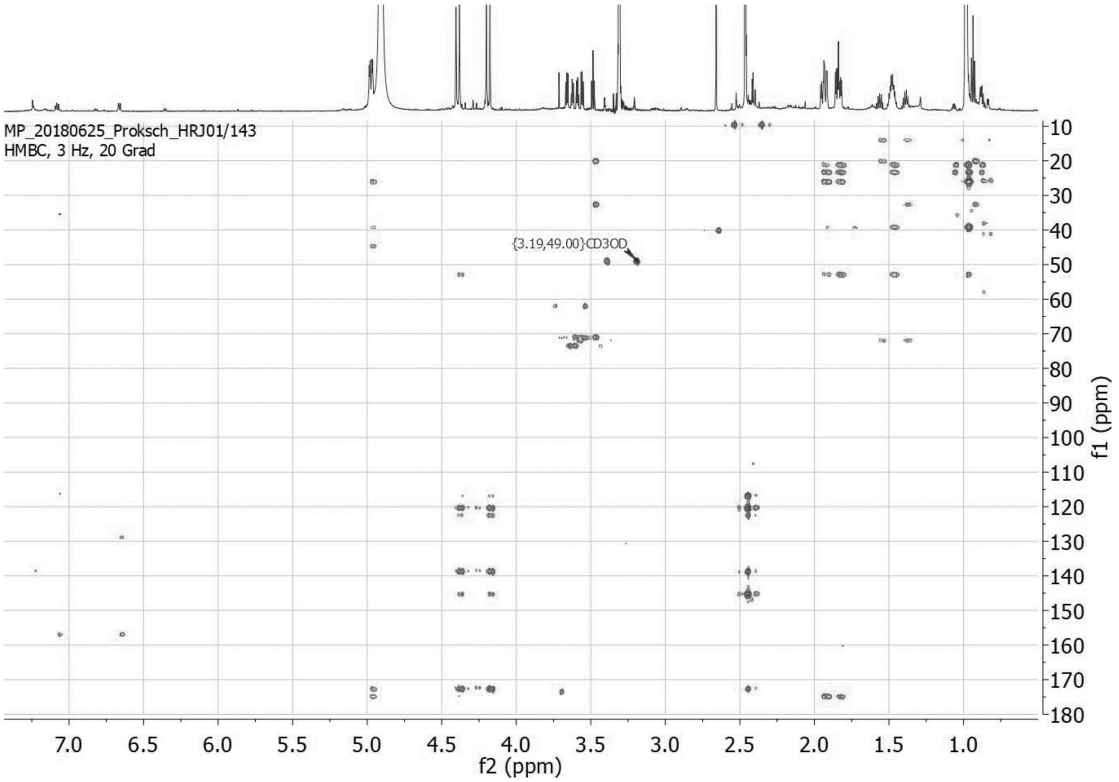

Figure S6. HMBC spectrum of **1** in MeOH-d_4 (700 and 176 MHz; T=293 K).

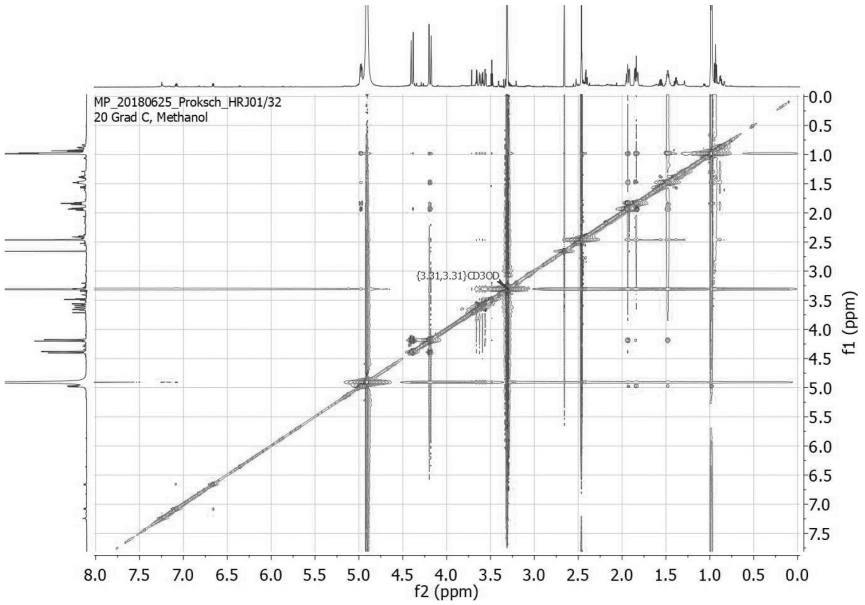

Figure S7. ROESY spectrum of **1** in MeOH-d_4 (700 MHz; T=293 K).

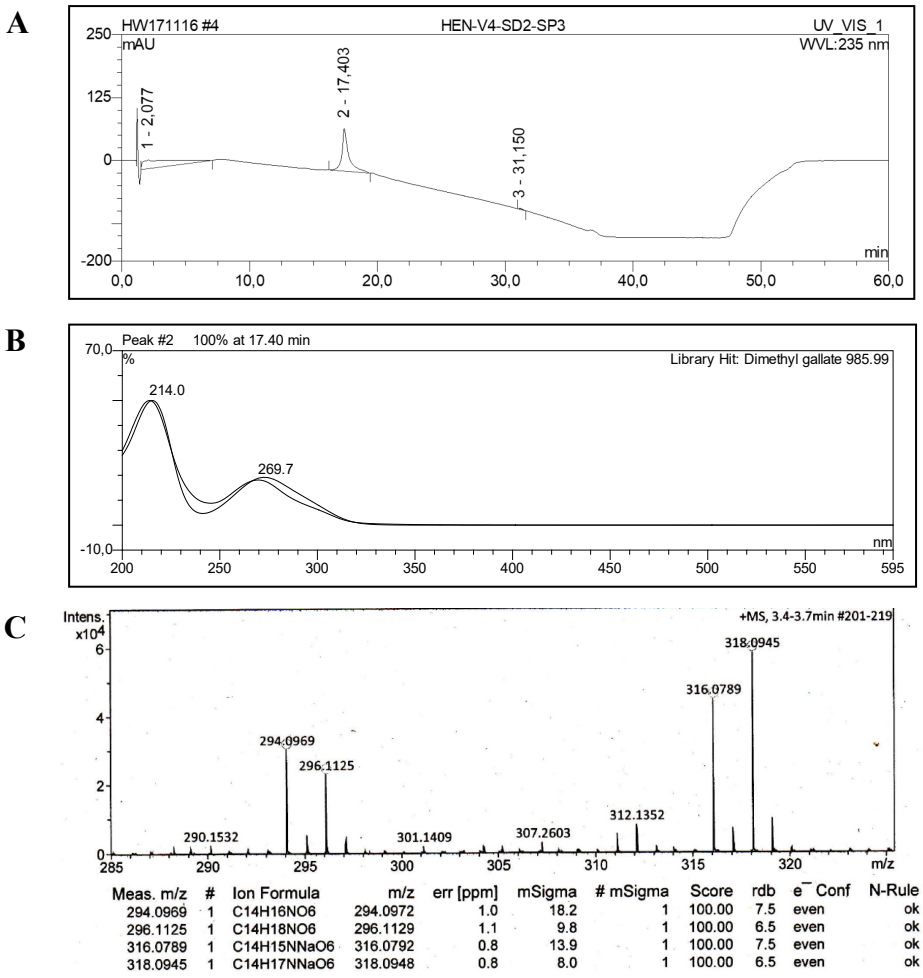

Figure S8. HPLC chromatogram (**A**), UV (**B**), and HRESIMS spectra (**C**) of **2**.

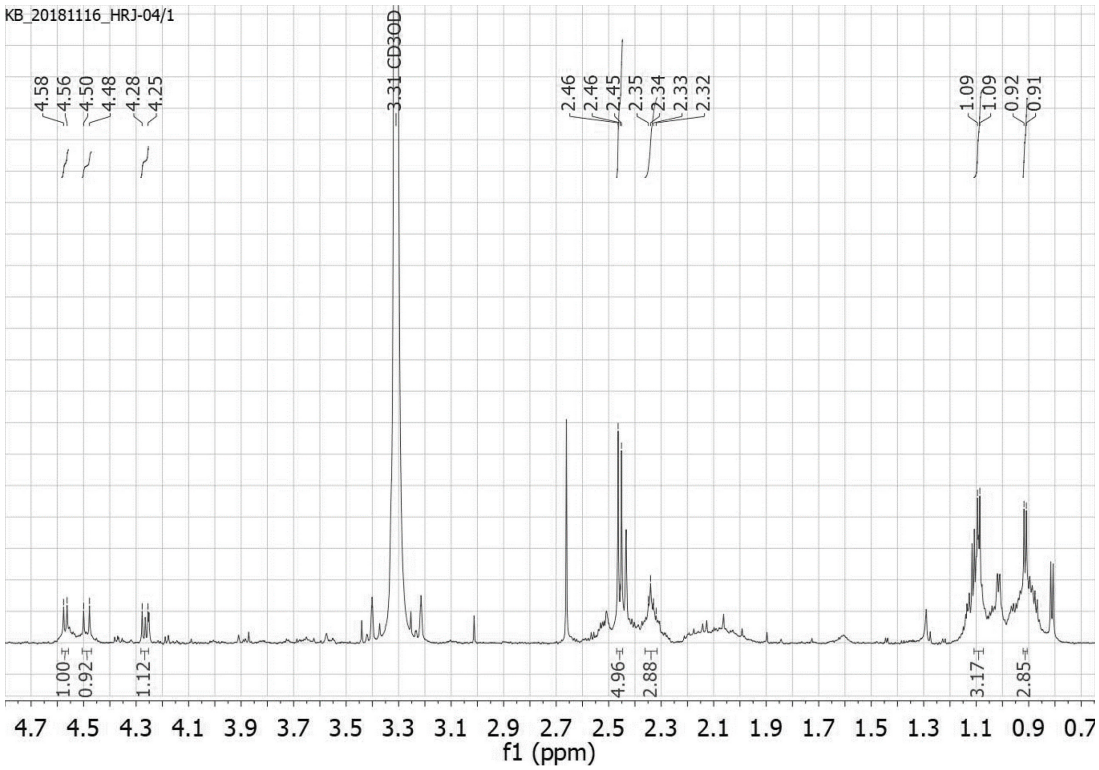

Figure S9. ^{1}H NMR spectrum of **2** in MeOH-d_4 (750 MHz; T=293 K).

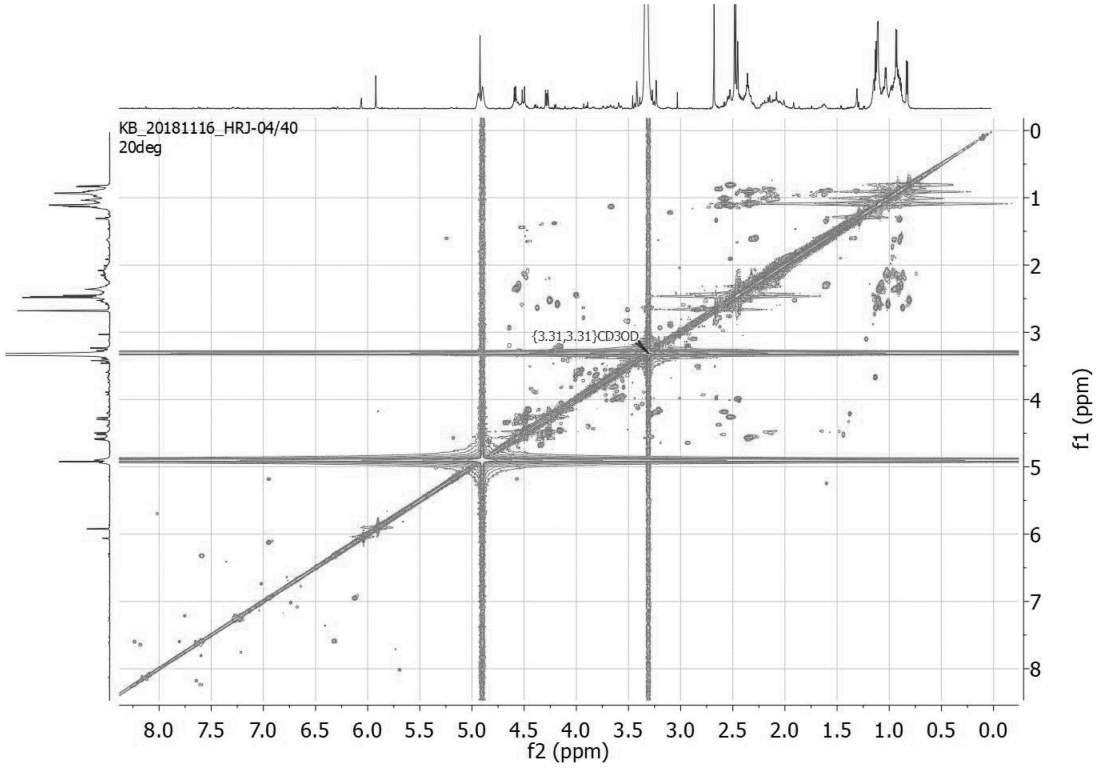

Figure S10. ^{1}H-^{1}H COSY spectrum of **1** in MeOH-d_4 (750 MHz; T=293 K).

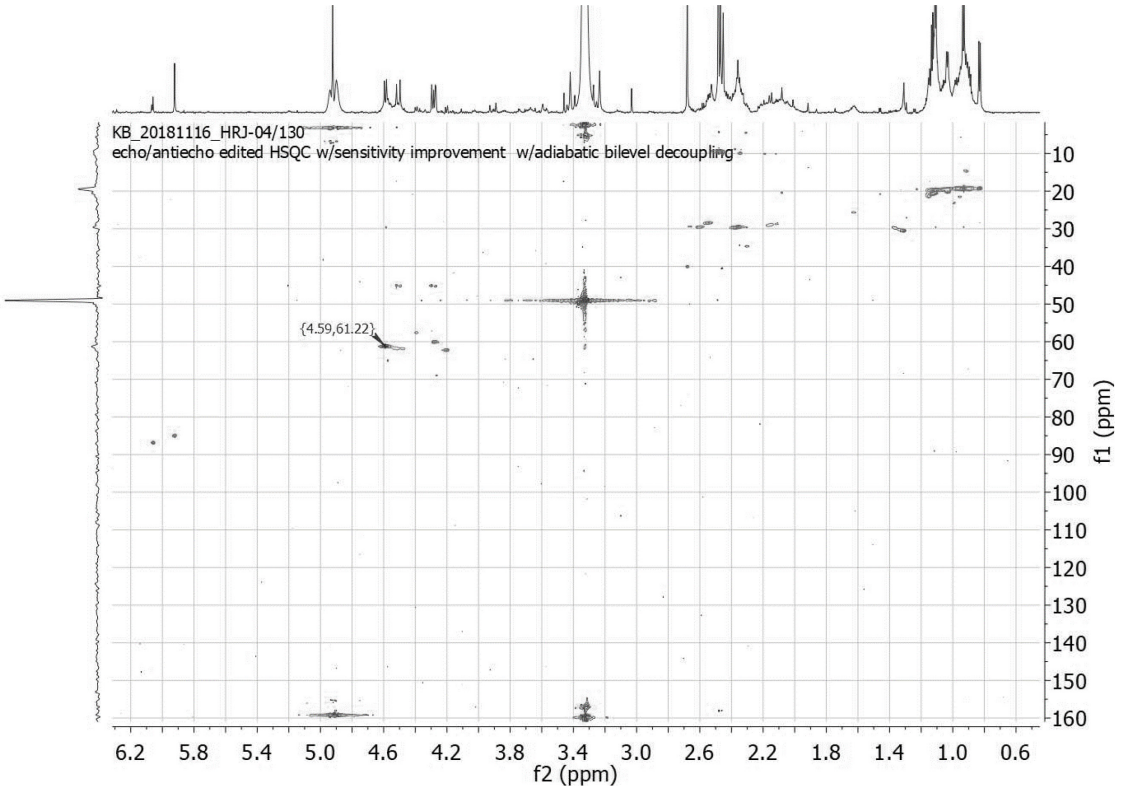

Figure S11. HSQC spectrum of **1** in MeOH-d_4 (750 and 189 MHz; T=293 K).

