Bioactive Fungal Natural Products from Extremophilic Endophytes

(Bioaktive, pilzliche Naturstoffe aus extremophilen Endophyten)

Inaugural-Dissertation

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Catalina Francis Pérez Hemphill
aus Duisburg

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Abstract (English)

To induce the production of new and bioactive secondary metabolites in endophytic fungi, several modern approaches were performed in the course of this dissertation, including standard fermentation on solid rice medium and implementation of OSMAC by changing and altering the fermentation medium.
All natural products described in this work were obtained by chromatographic purification of ethyl acetate extracts. The structures of purified compounds were unambiguously elucidated by state of the art MS, NMR, ORT, and CD analysis.

Chapter I - *Fermentation of Marine Endophytic Fungi on Solid Rice Medium*
This Chapter contains experiments and results from fermenting marine endophytic fungi on solid rice medium:
Section 1 - *Polyketides from the Mangrove-derived Fungal Endophyte Pestalotiopsis clavispora*
The cultivation of Mangrove-derived fungus *Pestalotiopsis clavispora* yielded a total of 14 polyketides, including the six new natural products pestalpolyol I, pestapyrones A and B, (*R*)-periplanetin D, pestaxanthone, norpestaphthalide A, the isolation artifact pestapyrone C, and seven known metabolites similanpyrone B, norpestaphthalide B, pestaphthalide A and B, 2-*epi*-herbarumin II, and pestalotiollides A and B. All compounds were tested for their cytotoxicity against the mouse lymphoma cell line L5178Y and pestalpolyol I exhibited an IC_{50} value of 4.1 µM. Accordingly, these findings were published in the international journal Tetrahedron Letters 57 [http://dx.doi.org/10.1016/j.tetlet.2016.03.101].
Section 2 - *Absolute Configuration and anti-tumor Activity of Torrubiellin B*
By cultivating *Acremonium* sp. on rice this Mangrove-derived endophyte produced a large quantity of the dimeric anthracene torrubiellin B. The hitherto unknown absolute configuration was established as (5'*R*,10'*S*,10a'*R*) via ECD measurements and exhibited strong anti-tumor activity against several human solid cancer cell lines with IC_{50} values of 0.2 - 2.6 µM. These results were published in Tetrahedron Letters 56 [http://dx.doi.org/10.1016/j.tetlet.2015.03.126].

Chapter II - *Induction and Enhancement of Secondary Metabolites with OSMAC*

This chapter covers all experiments and results performed with the implementation of OSMAC by an alteration or change of the cultivation medium:

Section 1 – *OSMAC Approach leads to New Fusarielin Metabolites from Fusarium tricinctum*

The cultivation of the endophyte *Fusarium tricinctum* on juice-supplemented solid rice medium, compared to standard rice medium, led to the 80-fold enhanced production of the new natural product fusarielin J, which exhibited an IC_{50} value of 12.5 µM against the human ovarian cancer cell line A2780 CisR. Additionally, two new fusarielins K and L, and the known fusarielins A and B were induced in the juice cultivars, and exhibited moderate cytotoxic activity, when tested on the same cell line. Finally, compounds inherently present in *F. tricinctum* were identified, namely (-)-citreoisocoumarin, (-)-citreoisocoumarinol, lateropyrone, enniatin B, B1, A, and A1, and fusaristatin A. The regarding manuscript was submitted to the Journal of Antibiotics.

Section 2 - *Optimization of Enniatin Production by Solid-Phase Fermentation of Fusarium tricinctum*

Cultivation of the endophytic fungus *F. tricinctum* on various fermentation media, including various solid media and one liquid medium with or without the addition of peptone or amino acids, exhibited the highest production of enniatins A, A1, B, and B1 on solid white beans medium. These results were published in Zeitschrift für Naturforschung 68C [http://dx.doi.org/10.1515/znc-2013-5-608].

Abstract (German)

Um die Produktion von neuen und bioaktiven Sekundärmetaboliten in endophytischen Pilzen zu aktivieren, wurden mehrere moderne Ansätze im Zuge dieser Dissertation durchgeführt, welche die Standardfermentation auf festem Reismedium und die Implementierung von OSMAC durch einen Wechsel oder eine Änderung des Fermentationsmediums umfassen.
Alle Naturstoffe, die in dieser Arbeit beschrieben sind, wurden mittels chromatographischer Trennverfahren aus Ethylacetat-Extrakten aufgereinigt. Die Strukturen der reinen Substanzen wurden mithilfe von hochmodernen MS-, NMR-, optischer Aktivitäts- und CD-Analysen aufgeklärt.

Kapitel I - *Fermentation von marinen endophytischen Pilzen auf festem Reismedium*
Dieses Kapitel beinhaltet Experimente und Ergebnisse von Fermentierungen endophytischer Pilze auf festem Reismedium:
<u>Abschnitt 1</u> - *Polyketide aus dem Mangroven-stämmigen pilzlichen Endophyten Pestalotiopsis clavispora*
Der Mangroven-stämmige Pilz *Pestalotiopsis clavispora* brachte insgesamt 14 Polyketide hervor, einschließlich der sechs neuen Naturstoffe Pestalpolyol I, Pestapyrone A und B, (*R*)-Periplanetin D, Pestaxanthon, Norpestaphthalid A, das Isolierungsartefakt Pestapyron C und die sieben bekannten Metaboliten Similanpyron B, Norpestaphthalid B, Pestaphthalid A und B, 2-*epi*-Herbarumin II und Pestalotiollid A und B. Alle Substanzen wurden auf ihre Zytotoxizität gegen die Maus-Lymphom-Zelllinie L5178Y getestet und Pestalpolyol I wies einen IC_{50}-Wert von 4,1 μM auf. Entsprechend wurden diese Erkenntnisse in Tetrahedron Letters 57 publiziert [http://dx.doi.org/10.1016/j.tetlet.2016.03.101].
<u>Abschnitt 2</u> - *Absolute Konfiguration und Anti-Tumor-Aktivität von Torrubiellin B*
Durch die Kultivierung von *Acremonium* sp. auf Reis produzierte dieser Endophyt eine große Menge des dimeren Anthracens Torrubiellin B. Die bisher unbekannte absolute Konfiguration wurde als (5'*R*,10'*S*,10a'*R*) via ECD-Messungen bestimmt und wies eine große Anti-Tumor-Aktivität gegenüber mehreren soliden Tumor-Zelllinien auf, mit IC_{50}-Werten von 0,2 bis 2,6 μM auf. Diese Ergebnisse wurden in der Fachzeitschrift Tetrahedron Letters 56 veröffentlicht [http://dx.doi.org/10.1016/j.tetlet.2015.03.126].

Kapitel II – *Induktion und verstärkte Produktion von Sekundärmetaboliten mit OSMAC*

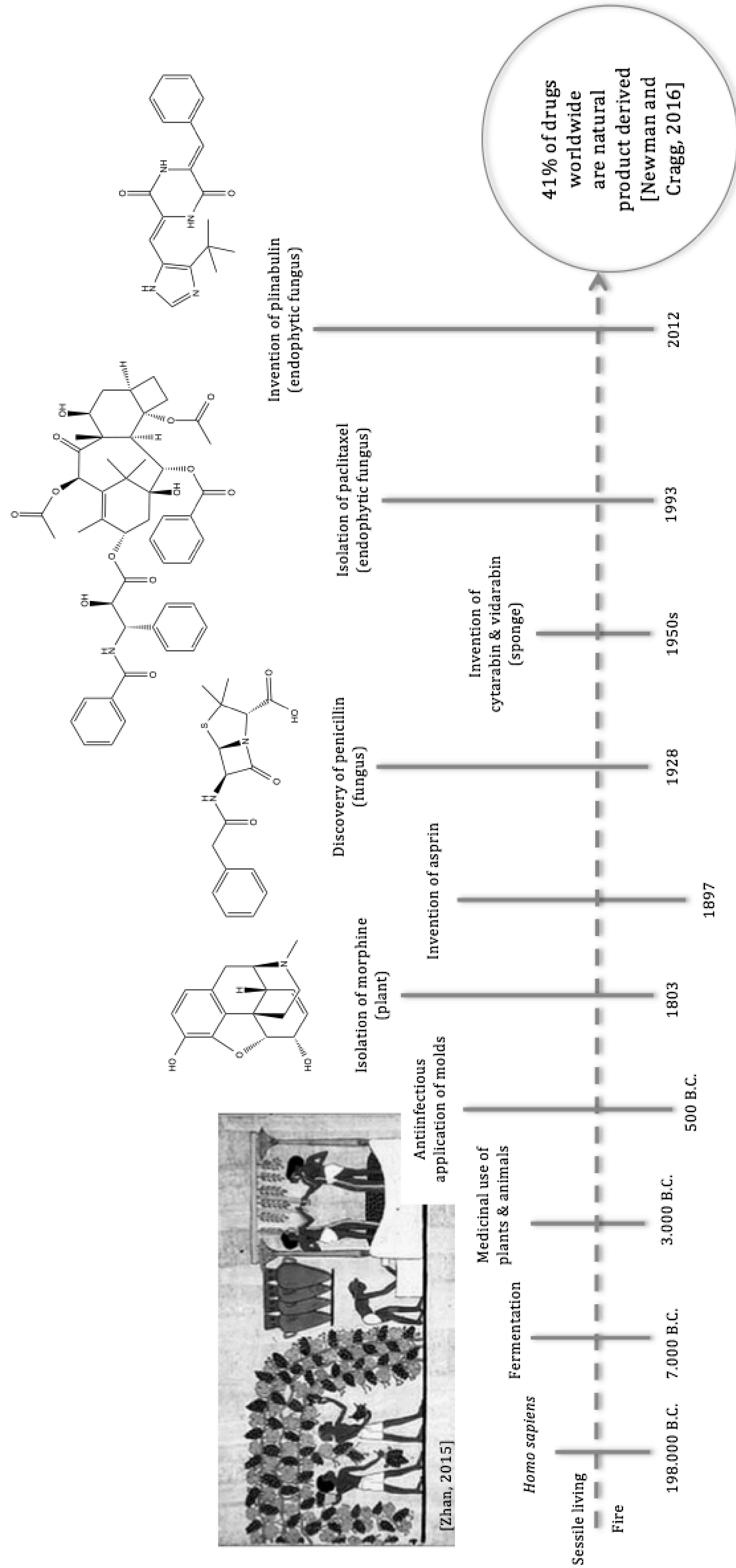
Dieses Kapitel deckt alle Experimente und Ergebnisse ab, die mit der OSMAC-Implementierung über eine Änderung oder einen Wechsel des Kultivierungsmediums ausgeführt wurden:

Abschnitt 1 – *OSMAC-Ansatz führt zu neuartigen Fusarielin-Metaboliten von Fusarium tricinctum*

Die Kultivierung des Endophyten *Fusarium tricinctum* auf Saft-supplementiertem festem Reismedium führte im Vergleich zu dem Standard-Reismedium zu einer 80-fach erhöhten Produktion des neuen Naturstoffes Fusarielin J, welcher einen IC_{50}–Wert von 12,5 µM gegen die humane Ovarialkarzinom-Zelllinie A2780 CisR aufwies. Zusätzlich wurde die Produktion zweier neuer Naturstoffe Fusarielin K und L und zweier bekannter Fusarieline A und B in den Saft-Kultivaren induziert. Abschließend wurden die inhärent vorhandenen Substanzen von *F. tricinctum* identifiziert, nämlich (-)-Citreoisocoumarin, (-)-Citreoisocoumarinol, Lateropyron, Enniatin B, B1, A und A1 und Fusaristatin A detektiert. Das entsprechende Manuskript wurde bei dem Journal of Antibiotics eingereicht.

Abschnitt 2 - *Optimierung der Enniatin-Produktion von Fusasrium tricinctum durch Festphasen-Fermentation*

Die Kultivierung des endophytischen Pilzes *F. tricinctum* auf verschiedenen Fermentationsmedien, einschließlich verschiedenen festen und einem Flüssig-Medium mit oder ohne Zusatz von Pepton oder Aminosäuren, zeigte die höchste Produktion von Enniatin A, A1, B und B1 auf festem Bohnenmedium. Diese Ergebnisse wurden in der Zeitschrift für Naturforschung 68C publiziert [http://dx.doi.org/10.1515/znc-2013-5-608].


[Zhan, 2015]
Sessile living
Fire
Homo sapiens
198.000 B.C.
Fermentation
7.000 B.C.
Medicinal use of plants & animals
3.000 B.C.
Antiinfectious application of molds
500 B.C.
Isolation of morphine (plant)
1803
Invention of asprin
1897
Discovery of penicillin (fungus)
1928
Invention of cytarabin & vidarabin (sponge)
1950s
Isolation of paclitaxel (endophytic fungus)
1993
Invention of plinabulin (endophytic fungus)
2012
41% of drugs worldwide are natural product derived [Newman and Cragg, 2016]

Our existence as modern human beings (*Homo sapiens*) dates back to about 200.000 years ago, which is how old the remains of our eldest ancestors are determined to be [McDougall *et al.*, 2005]. Today we are the last living representatives of the genus *Homo*. Amongst many complex factors, we apparently survived other hominis by evolving our abilities to adapt to changing environmental conditions. Our evolution includes using fire and hand-made weapons to prepare our food into a more caloric dense alimentation, and also to evolve to a sessile style of domicile, developing diverse habits to better utilize otherwise unusable nutritional sources, *e.g.* clearing woodlands to grow crops and to raise live stock [Wrangham, 2010].
Alongside using fire to unbar starches from otherwise indigestible foods, such as potatoes and grains, our ancestors have been implementing various fermentation techniques, *e.g.* for the production of bread and beer since about 9000 years [McGovern *et al.*, 2004].
Along with this development came the use of plants, sponges and other animals from terrestrial and marine origin to treat diseases. This is first documented ca. 5000 years ago, *e.g.* with fish-derived drugs in China around 2953 B.C. and Egyptian plant-derived medicines around 53 years later, many of which are still in use to this day [Halstead, 1992; Jia *et al.*, 2004; Cragg and Newman, 2005; Dias *et al.,* 2012; Petrovska, 2012].
Humans have perfected this medicinal utilization of nature, accumulating 1803 in the capability of isolating a single pharmacologically active compound from the dried latex of unripe seed capsules of the plant *Papaver somniferum*, morphine (Figure 1), an analgesic drug, which is still in medicinal use today [Sertürner, 1805].
Thereafter the term 'natural product' was chimed, covering all so called secondary metabolites, which differ from primary metabolites, such as carbohydrates, amino and fatty acids, in the sense that they are not essential for the survival of the concerning organism, but help it to proliferate, reproduce or adapt to changing environmental conditions [Drew and Demain, 1977]. Natural products were initially isolated from plants, later including all living organisms as sources, such as microorganisms and animals [Croteau *et al.*, 2000].

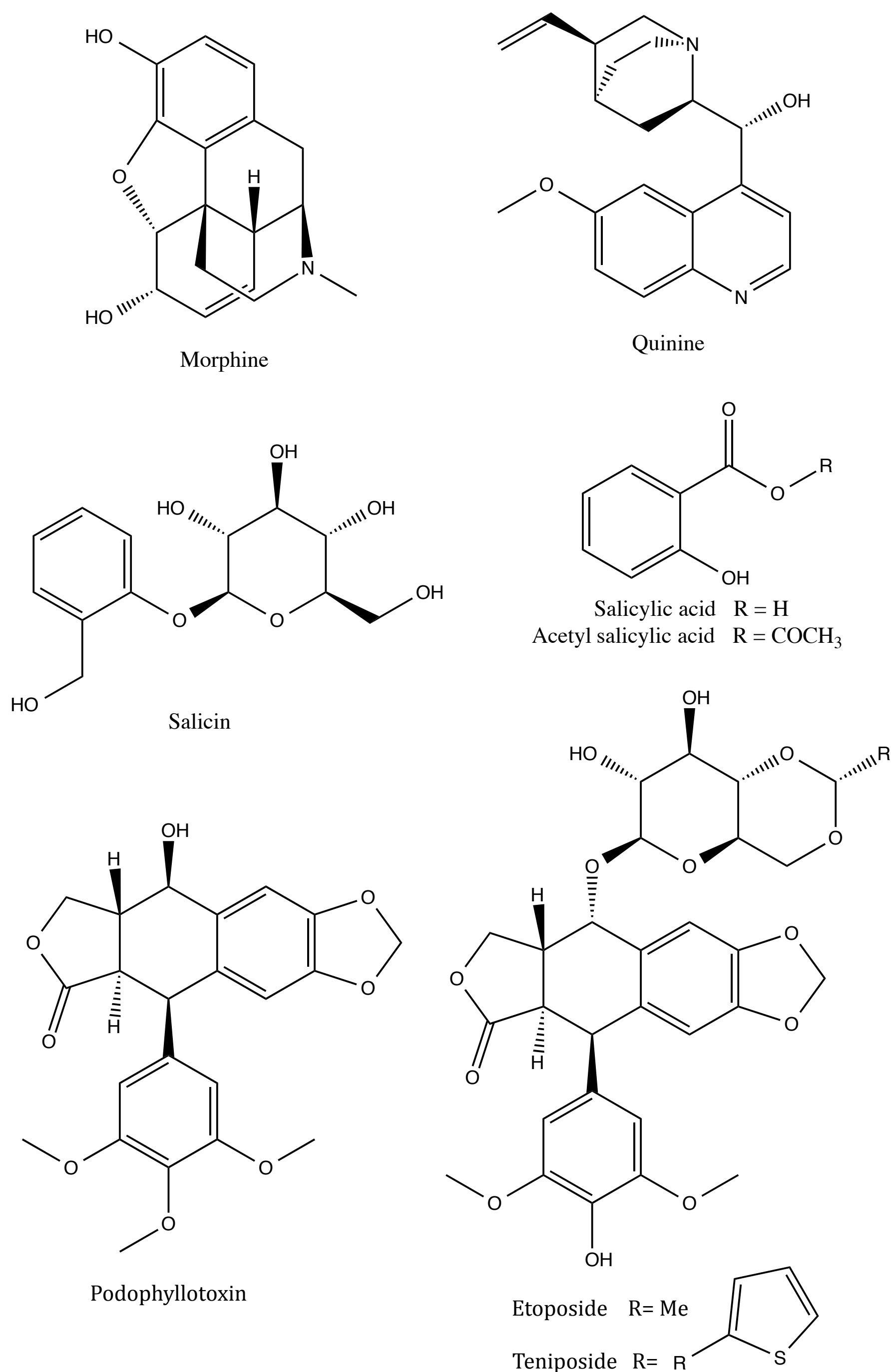


Figure 1. Some of the first plant-derived natural products and their semi-synthetic analogues in medicinal use.

And so, after the isolation and later the chemical and pharmacological characterization of morphine, other bioactive natural products from various sources followed, *e.g.* quinine from the bark of the tree *Cinchona pubescens* in 1820, which is also still in medicinal use today in malaria therapy [Gerhardt, 1853], and salicin from the willow bark in 1828, of which the aglycon salicylic acid is applied topically for the treatment of various skin conditions. Salicylic acid was chemically modified to acetylsalicylic acid in

1897 and is since known and therapeutically used as asprin, against pain, inflammation and fever [Hoffmann, 1898].

With asprin the medicinal evolution of humans shows a climax, as we have since then been taking products from nature and chemically modifying these to our benefit, *e.g.* the chemotherapeutic drugs etoposide and teniposide, which were semi-synthetically derived from the plant natural product podophyllotoxin in the 1960s (Figure 1) [Hande, 1998; Ikan, 2008; Cragg and Newman, 2013].

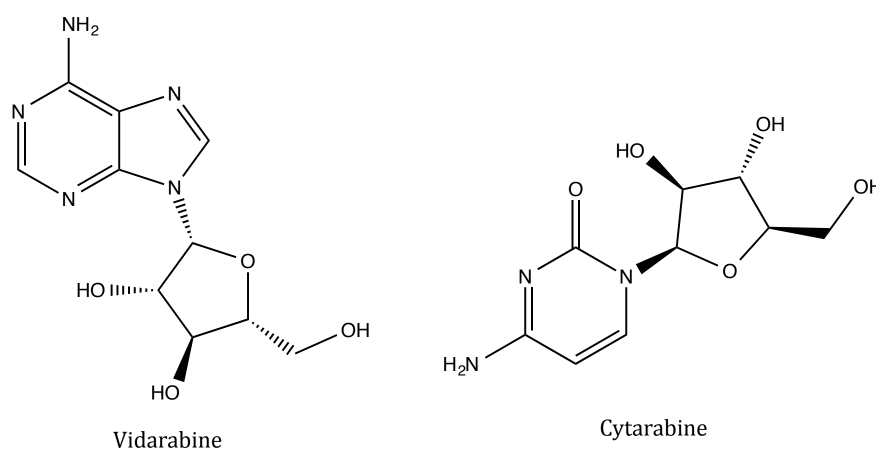


Figure 2. Some of the first sponge-derived natural products in medicinal use.

Throughout the years other sources for the discovery of new natural product drug leads were then utilized, *e.g.* sponges: Traditionally used by humans for mechanical purposes, these animals were now being screened for bioactive natural products. It was suspected that these sessile creatures must bear paramountly active compounds, as even given the lack of a mechanical defense system or the option of running away from predators, their surface remains unharmed by micro- and macroorganisms. And fair enough, bioactive and medicinally valuable natural products were isolated from sponge extracts, some of the first and most prominent products were C-nucleosides, isolated in the 1950s, which were later semi-synthetically modified to the anti-cancer drug cytarabine and the anti-viral agent vidarabine (Figure 2) [Cragg and Newman, 2005].

The next big example of new sources for drug discovery are microorganisms, in particular fungi. Microorganisms have unknowingly been assisting humans in everyday life, as mentioned above, through fermentation since 9000 years, and probably in the treatment of infections since ancient times, but the first documented medicinal use of microorganisms dates back to the mid 19th century [Guttmann and Rao, 2011]. The first isolation of a natural product from a filamentous fungus occurred later, after penicillin was discovered as an antibiotic metabolite from a *Penicillium* species in 1928 [Fleming, 1929].

Since then several microorganisms were systematically screened for potentially bioactive secondary metabolites in 1939, before penicillin was implied in therapeutic use against infections caused by gram-positive bacteria [Sahm *et al.*, 2013].
Later on an antibiotic agent was even found to be produced by a bacterium itself: streptomycin, which was isolated in 1943 from *Streptomyces griseus* (originally *Actinomyces griseus*) [Schatz *et al*., 1944].

After this revolution, natural product research increased, especially with fungi, leading to the isolation of many new medicinal agents, *e.g.* the antibiotic cephalosporin C from *Cephalosporium acremonium* in 1945 [Burton and Abraham, 1951; Abraham, 1962], the immunosuppressant ciclosporin A from *Trichoderma polysporum* in 1969 [Dreyfuss *et al.*, 1976], and compactin from the plant-associated fungus *Penicillium citrinum* in 1973, which served as a drug-lead for the development of statins, *e.g.* lovastatin (Figure 3), approved for the treatment of hypercholesterolemia and the prevention of cardiovascular disease [Endo, 2010].

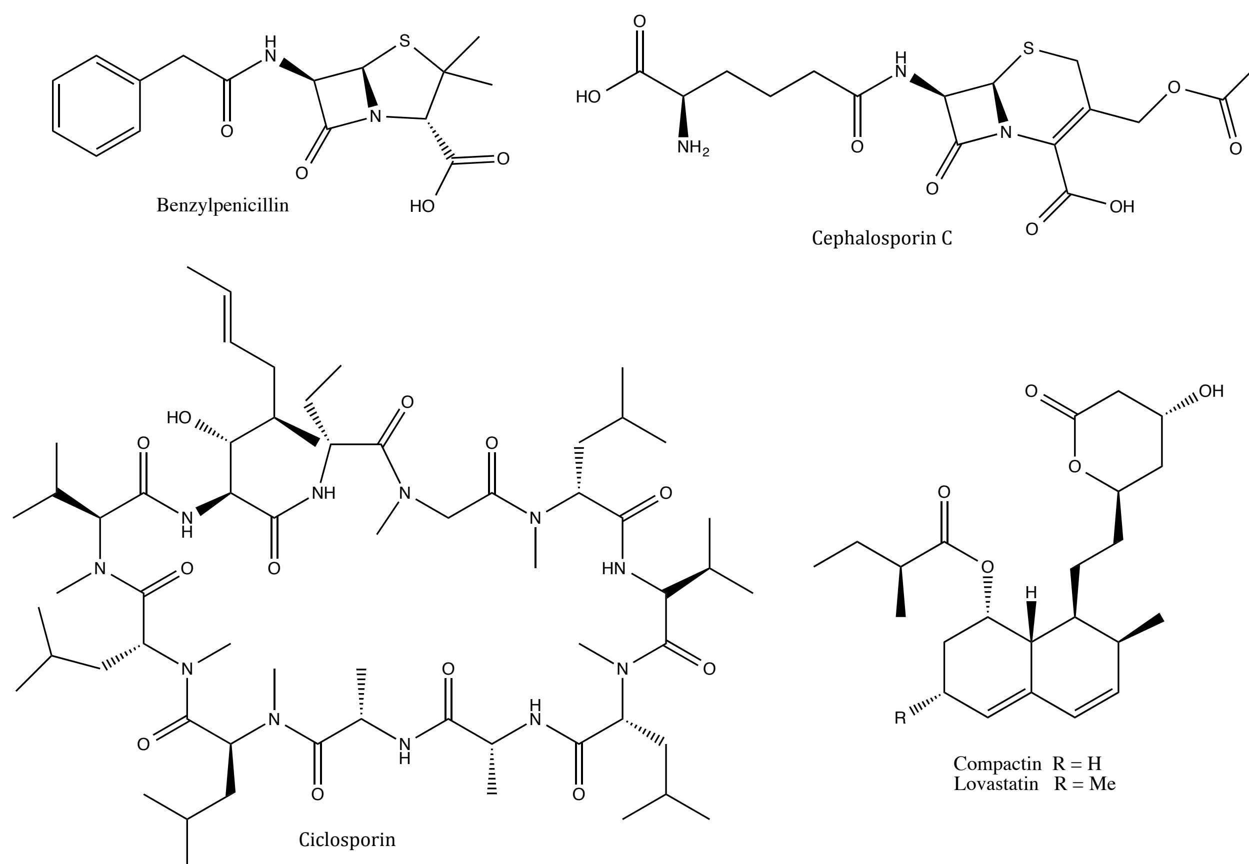


Figure 3. Some of the first natural products and derivatives in medicinal use, derived from fungi.

Thereafter various sources were explored to collect fungal species, which were cultivated and extracted in search for new bioactive compounds, such as contaminated foods, soils and surfaces, as well as secretions and internal tissues of different origins. But when the plant-derived, first billion dollar anti-cancer drug paclitaxel (Figure 4), first found in the bark of the pacific yew *Taxus brevifollia* [Wani *et al.*, 1971], was also isolated from the endophytic fungus *Taxomyces andreanae* in 1993 and other endophytic fungi thereafter [Stierle *et al.*, 1993; Strobel *et al.*, 1996], natural product research with endophytic fungi was ignited and more recent studies show, that by now more than half of all microbial-derived bioactive compounds are in fact isolated from endophytes [Schulz and Boyle, 2005; Nisa *et al.*, 2015].

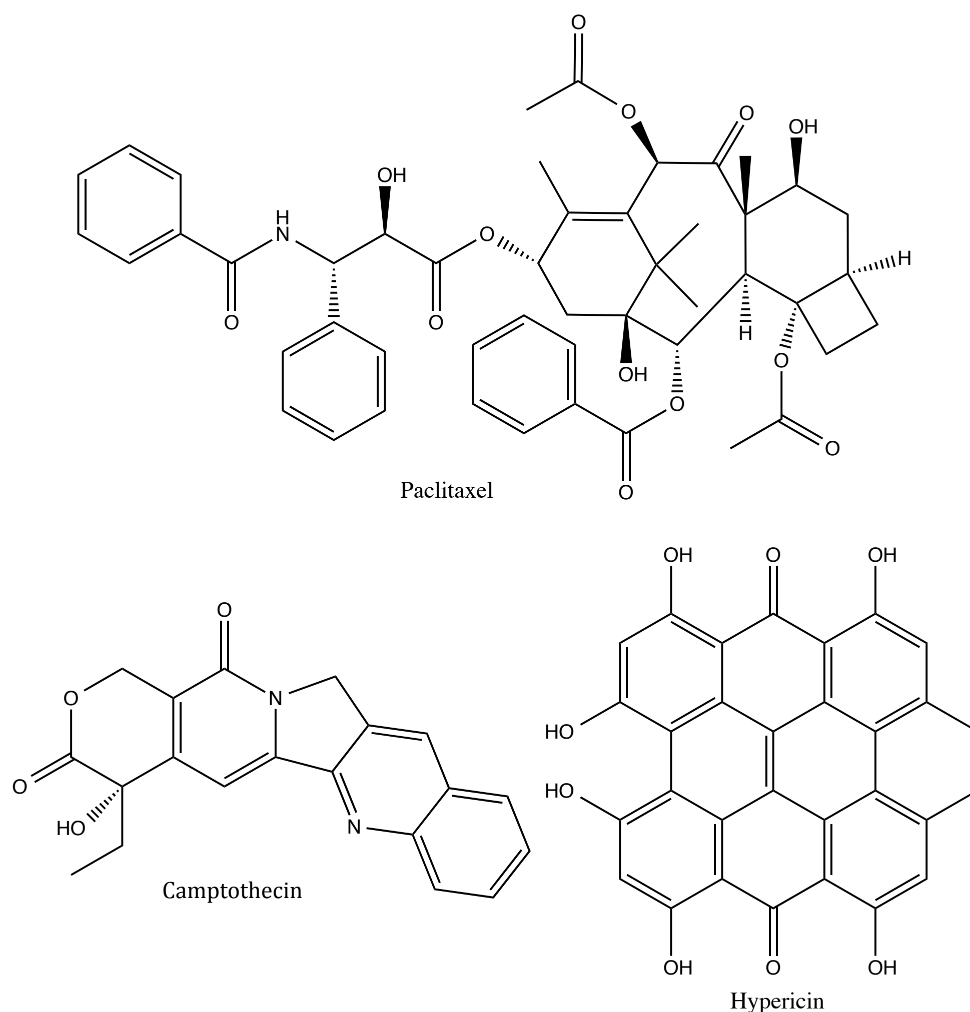


Figure 4. Medicinal drugs found in plants and endophytic fungi alike.

In essence endophytic (Greek: *endon* “inside”, *phyton* “plant”) fungi are described to reside in internal tissues of living plants, by definition causing no apparent harm. They live with the plant in a balanced symbiosis, with a mutualistic exchange of nutrients and secondary metabolites, *e.g.* growth hormones, such as gibberellins [Khan *et al.*, 2008], or antibiotic and anti-herbivore agents for the plant, *e.g.* peramine [Rowan *et al.*, 1990; Petrini, 1991; Marschner and Dell, 1994; Wilson, 1995]. In turn, the endophytic fungi prosper from a stable water and mineral source and are strongly influenced by the plant they inhabit, so that any environmental changes also have effects, *e.g.* on the proliferation and secondary metabolite production of these fungi [Jia *et al.*, 2016]. Besides an obligatory assertion amongst the present microbial community, these endophytes develop strategies to survive microbial-toxic plant agents, by producing more complex secondary metabolites [Chandra, 2012].

Since the isolation of paclitaxel from an endophytic fungus in the 90s, further plant-derived drugs, such as the chemotherapeutic camptothecin and the antidepressant hypericin (Figure 4), were likewise isolated from endophytic fungi isolated from plants producing these same compounds [Puri *et al.*, 2005; Kusari *et al.*, 2008]. This suggests that a horizontal gene transfer between plant and microorganism is probable, which enables the latter to biosynthesize metabolites, previously believed to have been derived only from plants [Wink, 2016], and thus underlines the importance and promising prospect of investigating endophytic fungi as sources for new and pharmacological active agents [Nicoletti and Fiorentino, 2015].
Natural product research from fungi has been most prosperous, when the microorganisms investigated stemmed from extreme habitats, as discussed for marine-derived fungi [Imhoff, 2016]. Likewise, endophytes isolated from extreme habitats, including extreme heat, humidity or salinity [Khan *et al.*, 2013], show a wider array of bioactive secondary metabolites [Banerjee, 2011; Cragg and Newman, 2013; Verma and Gange, 2013].

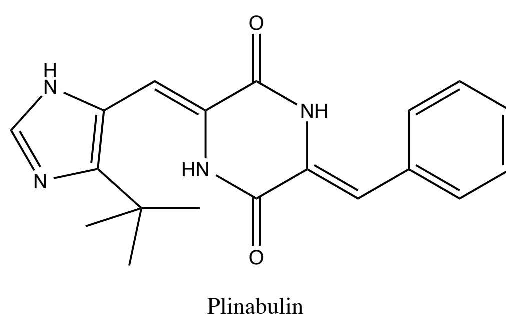


Figure 5. Promising anti-cancer agent derived from an endophytic fungal metabolite.

Since the discovery of paclitaxel from the endophyte *T. andreanae*, many interesting and bioactive secondary metabolites have been isolated from endophytic fungi [Shukla *et al.*, 2014]. The most important new bioactive metabolite is plinabulin (Figure 5), a semi-synthetic drug derived from the natural product phenylahistin, which was isolated from *Aspergillus ustus*, an endophyte derived from the marine alga *Halimeda lacrimosa* [Kanoh *et al.*, 1997; Kim, 2012]. Plinabulin is now in a phase III clinical trial as an anti-cancer drug [Kasunich and Swift, 2016].

In parallel, the fully chemical-based development of new medicinal agents in laboratories has played an equally important role:
One of the first medicinal agents produced in a test tube by man was chloral hydrate, which was synthesized in 1832 and used as a sedative anesthetic during operations ca. 37 years later [Liebreich, 1869]. A multitude more followed, with examples such as the infamous barbiturates [Baeyer, 1864], the asprin alternatives phenacetin and acetanilide [Cahn, 1886; Jones, 2011], and thalidomide, which was synthesized in 1954 and is in use today in the treatment of multiple myeloma and leprosy [Silverman, 2002; FDA, 2015].

In the 1950s thalidomide was applied as a racemic mixture, inter alia against nausea in pregnant women, tragically causing embryonal phocomelia with a mortality rate of 50%. Initial cause studies thereafter showed that only the (+)-(*R*)-enantiomer had the desired anti-emetic effect, and (-)-(*S*)-thalidomide was teratogenic, but in fact both enantiomers racemize after few hours of ingestion in the human body [Eriksson *et al.*, 1995].

The unfortunate history of thalidomide taught scientists the importance of distinguishing the 3D-structure of compounds with potential medicinal application and led to the progression of chemical and spectroscopic analytical methods, amongst other progressions, *e.g.* implementing thorough pre-clinical and clinical test phases for the approval of new drugs before these are approved for treatment.
The chemical field of drug development is now evolved to a state, in which new medicinal drugs can theoretically be designed on the computer, whereby after selecting a biological target, or active lead-structure, digital libraries containing thousands of compounds are screened for potential drugs. Based on certain traits of these compounds, such as bioavailability, toxicity, biostability, and chemical-physical affinity to the target, or the respective similarity to the lead-structure, the favored structures can be optimally designed according to the target or pharmacophor in question, before they are chemically synthesized and physically tested in cell biological assays [Merz *et al.*, 2010]. One of the first examples herefore is dorzolamide, which was approved as a glaucoma drug [Greer *et al.*, 1994].

But, already Paracelsus, who empirically used chemical agents, *e.g.* sulphur or mercury, to treat medical conditions back in the 16th century, recognized the importance of not neglecting healing isolates from plants [Wear, 1995]. And so it is now that even with the ability of using state of the art computer technology to design new medicinal drugs from scratch, we still heavily rely on the supply of drug-lead structures and yet undiscovered metabolites from nature, as the former method yields only a limited amount of "hits", restricted by the compound library in use, and is gravely fueled by natural drug discovery [Feher and Schmidt, 2003]. In fact, a review on the development and current status in the field of drug discovery highlights the importance of natural product research in the ongoing search for new drug leads [Newman and Cragg, 2016].

To this day approximately 40.000 natural products are listed in the Dictionary of Natural Products, not including derivatives or stereoisomers, which is "the only comprehensive and fully-edited database on natural products" [DNP].
Over the years 1981-2014 close to 41% of approved drugs worldwide were natural products or semi-synthetically derived from those [Newman and Cragg, 2016], and over half of anti-cancer drugs approved by the FDA were from microbial origin [Trendowski, 2015] .

Yet, during the last four to five decades, the discovery of new natural products from filamentous fungi has been investigated so extensively, the re-discovery rate of secondary metabolites isolated from cultures kept under standard laboratory conditions had become so high, that the research motivation in this direction subsided and was even argued to be ineffective in comparison to computational drug design [Koehn and Carter, 2005] .
But ongoing research on the genome of several microorganisms showed, that this holds many cryptic gene clusters, which potentially encode for many more biosynthetical enzymes in a dormant state, which are capable of synthesizing hitherto undiscovered natural products [Corre and Challis, 2009; Nett *et al.*, 2009]. On the basis that one microbial strain is able to produce many compounds (OSMAC), if manipulated enough [Bode *et al.*, 2002], several methods for the activation of the silent genetic pathways were thus proposed and implied into recent research, to induce the biosynthesis of new secondary metabolites. These methods include variating the culture conditions

following the OSMAC (One Strain Many Compounds) approach, treatment of strains with epigenetic modifiers, or the co-cultivation of more than one microbial strain.

Inducing new secondary metabolites by cultivating fungi in altered conditions, *e.g.* with different cultivation media, at changed temperatures and in shaken *versus* static state, has been termed the OSMAC approach [Bode *et al.*, 2002]. This approach is based on the observation that one single fungal strain can produce hitherto unknown natural products, if the outer conditions are changed in the slightest way, as shown *e.g.* for endophytic fungi, which were cultivated using tap water *versus* distilled water, where in the latter cultivation only by a change of ion concentrations in the cultivation medium the production of new natural products was exhibited [Paranagama *et al.*, 2007]. This effect can be explained with the knowledge from other studies, that proves the need and ability of fungi, especially those residing inside of plants, to adapt to their environment in order to survive. This is mainly expressed in the production of more, sometimes new and bioactive metabolites [Jia *et al.*, 2016].

The addition of so-called epigenetic modifiers to the culture media of endophytic fungi, has proved to have an up-regulating effect of so far undiscovered secondary metabolites. Epigenetic modifiers are able to interfere with the expression of genes, *e.g.* by inhibiting the deacetylation of histones with vorinostat (SAHA), which was recently approved as chemotherapeutic agent, or by inhibiting the methylation of DNA with azacytidine, which is also an approved drug for cancer therapy. In fungi the underlying mechanism is the same and leads to the activation of silent gene clusters, thus enabling the fungus to biosynthesize new natural products, which are not found under standard laboratory conditions [Williams *et al.*, 2008]. Examples for the successful implementation of this method can be found in the literature, *e.g.* the 'epigenetic modifier-induced biosynthesis of novel fusaric acid derivatives in endophytic fungi from *Datura stramonium* L' [Chen *et al.*, 2013], and as 'epigenetic modifiers alter the secondary metabolite composition of plant endophytic fungus, *Pestalotiopsis crassiuscula* obtained from the leaves of *Fragaria chiloensis*' [Yang *et al.*, 2014].