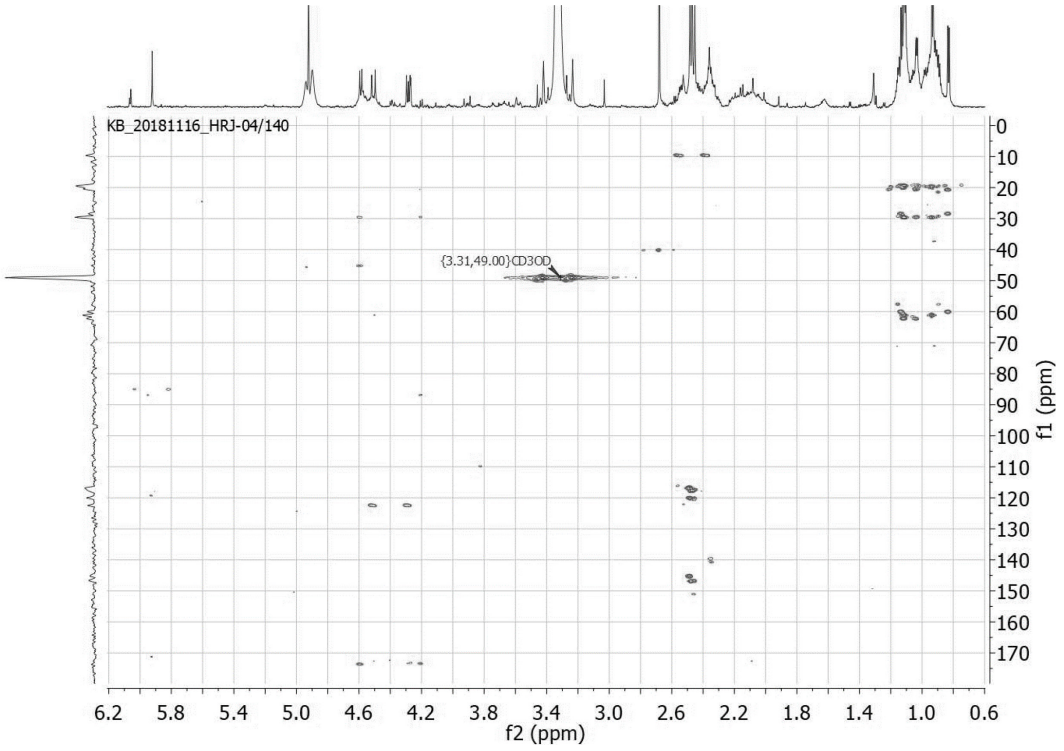

Figure S12. HMBC spectrum of **1** in MeOH-d_4 (750 and 189 MHz; T=293 K).

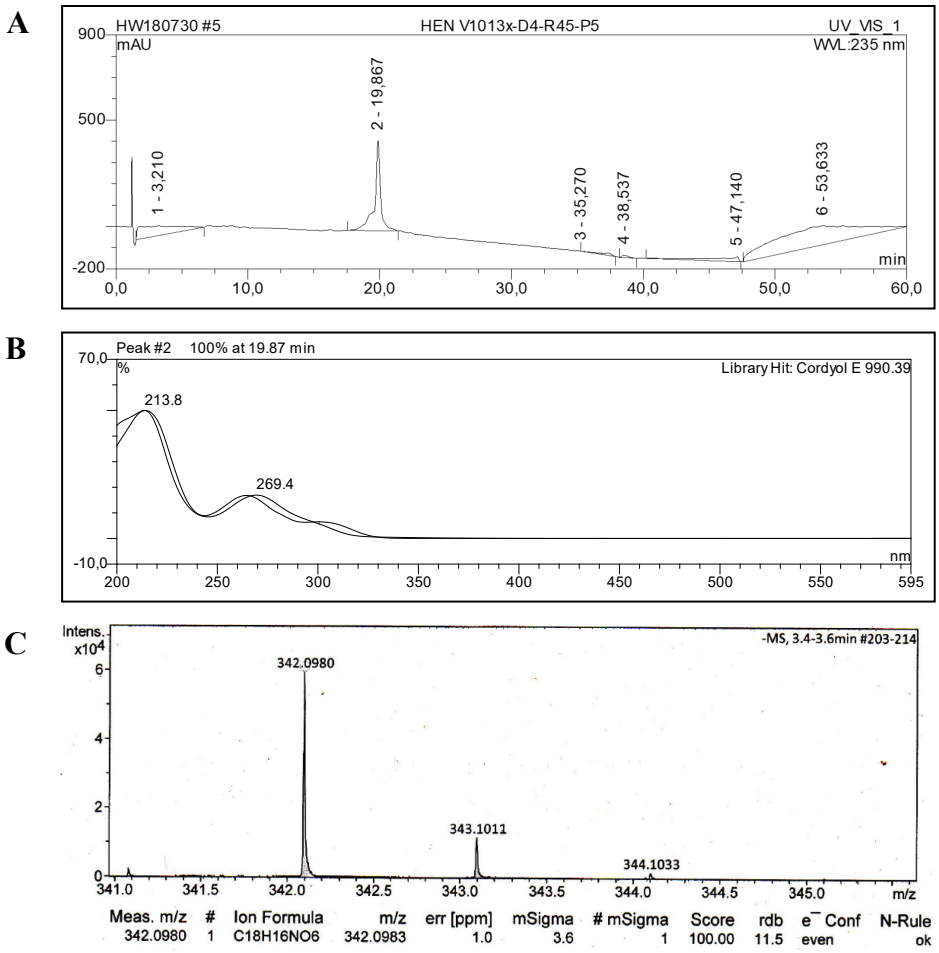

Figure S13. HPLC chromatogram (**A**), UV (**B**), and HRESIMS spectra (**C**) of **3**.

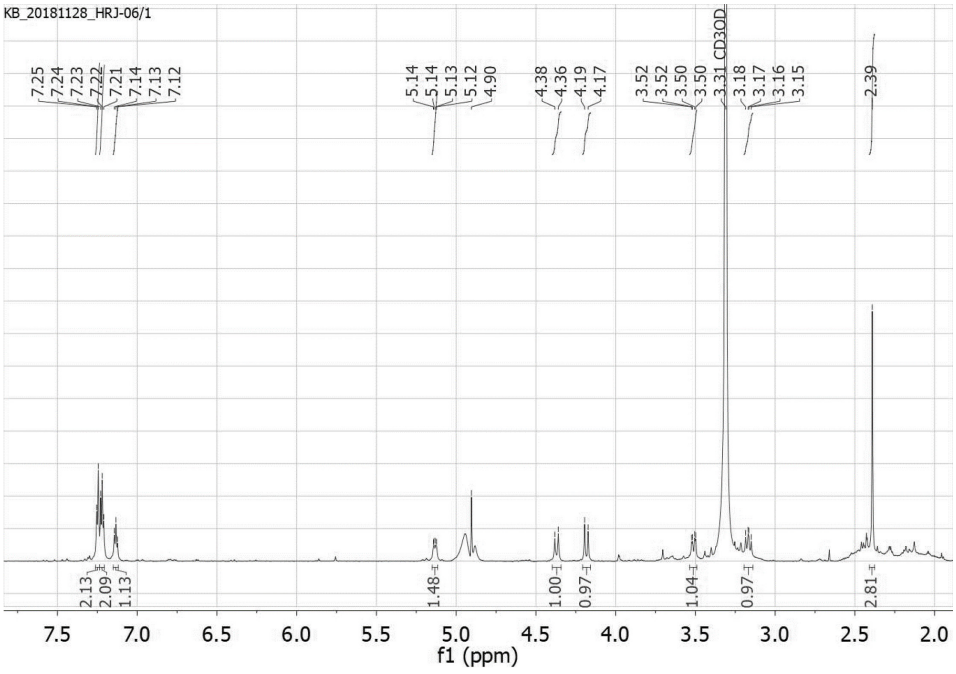

Figure S14. ^{1}H NMR spectrum of **3** in MeOH-d_4 (750 MHz; T=293 K).

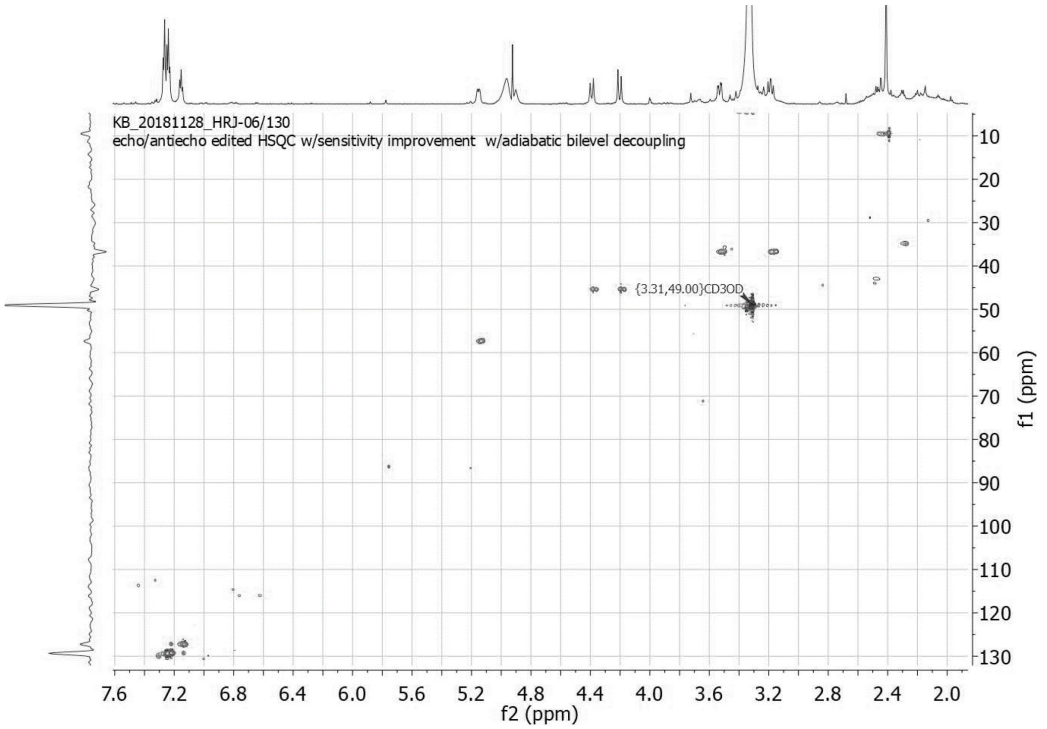

Figure S15. HSQC spectrum of **3** in MeOH-d_4 (750 and 189 MHz; T=293 K).

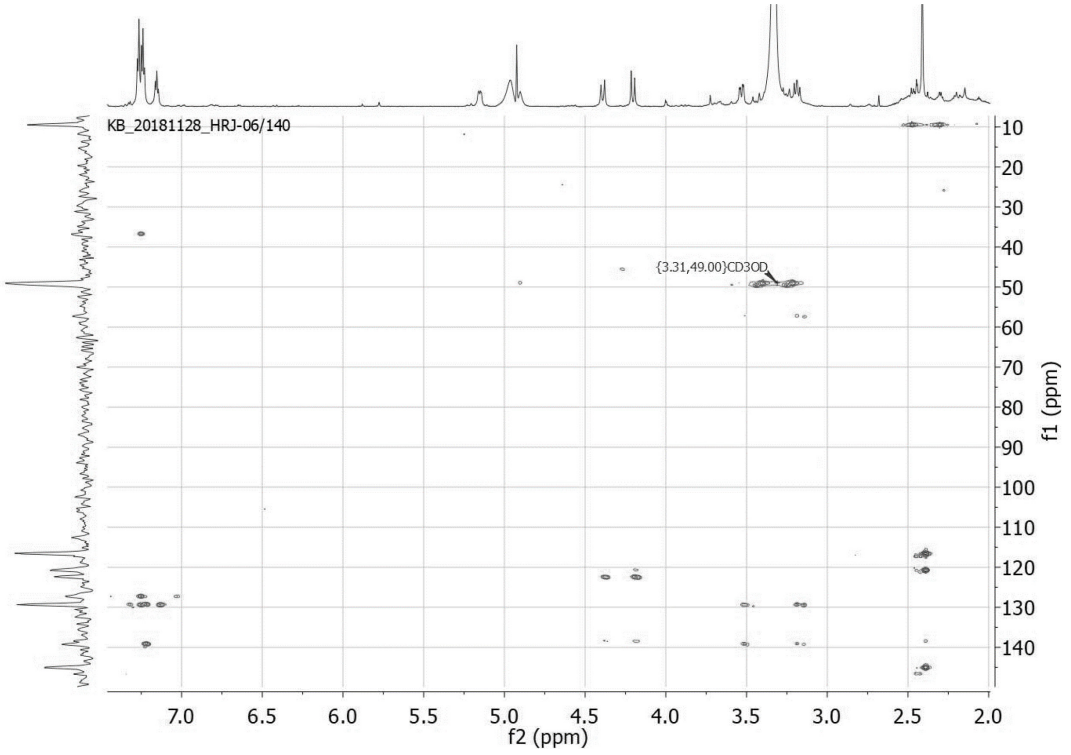

Figure S16. HMBC spectrum of **3** in MeOH-d_4 (750 and 189 MHz; T=293 K).

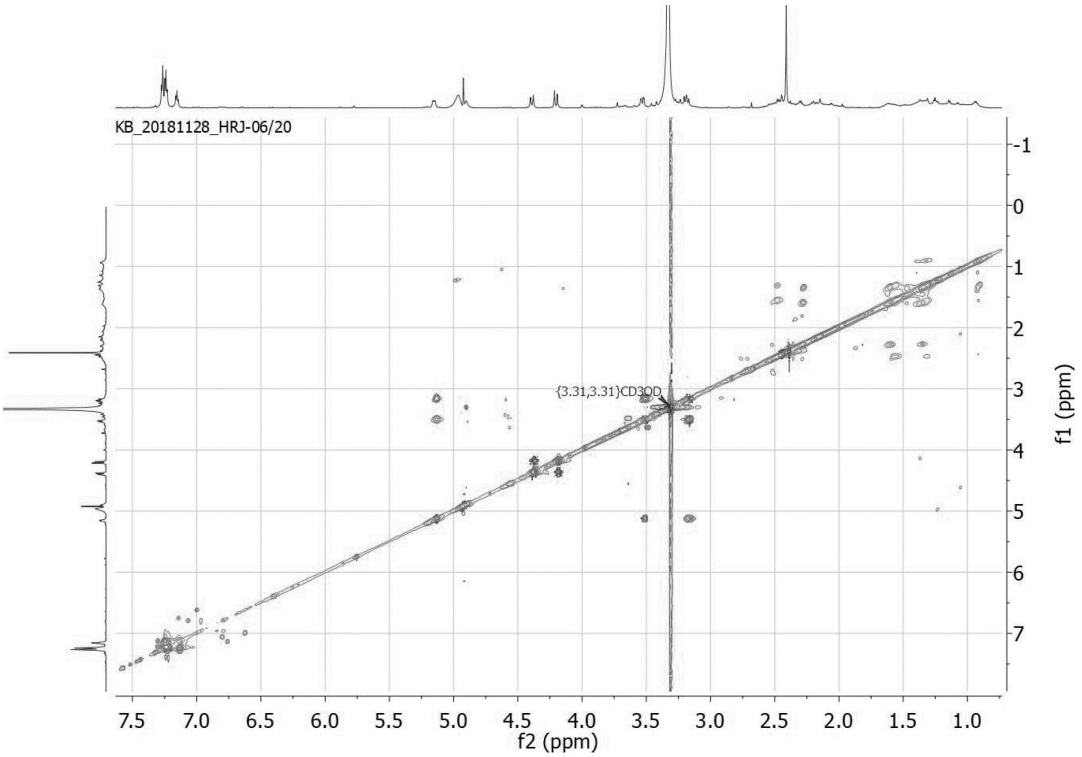

Figure S17. ROESY spectrum of **3** in MeOH-d_4 (750 MHz; T=293 K).

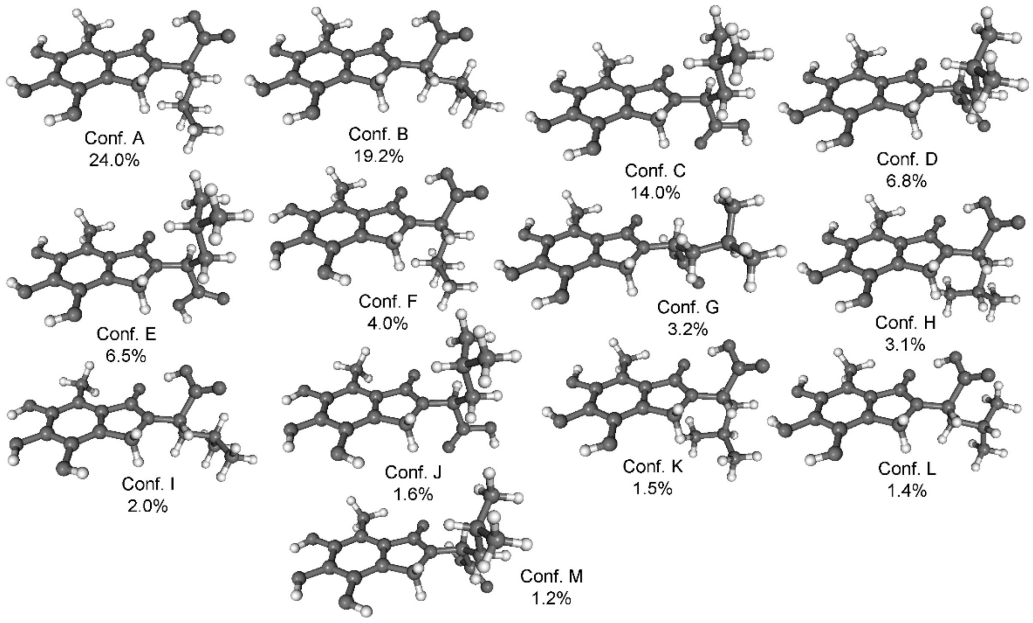

Fig. S18. Low-energy conformers (≥ 1%) of (*S*)-**1** obtained by ωB97X/TZVP PCM/MeCN reoptimization of the MMFF geometries.