The co-cultivation of two or more microbial strains (mixed fermentation) aims at mimicking the natural habitat of the respective species under investigation, as these dwell in environments rich in microbial cohabitants [Marmann *et al.*, 2014]. The

incubation of two or more microbial strains in the same cultivation vessel effects the metabolic profile of many microorganisms, as these are influenced by inter-species cross-talk, mediated through physical cell-to-cell contact or via soluble secondary metabolites of the coexisting other microorganism(s) [Marmann *et al.*, 2014]. Examples for this are the 'induction of secondary metabolite production by the soil-dwelling fungus *Aspergillus terreus* through bacterial co-culture' [Chen *et al.*, 2015], and the induction of 'secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through co-culture with *Bacillus subtilis*' [Ola *et al.*, 2013], which in both experiments led to the discovery of new natural products. These two publications, amongst others [Schroeckh *et al.*, 2009; Marmann *et al.*, 2014], show the enhancing effect on fungal metabolite production and the induction of new secondary metabolites, through the co-cultivation of fungi with bacteria.

Scope

As set out in the introduction, since the discovery of the prominent anti-cancer drug paclitaxel from an endophytic fungus, and with over 50% of the hitherto approved natural product based anti-cancer drugs being derived from microorganisms, natural product research with endophytic fungi has become a promising field of drug discovery. Also, as due to the development of resistances towards existing drugs, and the lack of existing drugs for some conditions, the demand for new potential drug leads is never ending. And so, in an ongoing investigation of endophytic fungi as natural sources for new and potentially bioactive drug leads, several plant-derived pure fungal strains from extreme habitats were cultivated on different fermentation media in the course of this dissertation, in order to induce and enhance secondary metabolite production of these fungi, following the OSMAC approach. By attaining crude ethyl acetate extracts of these cultivation experiments, secondary metabolites were chromatographically purified, using automated and manual, column and planar systems, and subsequently structurally elucidated, using state of the art spectrometric and spectroscopic techniques. The pharmacological potential of pure compounds was thus analyzed in antibiotic and cytotoxic cell assays and the new and promising results were published in relevant scientific journals.

Chapter I - *Fermentation of Marine Endophytic Fungi on Solid Rice Medium*

Section 1

Polyketides from the Mangrove-derived Fungal Endophyte *Pestalotiopsis clavispora*

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Polyketides from the Mangrove-derived fungal endophyte *Pestalotiopsis clavispora*

Catalina Francis Pérez Hemphill [a], Georgios Daletos [a], Zhen Liu [a], Wenhan Lin [b], Peter Proksch [a,*]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University Duesseldorf, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany
[b] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, 100191 Beijing, PR China

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ABSTRACT

Six new polyketide derivatives, including pestalpolyol I (**1**), pestapyrones A (**2**) and B (**3**), (*R*)-periplanetin D (**6**), pestaxanthone (**7**), norpestaphthalide A (**8**), and an isolation artifact pestapyrone C (**4**), were obtained from extracts of the endophytic fungus *Pestalotiopsis clavispora* isolated from the Mangrove plant *Rhizophora harrisonii*. In addition, seven known metabolites, including similanpyrone B (**5**), (*R,S*)-5,7-dihydroxy-3-(1-hydroxyethyl)phthalide (**9**), for which we propose the trivial name norpestaphthalide B, pestaphthalides A (**10**) and B (**11**), 2-*epi*-herbarumin II (**12**), and pestalotiollides A (**13**) and B (**14**) were isolated. The structures of the new compounds were unambiguously elucidated on the basis of one- and two-dimensional NMR spectroscopy, as well as by high-resolution mass spectrometry. All compounds were tested for their cytotoxicity against the mouse lymphoma cell line L5178Y. Compound **1** exhibited strong activity with an IC_{50} value of 4.10 μM. All other compounds (**2**–**14**) proved to be inactive (IC_{50} >10 μM) in this assay.

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Introduction

According to the WHO cancer is the second leading cause of deaths caused by non-communicable diseases.[1] The WHO's GLOBOCAN 2012 estimated a total of 8.2 million deaths accounted for by cancer worldwide and a total of 14.1 million new cases in 2012, which are expected to rise to 22 million in the next two decades.[2] Corresponding to the vast number of cancer types, the number of chemotherapeutics attacking various different targets is multifold, e.g., the mitotic inhibitor paclitaxel (Taxol®), the topoisomerase inhibitors topotecan (Hycamtin®) and etoposide (Vepesid®), and DNA alkylating agents such as streptozocin (Zanosar®).[3–5] Yet, besides numerous side effects, the limitations of this form of oncological therapy, such as insufficient bioavailability and the lack of sensitivity or the development of resistance, necessitate an ongoing research for the discovery and development of new and improved chemotherapeutic drugs.

One important source for the discovery of new drug leads in cancer chemotherapy is natural products.[6] To this day, a total of 35 naturally derived products are approved by the FDA and are in use in anticancer therapy,[7] which accounts for almost one fifth of all FDA approved antineoplastic agents (1995–2015).[8] Worldwide the number of small molecules that are approved as drugs for oncological therapy and that are derived or inspired from natural products even accounted for almost 50% (1940–2012).[9] More than half of those naturally derived drugs originated from microbial organisms,[7] which can be isolated from different sources.[10] Currently, nine naturally derived products with fungal origin are being evaluated in clinical trials against cancer, of which two are now in a Phase III study.[11a,b] Some of the most interesting fungi with regard to metabolite production with potent anticancer activity are found as endophytes in Mangroves.[12,13] Due to the need of these endophytes to adapt to extreme habitual conditions, their secondary metabolites are structurally diverse and often show biological activity, e.g., against cancer cells.[14,15]

One of the most promising genera of fungi, featuring secondary metabolites with anti-tumor activity, is *Pestalotiopsis*, which among other cytotoxic compounds, was shown to produce the anticancer drug paclitaxel, approved by the FDA, EMA, and TGA for the treatment of ovarian, breast and other cancers.[16–21]

In search of new potential antineoplastic drug leads, *Pestalotiopsis clavispora* was investigated in this study. The crude extract of fungal cultures grown on solid rice medium yielded a total of fourteen compounds (Fig. 1), all of which were submitted to a cytotoxicity assay against the mouse lymphoma L5178Y cell line. Of all compounds tested, **1**[22] with an IC_{50} value of 4.10 μM may be interesting for further investigation due to its strong cytotoxicity in this bioassay.

* Corresponding author. Tel.: +49 211 81 141 63; fax: +49 211 81 119 23.
E-mail address: proksch@uni-duesseldorf.de (P. Proksch).

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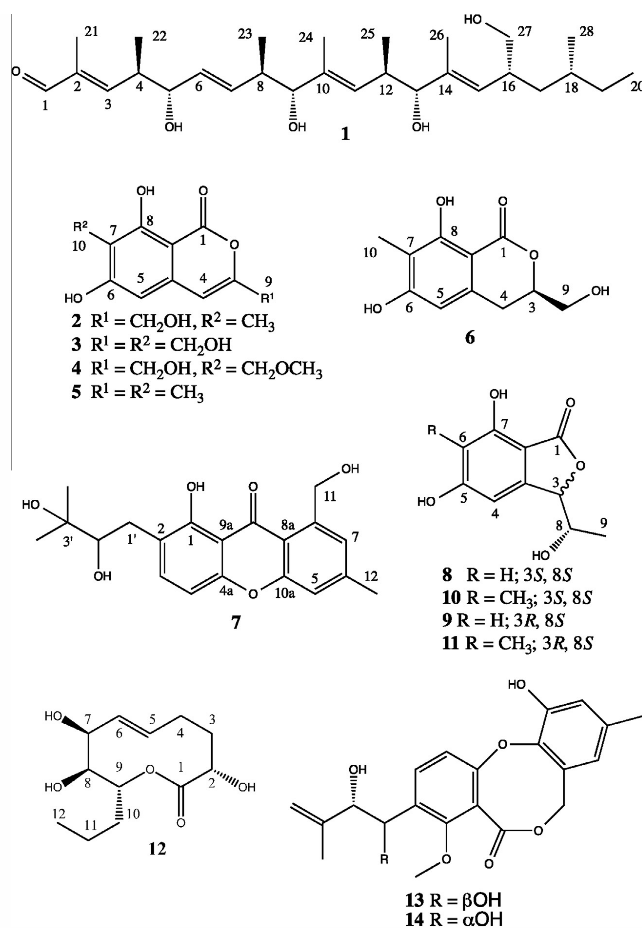

Figure 1. Structures of compounds **1–14**.

Results and discussion

P. clavispora was isolated from petioles of the Mangrove *Rhizophora harrisonii*, which was collected in Port Harcourt (Nigeria). The large-scale solid rice culture of *P. clavispora* was extracted with ethyl acetate, and subsequently evaporated to yield the dried extract (4 g). Chromatographic separation yielded fourteen compounds (**1**–**14**), from which pestalpolyol I (**1**), pestapyrones A (**2**) and B (**3**),[23,24] (*R*)-periplanetin D (**6**),[26] pestaxanthone (**7**),[27] and norpestaphthalide A (**8**)[28] proved to be new natural products, whereas pestapyrone C (**4**)[25] is most likely an artifact generated from **3** during extraction and/or isolation (Fig. 1).

Compound **1** was obtained as an amorphous, white powder with a HRESIMS prominent ion peak at *m*/*z* 465.3573 $[M+H]^+$ from which the molecular formula $C_{28}H_{48}O_5$ was established, revealing 5 degrees of unsaturation. It exhibited only one absorption maximum at λ_{max} 232 nm. The ^1H NMR spectrum of **1** revealed the presence of a deshielded proton at δ_H 9.39, belonging to an aldehyde function (H-1), five olefinic protons (δ_H 6.56, H-3; δ_H 5.49, H-6; δ_H 5.71, H-7; δ_H 5.27, H-11; and δ_H 5.09, H-15), three oxygenated aliphatic methine protons (δ_H 3.98, H-5; δ_H 3.67, H-9; and δ_H 3.69, H-13), one hydroxymethylene group at δ_H 3.45 and 3.37 (H_2-27), and eight methyl proton signals, including three olefinic (δ_H 1.75, H_3-21; δ_H 1.65, H_3-24; and δ_H 1.69, H_3-26) and five secondary (δ_H 0.87, H_3-20; δ_H 1.06, H_3-22; δ_H 0.85, H_3-23; δ_H 0.79, H_3-25; and δ_H 0.87, H_3-28) methyl groups. In addition, the resonances of four allylic methine protons (δ_H 2.82, H-4; δ_H 2.33, H-8; δ_H 2.65, H-12, and δ_H 2.65, H-16), and a cluster of aliphatic methylene (δ_H 1.22, H_2-17; and δ_H 1.21/1.29, H_2-19) and methine (δ_H 1.31, H-18) signals were observed. Detailed analysis of the COSY spectrum disclosed the presence of a long continuous spin system, which started from the olefinic proton H-3 and sequentially extended until the oxymethine proton H-9, with H-4 and H-8 further correlating with the methyl protons H_3-22 and H_3-23, respectively. Further inspection of the COSY spectrum allowed the assignment of two additional spin systems, corresponding to the fragments CH(11)CH(12)CH(13) and CH(15)CH(16)CH(17)CH(18)CH_2(19)CH_3(20) with H-12, H-16, and H-18 also

correlating with H_3-25, H_2-27, and H_3-28, respectively (Fig. 2). The HMBC spectrum confirmed the identified substructures and established their connection through key ^3J-correlations from H_3-24 to C-9, C-10, and C-11, from H_3-26 to C-13, C-14, and C-15, from H-9 to C-11 and C-24, and from H-13 to C-15 and C-26 (Fig. 2). In addition, the correlation from H-1 to C-2 and C-21, suggested the aldehyde group being located at C-1, thus, accounting for the remaining element of unsaturation in the structure of **1**. The configurations of the double bonds were established as *E* based on the large coupling constant between H-6 and H-7 ($^3J_{6,7}$ = 15.5 Hz), as well as the NOE correlations observed from H-3 to H-1 and H-5, from H-11 to H-9, and from H-15 to H-13 and H_2-17 (Fig. 2). This was further corroborated by the shielded carbon chemical shifts of CH_3-21 (δ_C 9.1), CH_3-24 (δ_C 11.0), and CH_3-26 (δ_C 11.2), along with their long-range COSY correlations to H-3, H-11, and H-15, respectively. Thus, the planar structure of **1** was elucidated (Fig. 1), revealing close structural analogy to those of pestalpolyols A–D and pestalpolyols E–H, which have been reported from *Pestalotiopsis* sp. cr013 and *Pestalotiopsis* sp. PG52, respectively.[20,29] The absolute configuration of pestalpolyols has previously been confirmed by single-crystal X-ray diffraction analysis. Therefore, based on the positive optical rotation value of **1** (+21.0°, *c* 0.02, MeOH), as well as on biogenetic considerations, the absolute configuration of **1** (Fig. 1) is assumed to be identical to that of pestalpolyols.[20,29] This assumption was further corroborated by their similar coupling constants and NOE correlations (Table 1). Thus, **1** was identified as a new long chain branched aldehyde for which the name pestalpolyol I is proposed.

Compound **2** was isolated as a yellowish powder. The molecular formula was determined as $C_{11}H_{10}O_5$ based on the prominent ion peak *m*/*z* 223.0601 $[M+H]^+$ in the HRESIMS spectrum. The UV spectrum was characteristic of an isocoumarin derivative with absorption maxima at λ_{max} 244, 279, and 331 nm. Comparison of **2** with the known similanpyrone B (**5**),[30] likewise isolated in this study, showed almost identical ^{1}H and ^{13}C NMR data for both compounds, the only difference being that compound **2** had a hydroxymethylene group instead of a methyl group as in **5** (Fig. 1). This hydroxymethylene group (H_2-9) was assigned at C-3, as it showed HMBC correlations to C-3 and C-4, which was further corroborated by the NOE correlation between H_2-9 and H-4. Hence, **2** was identified as a new natural product for which the name pestapyrone A is proposed.

Compound **3** was obtained as a yellow, amorphous solid, which exhibited the same UV spectrum as **2**. The molecular formula of **3** was determined as $C_{11}H_{10}O_6$ based on the prominent ion peak at *m*/*z* 239.0889 $[M+H]^+$ in the HRESIMS spectrum. Comparison of the ^{1}H and ^{13}C NMR spectra of **3** to those of **2** showed close similarity between both compounds, except for the absence of the methyl group signal at δ_H 2.09 (CH_3-10 in **2**) and the presence of a hydroxymethylene group (CH_2-10), as indicated by its deshielded signal at δ_H 4.91 (Table 2). The above spectral differences suggested that **3** features the same basic molecular framework as **2**, bearing an additional hydroxy substituent at C-10, which accounted for the molecular weight difference of 16 amu observed between both compounds. This assumption was further corroborated by the HMBC correlations of H_2-10 to C-6 (δ_C 165.8), C-7 (δ_C 113.5), and C-8 (δ_C 161.5). Accordingly, **3** was identified as a new natural product and was named pestapyrone B.

Table 1
NMR data of **1** measured in CD_3OD at 600 (^{1}H) and 150 (^{13}C) MHz

Position	δ_C, type	δ_H, mult. (*J* in Hz)	HMBC	NOESY
1	196.9, CHO	9.39, s	2, 21	3
2	139.5, C			
3	158.9, CH	6.56, dq (10.0, 1.4)	1, 21	1, 5, 4, 22
4	40.7, CH	2.82, dp (10.0, 7.0)		21
5	77.3, CH	3.98, dd (7.8, 7.0)	3, 7	3, 7, 22
6	131.9, CH	5.49, dd (15.5, 7.8)	4	8, 22, 23
7	137.7, CH	5.71, dd (15.5, 7.8)	5	5, 9, 23
8	40.7, CH	2.33, br h (7.2)	9	6, 24
9	83.7, CH	3.67, br d (9.0)	11, 24	7, 11, 23, 24
10	136.9, C			
11	133.4, CH	5.27, dd (9.8, 1.8)	9, 24	9, 25
12	36.3, CH	2.65, m	13	24
13	84.3, CH	3.69, br d (9.0)	15, 26	15, 25
14	138.6, C			
15	132.2, CH	5.09, br d (9.9)	13, 26	13, 17
16	39.5, CH	2.65, m		
17	39.4, CH_2	1.22, m		15, 27
18	33.0, CH	1.31, m		
19	31.5, CH_2	1.21, m		
		1.29, m		
20	11.5, CH_3	0.87, t (7.4)		
21	9.1, CH_3	1.75, d (1.3)	1, 2, 3	4
22	16.0, CH_3	1.06, d (6.8)	3, 4, 5	3, 5, 6
23	17.4, CH_3	0.85, d (7.2)	7, 8, 9	6, 7, 9
24	11.0, CH_3	1.65, d (1.3)	9, 10, 11	8, 9, 12
25	17.5, CH_3	0.79, d (6.8)	11, 12, 13	11, 13
26	11.2, CH_3	1.69, d (1.3)	13, 14, 15	
27	67.2, CH_2	3.45, dd (10.7, 6.0)	15	17
		3.37, dd (10.7, 7.3)		
28	19.0, CH_3	0.87, d (7.0)	18	

Compound **4** was isolated as a yellow, amorphous solid and displayed the same UV as **2** and **3**. The HRESIMS exhibited a prominent ion peak at *m*/*z* 253.0706 $[M+H]^+$ consistent with the molecular formula $C_{12}H_{12}O_6$, thus revealing a 14 amu increase in the molecular weight compared to **3**. The ^{1}H NMR data of **4** were similar to those of **3**, apart from the presence of an additional methoxy signal at δ_H 3.37, assigned to H_3-11, as indicated by its HMBC correlation to C-10 (Table 2). This assignment was corroborated by the 7.6 ppm downfield shift of the C-10 resonance of **4**, as compared to that of **3**, due to the inductive effect of the additional methoxy group (OCH_3-11). Notably, when **3** was kept in MeOH for 24 h, the formation of **4** was observed by HPLC analysis, thus indicating that the latter is probably an artifact arising through methylation of **3** during the isolation procedure. For compound **4**, the name pestapyrone C is suggested.

Compound **6** was isolated as a white, amorphous powder. It exhibited UV absorption maxima at λ_{max} 218 and 275 nm suggesting a dihydroisocoumarin as the basic structure. The molecular formula was determined as $C_{11}H_{12}O_5$, in accordance with the signal observed at 225.0758 $[M+H]^+$ in the HRESIMS. Comparison of the ^{1}H NMR data (Table 2) suggested that **6** is the dihydro derivative of **2** at C3/4, which is in accordance with the 2 amu molecular weight difference observed between both compounds. In the HMBC spectrum of **6**, the correlations observed from H_2-4 to

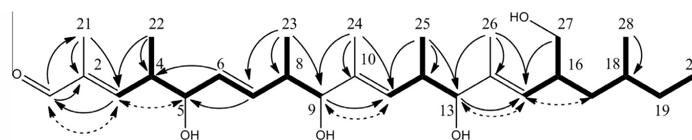

Figure 2. Key COSY (bold), HMBC (plain arrows), and NOE (dashed arrows) correlations of **1**.

Table 2
NMR data of compounds **2–4** and **6**

	2[a]		**3**[b]		**4**[b]		**6**[a]	
Pos.	δ_C[c], type	δ_H, mult.	δ_C[c], type	δ_H, mult.	δ_C[c], type	δ_H, mult.	δ_C[c], type	δ_H, mult. (J in Hz)
1	167.4, C		170.5, C		[d]		[d]	
3	156.3, C		158.3, C		158.1, C		[d]	4.54, dddd (12.0, 5.0, 4.0, 3.4)
4	104.4, CH	6.49, s	103.8, CH	6.59, s	103.3, CH	6.60, s	29.5, CH_2	2.80, dd (16.3, 3.4) 3.00, dd (16.3, 12.0)
4a	137.2, C		[d]		[d]		138.6, C	
5	102.8, CH	6.39, s	103.6, CH	6.50, s	103.4, CH	6.54, s	100.9, CH	6.26, s
6	164.8, C		165.8, C		165.6, C		162.5, C	
7	112.0, C		113.5, C		111.4, C		110.4, C	
8	161.7, C		161.5, C		162.7, C		164.2, C	
8a	99.2, C		99.9, C		99.7, C		100.8, C	
9	61.0, CH_2	4.34, s	60.3, CH_2	4.39, s	60.8, CH_2	4.40, s	[d]	3.76, dd (12.2, 5.0) 3.81, dd (12.2, 4.0)
10	7.7, CH_3	2.09, s	56.4, CH_2	4.91, s	64.0, CH_2	4.63, s	[d]	2.02, s
11					58.0, CH_3	3.37, s		
8-OH				11.49, s		11.56, s		

[a] Measured in CD_3OD at 600 (^1H) and 150 (^{13}C) MHz.
[b] Measured in $(CD_3)_2CO$ at 600 (^1H) and 150 (^{13}C) MHz.
[c] Values were extracted from HMBC data.
[d] Not observed.

C-4a and C-8a, and from H_2-9 to C-3 corroborated this assumption (Table S2). The planar structure of **6** was identical to that of the known periplanetin D, previously isolated from the American cockroach *Periplaneta americana*.[31] However, compound **6** had a positive optical rotation of +24.8° as opposed to the $[\alpha]_D^{27}$ value of −15.8° described for periplanetin D (both measured in MeOH at similar concentrations), for which the (*S*)-configuration had been demonstrated earlier. Compound **6** is thus the enantiomer of the previously reported (*S*)-periplanetin D and its absolute configuration was assigned as (*R*). For compound **6** the name (*R*)-periplanetin D is proposed.

Compound **7** was isolated as a yellow, amorphous solid. The molecular formula of **7** was determined as $C_{20}H_{22}O_6$ based on the prominent ion peak at m/z 359.1488 $[M+H]^+$ in the HRESIMS spectrum. It exhibited UV absorption maxima at λ_{max} 233, 259, and 300 nm typical for xanthone derivatives. The ^1H NMR spectrum of **7** showed signals representative of two *ortho*-coupled aromatic protons at δ_H 6.70 (d, J = 8.3 Hz, H-4) and 7.63 (d, J = 8.3 Hz, H-3), as well as two *meta*-coupled aromatic protons at δ_H 7.35 (d, J = 0.7 Hz, H-5) and 7.57 (d, J = 0.7 Hz, H-7). Additional signals included those of two methylene groups at δ_H 3.32 (dd, J = 14.0, 1.4 Hz, H_α-1′) and 2.68 (dd, J = 14.0, 10.4 Hz, H_β-1′), and 5.21 (H_2-11), two aliphatic methyl groups at δ_H 1.33 and 1.30 (H_3-4′ and H_3-5′, respectively), an aromatic methyl group at δ_H 2.53 (H_3-12), an oxymethine proton at δ_H 3.68 (dd, J = 10.4, 1.4, H-2′), and a chelated hydroxy group at δ_H 12.70 (OH-1) (Table 3). Inspection of the 2D NMR (COSY, HSQC, and HMBC) data of **7** (Fig. 3), revealed the same substitution pattern of the xanthone moiety as that of prenxanthone, previously isolated from an unidentified marine-derived member of the genus *Penicillium*.[32] Accordingly, the position of the chelated hydroxy group was assigned at C-1, as it showed HMBC correlations to C-1 and C-9a. Moreover, a hydroxymethylene group (CH_2-11) was located at C-8, as supported by its deshielded signal at δ_H 5.21 (δ_C 64.1), and by the HMBC correlations from H_2-11 to C-7 and C-8 and from H-7 to C-11. Further HMBC correlations from H_2-1′ to C-2, C-3, and C-3′, from H_3-4′ to C-2′, C-3′, and C-5′, and from H_3-5′ to C-2′, C-3′, and C-4′, in addition to the COSY correlation between H_2-1′ and H-2′, corroborated the presence of a 2-methylbutane-2,3-diol side chain and its connection to the xanthone moiety at C-2. The remaining methyl group CH_3-12 was positioned at C-6, based on its strong HMBC correlations to C-5, C-6, and C-7. These data suggested that **7** features the same basic skeleton as that of prenxanthone, apart from the isoprenoid substituent at C-2, the latter bearing a (*Z*)-2-methylbut-2-en-1-ol moiety.[32] Attempts to determine the absolute configuration of the stereogenic center at C-2′, using Mosher's reaction proved unsuccessful, probably, due to the presence of bulky substituents at C-3′. Accordingly, **7** was identified as a new natural product and was given the name pestaxanthone.

Table 3
NMR data of **7** measured in $(CD_3)_2CO$ at 600 (^1H) and 150 (^{13}C) MHz

Pos.	δ_C[a], type	δ_H, mult. (J in Hz)	HMBC
1	161.2, C		
2	119.1, C		
3	139.8, CH	7.63, d (8.3)	1, 4a, 1′
4	110.0, CH	6.70, d (8.3)	2, 9a
4a	154.4, C		
5	117.3, CH	7.35, d (0.7)	7, 8a, 10a, 12
6	147.9, C		
7	124.7, CH	7.57, d (0.7)	5, 8a, 11, 12
8	146.4, C		
8a	116.8, C		
9	[b]		
9a	110.2, C		
10a	158.4, C		
11	64.1, CH_2	5.21, s	7, 8
12	22.2, CH_3	2.53, s	5, 6, 7
1′	32.1, CH_2	3.32, dd (14.0, 1.4) 2.68, dd (14.0, 10.4)	2, 3 2, 3, 3′
2′	72.7, CH	3.68, dd (10.4, 1.4)	
3′	78.9, C		
4′	25.8, CH_3	1.33, s	2′, 3′, 5′
5′	25.4, CH_3	1.30, s	2′, 3′, 4′
1-OH		12.70, s	1, 9a

[a] Values were extracted from HMBC data.
[b] Not observed.

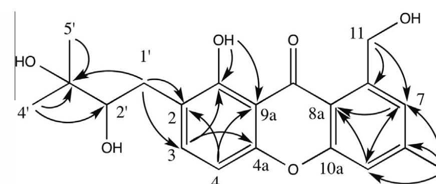

Figure 3. Key HMBC correlations of **7**.

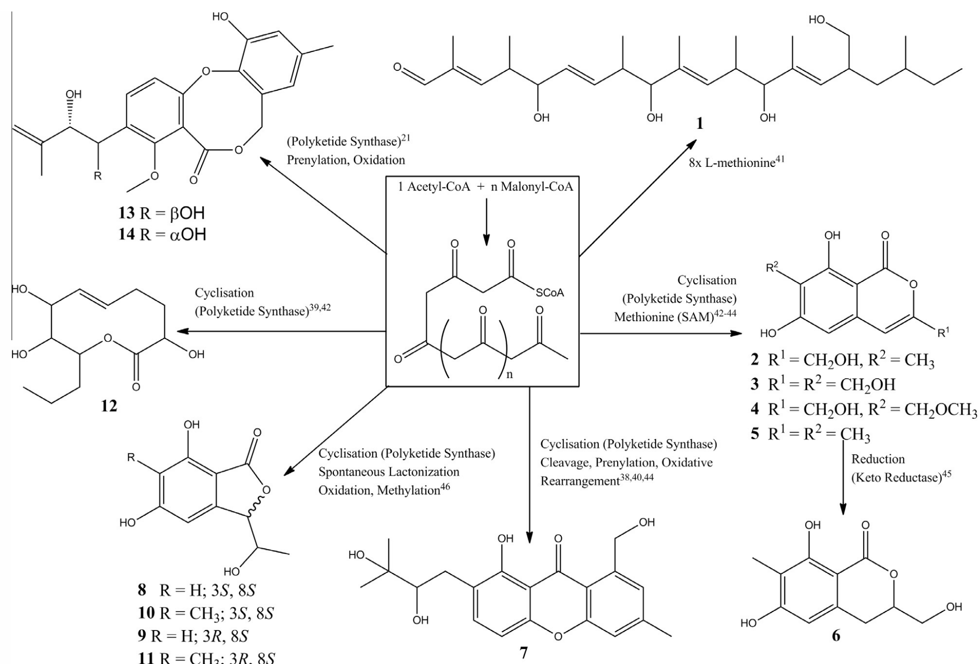

Figure 4. Putative biosynthesis of compounds **1–14**.

Compound **8** was obtained as a colorless oil and showed identical UV absorption maxima (λ_{max}, MeOH) to those observed for the known congener **9** (226, 258, and 292 nm),[33] likewise isolated in this study. Moreover, both compounds shared the same molecular formula ($C_{10}H_{10}O_5$) as indicated by HRESIMS (*m*/*z* 211.0601 $[M+H]^+$). Detailed analysis of the 2D NMR (COSY, HSQC, HMBC) data of **8**[28] established the same planar structure as for **9** (Fig. 1). However, the ^{1}H and ^{13}C NMR spectra of **8** were almost superimposable to those of **9**, thus suggesting that both compounds are epimers, differing in the configuration of either the C-3 or C-8 chirality center. It is worth mentioning that **8** and **9** are derivatives of the known epimers pestaphthalide A (**10**) and pestaphthalide B (**11**), which were likewise isolated in this study, the only difference being the presence of an aromatic proton at C-6, instead of a methyl group as in **10** and **11** (Fig. 1). The absolute configurations of **10** and **11** had previously been assigned as (3*S*,8*S*) and (3*R*,8*S*), respectively, by CD analysis, as well as by the modified Mosher method.[34] By taking into account the (3*R*,8*S*) configuration of **9** and the optical rotation value of **8** (+28.0°, *c* 0.05, MeOH), which is on the positive side as observed for **10** (+51.0°, *c* 0.05, MeOH), the absolute configuration of **8** was deduced as (3*S*,8*S*). This was further corroborated by the similar NMR data and coupling constants with those previously described for **10** and **11**.[34] Thus, **8**, previously described as a synthetic acetophthalidin derivative,[35] was identified as a new natural product for which the name norpestaphthalide A is proposed.

The known compounds similanpyrone B (**5**),[30] norpestaphthalide B (**9**),[33] pestaphthalides A (**10**) and B (**11**),[34] 2-*epi*-herbarumin II (**12**),[36] and pestalotiollides A (**13**) and B (**14**),[37] were identified on the basis of their MS and NMR data, as well as by comparison with the literature.

All compounds described in this study are biosynthetically derived from the polyketide pathway (Fig. 4). Plausible biogenetic pathways for the formation of compounds **1–14** can be found in the literature[21,38–46] and are summarized in Figure 4.

All compounds isolated in this study were submitted to an MTT assay with the murine lymphoma cell line L5178Y. Compound **1** was the most active compound encountered in this study and exhibited an IC_{50} value of 4.1 μM against the murine lymphoma cells, which is comparable to that of the positive control kahalalide F (4.3 μM). The strong cytotoxicity of **1** is in accordance with that of similar polyketides reported in the literature, e.g., pestalpolyol A, which was tested against the five human tumor cell lines HL-60, SMMC-7721, A-549, MCF-7, and SW480 with IC_{50} values of 10.4, 11.3, 2.3, 13.7, and 12.4 μM, respectively.[20] All other compounds isolated in this study exhibited no activity (IC_{50} >10 μM) in the respective assay.

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Supplementary data

Supplementary data (general experimental procedures and the NMR spectra for all new compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.03.101.

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22. Pestalpolyol I (**1**): white, amorphous powder; $[\alpha]_D^{27}$ +21.0° (*c* 0.02, MeOH); UV λ_{max} (PDA): 232 nm; ^{1}H, ^{13}C, and 2D NMR data, Table 1; HRESIMS *m*/*z* 465.3573 $[M+H]^+$ (calcd for $C_{28}H_{49}O_5$, 465.3575).
23. Pestapyrone A (**2**): yellowish powder; UV λ_{max} (PDA): 244, 279, and 331 nm; ^{1}H, ^{13}C, and 2D NMR data, Tables 2 and S2; HRESIMS *m*/*z* 223.0601 $[M+H]^+$ (calcd for $C_{11}H_{11}O_5$, 223.0602).
24. Pestapyrone B (**3**): yellow, amorphous solid; UV λ_{max} (PDA): 246, 279, and 330 nm; ^{1}H, ^{13}C, and 2D NMR data, Tables 2 and S2; HRESIMS *m*/*z* 239.0889 $[M+H]^+$ (calcd for $C_{11}H_{11}O_6$, 239.0887).
25. Pestapyrone C (**4**): yellow, amorphous solid; UV λ_{max} (PDA): 246, 279, and 330 nm; ^{1}H, ^{13}C, and 2D NMR data, Tables 2 and S2; HRESIMS *m*/*z* 253.0706 $[M+H]^+$ (calcd for $C_{12}H_{13}O_6$, 253.0707).
26. (*R*)-Periplanetin D (**6**): white, amorphous powder; $[\alpha]_D^{27}$ +24.8° (*c* 0.1, MeOH); UV λ_{max} (PDA): 218 and 275 nm; ^{1}H, ^{13}C, and 2D NMR data, Tables 2 and S2; HRESIMS *m*/*z* 225.0758 $[M+H]^+$ (calcd for $C_{11}H_{13}O_5$, 225.0757).
27. Pestaxanthone (**7**): yellow, amorphous solid; $[\alpha]_D^{27}$ +42.4° (*c* 0.1, MeOH); UV λ_{max} (PDA): 233, 259, and 300 nm; ^{1}H, ^{13}C, and 2D NMR data, Table 3; HRESIMS *m*/*z* 359.1488 $[M+H]^+$ (calcd for $C_{20}H_{23}O_6$, 359.1489).
28. Norpestaphthalide A (**8**): colorless oil; $[\alpha]_D^{27}$ +28.0° (*c* 0.05, MeOH); UV λ_{max} (PDA): 226, 258, and 292 nm; ^{1}H NMR (600 MHz, $(CD_2)_3CO$): δ_H 1.20 (d, *J* = 6.4 Hz, H_3-9), 4.21 (qd, *J* = 6.4, 3.4 Hz, H-8), 5.29 (d, *J* = 3.4 Hz, H-3), 6.37 (d, *J* = 1.3 Hz, H-6), 6.57 (d, *J* = 1.3 Hz, H-4); ^{13}C NMR (150 MHz, $(CD_2)_3CO$): δ_C 18.6 (C-9), 67.8 (C-8), 84.3 (C-3), 102.3 (C.4), 103.0 (C-6), 105.0 (C-7a), 155.9 (C-3a), 158.8 (C-7), 165.6 (C-5), 168.2 (C-1); HRESIMS *m*/*z* 211.0601 $[M+H]^+$ (calcd for $C_{10}H_{11}O_5$, 211.0602).
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Supporting information

Polyketides from the Mangrove-derived fungal endophyte *Pestalotiopsis clavispora*

Catalina Francis Pérez Hemphill[a], Georgios Daletos[a], Zhen Liu[a], Wenhan Lin[b], Peter Proksch[a,*]

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

[b]*State Key Laboratory of Natural and Biomimetic Drugs, Peking University, 100191 Beijing, PR China*

* Corresponding author. Tel.: +49 211 81 141 63; fax: +49 211 81 119 23. *E-mail address:* proksch@uni-duesseldorf.de (P. Proksch).

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General experimental procedures

General

The specific rotation of all optically active pure compounds was measured with a PerkinElmer 241MC polarimeter using Uvasol® spectroscopic grade solvents (Merck).

1D and 2D NMR (^{1}H and ^{13}C) spectra were recorded with a Bruker Avance III 600 MHz NMR spectrometer. LCMS spectra were measured using a HP110 Agilent Finnigan LCQDecaXP Thermoquest machine and for new compounds HRESIMS spectra were recorded with a UHR-TOF maxis 4G mass spectrometer. For analytical HPLC analysis a DIONEX p580 UVD340S PDA system with a Eurosphere-10 C18 column was used, which was coupled with a UVD340S PDA detector with a detection wavelength set at 285, 254, 280, and 340 nm. Separation was achieved with a flow rate of 1 mL/min and a linear gradient of 0.1% aqueous formic acid and MeOH. Semi-preparative HPLC was performed with a Merck Hitachi HPLC (I-7100 pump and I-7400 UV detector), using a prefilled Eurosphere-10 C18 column (5μm; 300 x 8 mm) with a linear gradient of 0.1% aqueous TFA and MeOH at a flow rate of 5 mL/min. Column chromatography was done with silica gel 60M (0.04-0.063 mm; Macherey-Nagel), or Sephadex LH-20 (Sigma) as stationary phases. TLC plates, which were precoated with silica gel 60 F254, were used for the analysis of fractions at 254 and 366 nm. Apart from spectral grade solvents for spectroscopic measurements, distilled solvents were used.

Fungal material

P. clavispora was isolated from petiole samples of the Mangrove *Rhizophora harrisonii*, which were collected from the Swamp Forest Research Station Onne in Port Harcourt, Rivers State (Nigeria), in June 2012. The isolation and identification procedure followed

the protocol published by Kjer *et al.* in 2010 and the accession number **JN002166.1** was assigned, after submitting the sequence data of the obtained endophyte *P. clavispora* to GenBank. The voucher specimen was deposited at the corresponding author's laboratory.

Extraction and isolation

Following the standard cultivation procedure[1] the pure fungal strain, identified as *P. clavispora*, was transferred to solid rice medium and cultivated for five weeks at 22°C, before being extracted with ethyl acetate and subsequently dried with a rotary evaporator, to gain the crude extract (4g). The first fractionation step consisted of a VLC separation with silica gel as stationary phase. The nonpolar fraction (465 mg), was obtained using the mobile phase n-hexane/ethyl acetate (2:3) and yielded compound **5** (5.1 mg) after further fractionation with Sephadex LH-20 (fraction 3), using methanol/dichloromethane (1:1) as mobile phase. The next polar VLC fraction (259 mg), which was obtained using the mobile phase n-hexane/ethyl acetate (1:4), was further separated via a Sephadex column with methanol as mobile phase and yielded compound **2** (fraction 4, 4.8 mg). Compound **12** (2.2 mg) was isolated from fraction 2 (90 mg) by subsequent purification with a Sephadex column, using methanol as eluent. Fraction 3 (30 mg) was further purified using semi-preparative RP-HPLC, and yielded compounds **10** (3.1 mg), **11** (2.9 mg), **13** (4.3 mg), and **14** (2.7 mg). From the VLC separation, the fractions obtained with ethyl acetate (100%) and dichloromethane/methanol (9:1 and 7:1) were combined (1072 mg). Half of this fraction was submitted to a Sephadex column with methanol as eluent. Fraction 3 (32 mg) was further submitted to semi-preparative RP-HPLC and yielded compounds **7** (1.1 mg), **8** (1.0 mg), and **9** (1.0 mg). Fraction 4 (10 mg) was also submitted to semi-

preparative RP-HPLC and yielded compounds **3** (1.2 mg), **4** (4.1 mg), and **6** (1.0 mg). The second half of the VLC fraction last mentioned was submitted to RP-VLC, yielding compound **1** (1.5 mg) after semi-preparative RP-HPLC.

Bioassay

All compounds isolated in this study were submitted to an MTT assay with the mouse lymphoma L5178Y cell line. The assay was performed as previously described.[2-4]

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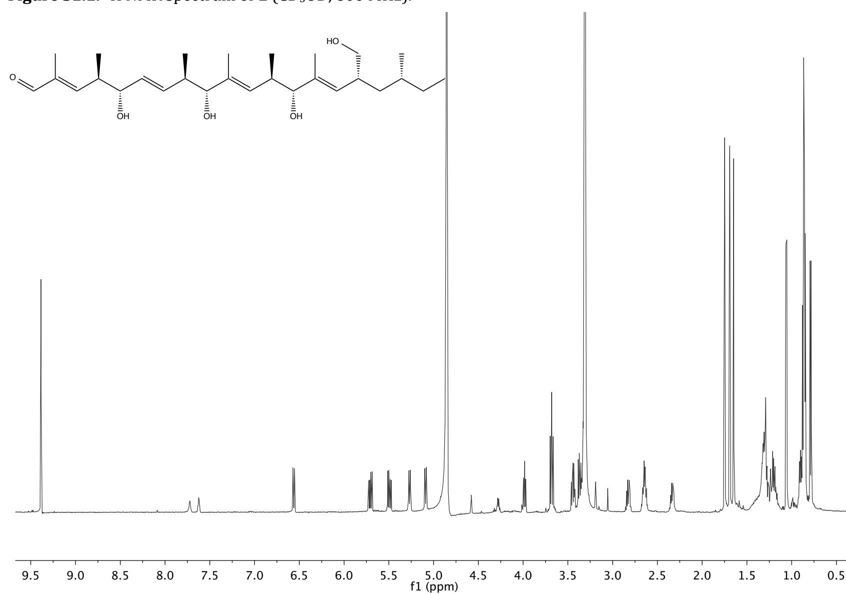
Table S2. NMR data of compounds **2-4** and **6**.