Table S1. Boltzmann populations and optical rotations of the low-energy conformers of (*S*)-**1** computed at various levels for the ωB97X/TZVP PCM/MeOH reoptimized MMFF conformers

Conformer	Boltzmann population	B3LYP/TZVP PCM/MeOH	BH&HLYP/TZVP PCM/MeOH	CAM-B3LYP/TZVP PCM/MeOH	PBE0/TZVP PCM/MeOH
Conf. A	24.10 %	171.95	132.19	145.38	163.70
Conf. B	19.23 %	159.60	122.42	134.22	152.71
Conf. C	14.03 %	28.47	22.23	24.13	27.56
Conf. D	6.82 %	-322.02	-267.00	-284.80	-316.45
Conf. E	6.51 %	-53.28	-47.48	-49.19	-54.40
Conf. F	3.89 %	173.61	133.39	146.27	165.15
Conf. G	3.19 %	-241.16	-195.79	-208.63	-235.35
Conf. H	3.13 %	196.48	152.98	168.16	187.94
Conf. I	1.94 %	142.21	109.83	118.15	134.80
Conf. J	1.59 %	40.23	30.69	34.62	39.70
Conf. K	1.50 %	202.19	161.64	176.96	193.93
Conf. L	1.44 %	146.22	112.20	123.10	141.05
Conf. M	1.19 %	-315.16	-262.36	-279.03	-309.28
Average	N/A	68.58	50.27	56.34	64.26

CHAPTER 4

Biotransformation of host plant flavonoids by the fungal endophyte *Epicoccum nigrum*

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Biotransformation of Host Plant Flavonoids by the Fungal Endophyte *Epicoccum nigrum*

Harwoko Harwoko,[a, b] Rudolf Hartmann,[c] Georgios Daletos,[a] Elena Ancheeva,[a] Marian Frank,[a] Zhen Liu,*[a] and Peter Proksch*[a]

Fermentation of the fungus *Epicoccum nigrum* isolated from leaves of *Salix* sp. on green lentil solid medium yielded the flavonol kaempferol (**3**) as well as two kaempferol *O*-diglycosides (**1** and **2**) including the new compound **1**. The fungal flavonoids bear strong structural similarities to kaempferol derivatives such as kaempferol *O*-glycoside (**4**) being present in green lentils. Furthermore, feeding experiments were conducted by adding flavonoids (kaempferol and rutin) as precursors to solid rice media followed by HPLC and LC-MS analyses. Fermentation of the fungus on flavonoid free solid rice medium afforded flavonoid free extracts indicating that the fungal flavonoids originate through hydrolytic cleavage of kaempferol glycosides such as **4** followed by glycosylation and acetylation. This study suggests that *E. nigrum* is capable of biotransformation reactions of plant derived flavonoids whereas *de novo* biosynthesis of flavonoids is less likely.

Introduction

Endophytic microorganisms, primarily fungi or bacteria, that inhabit the interior of the host plant without inciting disease symptoms or producing external structures, constitute promising sources of novel drugs or drug leads.[1] Some compounds that have been isolated from endophytic fungi in the past such as plant hormones or even pharmaceutically important drugs or drug leads like taxol,[2] camptothecin,[3] and podophyllotoxin[4] are structurally identical to compounds known from plants. Flavonoids are usually considered as typical secondary metabolites of plants, however in a few cases, flavonoids were also reported from endophytic fungi.[5,6] For instances, the Ascomycetes *Pestalotiopsis uvicola*, *Aspergillus flavus,* and *Annulohypoxylon squamulosum* have been shown to accumulate flavonoids either in the form of aglycones or as glycosides as commonly found in plants.[5] Similarly, our previous work also demonstrated that flavonoid monoglycosides or an unusual chlorinated flavonoid called chlorflavonin are found in *Nigrospora oryzae* or *Mucor irregularis* associated with the medicinal plants *Loranthus micranthus* or *Moringa stenopetala*, respectively.[6]

Epicoccum nigrum Link (synonym *Epicoccum purpurascens* Ehrenb. ex Schlecht.), is a fungicolous and mycoparasitic fungus, which is used as biocontrol agent for phytopathogens.[7] Recently, chemical investigation of fungi of the genus *Epicoccum* resulted in the isolation of structurally diverse bioactive compounds, including polyketides,[8] diketopiperazine alkaloids,[9] and diterpenes[10] which exhibited important biological activities.[8–10] Remarkably, even the well known antitumor drug taxol,[11] glycosylated flavonoids,[12] and anthraquinones[13] were reported from *E. nigrum*.

Cultivation of *E. nigrum*, an endophytic fungus isolated from leaves of willow (*Salix* sp., Salicaceae), on a solid medium composed of green lentils now yielded the flavonol kaempferol as well as two kaempferol diglycosides including a new natural product (**1–3**). The presence of flavonoid glycosides in *E. nigrum* had also previously been reported when the fungus was fermented on solid corn medium based on UV absorption spectra even though no individual compounds were reported.[12] The structures of the flavonoids isolated in this study were unambiguously elucidated on the basis of NMR and mass spectrometry data, as well as by comparison with the literature. Structural comparison of the fungal flavonoids with flavonoids present in green lentils as well as feeding studies involving kaempferol and the quercetin diglycoside rutin strongly suggest that *E. nigrum* is capable of biotransforming host plant derived flavonoids giving rise to the flavonoid derivatives **1–3**.

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Results and Discussion

The fungal strain *E. nigrum* isolated from willow leaves was fermented on green lentil medium for 14 days. The presence of flavonoids (**1**–**3**) in the fungal extract was detected based on HPLC-DAD analysis of the crude EtOAc extract due to typical UV absorption spectra of kaempferol derivatives as evident from a comparison with an *in house* library of UV absorption spectra. No flavonoids were detected in comparison when the fungus was grown on solid rice medium as a control.

Chromatographic separation of the EtOAc extract of the fungus following fermentation on green lentil solid medium resulted in the isolation of three kaempferol derivatives, including a new acetylated flavonoid glycoside (**1**) and two known derivatives, kaempferol 3-*O*-[α-rhamnopyranosyl-(1→6)-β-galactopyranoside] (biorobin, **2**)[14] and kaempferol (**3**).[15] Compound **1**, a yellow amorphous powder, had the molecular formula $C_{29}H_{32}O_{16}$ as determined by positive-ion HRESIMS at *m/z* 637.1758 $[M + H]^+$, 42 amu higher than that of biorobin (**2**). The ^{1}H and ^{13}C NMR data of **1** (Table 1) were similar to those of co-isolated known kaempferol *O*-diglycoside, biorobin (**2**),[14] except for the presence of an additional acetyl group at δ_H 2.14 and at δ_C 20.6 and 172.1. The meta-coupled protons at δ_H 6.21 (d, J=2.1 Hz) and 6.41 (d, J=2.1 Hz) were assigned to H-6 and H-8 of ring A, while proton resonances at δ_H 8.09 (2H, d, J= 8.9 Hz) and 6.90 (2H, d, J=8.9 Hz) were in agreement with a 4′-monosubsituted ring B. A kaempferol skeleton was further confirmed by detailed analysis of the 2D NMR spectra of **1**. The presence of two anomeric protons at 5.17 (d, J=7.8 Hz) and 4.53 (d, J=1.7 Hz), along with HSQC and TOCSY spectra of **1** and comparison of ^{13}C NMR data with those reported in the literature confirmed the presence of galactose and rhamnose, respectively.[14,16,17] Furthermore, the configurations of the anomeric protons for galactose as β and for rhamnose as α were determined by the large $^3J_{H1,\ H2}$ value (7.8 Hz) and the small $^3J_{H1,\ H2}$ value (1.7 Hz), respectively.[16–18]

Crosspeaks observed in the HMBC spectrum from δ_H 5.17 (H-1″, galactose) to δ_C 135.2 (C-3, kaempferol) and from δ_H 4.53 (H-1‴, rhamnose) to δ_C 66.4 (C-1′, galactose) confirmed that the galactose moiety is connected at the C-3 position of kaempferol, whereas the rhamnose unit was linked to C-6 of the galactose moiety. The sugar sequence was further confirmed to be (1→6) by ROESY correlation from methine proton at δ_H 4.53 (H-1‴, rhamnose) to both methylene protons of galactose at H_2-6″. The spectral features supported that compound **1** was the mono- acetylated derivative of compound **2**, where ^{1}H-^{1}H COSY cross- peaks established a spin system starting from H-1″ (δ_H 5.17) and extending till H-4″ (δ_H 3.96). Moreover, the HMBC correlation from the methyl protons of the acetyl function at δ_H 2.14 to the carbonyl carbon at δ_C 172.1 was clearly observed, although the cross-peak between the signals at δ_H 4.76 (H-3″, galactose) and carbonyl (3″-OAc) was weak. However, a proton at H-3″ of galactose in **1** resonated at a lower field ($\triangle\delta$: +1.00 ppm) when compared with that of in **2**, indicating that the acetyl group was located at the C-3 position of galactose as reported for similar compounds (Figure 1).[18–20] In order to prove whether compound **1** is a true natural product or an artefact generated during extraction and/or isolation with ethyl acetate, the postulated parent compound **2** was incubated with ethyl acetate for one week at room temperature. However, no acetylated products were detected by HPLC. Hence, compound **1** was established as kaempferol 3-*O*-[α-rhamnopyranosyl-(1→6)-3-*O*-acetyl-β-galactopyranoside] and named 3″-*O*-acetyl biorobin. Remarkably, glycosylation reactions by adding galactose vs. glucose to precursors are rare for fungi.[6a,21,22] Galactose, however, is also found as a structural moiety of the pyrenopolyene *C*-glycoside epipyrone A[23] which is present in all extracts of *E. nigrum* analyzed in this study (Figures 2–3) indicating the capacity of the fungus to use galactose for glycosylation reactions.[22]

Chemical investigation of green lentil solid medium that was used in this study for fermentation of *E. nigrum* afforded kaempferol 7-*O*-rhamnoside (**4**)[24] which even though missing

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (188 MHz) data for compound **1** measured in CD_3OD (δ, *J* in Hz)

Position	1	
	δ_H	δ_C*
2		159.0 s
3		135.2 s
4		179.0 s
5		162.7 s
6	6.21, d (2.1)	99.7 d
7		166.1 s
8	6.41, d (2.1)	94.6 d
9		158.2 s
10		105.2 s
1′		122.2 s
2′, 6′	8.09, d (8.9)	132.1 d
3′, 5′	6.90, d (8.9)	115.8 d
4′		161.4 s
D-Gal		
1″	5.17, d (7.8)	104.9 d
2″	4.01, dd (10.2, 7.8)	70.1 d
3″	4.76, dd (10.2, 3.3)	77.1 d
4″	3.96, d (3.3)	67.1 d
5″	3.70, m*	74.6 d
6″	3.72, m*	66.4 t
	3.35, m*	
Acetyl		
CO	-	172.1 s
CH_3	2.14, s	20.6 q
L-Rha		
1‴	4.53, d (1.7)	101.5 d
2‴	3.61, dd (3.3, 1.7)	71.8 d
3‴	3.49, dd (9.5, 3.3)	72.0 d
4‴	3.28, m*	73.4 d
5‴	3.52, dq (9.5, 6.2)	69.4 d
6‴	1.19, d (6.2)	17.6 q

[a] Data extracted from HSQC and HMBC * Overlapping signals

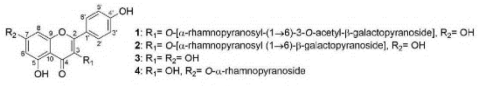

Figure 1. Flavonoids produced by the endophytic fungus *Epicoccum nigrum* (**1**–**3**) and those found in green lentil extract (**4**).

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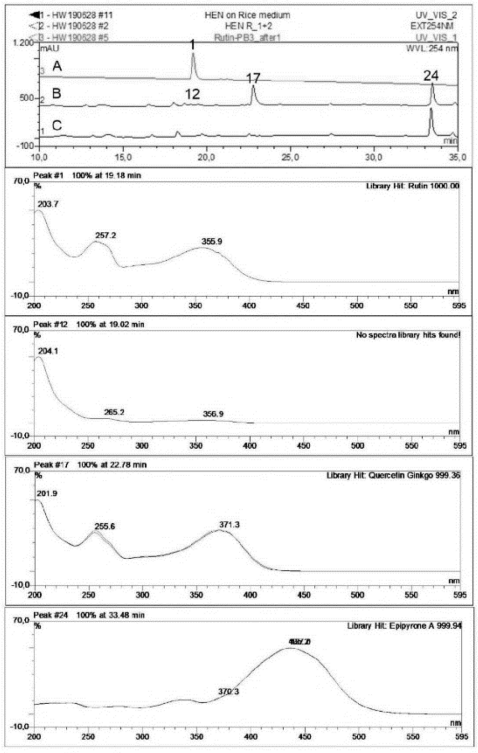

Figure 2. HPLC chromatograms and UV spectra of rutin (A), *E. nigrum* extract of feeding experiment by adding rutin 0.5% (B) and of control (C).

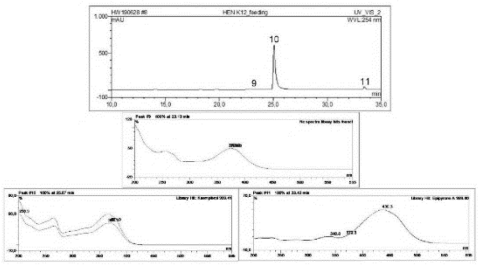

Figure 3. HPLC chromatogram and UV spectra of *E. nigrum* extract from feeding experiments by adding kaempferol 0.5%.

in the fungal extract is closely related to the fungal flavonoids **1–3** that were isolated in this study. The structural similarity of the green lentil flavonoid **4** vs. the fungal compounds **1–3** and the absence of flavonoids in the fungus following fermentation on solid rice medium suggest that the fungal flavonoids are formed through biotransformation of compound **4** rather than by *de novo* biosynthesis. This hypothesis was tested by adding the quercetin *O*-diglycoside rutin or the flavonol aglycone kaempferol to flavonoid free solid rice medium on which *E. nigrum* was subsequently grown. Chromatographic analysis of the resulting fungal extracts by HPLC indicated that rutin had been completely hydrolyzed by *E. nigrum* yielding the flavonol aglycone quercetin (Figure 2) as sole detectable flavonoid indicating that enzymatic hydrolysis of flavonol *O*-glycosides presumably through β-glucosidases occurs in the fungus.[25] Following addition of kaempferol to solid rice medium two more polar, small peaks with UV absorption spectra matching kaempferol (**3**) were detected by HPLC-DAD analysis (Figure 3) in addition to the added flavonol precursor.

LC-ESI-MS analysis (positive mode) yielded pseudomolecular ions at *m/z* 469 and *m/z* 483, respectively whereas the presence of kaempferol was indicated by the fragment ion at *m/z* 287 (Figure S7). When investigating both metabolites in the negative ESI-HRMS mode (Figure S8), the larger pseudomolecular ion peak at *m/z* 481.0769 (calculated for $C_{24}H_{17}O_{11}$) was determined to account for the methyl derivative of the first kaempferol derivative which featured the pseudomolecular ion peak at *m/z* 467.0613 (calculated for $C_{23}H_{15}O_{11}$). Furthermore, both compounds showed a fragment at *m/z* 423.0761 (calculated for $C_{22}H_{15}O_9$), indicating a mass loss of 44 amu for carboxylate and 58 amu for methyl carboxylate, respectively. The fragment peak at *m/z* 285.0404 (calculated for $C_{15}H_9O_6$) confirmed the presence of a kaempferol unit. A plausible fragmentation scheme of kaempferol derivatives is shown in Figure 4. To determine the molecular formula of the sidechains, one H_2O was added to account for the presence of esters or glycosides. The molecular formula of the kaempferol aglycone and those of the pseudomolecular ion peaks suggested the presence of substituents with molecular formula $C_9H_{10}O_6$ or $C_8H_8O_6$, respectively. Fumarylacetoacetate and its monomethylester are possible candidates for these substituents, as they fit with the observed decarboxylation pattern and are accessible by fungal phenylalanine/tyrosin degradation[26] via homo-

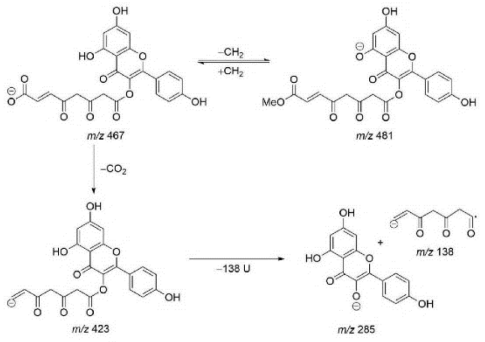

Figure 4. Proposed fragmentation pathways of kaempferol derivatives. Attachment of sidechains to kaempferol is arbitrarily chosen.

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gentisate pathway.[27] Recently, the strategies to produce or diversify valuable flavonoids apply enzymatic approaches (*e.g.* biocatalysis via flavonoids glycosyl-/rhamnosyltransferases) and metabolic engineering (*e.g.* bioconversion with engineered *Escherichia coli*), instead of microbial biotransformation.[22,28,29]

Conclusion

This study reports for the first time the capability of *E. nigrum* to biotransform plant-derived flavonoids into unprecedented metabolites, including a new kaempferol *O*-diglycoside (**1**). The fungal flavonoids (**1**–**3**) produced through an OSMAC (One Strain MAny Compounds) experiment on green lentil medium, originate via hydrolytic cleavage followed by glycosylation and acetylation of plant derived precursors such as **4**. This hypothesis was supported by adding the quercetin diglycoside rutin or kaempferol to solid rice media followed by HPLC and LC-MS analyses. Rutin was completely hydrolyzed by the fungus affording the aglycone quercetin as sole detectable biotransformation product. Meanwhile, kaempferol was partly metabolized yielding several peaks with higher polarity that were tentatively identified as unknown kaempferol metabolites based on LC-HRMS approach.

Supporting Information Summary

Experimental section and spectroscopic data of the new compound are provided. In addition, supporting information also describes the procedures for fermentation through OSMAC and feeding experiments, as well as for isolation of the compounds.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biotransformation · Endophytic fungus · *Epicoccum nigrum* · Flavonoids · Kaempferol derivatives

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Supporting Information

Biotransformation of Host Plant Flavonoids by the Fungal Endophyte *Epicoccum nigrum*

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Experimental Section

1. General experimental procedures

HPLC analysis was performed on a Dionex UltiMate3400 SD with an LPG-3400SD pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. ^{1}H, ^{13}C, and 2D NMR spectra were recorded at 297.9°K in CD_3OD on Bruker Avance III 600 or AV III HD 750 NMR spectrometers (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts are in ppm referring to the deuterated solvent peaks at δ_H 3.31 and δ_C 49.0 (MeOH-d_4) for ^{1}H and ^{13}C, respectively. LC-MS analysis was performed on a Dionex UltiMate3000 RS coupled to a Finnigan LCQ Deca mass spectrometer for low-resolution ESI mass spectra, meanwhile high-resolution ESI mass spectra were recorded on a UHR-QTOF maXis 4G (Bruker Daltonics) mass spectrometer. Optical rotations were determined on a Jasco P-2000 digital polarimeter (Jasco International, Tokyo, Japan). The separation column (125×4 mm, L×ID) was prefilled with Eurosphere-10C_{18} (Knauer, Germany), and the following gradient was used (MeOH, 0.1% HCOOH in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semi-preparative RP-HPLC was performed using a Merck Hitachi system (UV detector L-7400; pump L-7100; Eurosphere-100C_{18}, 300×8 mm, Merck KGaA, Darmstadt, Germany). Column chromatography included LH-20 Sephadex, and Merck silica gel 60 M (0.04 - 0.063 mm). TLC plates pre-coated with silica gel 60 F_{254} (Macherey-Nagel, Dueren, Germany) were used to monitor fractions, followed by detection under UV 254 and 366 nm or after spraying with anisaldehyde-sulfuric acid reagent.

2. Fungal material

The fungus *Epicoccum nigrum* was isolated from fresh and healthy leaves of *Salix* sp. (Salicaceae), collected in Caucasus mountain, Lago Naki, Russia, in May 2017. Isolation of fungi was achieved by the dilution plate method employing isolation medium (15 g/L bacto agar, 15 g/L malt extract in distilled water, at pH 7.4–7.8) supplied by chloramphenicol (0.20 g/L) and streptomycin sulfate (0.25 g/L) in order to inhibit the growth of bacteria and actinomycetes. The fungal strain was identified as *Epicoccum nigrum* (GenBank accession No. MK214079)[1] by amplification and sequencing of the ITS-Region including the 5.8S ribosomal DNA and subsequent BLAST search as previously described.[2] Voucher strain was deposited at the corresponding authors' laboratory (P.P.).

3. Fermentation and isolation

The fungal strain was cultivated on green lentil medium for the OSMAC (One Strain MAny Compounds) experiment, which was prepared by autoclaving 100 g of green lentil seeds and 110 mL of water in a 1 L Erlenmeyer flask prior to autoclaving. Large scale fermentation of this fungus was performed in 8 flasks for 14 days at room temperature under static condition. The culture was diced and extracted with EtOAc (5 L). The crude extract of *E. nigrum* (7.20 g) was partitioned by liquid-liquid extraction (LLE) using 90%MeOH-H_2O and n-hexane to yield the MeOH fraction (1.30 g), which was then directly subjected to size exclusion chromatography using a Sephadex LH-20 column (100×2.5 cm) with 100% MeOH as eluting solvent to yield six subfractions. Four subfractions HEN-D2−5 containing kaempferol derivatives (TLC monitoring) were selected for further purification via semi-preparative RP-HPLC using gradient elution of water and methanol yielding compounds **1** (0.56 mg), **2** (7.60 mg), and **3** (2.50 mg). In addition, green lentil seeds (*Teller Linsen*®) was used as a medium control and was prepared in the same manner as fungal fermentation and extraction steps. The green lentil extract (1.5 g) was partitioned by liquid-liquid extraction using 90%MeOH-H_2O and n-hexane to yield the MeOH fraction (0.50 g), which was then directly purified by semi-preparative RP-HPLC to afford compound **4** (0.83 mg). Furthermore, for the feeding experiments, cultures were grown in two flasks containing 10 g of solid rice medium each, which was spiked with 0.5% of either kaempferol (Sigma-Aldrich, Steinheim, Germany) or rutin (isolated from the dried flower buds of *Sophora japonica*) before autoclaving. Fungal control cultures were grown under the same conditions, along with the blanks containing only kaempferol or rutin on solid rice medium were also prepared. All samples were extracted with EtOAc and then analyzed chromatographically (HPLC-DAD). The EtOAc extract (118 mg) from feeding experiment with kaempferol was partitioned by LLE using 90%MeOH-H_2O and n-hexane to yield the MeOH fraction. This MeOH fraction was repartitioned by LLE using 50%EtOAc/H_2O to afford the EtOAc fraction. The latter fraction was subsequently applied on a C-18 reverse phase column using a step gradient of MeOH/H_2O, then subfraction HEN+K-R5 was purified by semi-preparative RP-HPLC using gradient elution of water and acetonitrile. The last purified sample HEN+K-R5-P1 containing kaempferol metabolites (HPLC monitoring) was selected for further LC-MS analysis using a step gradient of MeOH/H_2O starting from 5% to 95% MeOH for 10 min.

*3''-O-acetyl biorobin (**1**)*

yellow amorphous powder; $[\alpha]^{20}_{D}$: − 21.0° (*c* 0.20, MeOH); UV λ_{max} (MeOH) 265 nm and 345 nm; ^{1}H and ^{13}C NMR spectral data in MeOH-d_4, see Table S1; HRESIMS (+) *m*/*z* 637.1758 $[M + H]^+$ (calculated for $C_{29}H_{32}O_{16}$, 637.1763, Δ 0.5 mmu).

Table S1. NMR spectroscopic data of **1** at 600/750 (^{1}H) and 188 (^{13}C) (MeOH-d_4, δ in ppm).

Position	δ_H, mult. (*J* in Hz)	δ_C*	COSY	ROESY	HMBC
2		159.0			
3		135.2			
4		179.0			
5		162.7			
6	6.21, d (2.1)	99.7			5, 8, 10
7		166.1			
8	6.41, d (2.1)	94.6			6, 7, 9, 10
9		158.2			
10		105.2			
1’		122.2			
2’, 6’	8.09, d (8.9)	132.1	3’, 5’	3’, 5’	2’, 4’, 6’
3’, 5’	6.90, d (8.9)	115.8	2’, 6’	2’, 6’	1’, 3’, 4’, 5’
4’		161.4			
D-Gal					
1”	5.17, d (7.8)	104.9 d	2”	3”, 5”	3
2”	4.01, dd (10.2, 7.8)	70.1 d	1”, 3”	-	1”, 3”
3”	4.76, dd (10.2, 3.3)	77.1 d	2”, 4”	1”, 4”, 5”	2”
4”	3.96, d (3.3)	67.1 d	3”	3”	2”, 3”
5”	3.70, m*	74.6 d		1”, 3”	
6”	3.72, m*	66.4 t		4’’, 6”b, 1”’	5”
	3.35, m*			6”a, 1”’	
Acetyl					
CO	-	172.1			
CH_3	2.14, s	20.6			3”-OAc
L-Rha					
1”’	4.53, d (1.7)	101.5 d	2”’	6”a, 3”’	6”, 5”’
2”’	3.61, dd (3.3, 1.7)	71.8 d	1”’	3”’	3”’
3”’	3.49, dd (9.5, 3.3)	72.0 d		-	4”’
4”’	3.28, m*	73.4 d		6”’	3”’, 5”’, 6”’
5”’	3.52, dq (9.5, 6.2)	69.4 d	6”’	6”’	4”’
6”’	1.19, d (6.2)	17.6 q	5”’	4”’, 5”’	4”’, 5”’

[a] Data extracted from HSQC and HMBC, * Overlapped.

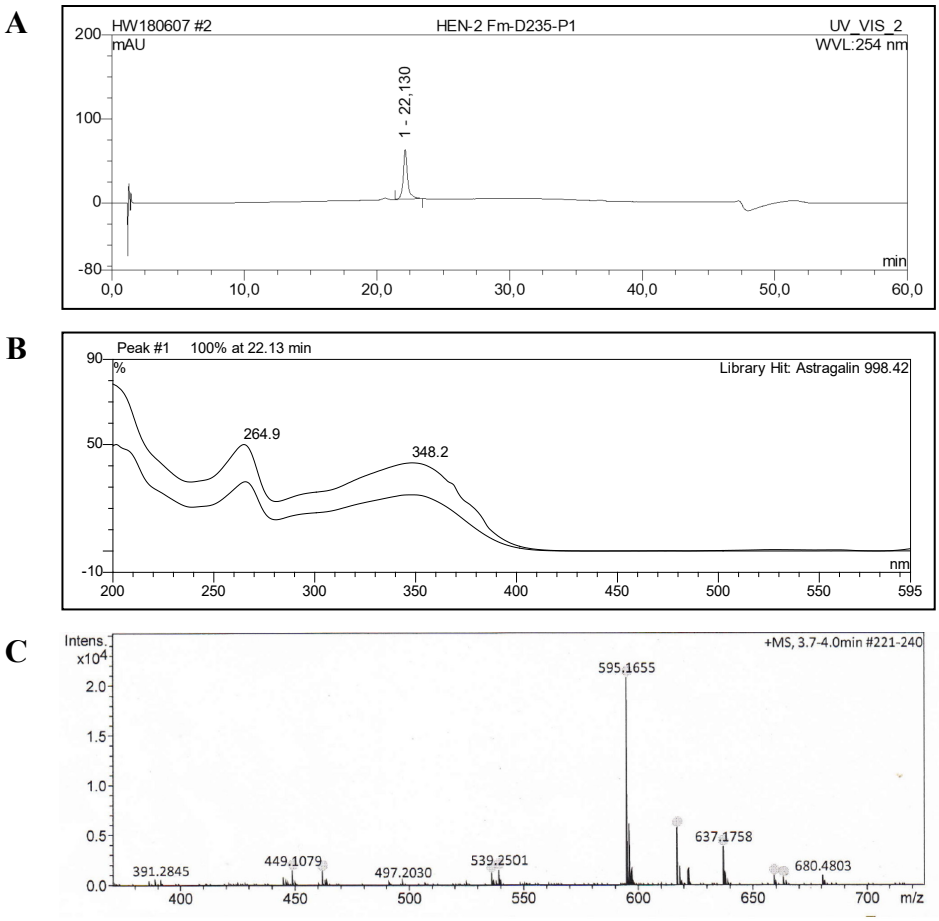


Figure S1. HPLC chromatogram (**A**), UV (**B**), and HRESIMS spectra (**C**) of **1**.

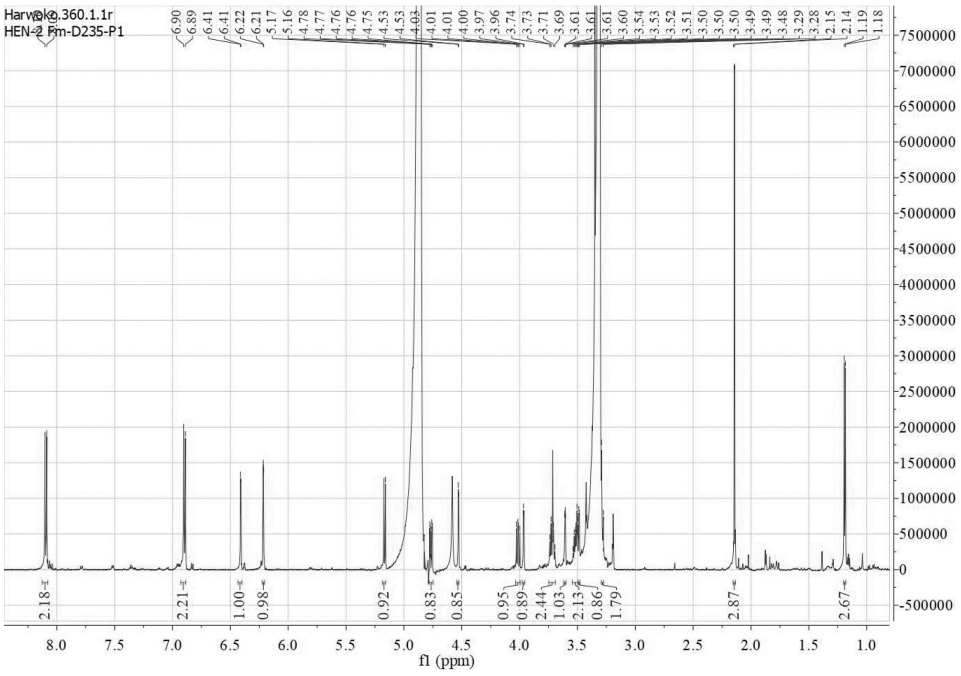


Figure S2. ^{1}H NMR spectrum of **1** in MeOH-d_4 (600 MHz; T=298 K).

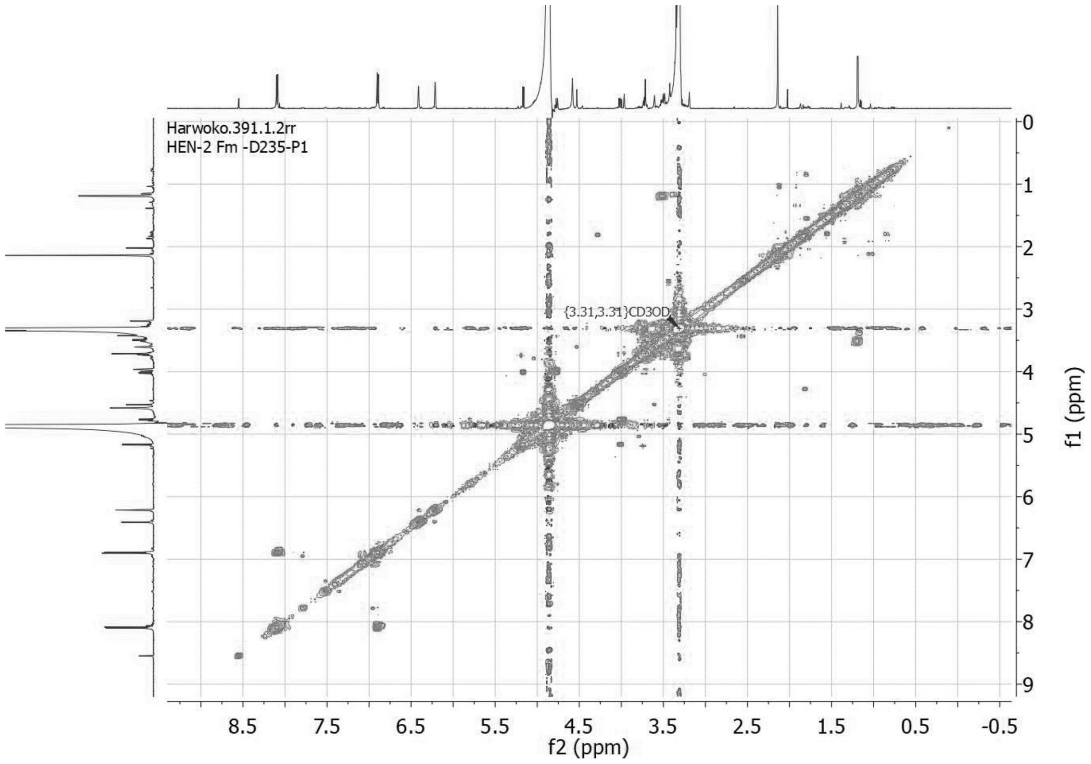


Figure S3. ^{1}H-^{1}H COSY spectrum of **1** in MeOH-d_4 (600 MHz; T=298 K).