	2[a]			**3**[b]			**4**[b]			**6**[a]		
Pos.	δ_C[c], type	δ_H, mult.	HMBC	δ_C[c], type	δ_H, mult.	HMBC	δ_C[c], type	δ_H, mult.	HMBC	δ_C[c], type	δ_H, mult. (J in Hz)	HMBC
1	167.4, C			170.5, C			[d]			[d]		
3	156.3, C			158.3, C			158.1, C			[d]	4.54, dddd (12.0, 5.0, 4.0, 3.4)	
4	104.4, CH	6.49, s	3, 4a, 5, 8a, 9	103.8, CH	6.59, s	3, 8a	103.3, CH	6.60, s	3, 5, 8a, 9	29.5, CH_2	2.80, dd (16.3, 3.4) 3.00, dd (16.3, 12.0)	4a, 8a
4a	137.2, C			[d]			[d]			138.6, C		
5	102.8, CH	6.39, s	4, 6, 7, 8a	103.6, CH	6.50, s	4, 7, 8a	103.4, CH	6.54, s	4, 6, 7, 8a	100.9, CH	6.26, s	4, 7, 8a
6	164.8, C			165.8, C			165.6, C			162.5, C		
7	112.0, C			113.5, C			111.4, C			110.4, C		
8	161.7, C			161.5, C			162.7, C			164.2, C		
8a	99.2, C			99.9, C			99.7, C			100.8, C		
9	61.0, CH_2	4.34, s	3, 4	60.3, CH_2	4.39, s	3, 4	60.8, CH_2	4.40, s	3	[d]	3.76, dd (12.2, 5.0) 3.81, dd (12.2, 4.0)	3
10	7.7, CH_3	2.09, s	6, 7, 8	56.4, CH_2	4.91, s	6, 7, 8	64.0, CH_2	4.63, s	6, 7, 8, 11	[d]	2.02, s	6, 7, 8
11							58.0, CH_3	3.37, s	10			
8-OH					11.49, s			11.56, s	7, 8, 8a			

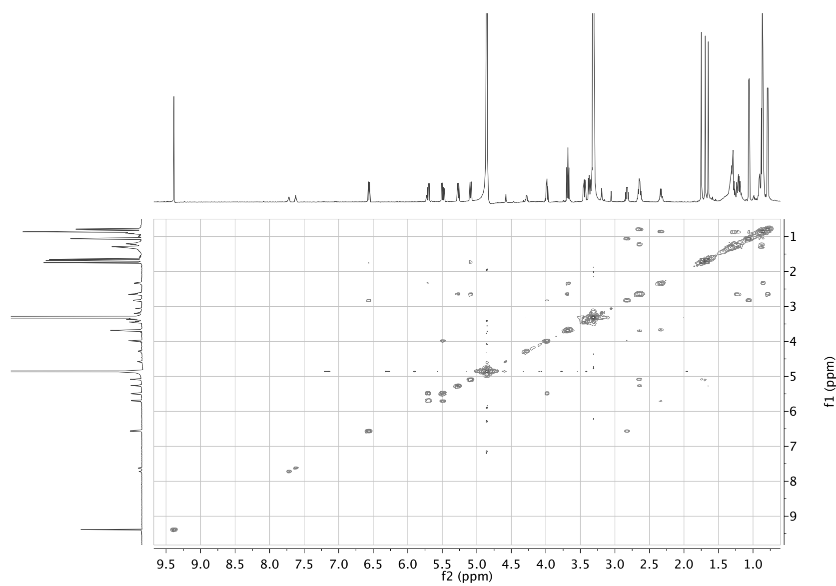
[a] Measured in CD_3OD at 600 (^1H) and 150 (^{13}C) MHz
[b] Measured in $(CD_3)_2CO$ at 600 (^1H) and 150 (^{13}C) MHz
[c] Values were extracted from HMBC data
[d] Not observed

Figure S1.1. ^{1}H NMR spectrum of **1** (CD_3OD, 600 MHz).



7

Figure S1.2. ^{1}H,^{1}H-COSY spectrum of **1** (CD_3OD, 600 MHz).



8

Figure S1.3. HSQC spectrum of **1** (CD_3OD, 600 MHz).

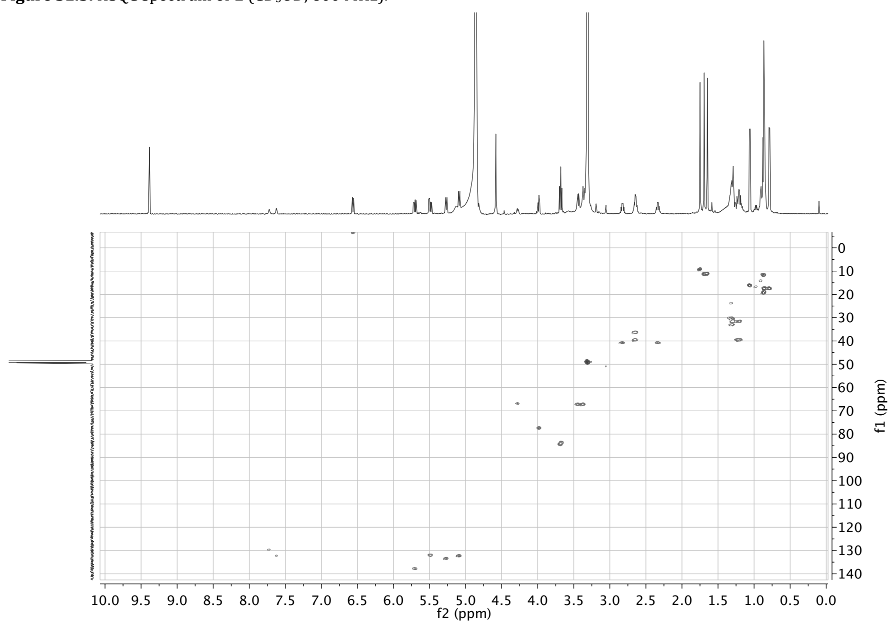


Figure S1.4. HMBC spectrum of **1** (CD_3OD, 600 MHz).

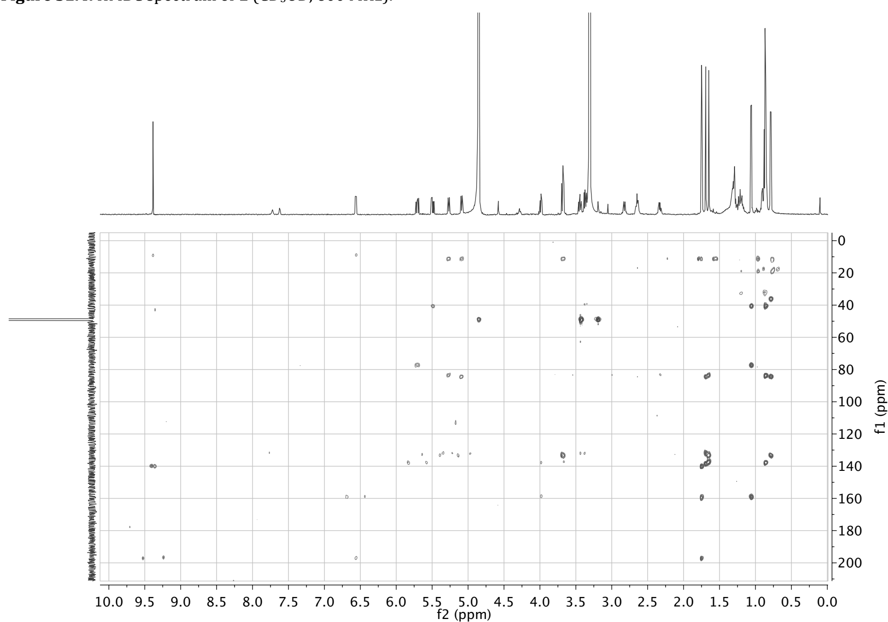


Figure S1.5. NOESY spectrum of **1** (CD_3OD, 600 MHz).

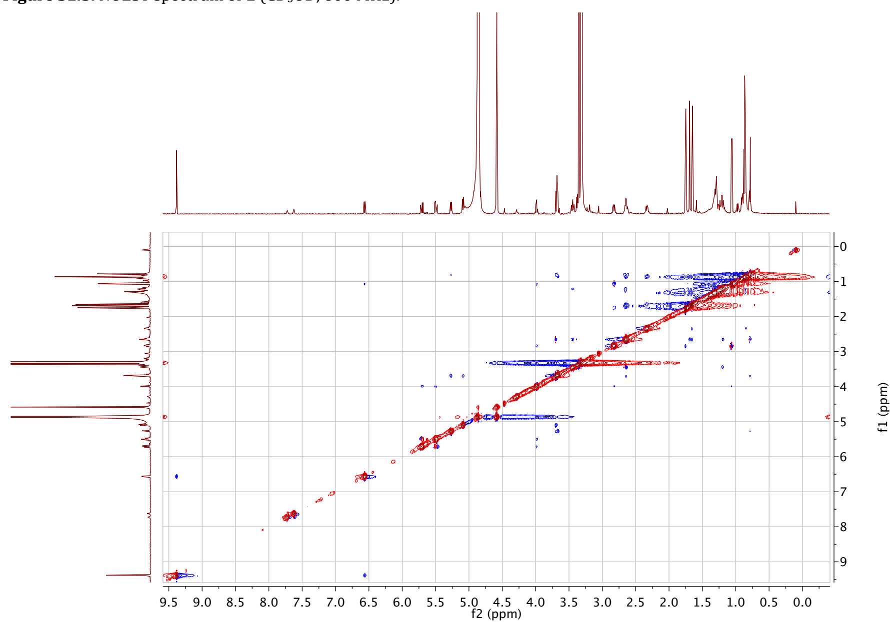


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Figure S2.1. ^1H NMR spectrum of **2** (CD_3OD, 600 MHz).

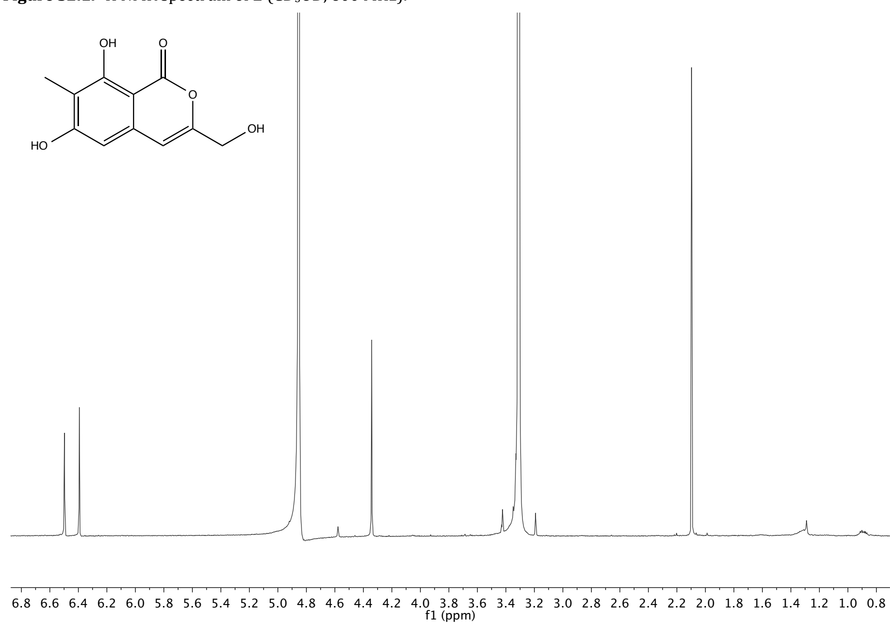

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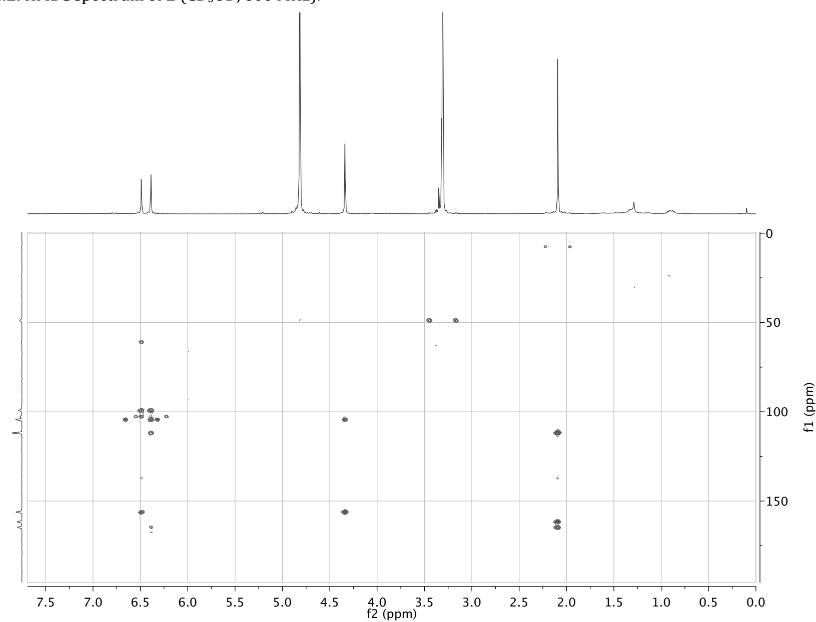
Figure S2.2. HMBC spectrum of **2** (CD_3OD, 600 MHz).



13

Figure S3.1. ^{1}H NMR spectrum of **3** (CD_3OD, 500 MHz).

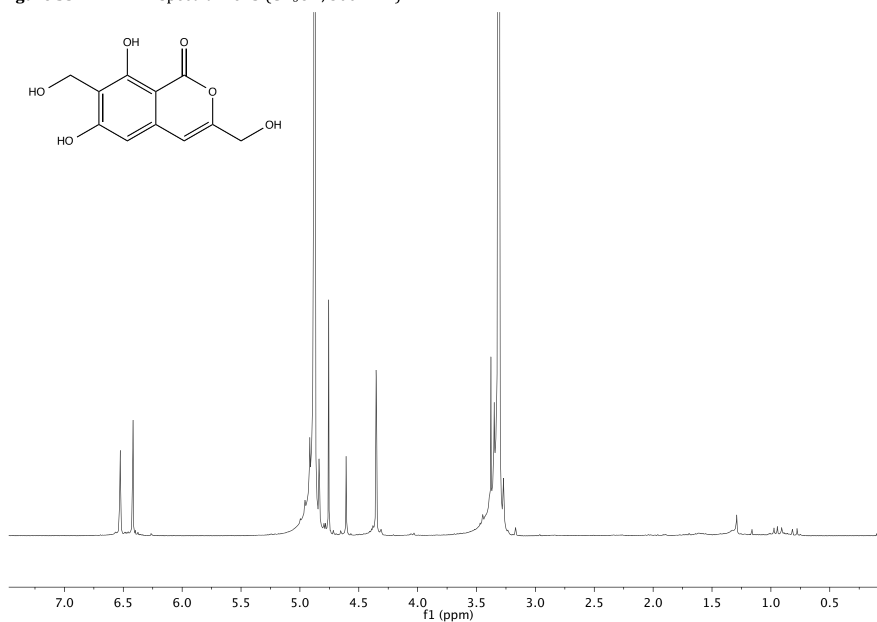


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Figure S3.2. ^{1}H NMR spectrum of **3** (red) and **4** (purple) as a 1:0.7 mixture ($(CD_3)_2CO$, 600 MHz).

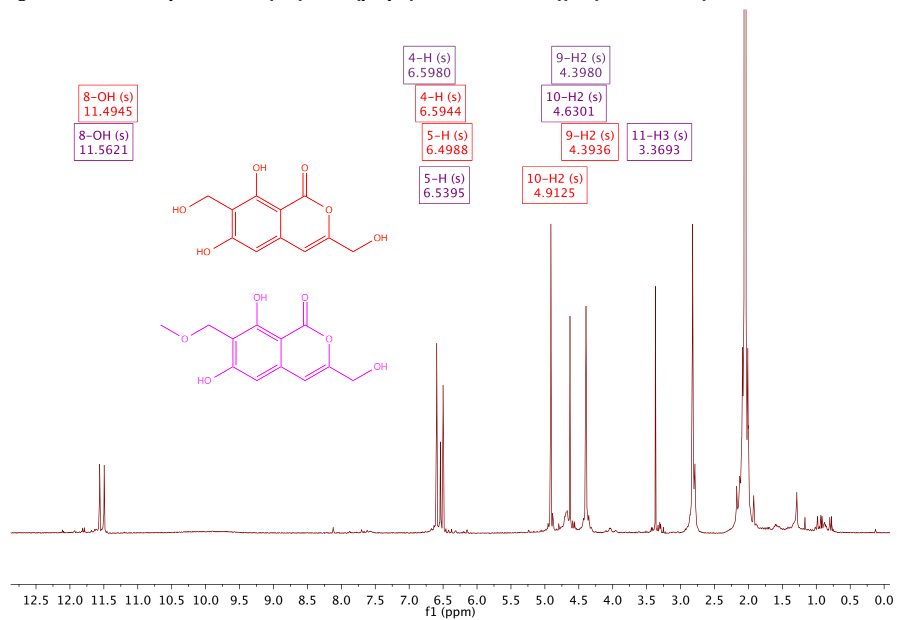

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Figure S3.3. HMBC spectrum of **3** ($(CD_3)_2CO$, 600 MHz).

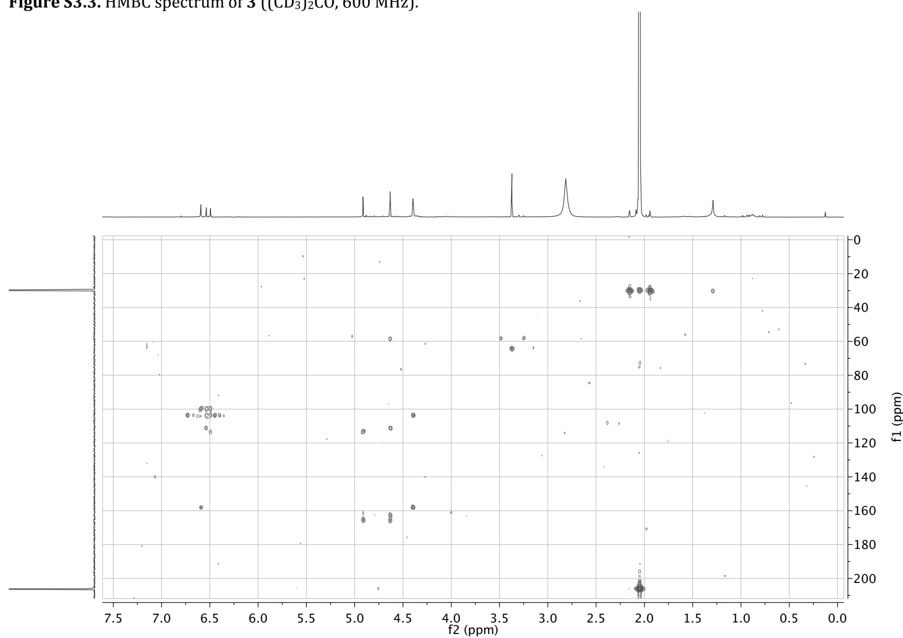

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Figure S4.1. ^{1}H NMR spectrum of **4** ($(CD_3)_2CO$, 600 MHz).