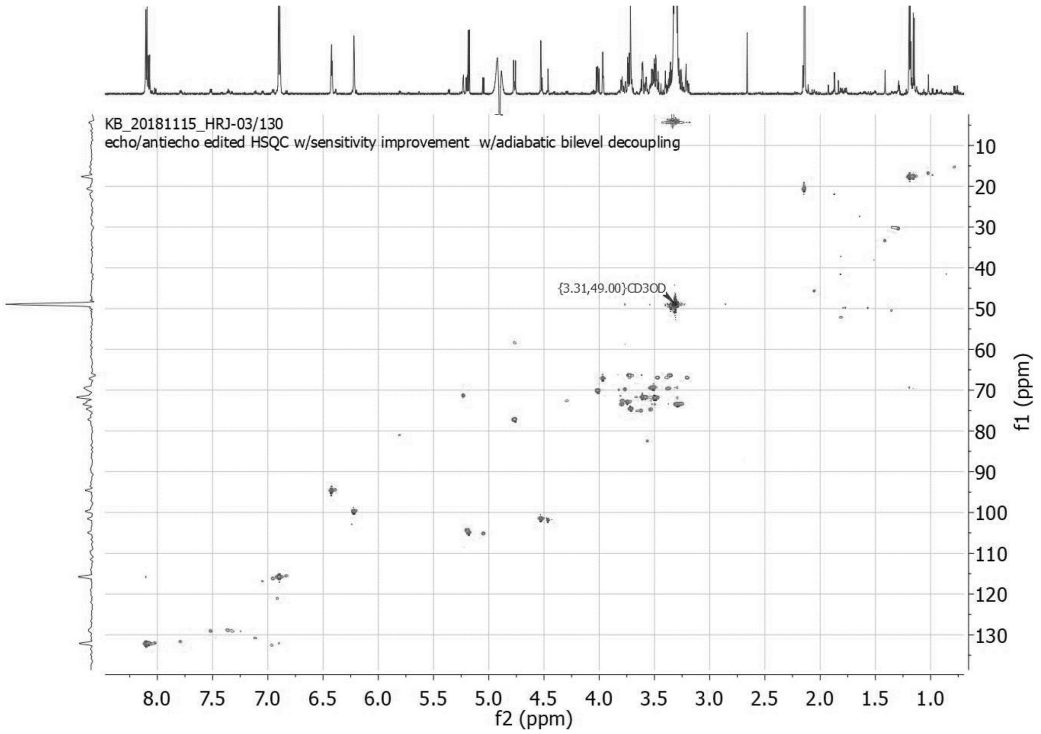


Figure S4. HSQC spectrum of **1** in MeOH-d_4 (750 and 188 MHz; T=293 K).

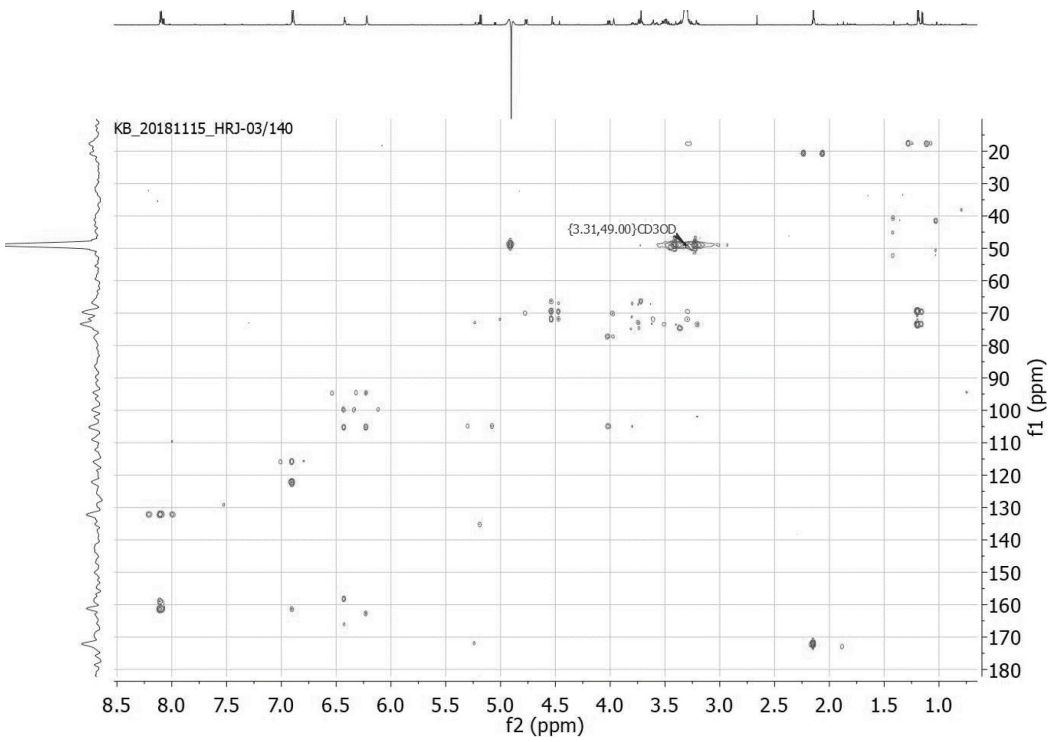


Figure S5. HMBC spectrum of **1** in MeOH-d_4 (750 and 188 MHz; T=293 K).

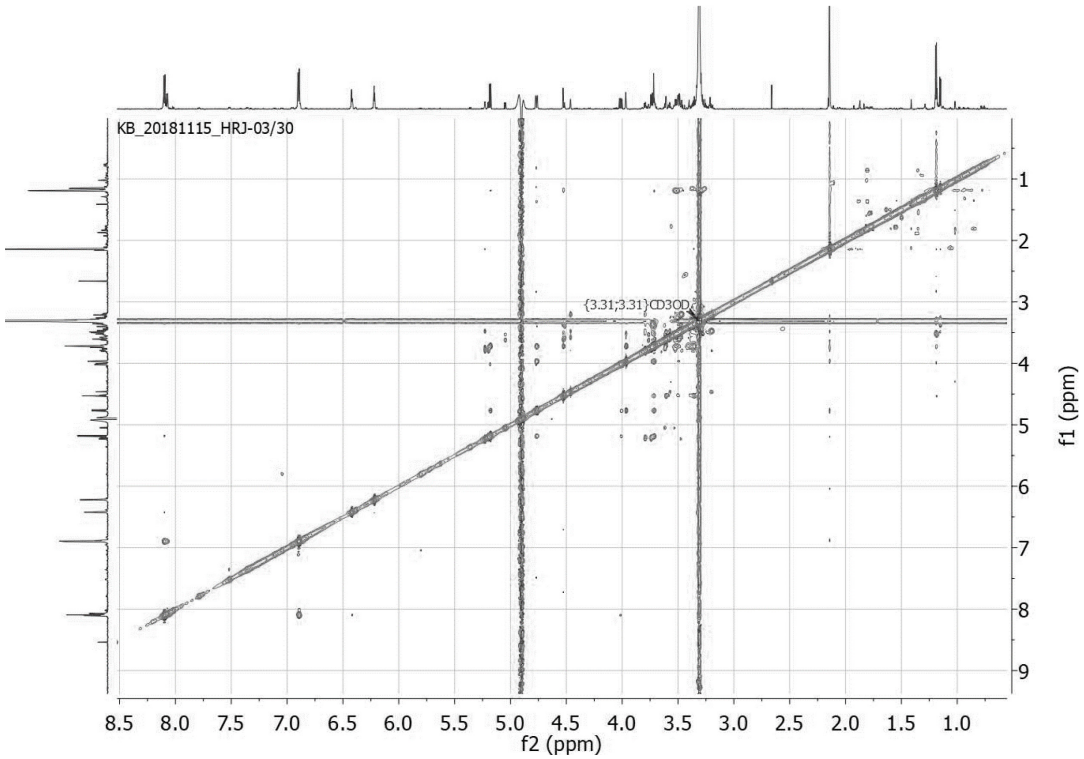


Figure S6. ROESY spectrum of **1** in MeOH-d_4 (750; T=293 K).

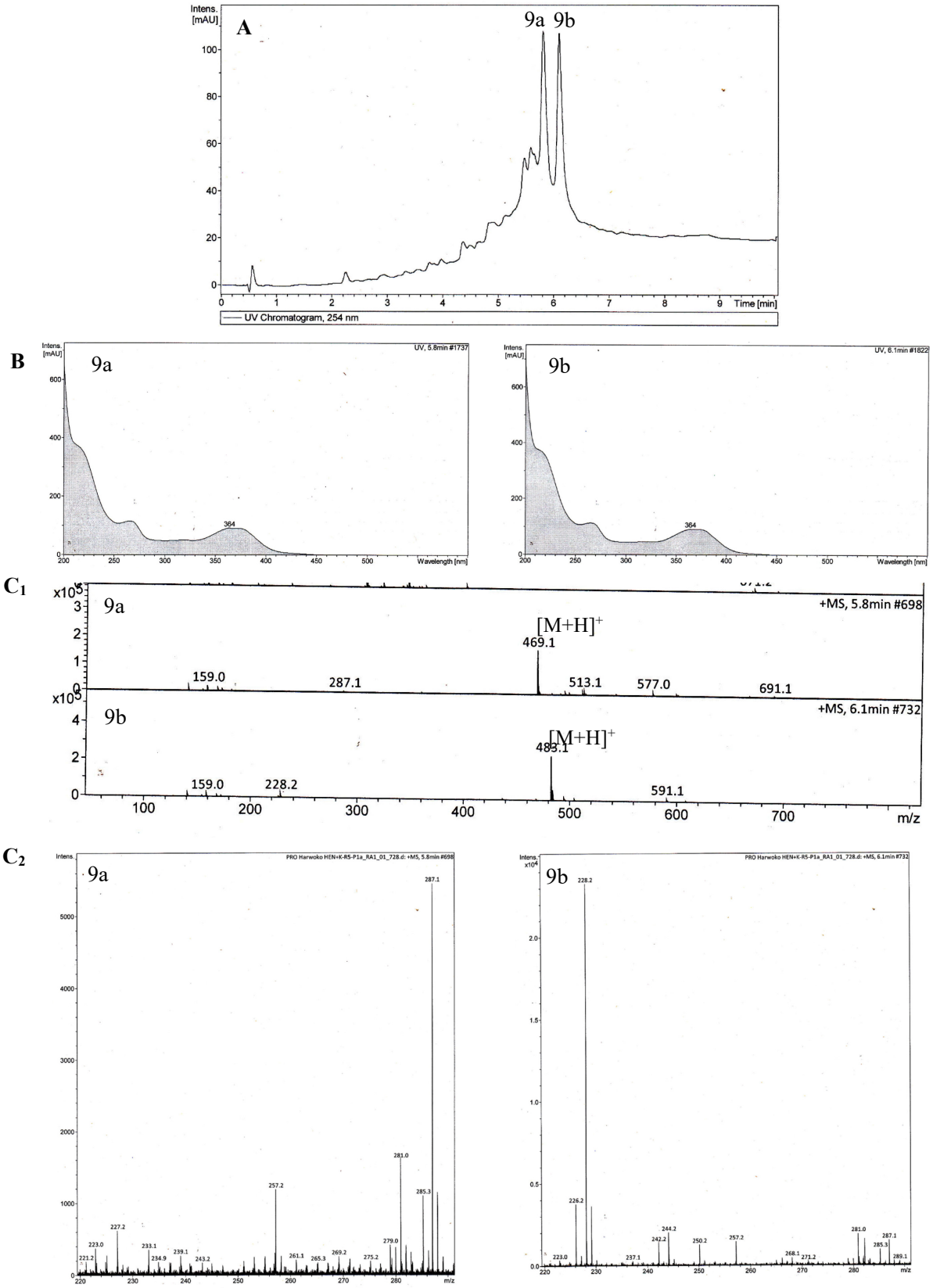
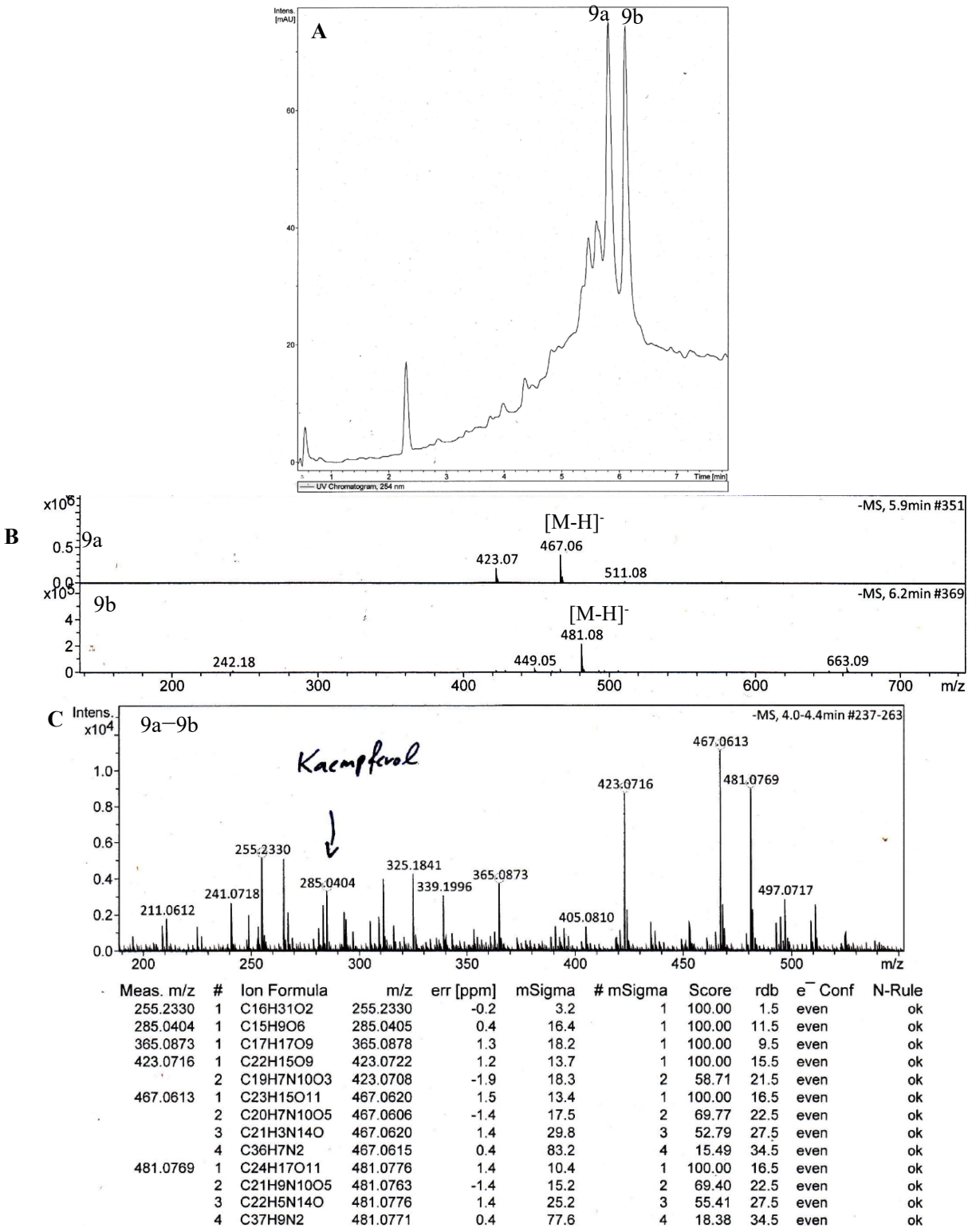


Figure S7. LC-MS elution time profile (**A**), UV (**B**), and ESIMS spectra at *m/z* 0−800 (**C**$_1$) or at *m/z* 220−290 (**C**$_2$) of peaks 9a−9b in positive mode.



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
255.2330	1	C16H31O2	255.2330	-0.2	3.2	1	100.00	1.5	even	ok
285.0404	1	C15H9O6	285.0405	0.4	16.4	1	100.00	11.5	even	ok
365.0873	1	C17H17O9	365.0878	1.3	18.2	1	100.00	9.5	even	ok
423.0716	1	C22H15O9	423.0722	1.2	13.7	1	100.00	15.5	even	ok
	2	C19H7N10O3	423.0708	-1.9	18.3	2	58.71	21.5	even	ok
467.0613	1	C23H15O11	467.0620	1.5	13.4	1	100.00	16.5	even	ok
	2	C20H7N10O5	467.0606	-1.4	17.5	2	69.77	22.5	even	ok
	3	C21H3N14O	467.0620	1.4	29.8	3	52.79	27.5	even	ok
	4	C36H7N2	467.0615	0.4	83.2	4	15.49	34.5	even	ok
481.0769	1	C24H17O11	481.0776	1.4	10.4	1	100.00	16.5	even	ok
	2	C21H9N10O5	481.0763	-1.4	15.2	2	69.40	22.5	even	ok
	3	C22H5N14O	481.0776	1.4	25.2	3	55.41	27.5	even	ok
	4	C37H9N2	481.0771	0.4	77.6	4	18.38	34.5	even	ok

Figure S8. LC-MS elution time profile (**A**), ESIMS (**B**), and HRESIMS (**C**) spectra of peaks 9a−9b in negative mode.