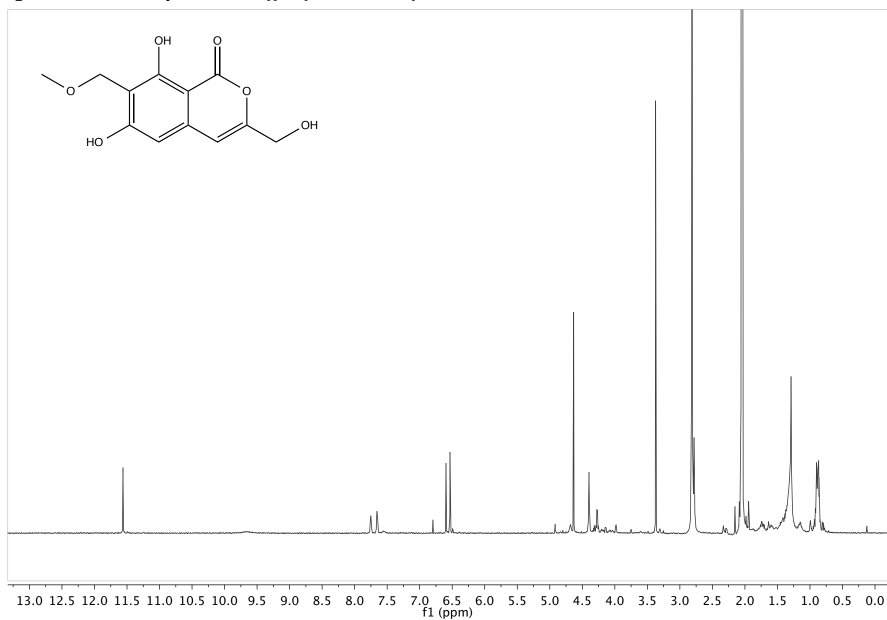

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Figure S4.2. HMBC spectrum of **4** ($(CD_3)_2CO$, 600 MHz).

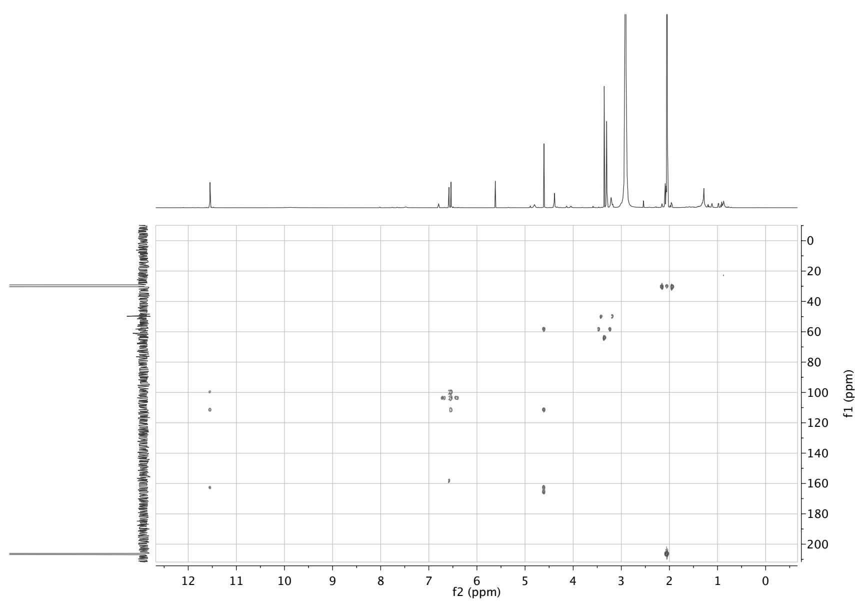

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Figure S5.1. ^{1}H NMR spectrum of **6** (CD_3OD, 600 MHz).

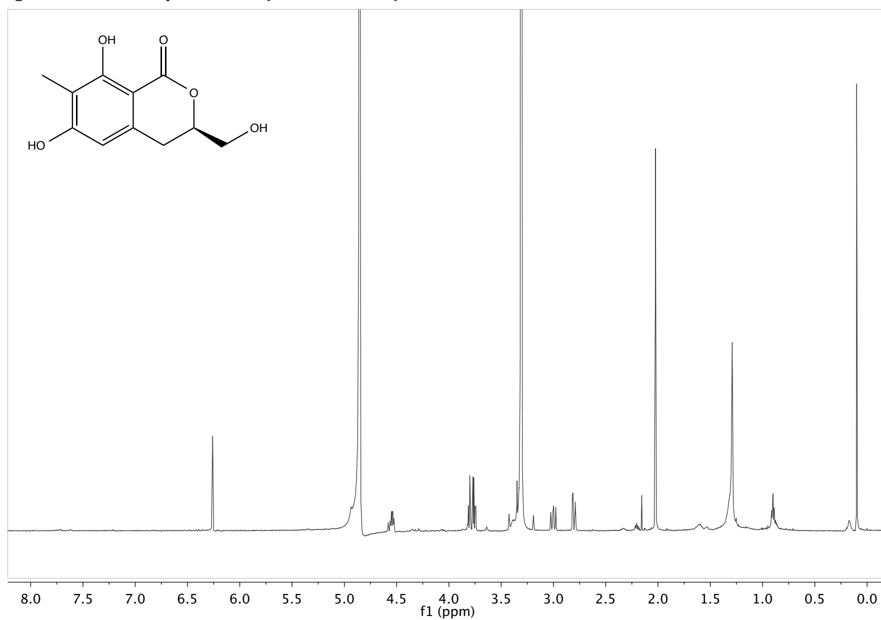

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Figure S5.2. HMBC NMR spectrum of **6** (CD_3OD, 600 MHz).

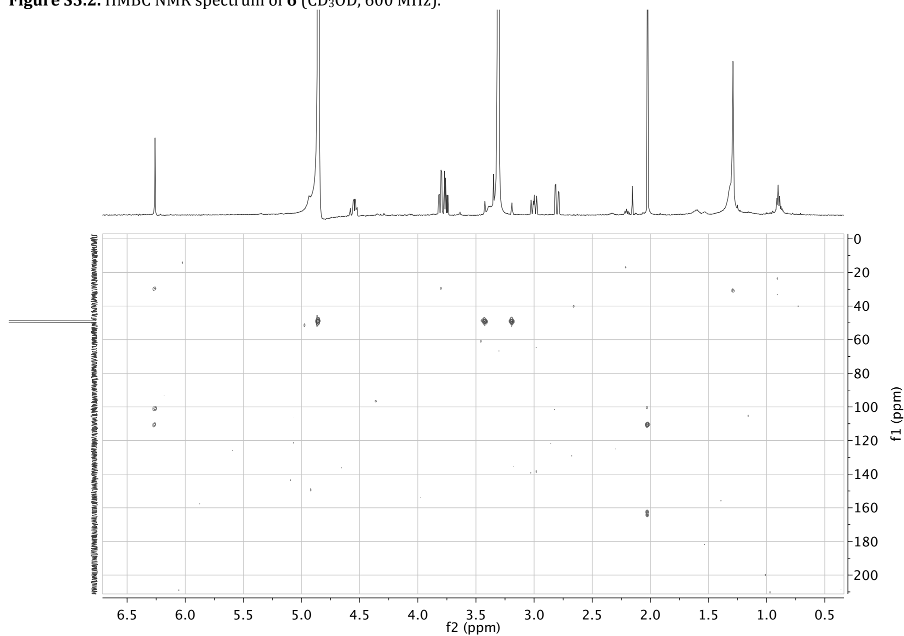

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Figure S6.1. ^{1}H NMR spectrum of **7** ($(CD_3)_2CO$, 600 MHz).

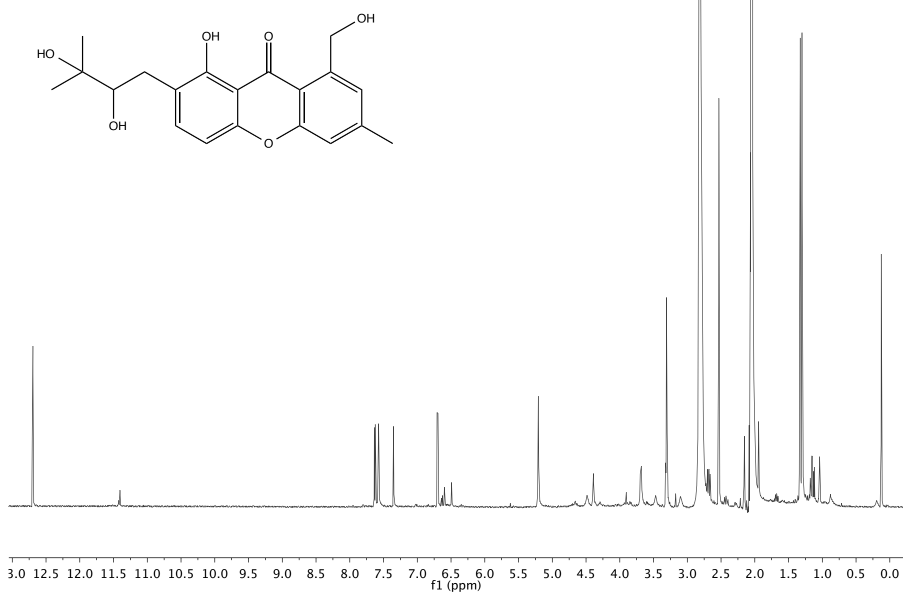

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Figure S6.2. HMBC spectrum of **7** ($(CD_3)_2CO$, 600 MHz).

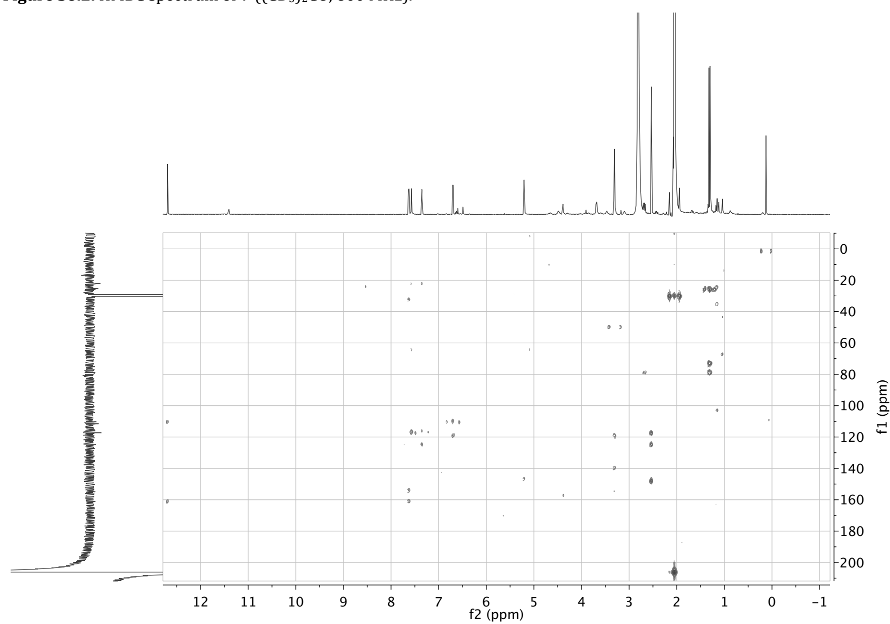

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Figure S7.1. ^{1}H NMR spectrum of **8** ($(CD_3)_2CO$, 600 MHz).

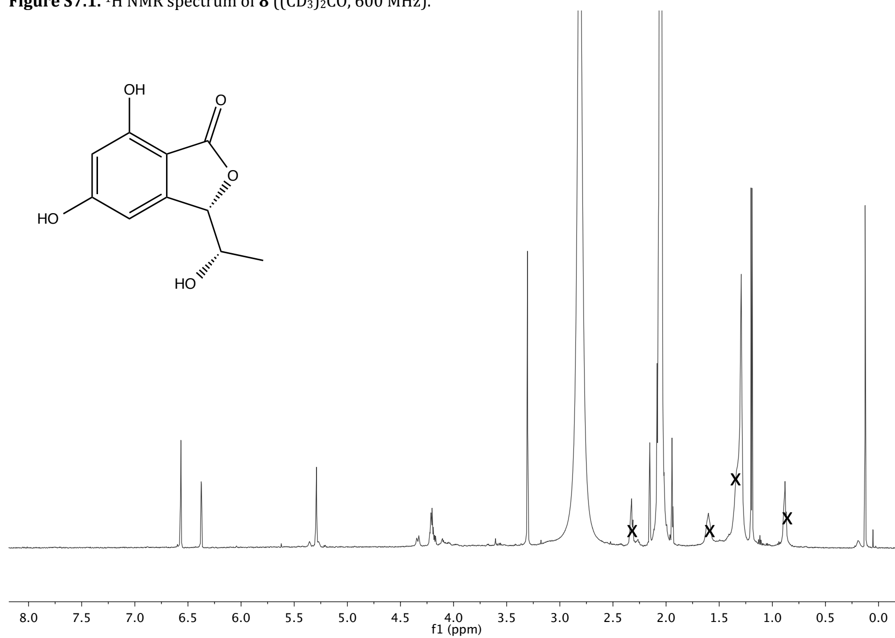

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Figure S7.2. HSQC spectrum of **8** ($(CD_3)_2CO$, 600 MHz).

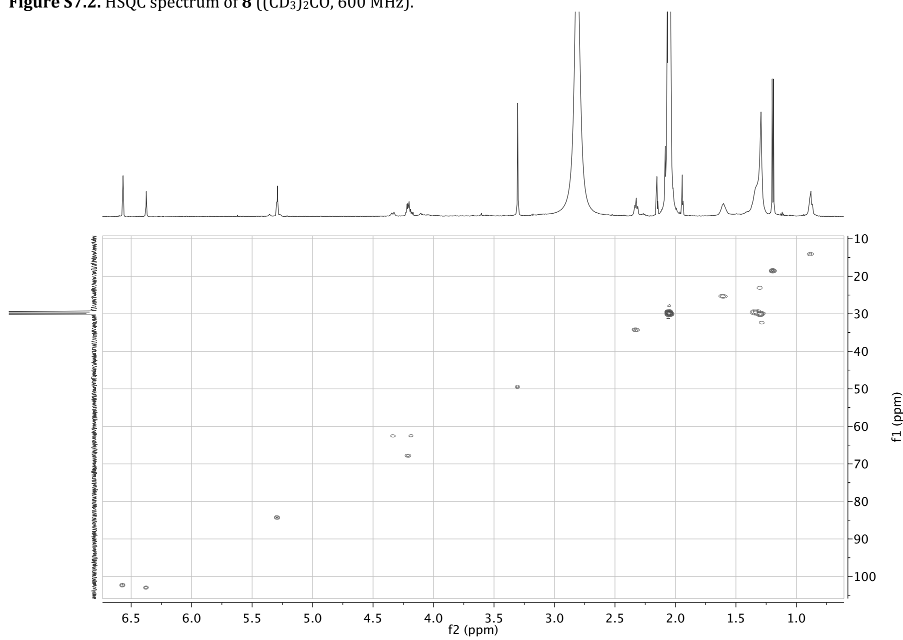

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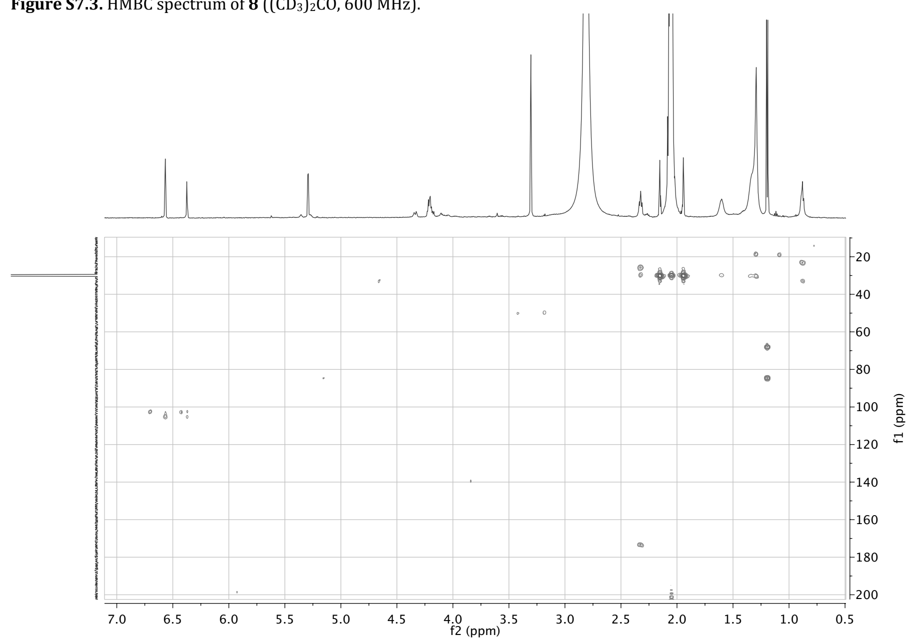
Figure S7.3. HMBC spectrum of **8** ($(CD_3)_2CO$, 600 MHz).



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Section 2

Absolute Configuration and anti-tumor Activity of Torrubiellin B

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Absolute configuration and anti-tumor activity of torrubiellin B

Catalina Francis Pérez Hemphill [a], Georgios Daletos [a], Alexandra Hamacher [b], Matthias Ullrich Kassack [b], Wenhan Lin [c], Attila Mándi [d], Tibor Kurtán [d], Peter Proksch [a,*]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University Duesseldorf, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany
[b] Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Duesseldorf, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany
[c] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, 100191 Beijing, PR China
[d] Department of Organic Chemistry, University of Debrecen, POB 20, 4010 Debrecen, Hungary

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A B S T R A C T

The dimeric anthracene derivative torrubiellin B (**1**) was isolated from the endophytic fungus *Acremonium* sp. that had been obtained from leaves of the Mangrove plant *Sonneratia caseolaris*. The absolute configuration of **1** was established as (5′*R*,10′*S*,10a′*R*) for the first time on the basis of its electronic circular dichroism (ECD) spectra aided with TDDFT-ECD calculations. Torrubiellin B (**1**) exhibited strong anti-tumor activity when tested in vitro against several solid cancer cell lines including cells that are resistant against the widely used cytostatic drug cisplatin. The IC_{50} values of **1** against cisplatin sensitive and cisplatin resistant cells were in the range of 0.2–2.6 μM depending on cell line investigated.
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Introduction

Endophytic fungi are known as a rich source of structurally diverse bioactive compounds that are often unprecedented in other organisms.[1–7] Endophytes which inhabit plants that are subject to severe stress conditions are especially interesting for bioprospecting since the fungi are assumed to contribute to the survival of their hosts, for example, through accumulation of defense metabolites.[8–10] We and others have repeatedly shown that Mangrove associated endophytes are valuable sources of new bioactive metabolites such as the anti-tumor metabolite phomoxanthone A isolated from the Mangrove endophyte *Phomopsis longicolla*, which was found to strongly inhibit tumor cells.[11] Mangroves are prone to numerous stress factors such as changing levels of salinity, periodical flooding, and a high incidence of herbivores and of pathogenic microbes. Apparently these factors have shaped not only the secondary metabolites of the hosts but also those of their associated endophytes.[12,13]

In the course of our ongoing studies on natural products from Mangrove derived endophytes we investigated the endophytic fungus *Acremonium* sp. that was isolated from leaves of the Mangrove plant *Sonneratia caseolaris* from the island of Hainan (PR China). Our interest in this fungus had been aroused by the strong anti-tumor activity of its crude EtOAc extract when tested in vitro against solid cancer cell lines including cells that are highly resistant against the widely used cytostatic drug cisplatin (severe growth inhibition of A2780sens cells observed at 10 μg/mL, and of A2780CisR cells at 100 μg/mL). Resistance of cancer cells that emerges frequently during chemotherapy is a serious problem for therapy of tumor patients and is responsible for the ultimate failure of this therapeutic approach in spite of reduction of tumor load during early phases of treatment.[14,15] Therefore, new therapeutics that are able to break resistance against currently employed cytostatic drugs are urgently needed.

During bioassay guided fractionation of the extract of *Acremonium* sp. torrubiellin B (**1**),[16] an asymmetric anthracene derivative, was isolated as the active ingredient that is responsible for the anti-tumor activity of the fungal extract. Torrubiellin B had first been reported from *Torrubiella* sp. BCC 28517 and the relative configuration of the compound had been determined through NOE experiments.[17] However, the absolute configuration of the compound was unknown so far. The determination of the absolute configuration of torrubiellin B (**1**) especially in light of its prominent activity against cancer cells is an important issue during structure determination. Here we report for the first time the absolute configuration of torrubiellin B (**1**) together with its pronounced anti-tumor activity against cisplatin sensitive and cisplatin resistant solid cancer cell lines.

* Corresponding author. Tel.: +49 2118114170; fax: +49 2118111923.
E-mail address: proksch@uni-duesseldorf.de (P. Proksch).

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Results and discussion

The ethyl acetate extract of the endophytic fungus *Acremonium* sp. grown on solid rice medium was submitted to a bioassay guided chromatographic separation using different stationary phases including silica gel, Sephadex LH-20 and semi-preparative HPLC on a C18 reversed phase column. Torrubiellin B (**1**) was isolated as the major constituent of the extract (Fig. 1). Compound **1** exhibited a UV/Vis spectrum with three absorption maxima at 206, 283, and 397 nm that is typical for a conjugated system. In the HRESIMS the pseudomolecular ion was detected at *m*/*z* 541.1138 $[M+H]^+$ and the molecular formula was determined as $C_{30}H_{20}O_{10}$. Comparison of the MS and NMR data of **1** with those previously reported for torrubiellin B suggested that they are identical.[17] The structure of **1** was finally confirmed as torrubiellin B by careful analysis of COSY, HMBC, and ROESY correlations. Interpretation of the COSY and HMBC correlations shown in Figure 2 revealed two anthracene moieties being fused via a cyclopentene ring. The HMBC correlations from H-5′ to C-4, C-3, C-10a′, and to C-11 established the connectivity of both monomers. The formation of this rare linkage between C-4 and C-5′ and between C-3 and C-10a′ through C-11 is a structural feature that is inherent to torrubiellins. The relative configuration of C-5′ and C-10′ was deduced from ROESY experiments (Fig. 4). H-10′ showed ROE correlations to H-5′ and $H\text{-}11_{eq}$ and furthermore $H\text{-}11_{eq}$ had a correlation to H-2, thus indicating the (5′*S**,10′*R**,10a′*S**) relative configuration of **1** as shown in Figure 4, which is in agreement with that previously reported.[17]

Torrubiellin B may be viewed as a 4,5′-linked heterodimeric biaryl of 7-hydroxyemodin and 1,8,10-trihydroxy-6-methyl-4a,10-dihydroanthracen-9(4*H*)-one, in which the rotation along the biaryl axis is blocked by the methylene linker between C-3 and C-10a. In contrast to axially chiral biaryls of anthracene-9,10-dione monomers,[18] torrubiellin B has only central chirality elements due to the C-5′, C-10,′ and C-10a′ chirality centers and the projection angle between the planes of the two monomers is governed by the central chirality. The solution ECD spectra of torrubiellin B showed intense negative Cotton effect (CE) at 435 nm and a positive one at 395 nm accompanied by shoulders and quite a number of other overlapping high-energy ECD transitions (Fig. 3). For the configurational assignment, solution conformational analysis and TDDFT-ECD calculations were performed. The initial MMFF conformational analysis of the arbitrarily selected (5′*S*,10′*R*,10a′*S*)-**1** resulted in 13 conformers, which were reoptimized at B3LYP/6-31G(d) level in vacuo. The reoptimization afforded 2 conformers (96.8% and 2.9% populations) above 1% Boltzmann-population, which differed only in the orientation of hydrogens of the 10′-OH and 3′-Me groups (Fig. 4). The $\omega_{C\text{-}3,C\text{-}4,C\text{-}5',C\text{-}6'}$ dihedral angle, a parameter describing the relative orientation of the electric transition moments of the two subunits, was found to be +103.17°. TDDFT-ECD calculations of the two low-energy conformers were carried out at 3 different levels of theory and the Boltzmann-averaged spectra were observed as the mirror-image of the experimental ECD curve. The PBE0/TZVP method showed the best agreement,

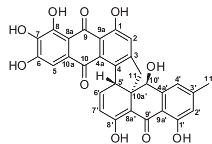

Figure 1. Structure of torrubiellin B (**1**).

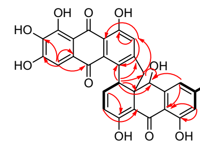

Figure 2. Key COSY (bold lines) and HMBC (arrows) correlations of torrubiellin B (**1**).

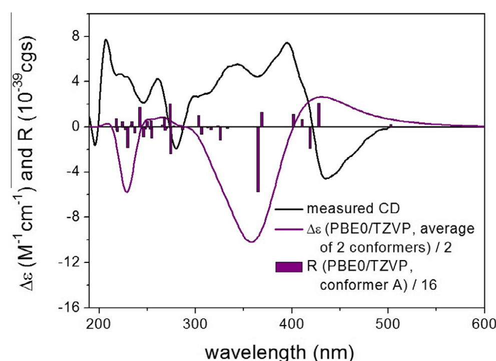

Figure 3. Experimental spectrum of **1** in MeCN and PBE0/TZVP-calculated ECD spectrum of the two low-energy conformers (>1%) of (5′*S*,10′*R*,10a′*S*)-**1** in vacuo. Bars represent the calculated rotational strength values of the lowest-energy conformer.

the result of which is shown in Figure 4. This allowed determining the absolute configuration of torrubiellin B (**1**) as (+)-(5′*R*,10′*S*,10a′*R*).

The putative biogenetic formation of **1** and of the structurally related 6,7-dideshydroxy derivative torrubiellin A (**2**) has been suggested to occur through oxidative coupling of emodin (**5**), aloe-emodin (**6**) or of chrysophanol (**7**) on the basis of the co-occurrence of these monomers in the respective investigated fungal extracts.[17] After thorough UV and LCMS analysis of the crude ethyl acetate extract of *Acremonium* sp. analyzed in this study typical UV absorption maxima at 223, 250, 273, 293, and 442 nm, as well as a prominent pseudomolecular ion peak at 269.5 $[M-H]^-$ were detected that account for emodin, but no signals were found for aloe-emodin or for chrysophanol. These findings suggest that **1** may biogenetically arise from two emodin (**5**) moieties through subsequent reduction and hydroxylation.

Compound **1** showed pronounced cytotoxic activity against several human solid cancer cell lines including Cal27 (head-neck cancer), Kyse510 (esophageal squamous cell carcinoma), HCC38 (breast cancer), A2780 (ovarian cancer) and MDA-MB-231 (breast cancer) (Table 1). The cell lines included pairs of cells that are either sensitive (sens) or resistant (CisR) toward the well known cytostatic drug cisplatin as indicated by their significantly different IC_{50} values for cisplatin. Torrubiellin B (**1**) proved to be strongly active against all cell lines investigated with IC_{50} values ranging from 0.3 to 1.5 μM against the cisplatin sensitive cells, and from 0.2 to 2.6 μM against the cisplatin resistant cells, dependent on the cell line investigated. For all cell lines investigated the activity of torrubiellin B (**1**) was superior to that of cisplatin, which makes **1** an interesting candidate for further studies.

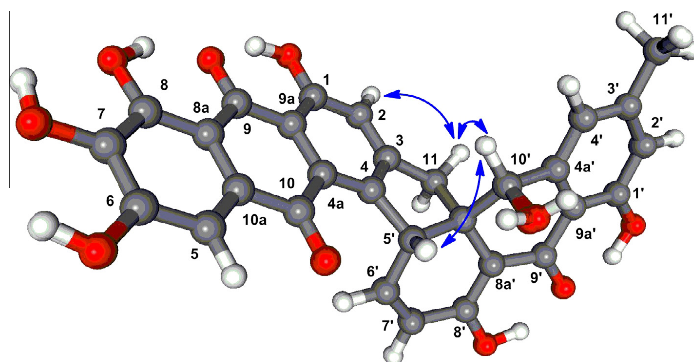

Figure 4. Key long-range ROE correlations shown on the overlapped lowest-energy computed conformers of (5′*R*,10′*S*,10a′*R*)-**1**.

Table 1
Cytotoxic activities of **1**

Compound	IC50 (μM) in various cell lines[a,b]									
	Cal27		Kyse510		HCC38		A2780		MDA-MB-231	
	sens	CisR	sens	CisR	sens	CisR	sens	CisR	sens	CisR
1	0.3	2.1	1.2	0.2	0.4	0.7	0.3	0.5	1.5	2.6
Cisplatin	2.9	6.7	1.8	6.8	3.5	27.9	1.5	11.7	13.9	38.1

[a] Incubation for 72 h.
[b] Abbreviations: sens, sensitive to cisplatin; CisR, cisplatin-resistant.

Experimental

General

The optical rotation was determined with a PerkinElmer 241MC Polarimeter. The ^{1}H, ^{13}C, and 2D NMR spectra were recorded in DMSO-d_6 on a Bruker Avance III 600 NMR spectrometer. Mass spectra were measured with a HP110 Agilent Finnigan LCQ Deca XP Thermoquest and high-resolution mass spectrometry (HRESIMS) spectra were recorded using a UHR-TOF maxis 4G mass spectrometer. ECD spectra were recorded on a J-810 spectropolarimeter. HPLC analysis was performed with a HPLC system Dionex p580 coupled to a UVD340S photodiode array detector; routine detection was at 235, 254, 280, and 340 nm. The separation column was prefilled with Eurosphere-10 C18 using a linear gradient of 0.1% aqueous HCOOH and MeOH with a flow rate of 1 mL/min. Final purification was performed via semi-preparative HPLC using a linear gradient of 0.1% aqueous TFA and MeOH over a Eurosphere-100 C18 RP column (5 μm; 300 × 8 mm) with a flow rate of 5 mL/min using a Merck Hitachi HPLC system (I-7400 UV detector; L-7100 pump). Column chromatography included silica gel 60M (Macherey-Nagel) and Sephadex LH-20 (Sigma). Precoated TLC plates (Silica gel 60 F254) were used to monitor fractions; detection was under UV at 254 and 366 nm. Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements.

Computational section

Mixed torsional/low mode conformational searches were carried out by means of the MACROMODEL 9.9.223[19] software using Merck Molecular Force Field (MMFF) with implicit solvent model for chloroform applying a 42 kJ/mol energy window. Geometry reoptimizations of the resultant conformers [B3LYP/6-31G(d) level in vacuo] and TDDFT calculations were performed with GAUSSIAN 09[20] using various functionals (B3LYP, BH&HLYP, PBE0) and TZVP basis set. ECD spectra were generated as the sum of Gaussians[21] with 2400 cm^{-1} half-height width (corresponding to ca. 29 nm at 350 nm), using dipole-velocity computed rotational strengths. Boltzmann distributions were estimated from the ZPVE corrected B3LYP/6-31G(d) energies. The MOLEKEL[22] software package was used for visualization of the results.

Fungal material

Acremonium sp. was isolated from leaves of the Mangrove plant *Sonneratia caseolaris*, collected in October 2005 in Dong Zhai Gang Mangrove Garden, Hainan, PR China, and cultivated in large-scale following the respective protocol described in the literature.[23] The fungal strain could be identified after DNA extraction, amplification and sequencing of its ITS region according to the literature.[24] The sequence data were submitted to GenBank, accession number FR822815.1.

Extraction and isolation

After 4 weeks of cultivation the fungus was extracted with EtOAc. The dried crude extract (1 g) was submitted to VLC separation. The last fraction (0.1% methanolic TFA) containing **1** was further separated over a Sephadex LH-20 column affording F1–F10 fractions. Fractions 6 and 7 were combined and purified using semi-preparative HPLC with an eluting gradient of MeOH/0.1% aqueous TFA to yield **1** (20 mg).

Bioassay

The cytotoxicity was tested against the following human carcinoma cell lines obtained from DSMZ: A2780 (ovarian), Cal27

(tongue), Kyse510 (esophagus), HCC38 (triple-negative mamma carcinoma), and MDA-MB-231 (triple-negative mamma carcinoma). The CisR cell lines were generated as previously described.[25,26]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tetlet.2015.03.126.

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16. Data for **1**: Orange amorphous powder. $[\alpha]_D^{27}$ +189 (*c.* 0.025, $CHCl_3$). UV λ_{max} (PDA): 458sh, 417sh, 397, 373sh, 307sh, 283, 206sh nm. HRESIMS *m/z* 541.1138 $[M+H]^+$ (calcd for $C_{30}H_{21}O_{10}$, 541.1138). ECD (MeCN, λ [nm] ($\Delta\varepsilon$), $c = 3.08 \times 10^{-4}$ M): 465sh (−2.12), 435 (−4.13), 415sh (2.50), 395 (6.85), 343 (4.96), 296 (2.30), 281 (−2.39), 260 (4.08), 231sh (4.56), 221sh (5.05), 206 (8.55), 195 (−2.51).
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Supporting information

Absolute configuration and anti-tumor activity of torrubiellin B

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Figure S1. ^{13}C NMR spectrum of torrubiellin B (**1**) (DMSO-d_6, 150 MHz).

Figure S2. ^{1}H NMR spectrum of torrubiellin B (**1**) (DMSO-d_6, 600 MHz).

Figure S3. HMBC spectrum of torrubiellin B (**1**) (DMSO-d_6, 600 MHz).

Figure S4. ROESY spectrum of torrubiellin B (**1**) (DMSO-d_6, 600 MHz).

Table S1. 1D and 2D NMR data of **1** at 600 (^{1}H) or 150 (^{13}C) MHz (DMSO-d_6, δ in ppm, *J* in Hz).

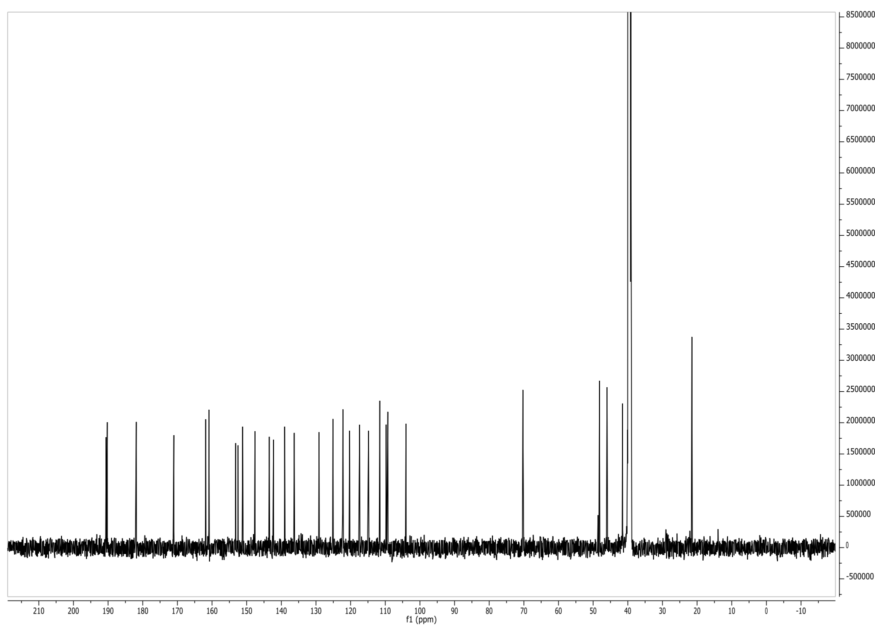

Figure S1. ^{13}C NMR spectrum of torrubiellin B (**1**) (DMSO-d_6, 150 MHz).

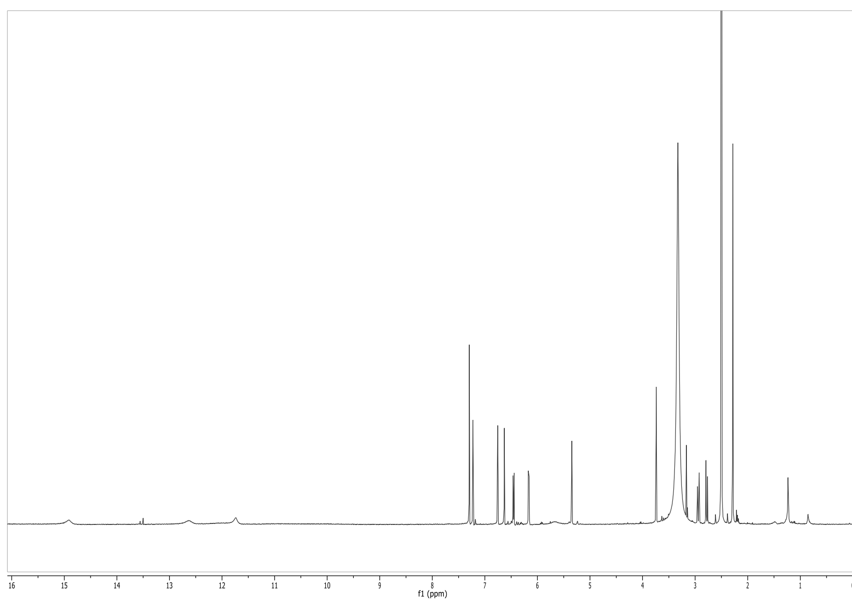

Figure S2. ^{1}H NMR spectrum of torrubiellin B (**1**) (DMSO-d_6, 600 MHz).

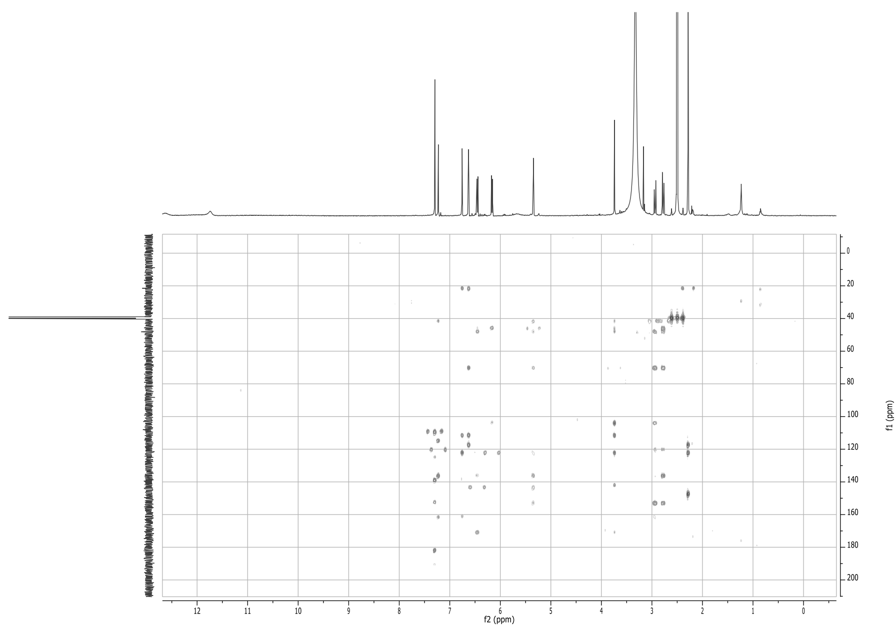

Figure S3. HMBC spectrum of torrubiellin B (**1**) (DMSO-d_6, 600 MHz).