References

(1) H. Harwoko, G. Daletos, F. Stuhldreier, J. Lee, S. Wesselborg, M. Feldbrügge, W. E. G. Müller, R. Kalscheuer, E. Ancheeva, P. Proksch. *Nat. Prod. Res*. **2019** doi: 10.1080/14786419.2019.1627348.
(2) J. Kjer, A. Debbab, A. H. Aly, P. Proksch. *Nat. Protoc*. **2010**, *5*, 479−490.

CHAPTER 5
Discussion

5.1 Chemical diversity of novel compounds from two investigated fungi

Two investigated fungi belonging to the species of *T. harzianum* and *E. nigrum* were isolated from the medicinal plants ginger (*Zingiber officinale*) and willow (*Salix* sp.), respectively. The secondary metabolites that were obtained from fermentations of these endophytic fungi belong to rare classes such as epidithiodiketopiperazines (ETPs), flavipin-derived alkaloids and flavonoid glycosides. ETPs were produced by both species, while the other two classes are rarely found in fungi and were isolated only from *E. nigrum*. The structures of new compounds produced by these fungi are shown in Fig. 5.1.

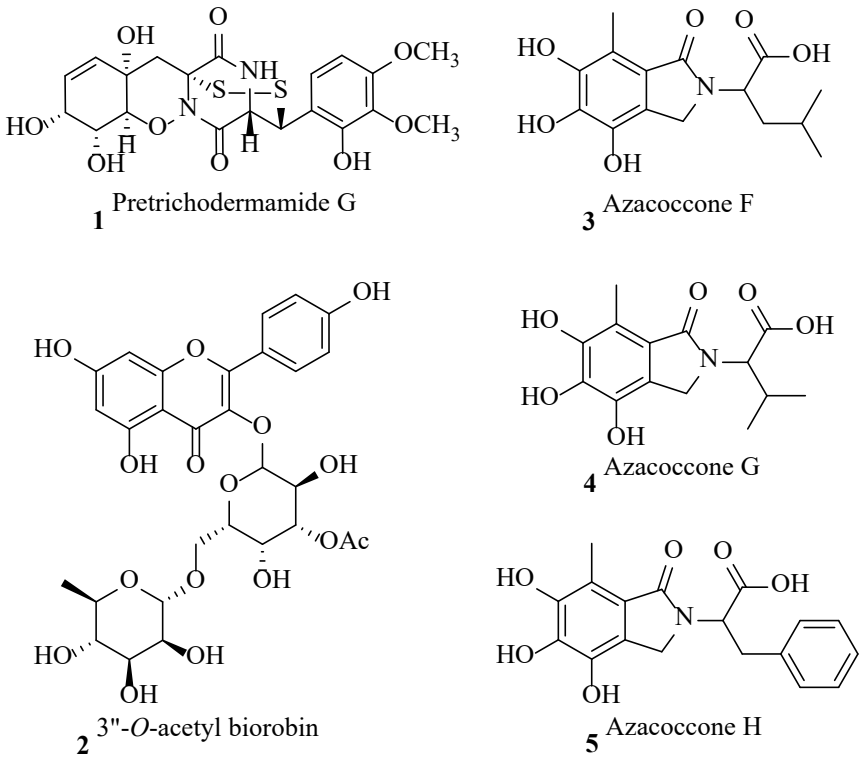


Figure 5.1. Chemical diversity of new compounds isolated from *Trichoderma harzianum* MK213940 (**1**) and *Epicoccum nigrum* MK214079 (**2**–**5**).

ETPs are generally characterized by the occurrence of a disulfide bridge or alternatively two methylsulfides, with the sulfur-atoms most commonly attached to the α-positions of a cyclic dipeptide (Seephonkai *et al.*, 2006). This study discovered unusual ETPs featuring a rare 1,2-oxazadecaline skeleton with the disulfide bridge attached to the α- and β-positions of the

amino acid residues, as in the case of pretrichodermamides A and G isolated from *T. harzianum* MK213940 (chapter 2). It is noteworthy to mention that only few metabolites feature this unusual scaffold and only nineteen such ETPs were described until now. Most of the reported examples originate from the genera *Trichoderma* spp. and *Penicillium* spp. Examples from the genus *Trichoderma* include: Gliotoxin, gliovirin, DC1149B, DC1149R, iododithiobrevamide, chlorotrithiobrevamide, as well as pretrichodermamides A and G (Weindling and Emerson, 1936; Seephonkai *et al.,* 2006; Yamazaki *et al.,* 2015[ab]; Yurchenko *et al.,* 2016; Harwoko *et al.*, 2019[a]). Other ETPs such as N-methylgliovirin (FA-2097), N-methylpretrichodermamide B, pretrichodermamides C–F, outovirins A–C and peniciadametizine A are produced by the genus *Penicillium* (Yokose *et al.,* 1984; Liu *et al.*, 2015; Orfali *et al.,* 2015; Kajula *et al.,* 2016; Yurchenko *et al.,* 2016). To date, to the best of our knowledge only two structurally related compounds were discovered in another fungal genus: Aspirochlorine and pretrichodermamide A were isolated from *Aspergillus* sp. (Sakata *et al.*, 1982; Zhou *et al.*, 2013).

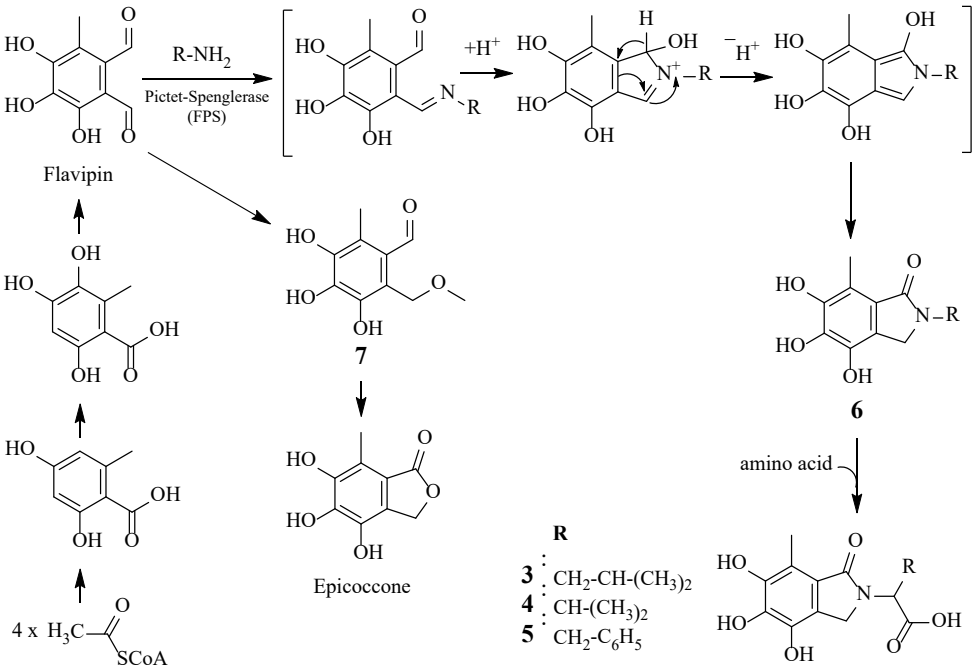
The second compound class discussed in this study are flavipin-derived alkaloids, which belong to atypical fungal alkaloids. Their rather simple structures are comprised of an epicoccone core that is forming an isoindolin-1-one ring system with the nitrogen of an attached amino acid. The structures of the unusual alkaloids azacoccones F−H (**3**–**5**) that were discovered from *E. nigrum* MK214079 residing in leaves of *Salix* sp. are displayed in Fig. 5.1. These compounds, impart unique because of their biosynthetic origin via Pictet-Spengler (PS) reaction, are rarely found in fungi. This PS-reaction is characterized by a condensation of a *β*-arylethylamine with an aldehyde to form a piperidine ring (Yan *et al.*, 2014[b]). Evidence for the biosynthetic formation of PS-products has been presented in the form of a published fungal Pictet-Spenglerase (FPS) gene that was detected in *Chaetomium globosum* 1C51 and is involved in the biosynthesis of several PS-derived natural products. This finding underlines the distinctive nature of fungal metabolites such as chaetoglines A–H and azacoccones A–E which are respectively produced by *C. globosum* and *A. flavipes* (Yan *et al*., 2014[b]; Zou *et al.*, 2018). Interestingly, this PS-reaction is initially involved in the biosynthesis of remarkable plant-alkaloids, such as morphine, reserpine and camptothecin. Moreover, this reaction has been shown to be naturally occurring in bacteria and marine sponges during the biosynthesis of tetrahydroisoquinoline antibiotics and hyrtioreticulin F, respectively (Yan *et al*., 2014[b]).

The present study classifies these new azacoccones F−H as flavipin-derived alkaloids, thereby contributing to the small number of known examples of this rare compound class (Zou *et al.*, 2018). The new compounds **3**−**5** are presumably generated via unusual PS-reaction from

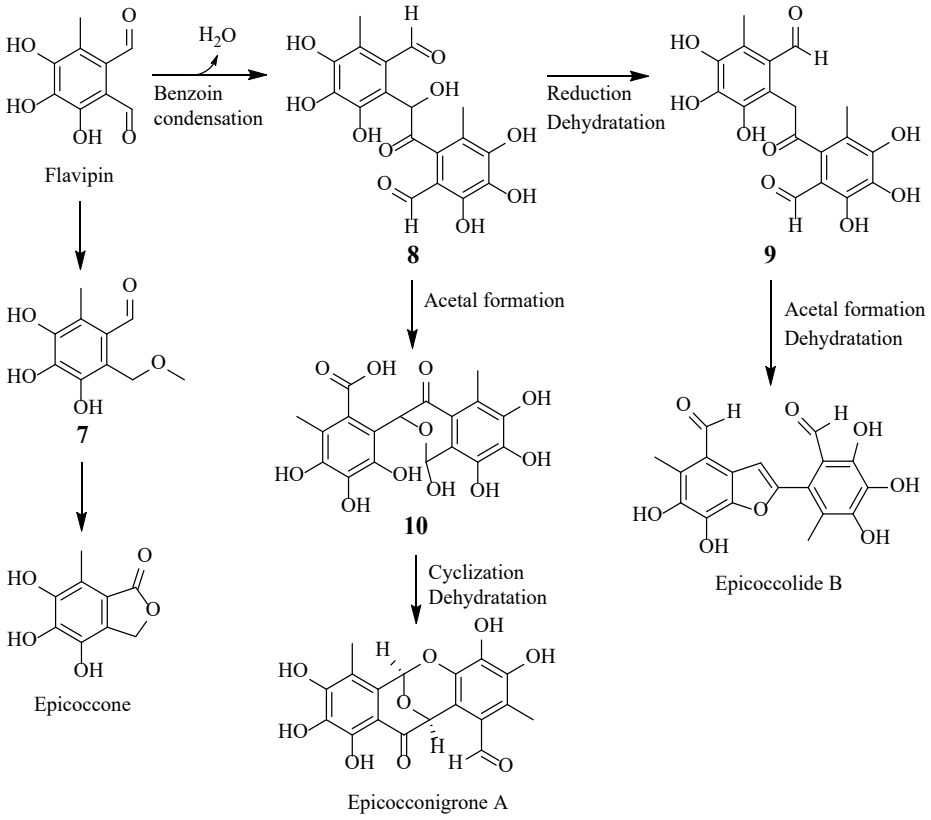
the major precursor flavipin, similar to the biosynthesis of chaetoglines and azacoccones (Yan *et al.*, 2014[b]; Zou *et al.*, 2018). An inspiring hypothesis proposed by Zou *et al*. (2018) leads us to provide further insight into a postulated biosynthesis of the flavipin-derived alkaloids (**3**−**5)** as illustrated in Scheme 1. The proposed biosynthetic pathway of azacoccones F−H starts from flavipin as precursor and undergoes a PS-reaction catalyzed by an FPS to afford **6** as key intermediate (Scheme 1). The core structure of key intermediate (**6**) was proposed as aza-epicoccone, as it is identical to epicoccone except for the replacement of an oxygen atom by a nitrogen atom (Zou *et al.*, 2018). This is further supported by the structural similarity of **3**−**5** to other compounds such as chaetoglines C and D, which were produced during the co-exposure of flavipin and 1-methyl-L-tryptopan to the fungal enzyme (Yan *et al.*, 2014[b]). In fact, the new alkaloids **3**−**5** are leucine, valine and phenylalanine analogues of chaetoglines C/D. Hence, they are likely constructed via the same route with the final step presumably being the fusion of key intermediate (**6**) with the respective amino acid.

The biosynthetic pathways of polyketides that originate from flavipin, such as epicoccones, epicocconigrones and epicoccolides have previously been postulated (Talontsi *et al.*, 2013; El Amrani, 2014). Because epicoccone originates from flavipin as well, related compounds epicocconigrone A and epicoccolide B should have the same biogenetic origin. Accordingly, this study proposes the plausible biogenetic pathways for epicocconigrone A and epicoccolide B as illustrated in Scheme 2. Both polyketides are formed through asymmetrical benzoin condensation of flavipin catalyzed by thiamin to yield intermediate (**8**). The reductive cyclization of **8** via a compound such as **9** followed by acetalization and loss of water would afford epicoccolide B. Initially, following the same route to afford intermediate compound **8** and subsequent ring closure via **10**, continued by cyclization and loss of water would be the last steps to yield epicocconigrone A (Talontsi *et al.*, 2013). Another hypothesis proposed the involvement of two possible intermediates, such as orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid to construct this latter compound (El Amrani, 2016).

The last discussed compound class of this study are flavonoids, which are hitherto mostly known from plants. The discovery of flavonoids from fungal endophytes highlights the remarkable biosynthetic capability of fungi in mimicking plant-derived natural products (Ebada *et al.*, 2016). One of the flavonoid-producing endophytic fungi is *E. nigrum* that afforded three flavonoids, including 3”-*O*-acetyl biorobin, biorobin and kaempferol (Harwoko *et al*., 2019[b]).



Scheme 1. A plausible biogenetic pathway of flavipin-derived alkaloids (**3**−**5**) as postulated by Zou *et al.* (2018) for related compounds.



Scheme 2. A plausible biogenetic pathways of flavipin-derived polyketides as proposed by Talontsi *et al.* (2013) and El Amrani (2016).

All isolated compounds feature the same kaempferol aglycone, while the glycosidic part is composed of two additional sugar moieties such as galactose and rhamnose. Moreover, this study indicates that in fungi attachment of galactose vs. glucose to the aglycone rarely occurs via glycosylation as exemplified by hyperin from previous work (Ebada *et al.*, 2016). The presence of flavonoid glycosides during fungal fermentation on green lentil solid medium is congruent with previous descriptions of *E. nigrum* fermented on solid corn medium (Barbu *et al.*, 2006). Unlike the aforementioned classes of alkaloids that were naturally formed via *de novo* biosynthetis, noteworthy these flavonoids originated via biotransformation reactions (chapter 4).

5.2 Bioactive secondary metabolites isolated from two biocontrol fungi

Fungal strains of the genus of *Trichoderma* and *Epicoccum* are largely used as biological control agents and they are even commercially available worldwide as products for agricultural purposes (Harman, 2000; Koutb and Ali, 2010). As already described, the ETP class is derived from diketopiperazines (DKPs) featuring a unique scaffold containing a six-membered ring separated by amide linkages along with two nitrogen atoms of a piperazine, which is overarched by a disulfide bridge (Fatima *et al.,* 2016). DKPs are biosynthesized via condensation of two α-amino acids by nonribosomal peptide synthetases (NRPSs) or cyclodipeptide synthases and they are frequently found in the fungal kingdom (Braga *et al*., 2018). This study re-isolated DKP derivatives with the disulfide functionality attached to the adjacent amides as is typical for epicorazines A–C, epicoccins A–T and ent-epicoccin G (Kleinwächter *et al*., 2001; Zhang *et al.*, 2007; Guo *et al.,* 2009; Wang *et al.,* 2010). While these compounds have been previously isolated from *E. nigrum*, they are still chemically and pharmacologically interesting due to the diversity of scaffolds and/or biological activities.

Nowadays, over fifty percent of small molecules approved for anticancer drugs worldwide are natural products or derived from natural products (Newman and Cragg, 2016). For instance, epicorazine A obtained from *E. nigrum* MK214079 revealed pronounced antiproliferative effects in different tumor cell lines as supported by previous studies (Kleinwächter *et al*., 2001; Kong *et al.*, 2014). Cytotoxic activity against mouse and human carcinoma cell lines exhibited by epicorazine A ranges between IC_{50} values of 1.3 to 28 µM (chapter 2). Therefore, we carried out further mechanistic studies and newly provided evidence

that epicorazine A induced apopotic cell death through the intrinsic and extrinsic pathway (chapter 2). Moreover, epicorazine A exhibited antifungal activity against corn smut (Harwoko *et al*., 2019[a]) and was also reported active against *Candida albicans* (Kleinwächter *et al*., 2001). Meanwhile, ent-epicoccine G was inactive towards tumor cell lines and microbial pathogens tested in this study (Harwoko *et al.*, 2019[a]). Nonetheless, it possessed anti-inflammatory property through *in vitro* inhibition of the release of *β*-glucuronidase in rat polymorphonuclear leukocytes induced by platelet activating factor (Wang *et al*., 2010). Among the isolated ETPs, pretrichodermamide A selectively inhibited the growth of *Ustilago maydis* and furthermore exhibited mild antitubercular effect as described in the literature (Seephonkai *et al.,* 2006). However, it did not reveal cytotoxic activity against both cancer cell lines and against Vero cells (Seephonkai *et al.,* 2006).

The known bioactive compounds from *E. nigrum* include epipyrone A, epicocconigrone A, epicoccamide A and epicoccolide B. They showed antimicrobial and antinematode activities as displayed in Table 5.1. The major orange pigment from the extract of *E. nigrum*, epipyrone A, displays antibacterial and antifungal properties. In addition, epipyrone A has been reported as an NF-κB inhibitor, and as anti-viral (influenza A) and fungicidal agents (Peng *et al*., 2012; Van Ginkel *et al*., 2012). The flavipin-derived polyketides such as epicocconigrone A and epicoccolide B revealed antinematode activity, which is plausible because their biosynthetic precursor flavipin is well known to antagonize plant parasitic nematodes (Nitao *et al*., 2002). Besides pretrichodermamide A, it is noteworthy to mention that epicoccamide A is reported for the first time to reveal selective antifungal activity towards *U. maydis*, as this tetramic acid derivative has been reported as being inactive in various bioassays performed during previous research. However, only epicoccamide D possessing the longest chain exhibited cytotoxic potential (Wright *et al*., 2003; Wangun *et al*., 2007).

The new compounds showed no significant effects in several bioactivity studies (chapters 2–3). Similarly, none of the secondary metabolites in this study reveals cytotoxic activity when tested at doses of 20–30 μM, except for epicorazine A. However, epicocconigrone A and epicoccolide B have potential as *in vitro* HDACs inhibitors in order to suppress cancer cell growth and should be considered as potential anticancer agents (El Amrani *et al*., 2014). Even though, epipyrone A is patented as telomerase inhibitor aiming to limit cancer cell proliferation (Kanai *et al*., 2007; Peng *et al*., 2012), it showed no cytotoxicity neither in this work nor in the literature (Hufendiek, 2017). Among the aza-epicoccone derivatives, only azacoccones C and E possessed strong anti-oxidant properties (Zou *et al*., 2018). Unexpectedly,

the latter compound showed cytotoxic potential mainly towards breast cancer through selective inhibition of 3-phosphoglycerate dehydrogenase (PHGDH) (Guo *et al*., 2019[a]). In addition, both chaetoglines B and F exhibited better antibacterial activity than the clinically used antibiotic, tinidazole (Yan *et al.*, 2014[b]). Remarkably, only chaetogline F can *potentially* be developed as acetylcholinesterase (AChE) inhibitor for treating Alzheimer's disease (Ge *et al*., 2008).

Table 5.1. Summary of bioactive compounds produced by *Trichoderma harzianum* and *Epicoccum nigrum*

Compound	Bioactivity			
	Antibacterial	**Antifungal[c]**	**Antinematode[d]**	**Anticancer[e]**
Pretrichodermamide A*	√ (a)	√	-	-
Epicorazine A	-	√	-	√
Epicoccamide A	-	√	-	-
Epipyrone A	√ (b)	√	√	-
Epicocconigrone A	√ (b)	√	√	-
Epicoccolide B	√ (b)	-	√	-

a : *Mycobacterium tuberculosis* H37Rv, b : *Staphylococcus aureus* ATCC 29213,

c : *Ustilago maydis* AB33, d : *Heterodera schachtii*,

e : Mouse and human carcinoma cell lines,

* Data is adapted from Harwoko *et al*., 2019[a], √ : active, - : inactive

5.3 Preliminary structure-activity relationships of the isolated compounds

A preliminary structure-activity relationship is proposed based on the described results as well as based on comparison with the literature. All bioactive compounds (Table 5.1) feature a *β*-hydroxy-*α,β*-unsaturated carbonyl moiety, which is absent in the new and inactive metabolites. The study implies that this moiety is likely to be responsible for the biological properties. The previous studies on another *E. nigrum* (syn. *E. purpurascens*) strain confirm that the bioactive metabolites possess this pharmacopore, i.e. in epicoccones A−B, epicoccolides A−B and epicocconigrones A−B that all have a broad spectrum of bioactivities (Abdel-Lateff *et al*., 2003; Talontsi *et al.*, 2013; El Amrani *et al*., 2014). Meanwhile, the bioactive compound from *T. harzianum* pretrichodermamide A exhibits antimicrobial properties either as fungicidal or antibacterial agent (chapter 2). Antifungal effects of pretrichodermamide A are presumably related to the structure of the cyclohexene unit, suggesting that it is important

as pharmacophore. Hence, substitution pattern and/or location of double bonds in this unit might influence its activity. Few fungal natural products belonging to the ETPs class show antifungal activity, for instance peniciadametizine B, obtained from a *Penicillium* strain exhibited selective antifungal activity against *Alternaria brassicae* (Liu *et al*., 2015). Similar to pretrichodermamide A, the active site of this compound is probably located in the *β*-hydroxy-*α,β*-unsaturated carbonyl fragment. Another example, aspirochlorine is firstly known as antibiotic A30641 isolated from *Aspergillus* sp. revealed *in vitro* antimicrobial effects against fungi and Gram-positive bacteria (Berg *et al*., 1976). Remarkably, antibiotic properties of aspirochlorine might be affected by the presence of a chlorine atom. Chlorine atoms can be vital for the antibacterial activity, as exemplified by chlorflavonin, which is strongly active against *Mycobacterium tuberculosis* (MIC_{90} 1.56 μM), while dechlorflavonin was completely inactive (Rehberg *et al.,* 2018).

Epipolythiodioxopiperazines (ETPx) are well known as mycotoxins produced by fungi and they feature an internal disulfide bond, which causes toxicity to animals or humans, e.g. gliotoxin is the best example. The toxicity mechanism of ETPx is mediated by inactivating proteins via reaction with thiol groups and subsequent by producing reactive oxygen species via redox cycling. The unique nature of a disulphide bridge and the mode of action of ETPx molecules attracts attention for design of future chemopreventive and chemotherapeutic agents (Gardiner *et al*., 2005). A functional group such as *β*-hydroxy-*α,β*-unsaturated carbonyl contributes to the cytotoxic properties of structurally distinct compounds, which is well documented in the literature (Nakayachi *et al.,* 2004). Interestingly, the *α,β*-unsaturated carbonyl compounds are intensively studied for drug design related to their cytotoxicity, anti-inflammatory, antioxidant and many other properties (Amslinger, 2010).

The initial screening for cytotoxicity of the isolated compounds indicated that only epicorazine A has potent cytotoxicity by inducing apoptosis (chapter 2). However, the new and related compounds, e.g. pretrichodermamides A–G and azacoccones A–H show no or low cytotoxicity (Yurchenko *et al.,* 2016; Zou *et al.*, 2018; Harwoko *et al*., 2019[a]). Structures of the cytotoxic compounds include trichodermamide B, N-methylpretrichodermamide B, chlorotrithiobrevamide, and chloro-ETP (DC1149B). They bear a chlorine atom that plays a crucial role for their cytotoxicity (Garo *et al.,* 2003; Orfali *et al.,* 2015; Yamazaki *et al.,* 2015[ab]; Jans *et al.,* 2017). It implies that a chlorohydrin moiety would be the precursor to a reactive epoxide in the active form of the ETP derivatives (Garo *et al.,* 2003). However, when this reactive moiety is replaced by a trans vicinal diol in ETPs such as pretrichodermamides A–G

or trichodermamides A and C, their cytotoxicity would be reduced or even eliminated (Orfali *et al.,* 2015; Yurchenko *et al.,* 2016; Jans *et al.,* 2017; Harwoko *et al*., 2019[a]).

5.4 The ability of certain fungal endophytes to produce plant flavonoids

The fact that almost 70% of microbial biosynthetic gene clusters remain silent under standard laboratory culture conditions, severely limits the diversity of discovered microbial secondary metabolites (MSMs) (Fisch *et al*., 2009). This bottleneck can be overcome by applying an OSMAC (One Strain MAny Compounds) technique in order to stimulate the biosynthesis of MSMs. Biosynthesis of fungal metabolites can be influenced by modifying the culture parameters, e.g., alterations in media composition, pH value, temperature, oxygen, light, even the shape of the cultural vessel (Pan *et al*., 2019). Nowadays, OSMAC method can even be combined with modern tools such as molecular networking coupled with bioactivity mapping (Fan *et al*., 2019). These tandem approaches have been proven to be effective in setting up the optimal culturing conditions as well as to foresee a visual identity of the structural and functional diversity of the fungal extracts before proceeding to the purification and structure elucidation (Fan *et al*., 2019).

Flavonoids are generally accepted by the scientific community as plant secondary metabolites. An OSMAC approach was implemented in this study through cultivation of *E. nigrum* on green lentil solid medium. Interestingly, kaempferol derivatives are produced from fermentation of this fungal species on solid medium. However, flavonoids are rarely reported so far to be produced by endophytic fungi as proven unequivocally in this study. Similarly, culture broth of *E. nigrum* on solid corn medium afforded flavonoid glycosides, even though solely analyzed by UV-Vis spectrophotometry and HPLC (Barbu *et al.*, 2006). In a very rare case, the previous work with *Nigrospora oryzae* and *Mucor irregularis* was able to yield quercetin monoglycosides and chlorflavonin, even though these fungi were fermented on solid rice medium (Ebada *et al.*, 2016; Rehberg *et al.,* 2018). In contrast, the flavonoid-producing fungi are commonly cultivated on liquid media to produce flavonoid and phenolic compounds. For example, the plant-associated fungi *Aspergillus* sp. and *Pestalotiopsis* sp. that were cultured on liquid potato dextrose agar (PDA), CzapekDox broth and liquid Sabouraud broth (SDB) were able to produce plant flavonoids (Qiu *et al*., 2010; Patil *et al*., 2015; Qian *et al*., 2017). However, in some cases it is confirmed that the presence of flavonoids in fungal

endophytes mimics those found found in host plants (Cheng *et al.,* 2012; Cui *et al*., 2012; Patil *et al*., 2015; Ebada *et al.*, 2016).

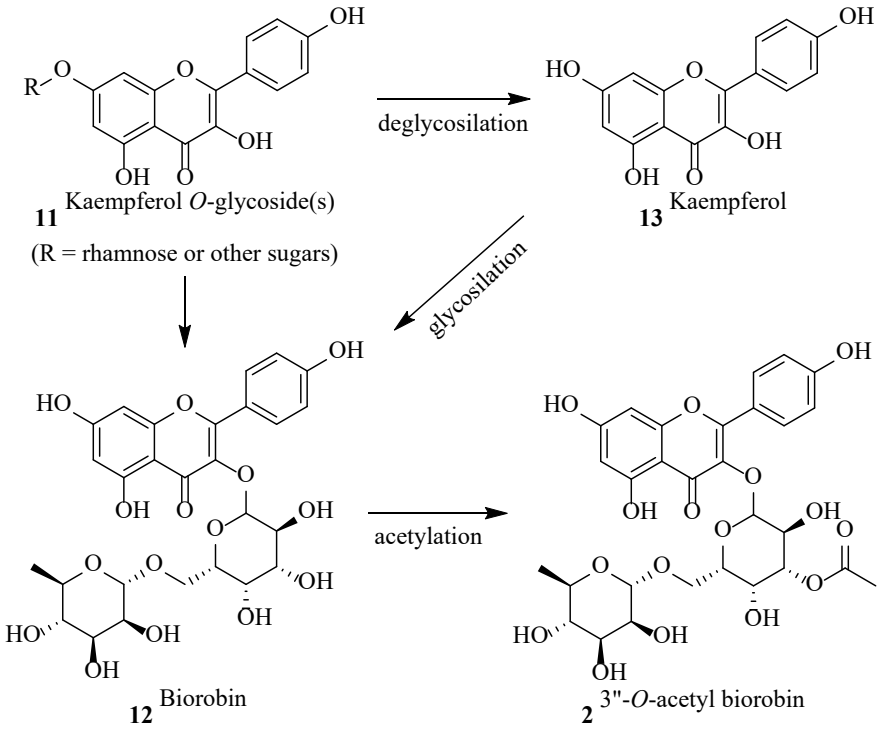
In fact, most flavonoid-producing fungi are associated with medicinal plants (Huang *et al*., 2007). For example, ginkgolide B is the bioactive flavonoid produced by *Fusarium oxysporum* obtained from the root bark of *Gingko biloba* (Cui *et al*., 2012). Other fungal species include *Pestalotiopsis uvicola* and *Annulohypoxylon squamulosum*, which were respectively isolated from *Artemisia japonica* and *Cinnamomum* sp. and yielded rutin, kaempferol and quercetin (Cheng *et al.,* 2012; Qian *et al.,* 2017). The previous research on the fungus *N. oryzae* isolated from the Nigerian mistletoe *Loranthus micranthus* yielded hyperin, isoquercetrin and guaijaverin as fungal constituents. Those are flavonoid monoglycosides bearing different sugar moieties (Ebada *et al.,* 2016). Remarkably, an eminent fungal flavonoid is chlorflavonin produced by *M. irregularis* residing in leaves of the Cameroonian medicinal plant *Moringa stenopetala* as recently published (Rehberg *et al.,* 2018). Interestingly, it may be possible to develop chlorflavonin as an anti-TB agent due to its potent antitubercular activity at non-toxic doses by inhibiting acetohydroxyacid synthase catalytic subunit IlvB1 (Rehberg *et al.,* 2018). Thus, those examples corroborate the plasticity of secondary metabolism of endophytic fungi exhibiting their capability to generate the same or similar compounds as present in plants (Ebada *et al.,* 2016).

5.5 Biotransformation reactions performed by the fungus *Epicoccum nigrum*

Biotransformation of natural products by filamentous fungi is a powerful strategy to provide valuable MSMs with promising bioactivities. In most cases such as drug synthesis, it is used for catalysis of particular steps from a drug lead to the final product. Biotransformation processes have been used to produce foods and beverages, in addition to metabolysing xenobiotics as well as to break down toxic and hazardous substances in bioremediation. Production or modification of value-added products through derivatization can be obtained by biotransformation using precursor-directed biosynthesis (Schneider *et al*., 2008; Hüttel and Hoffmeister, 2010).

Epicoccum nigrum was recently reported to yield the cardiac glycoside digoxin (El-Sayed *et al.*, 2020), in addition to its remarkable ability to produce paclitaxel (Somjaipeng *et al*., 2016). Fermentation of *E. nigrum* on a solid medium composed of green lentils afforded kaempferol derivatives including a new acetylated flavonol (**2**) as shown in Fig. 5.2.

Unexpectedly, the extract of green lentils as solid medium was dominated by kaempferol 7-*O*-rhamnoside (**11**) which was missing in the fungal extract. However, the isolated flavonoids were absent in cultivation of *E. nigrum* on solid rice medium used as control. Hence, the study suggests that kaempferol derivatives are produced via fungal biotransformation of host plant flavonoids rather than by *de novo* biosynthesis as illustrated in Fig. 5.2. Collectively, the fungal flavonoids in this study may be formed through hydrolytic cleavage of kaempferol glycosides such as **11** followed by glycosylation and acetylation. It is noteworthy to mention that glycosylation reactions by adding galactose to kaempferol as the building block are rarely found in fungi (Xiao *et al*., 2014; Ebada *et al*., 2016).



11 **13** **12** **2**

Figure 5.2. Biotransformation mediated by *Epicoccum nigrum* to obtain flavonoids (**2**, **12** and **13**).

A remarkable finding from an OSMAC approach lead us to conduct further feeding experiments by adding precursors rutin or kaempferol to cultures of *E. nigrum* on solid rice medium. It was aimed to prove our hypothesis that the isolated kaempferol derivatives (**2**, **12**, **13** in Fig. 5.2) are biotransformation products of flavonoids from the green lentil medium rather than being formed *de novo* by the fungus. These precursors represented the flavonol diglycoside and the flavonol aglycone, while the solid rice media was free from flavonoid. Concisely, these feeding experiments proved that the fungal biotransformation was able to hydrolyze flavonol *O*-glycosides (rutin) into its aglycone (quercetin) probably through *β*-glucosidases produced by

the fungus (Ajila *et al*., 2012). Fungal polysaccharide-degrading enzymes have been described in the literature such as polygalacturonase, pectin-lyase, cellulase, arabanase and xylanase produced by *E. nigrum* that grew on flax stem tissue (Brown *et al*., 1984). Meanwhile, kaempferol as a precursor was biotransformed into two small peaks with high polarity and UV absorption spectra matching kaempferol based on HPLC-DAD and LC-MS analysis. This study proposes a plausible fragmentation scheme of kaempferol derivatives as produced in the latter work and represented in Fig. 5.3. Noteworthy, *E. nigrum* has potential application in biotechnological through biotransformation of ketones and ionones, even applicable in drug metabolism like diclofenac (Braga *et al*., 2018).

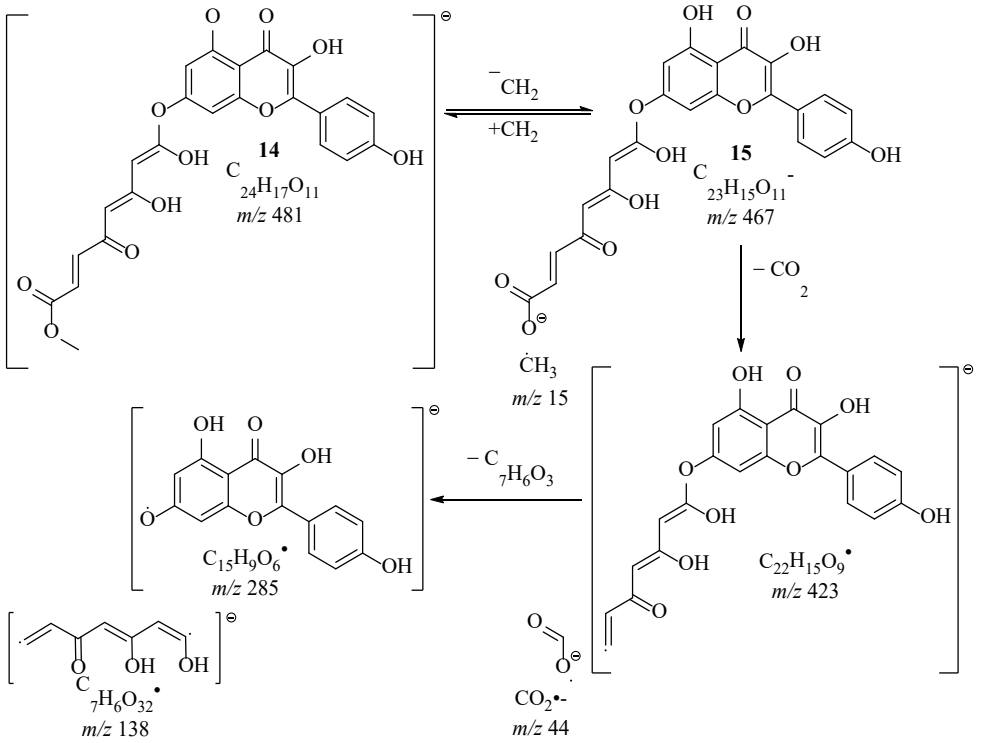


Figure 5.3. Proposed fragmentation patterns of kaempferol derivatives (**14**–**15**) obtained from feeding of kaempferol on *Epicoccum nigrum* (MS data in Chapter 4-Supporting Information Figs. S7–S8).

The feeding of kaempferol on *E. nigrum* followed by extraction and purification led to the presence of two kaempferol derivatives (**14**–**15**). Unfortunately, very low yields of the purified fractions prohibited NMR investigations. Nevertheless, this study attempts to characterize the biotransformation products by analyzing LC-MS/MS data on the basis of the MS fragmentation pathways (Fig. 5.3). The study proposed the structures featuring the pseudomolecular ion peaks at *m/z* 481.0769 (calculated for $C_{24}H_{17}O_{11}$) and *m/z* 467.0613 (calculated for $C_{23}H_{15}O_{11}$). The presence of a kaempferol unit was confirmed by the fragment peak at *m/z* 285.0404 (calculated

for $C_{15}H_9O_6$). The side chain attached to kaempferol is constructed from a typical substituent, which can be determined from the molecular formula $C_9H_{10}O_6$ (**14**) or $C_8H_8O_6$ (**15**). This small molecule is proposed as fumarylacetoacetate or its monomethylester, considering that it is probably derived from degradation of phenylalanine/tyrosin by fungi via homogentisate pathway (Fernández-Cañón and Peñalva, 1995; Gertsman *et al*., 2015). Thus, kaempferol as a precursor could be metabolized by this fungus, yielding compounds **14** and **15** which were identified as kaempferol derivatives albeit not with sugar substituents.

Feeding experiments demonstrate an effective approach to mediate fungal biotransformation. Recent advances deal with feeding experiments that probably result in fungal biotransformation in order to obtain new compounds or to optimize known ones. Current example include the transformation of colletoketol, where feeding of tryptophan to *Trichocladium* sp. resulted in the isolation of a new macrolide linked to an anthranilic acid moiety, namely 13-*N*-(2-carboxyphenyl)colletoketol (Tran-Cong *et al*., 2019). In addition, feeding of anthranilic acid or tryptophan to *A. ochraceus* led to the isolation of ochraspergillic acids A and B, suggested as a biotransformation product of penicillic acid with building blocks of those precursors (Frank *et al*., 2009[b]). Advanced strategies have been applied to yield valuable flavonoids or polyphenols, by biocatalysis via flavonoids glycosyltransferases (enzymatic approach) or bioconversion with engineered *Escherichia coli* or *Corynebacterium glutamicum* (metabolic engineering), instead of microbial biotransformation (Xiao *et al*., 2014; Kallscheuer *et al*., 2017; Wang *et al*., 2019).

CHAPTER 6
Summary and Future Prospects

6.1. Summary

Two endophytic fungi were studied: *Trichoderma harzianum* and *Epicoccum nigrum*, which reside in leaves of the medicinal plants *Zingiber officinale* and *Salix* sp., respectively. The investigation provided a wide variety of structurally unique and biologically potent natural products. The structural diversity of those fungal endophytes was exemplified by unusual epidithiodiketopiperazines with a rare 1,2-oxazadecaline skeleton and disulfide functionality attached to both α- and β-positions of the amino acid residues as in the case of pretrichodermamides A and G isolated from *T. harzianum*. Moreover, the Pictet-Spengler-based fungal biosynthetic machinery present in *E. nigrum* was able to produce unprecedented natural products such as azacoccones F–H.

This study adds further evidence that *E. nigrum* is able to mediate biotransformation of host plant flavonoids through an OSMAC approach with solid-state fermentation on green lentil medium, yielding a new acetylated flavonol along with known flavonoids. Furthermore, feeding experiments with flavonoids used as precursors prove that kaempferol derivatives as isolated in this study are formed through biotransformation of precursor flavonoids rather than by *de novo* biosynthesis.

Compounds containing the *α,β*-unsaturated carbonyl fragment possess multiple biological activities that play crucial roles in drug design as they could be transferred into novel bioactive compounds (Amslinger, 2010). Interestingly, all bioactive compounds obtained in this study feature a *β*-hydroxy-*α,β*-unsaturated carbonyl moiety as found in pretrichodermamide A, epicorazine A, epipyrone A, epicoccamide A, epicocconigrone A and epicoccolide B, that is likely responsible for their biological activities.

6.2. Future Prospects

To date, multiple drug resistant (MDR) infections attract the attention of the WHO and similar communities worldwide. Nowadays, discovery of new antimicrobial agents is urgently needed to control emerging diseases or resistant strains of microorganisms. Endophytic fungi constitute a promising source of structurally diverse and/or biologically active metabolites as a

starting point in drug discovery. However, the historical and complex bottlenecks are emerging and even challenging, for example the timescale and complexity of traditional procedures, small amounts of pure metabolites, supply issue of biological samples, the silent biosynthetic gene clusters under standard laboratory culture conditions, as well as reliable validation of bioactivity and the best mechanisms of flow-through into exploitation.

A comprehensive strategy in drug discovery and development today can be focused to overcome these obstacles through application of innovative approaches such as transcriptomics and metabolomics-based studies (Covington *et al*., 2017), MS/MS-based molecular networking analysis (Wang *et al*., 2016) or microbe genome mining (Hug *et al*., 2018). Nowadays, biotransformation is an effective option to produce either novel compounds and/or bioactive ones. These recent advances are reliable to complement the conventional approaches such as investigation of the unexplored microorganisms and improving methods of their cultivation by applying OSMAC, co-cultivation or epigenetic modification (Romano *et al*., 2018; Pan *et al*., 2019). Since MSMs represent a major source for novel drugs or drug leads, it is expected that future studies open up new and exciting area for the discovery of new drug candidates circumventing time-consuming techniques.

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(Chapters 1 and 5–6)

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List of Abbreviations

$[\alpha]^{20}_{D}$	specific rotation at the sodium D-line
µg	microgram
µL	microliter
µM	micromolar
δ	chemical shift
^{13}C-NMR	carbon-13 nuclear magnetic resonance
1D/ 2D	One dimensional/ two dimensional
^{1}H-NMR	proton nuclear magnetic resonance
5-HT2	5-OH tryptamine
6-PP	6-pentyl-α-pyrone
ABSSSI	acute bacterial skin and skin structure infection
Ac-DEVD-AMC	caspase-3 fluorogenic substrate
AChE	acetylcholinesterase
ADP	adenosine 5'-diphosphate
AMR	antimicrobial resistance
amu	atomic mass unit
BCA	biological control agent
BLAST	basic local alignment search tool
BLI	*β*-lactamase inhibitor
c	concentration
CD_3OD	deuterated methanol
CH_2Cl_2	dichloromethane
Cl	chlorine
clonNAT	nourseothricin sulfate
CLSI	clinical and laboratory standards institute
CM	complete minimal
COSY	correlation spectroscopy
CRAB	*Acinetobacter baumannii*, carbapenem-resistant
CRE	*Enterobacteriaceae*-, carbapenem- and third-generation cephalosporin-resistant
CRPA	*Pseudomonas aeruginosa*, carbapenem-resistant
cUTI	complicated urinary tract infection
d	doublet
DAAD	german academic exchange service (*deutscher akademischer austausch dienst*)
DBO	diazabicyclooctane
DCM	dichloromethane
dd	doublet of doublet signal
ddH_2O	double distilled water

DKP	diketopiperazine
DMSO	dimethyl sulfoxide
DMSO-*d*6	deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECD	electronic circular dichroism
EGMME	ethylene glycol monomethyl ether
EI	electron impact ionization
EMA	european medicines agency
ESI	electron spray ionization
ESI-MS	electrospray ionisation mass spectrometry
et al.	et altera (and others)
EtOAc	ethyl acetate
ETP	epidithiodiketopiperazine
ETPx	epipolythiodioxopiperazine
FDA	US food and drug administration
FPS	fungal pictet-spenglerase
g	gram
h	hour
HCl	hydrochloric acid
HCV	hepatitis C virus
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond connectivity
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	high performance liquid chromatography
HPLC-DAD	high performance liquid chromatography-diode-array detector
HPLC-MS	high performance liquid chromatography-mass spectrometry
HRESIMS	high-resolution electrospray ionisation mass spectrometry
HR-MS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
Hz	hertz
IC_{50}	half maximal inhibitory concentration
IMPDH	inosine-5'-monophosphate dehydrogenase
ITS	internal transcriber spacers
J	coupling constant
JNK	Jun N-terminal kinase
Jurkat J16	lymphoblastic leukemia T cells
K	kelvin
L or l	liter
L5178Y	mouse lymphoma cell line
LC	liquid chromatography

LC-MS	liquid chromatography-mass spectrometery
LLE	liquid-liquid extraction
m	multiplet
M	molar
m/z	mass per charge
MAA	marketing authorisation application
MDR	multi (multiple) drug resistance
MeOD	deuterated methanol
MeOH	methanol
mg	milligram
MHz	mega hertz
MIC	minimum inhibitory concentration
min	minute
mL	milliliter
mM	millimolar
mm	millimeter
mmu	milli mass unit
MS	mass spectrometry
MSM	microbial secondary metabolite
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NCE	new chemical entity
NDA	new drug application
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated b-cells
nm	nanometer
nM	nanomolar
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase
OPP	other priority pathogens
OSMAC	one strain many compounds
p38	one of mitogen-activated protein kinases
PARP-1	poly(ADP-Ribose)-polymerase 1
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PDA	potato dextrose agar
PHGDH	3-phosphoglycerate dehydrogenase
ppm	parts per million
PS	pictet-spengler
q	quartet signal

Q-VD-OPh	quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone), a pan caspase inhibitor
Ramos	Burkitt's lymphoma B lymphocytes
ROESY	rotating frame overhauser enhancement spectroscopy
RP 18	reversed phase C-18
s	singlet
SAHA	suberoylanilide hydroxamic acid
SDB	sabouraud broth
SI	supporting information
sp.	species (singular)
spp.	(several) species
T	temperature
t	triplet
TB	tuberculosis
TDDFT	time-dependent density functional theory
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
UV	ultra-violet
VLC	vacuum liquid chromatography
WHO	world health organization
YPD or YEPD	yeast extract peptone dextrose

Research Contributions

Publications

(1) **Harwoko Harwoko**, Georgios Daletos, Fabian Stuhldreier, Jungho Lee, Sebastian Wesselborg, Michael Feldbrügge, Werner E. G. Müller, Rainer Kalscheuer, Elena Ancheeva, Peter Proksch (2019) Epidithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and *Epicoccum nigrum*. *Nat. Prod. Res.*, *in press*. [DOI: 10.1080/14786419.2019.1627348].

The first author contributed 75% to this publication. The first author's work involves all laboratory works including the sample collection, compounds isolation, structure elucidation, as well as manuscript writing.

(2) **Harwoko Harwoko**, Jungho Lee, Rudolf Hartmann, Attila Mándi, Tibor Kurtán, Werner E. G. Müller, Michael Feldbrügge, Rainer Kalscheuer, Elena Ancheeva, Georgios Daletos, Marian Frank, Zhen Liu, Peter Proksch (2020) Azacoccones F – H, new flavipin-derived alkaloids from an endophytic fungus *Epicoccum nigrum* MK214079. [Manuscript in preparation].

However, most of the results have been presented as poster at the conference of "Natural Products in Drug Discovery and Human Health (NatProdDDH)" in July 28–31th, 2019, Lisbon, Portugal. (Abstract book)

The first author contributed 60% to this publication. The first author's work involves all laboratory works including the compounds isolation, structure elucidation, as well as the manuscript preparation.

(3) **Harwoko Harwoko**, Rudolf Hartmann, Georgios Daletos, Elena Ancheeva, Marian Frank, Zhen Liu, Peter Proksch (2019) Biotransformation of host plant flavonoids by the fungal endophyte *Epicoccum nigrum*. *ChemistrySelect*, 4(45): 13054–13057. [DOI: 10.1002/slct.201903168].

The first author contributed 70% to this publication. The first author's work involves all laboratory works including the compounds isolation, LC-MS/MS experiments, as well as manuscript writing.

Declaration of Academic Honesty/ Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel „Metabolite Diversity of Endophytic Fungi Associated with Medicinal Plants“ selbst angefertigt habe.
Außer den angegebenen Quellen und Hilfsmitteln wurden keine weiteren verwendet. Diese Dissertation wurde weder in gleicher noch in abgewandelter Form in einem anderen Prüfungsverfahren vorgelegt. Weiterhin erkläre ich, dass ich früher weder akademische Grade erworben habe, noch dies versucht habe.

Düsseldorf, den 19.02.2020

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Selected Publications

- Harwoko H, Hartmann R, Daletos G, Ancheeva E, Frank M, Liu Z, Proksch P. (**2019**) Biotransformation of host plant flavonoids by the fungal endophyte *Epicoccum nigrum*. *ChemistrySelect*, 4(45): 13054–13057. [DOI: 10.1002/slct.201903168]

- Harwoko H, Daletos G, Stuhldreier F, Lee J, Wesselborg S, Feldbrügge M, Müller WEG, Kalscheuer R, Ancheeva E, Proksch P. (**2019**) Epidithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and *Epicoccum nigrum*. *Natural Product Research*, *in press*. [DOI: 10.1080/14786419.2019.1627348]
- Abdel-Wahab NM, Harwoko H, Müller WEG, Hamacher A, Kassack MU, Fouad MA, Kamel MS, Lin W, Ebrahim W, Liu Z, Proksch P. (**2019**) Cyclic heptapeptides from the soil-derived fungus *Clonostachys rosea. Bioorganic & Medicinal Chemistry*, 27(17): 3954–3959.
- Fareza MS, Ayoesty LT, Wargiyanti SR, Choironi NA, Harwoko, Sunarto (**2017**) Antibacterial activity of ethyl acetate culture broth extract from endophytic fungi of *Nigrospora oryzae* associated with *Rhizophora mucronata. Indonesian Journal of Pharmaceutical Science,* 15(2): 191–195.

Joint Programme and Conference

- Member of Interdisciplinary Graduate and Research Academy Duesseldorf (iGRAD).
- Natural Products in Drug Discovery and Human Health (NatProdDDH) conference, Lisbon, Portugal, 07/2019 – Poster Presentation: "Bioactive secondary metabolites produced by the endophytic fungus *Epicoccum nigrum* MK214079."