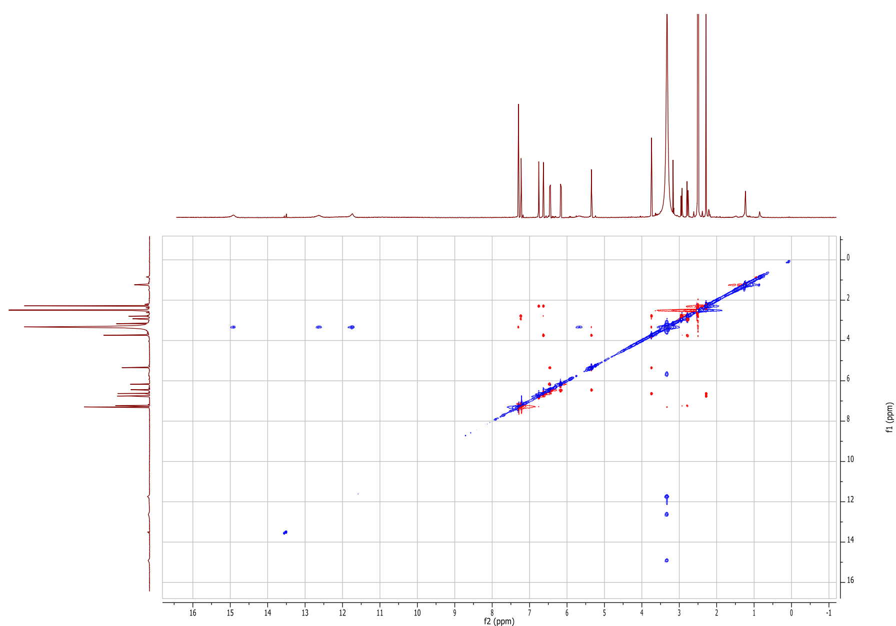

Figure S4. ROESY spectrum of torrubiellin B (**1**) (DMSO-d_6, 600 MHz).

Table S1. 1D and 2D NMR data of **1** at 600 (^{1}H) or 150 (^{13}C) MHz (DMSO-d_6, δ in ppm).

Pos.	$\delta_{C,}$ type	δ_H (J in Hz)	HMBC	ROESY
1	161.8, qC			
2	120.3, CH	7.23 s	1, 4, 9a, 11	H2-11ax, H2-11eq
3	153.2, qC			
4	136.3, qC			
4a	129.2, qC			
5	109.2, CH	7.30 s	10, 6, 7, 10a	
6	152.5, qC			
7	139.0, qC			
8	151.2, qC			
8a	109.8, qC			
9	190.2, qC			
9a	114.9, qC			
10	181.9, qC			
10a	125.1, qC			
11ax	41.5, CH_2	2.94 dd (17.4,1.6)	3, 2, 8a’, 10’, 10a’	-2, -10’
11eq		2.78 d (17.4)	3, 4, 2, 10’, 5’	-2, -10’
1’	160.9, qC			
2’	117.4, CH	6.76 d (1.6)	1’, 4’, 9a’, 11’	-11’
3’	147.6, qC			
4’	122.3, CH	6.63 d (1.6)	2’, 9a’, 11’, 10’	-11’, -11eq, -10’
4a’	142.2, qC			
5’	46.0, CH	5.35 t (2.7)	11, 10a’, 10’, 3, 6’, 4, 7’	2-11, -6’, -10’
6’	143.2, CH	6.45 dd (10.0, 2.7)	8’, 4, 10a’	-5’
7’	122.2, CH	6.14 dd (10.0, 2.7)	8a’, 5’, 8’	
8’	171.0, qC			
8a’	104.0, qC			
9’	190.6, qC			
9a’	111.6, qC			
10’	70.3, CH	3.74 s	8’, 4a’, 6’, 9a’, 8a’, 10a’, 5’, 11	2-11, -4’, -5’
10a’	48.2, qC			
11’	21.5, CH_3	2.28 s	3’, 4’, 2’	-2’, -4’
1-OH		12.64 br s		
8-OH		12.08 br s		
1’-OH		11.74 br s		
8’ -OH		14.92 br s		

Chapter II - *Induction and Enhancement of Secondary Metabolites with OSMAC*

Section 1

OSMAC Approach leads to New Fusarielin Metabolites from *Fusarium tricinctum*

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OSMAC Approach leads to New Fusarielin Metabolites from *Fusarium tricinctum*

Catalina F. Pérez Hemphill[1], Parichat Sureechatchaiyan[2], Matthias U. Kassack[2], Raha S. Orfali[3], Wenhan Lin[4], Georgios Daletos[1], and Peter Proksch[1]

[1] Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Duesseldorf, Germany; [2]Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University, Duesseldorf, Germany; [3]Pharmacognosy Department, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia; [4]State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, People's Republic of China.

Correspondence: Dr G Daletos or Professor Proksch, Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University Duesseldorf, Universitaetsstrasse 1, D-40225, Germany.
E-mail: georgios.daletos@hhu.de or proksch@hhu.de.

Keywords

Fusarielins

Fusarium tricinctum

OSMAC

Anti-cancer activity

Abstract

Using the OSMAC approach, the fungal endophyte *Fusarium tricinctum* was cultivated on fruit juice-supplemented solid rice media. This led to an up to 80-fold increase in the accumulation of the new natural product fusarielin J (**1**), as well as to the induction of two new natural products fusarielin K (**2**) and fusarielin L (**3**) and the known derivatives fusarielins A (**4**) and B (**5**). The highest increase in the accumulation of compound **1** was observed in the presence of apple and carrot juice whereas the stimulating effect was weaker for banana juice. Compound **1** exhibited cytotoxicity against the human ovarian cancer cell line A2780, with an IC_{50} value of 12.5 μM.

Introduction

The fungal genus *Fusarium* is most prominently known for its phytopathogenic effects on various staple food crops, causing depletory '*Fusarium* wilt' (Panama Disease) in bananas[1] and '*Fusarium* head blight' in cereals, such as wheat, oat, barley and maize.[2] *Fusarium* species, *e.g. F. graminearum* and *F. tricinctum*, produce mycotoxins, that are potentially harmful to humans, whereas some of them can also be used as drugs or drug candidates. These mycotoxins include beauvericins, trichothecenes, and most importantly enniatins,[3,4] which were medicinally used (fusafungine) up to the beginning of this year as local antibiotics in nasopharyngitis treatment.[5] Enniatins are currently being investigated as potential anti-cancer agents,[6,7] and so are semi-synthetic derivatives of the cyclic depsipeptide sansalvamide A, another *Fusarium* metabolite with strong *in vitro* cytotoxicity against pancreatic, colon and breast cancer cell lines.[8-11] Additionally, *Fusarium* strains have been shown to produce several antineoplastic agents formerly known from plants such as paclitaxel, camptothecin, and L-asparaginase,[12-14] as well as podophyllotoxin,[15] the latter being the lead compound, which led to the clinically used semisynthetic drugs etoposide and teniposide.

These examples demonstrate how auspicious secondary metabolites from *Fusarium* species are and that ongoing natural product research on this genus promises to be a highly rewarding strategy for the discovery of new bioactive compounds.[16]

One of the approaches for drug discovery from fungi involves the application of the OSMAC method, which attempts to induce silent biogenetic clusters and hence may lead to accumulation of compounds not accumulated during more conventional fermentations. OSMAC (One Strain MAny Compounds) terms the modulating effect that altered culture conditions (*i.e.* temperature, osmolarity, pH) may have on the secondary metabolite production of microorganisms.[17,18] Examples for such culture variations for filamentous fungi include the use of different liquid or solid media, as applied in a previous study with *F. tricinctum* fermented on solid beans and liquid Wickerham medium *versus* cultivation on solid rice medium, leading to an enhanced production of enniatins on beans.[19,20] Another example is the mimicry of extreme habitats, by cultivating at colder temperatures or using highly saline media, *e.g.* growing *Gymnascella dankaliensis* on either NaCl- or NaBr-enriched rice medium, which led to the induction of chlorinated or brominated metabolites.[21-23]

With *Fusarium* species, *e.g. F. graminearum* and *F. tricinctum*, addition of $CaBr_2$ to the cultivation medium led to an induced production of the bioactive compound fusarielin A.[24,25] Fusarielin-type polyketides are a therapeutically promising class of *Fusarium* metabolites: With seventeen known derivatives identified so far (fusarielin A-I, ICM0301 A-H),[26-30] fusarielins display antifungal,[28] mild antibacterial,[24,31] and anti-angiogenic activities,[27,32] in addition to weak cytotoxicity against HeLaS3 and NCI-H69 cells.[26] Interestingly, previous cultivation experiments have shown enhanced fusarielin yields by cultivating *F. tricinctum* on complex carbon sources.[25] Therefore having an endophytic *F. tricinctum* strain at hand, which was previously isolated from the rhizome of *Aristolochia paucinervis*, we decided to implement the OSMAC concept by cultivating it on solid rice medium supplemented with three types of commercially available fruit juices, namely apple, banana, and carrot juice, as these naturally contain a high percentage of various carbohydrates. Herein, cultivating *F. tricinctum* on rice containing juice had a starkly enhancing and inducing effect on the production of fusarielins: the new fusarielin J (**1**) was enhanced 80-fold compared to control cultures lacking fruit juice and the production of the new fusarielins K (**2**) and L (**3**) and the known fusarielins A (**4**) and B (**5**) was induced. A total of fourteen natural products was identified based on their NMR and MS spectra and all fusarielins (Figure 1) were investigated for their cytotoxicity against A2780 human ovarian cells.

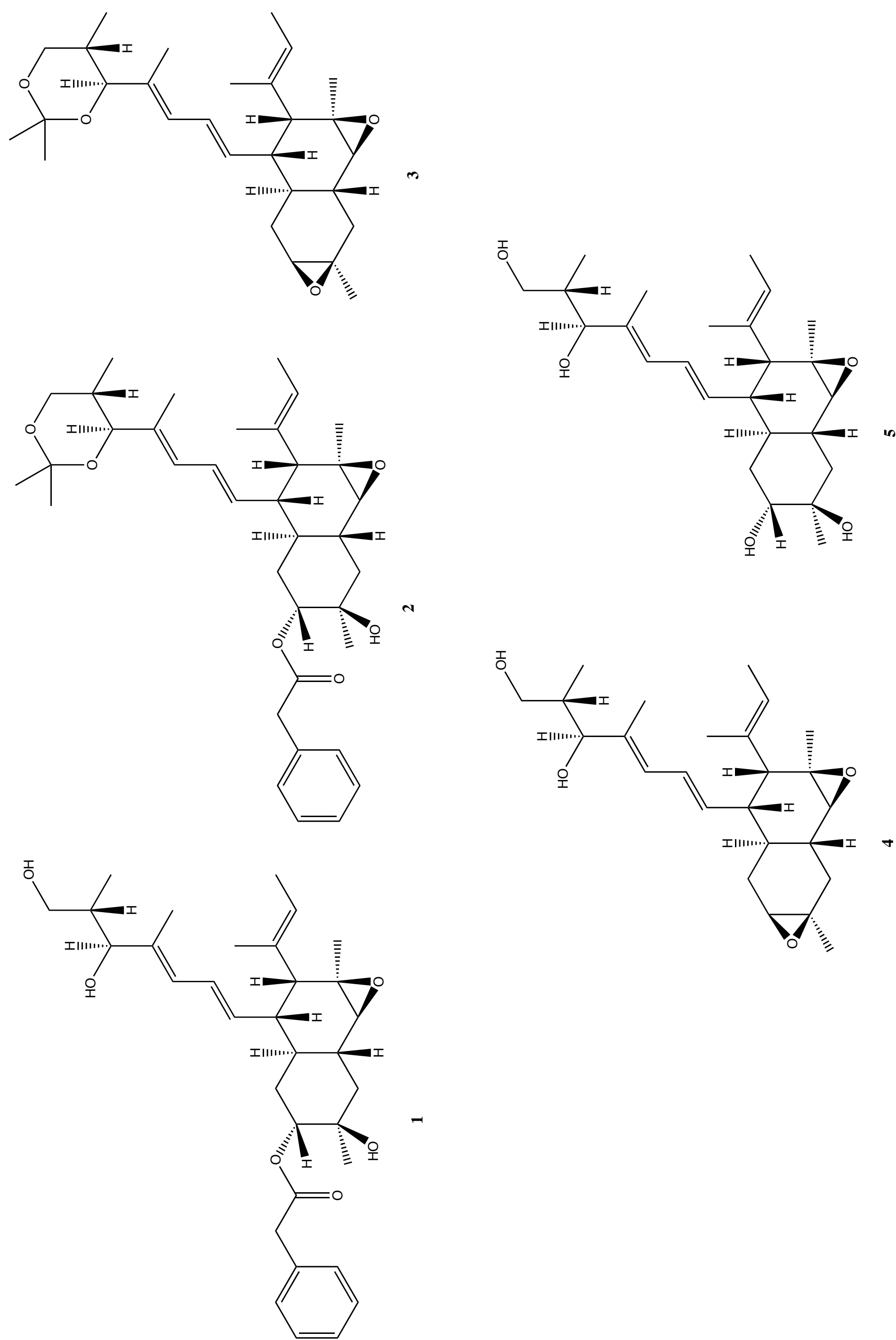

Figure 1 Structures of compounds **1-5**.

Results

F. tricinctum was cultivated on solid rice medium with or without fruit juice, in two flasks per medium variation. In each case the fermentation was stopped by addition of ethyl acetate after 14 days and all experiments were carried out in triplicate, resulting in 6 cultivation flasks per medium.

The ethyl acetate extracts of all treatments presented typical metabolic profiles in HPLC analysis (Figure 2): the known compounds (-)-citreoisocoumarin (**6**), (-)-citreoisocoumarinol (**7**), lateropyrone (**8**), enniatin B (**9**), enniatin B1 (**10**), enniatin A1 (**11**), enniatin A (**12**), and fusaristatin A (**13**) were readily identified by LC-MS analysis and by comparison with authentic standards, which had either previously been isolated from the same fungal strain,[33] or in the case of phenyl acetic acid (**14**) by comparison with a commercially available standard. The amounts of **6-13** per flask irrespective of the presence or absence of fruit juice were comparable to those of previous experiments (Table 1).[33] Compound **14** was isolated from extracts of juice cultivars; however, it was not detectable in the HPLC chromatograms. Interestingly, the juice-based cultivations enhanced the production of the new natural product fusarielin J (**1**) up to 80-fold compared to fungal cultures lacking juice (Table 1). Furthermore, the cultivation of *F. tricinctum* with juice induced the production of two new natural products fusarielin K (**2**) and fusarielin L (**3**), and of two known natural products fusarielins A (**4**) and B (**5**) that were not detected in fungal controls. None of the investigated compounds including the new natural products **1 – 3** were detected when only the juices used for the described experiments were analyzed.

Table 1 Compound yields per flask (n=6)

	Rice medium composition						
Compound	*Water (mg/flask)*	*Banana juice (mg/flask)*	*Increase (fold)*	*Apple juice (mg/flask)*	*Increase (fold)*	*Carrot juice (mg/flask)*	*Increase (fold)*
1	0.1 ± 0.0	1.4 ± 0.4	14.0	8.0 ± 2.1	80.0	7.8 ± 2.8	78.0
2	[1]	[2]		0.1 ± 0.1		0.1 ± 0.1	
3	[1]	[1]		0.1 ± 0.0		[2]	
4	[1]	[1]		0.1 ± 0.1		0.1 ± 0.1	
5	[1]	[1]		0.2 ± 0.1		0.1 ± 0.1	
6	[2]	[2]		[1]		[1]	
7	[2]	[2]		[1]		[2]	
8	0.2 ± 0.2	0.9 ± 0.2	4.5	1.5 ± 1.4	7.5	0.6 ± 0.5	3.0
9	5.9 ± 1.9	8.9 ± 2.4	1.5	2.7 ± 0.7	0.5	9.5 ± 0.6	1.6
10	6.5 ± 2.1	7.8 ± 1.5	1.2	3.2 ± 0.8	0.5	10.6 ± 0.9	1.6
11	3.1 ± 1.1	3.0 ± 0.5	1.0	1.3 ± 0.4	0.4	4.9 ± 0.3	1.6
12	0.5 ± 0.2	0.6 ± 0.2	1.2	[1]		0.7 ± 0.1	1.4
13	15.5 ± 7.7	9.2 ± 3.4	0.6	12.4 ± 4.3	0.8	21.4 ± 3.8	1.4
14	[1]	[1]		[1]		[1]	

[1]not detected; [2]< 0.05 mg

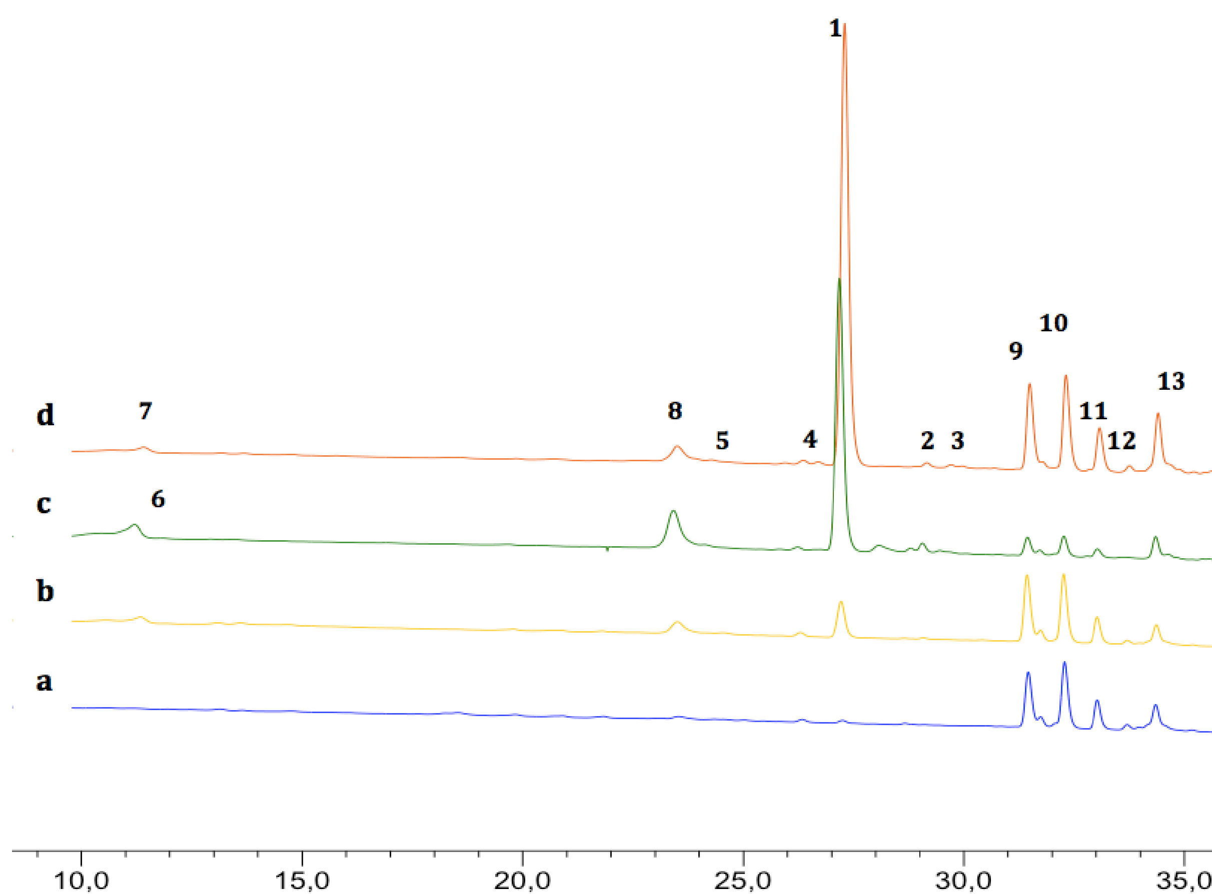

Figure 2 HPLC chromatograms (min vs. mAU) overlay of EtOAc extracts: *F. tricinctum* on solid rice medium prepared with water (a), banana (b), apple (c), and carrot juice (d); compound **14** was not detectable.

Compound **1** was isolated as a white, amorphous solid and exhibited a UV absorption maximum at 241 nm. The HRESIMS spectrum of **1** displayed a prominent ion peak at *m/z* 561.3182 $[M+Na]^+$ indicating the molecular formula $C_{33}H_{46}O_6$, consistent with eleven degrees of unsaturation. In the ^{1}H NMR spectrum of **1**, 43 protons were detected, including two pairs of overlapping aromatic protons at δ_H 7.26 (brd, 7.4, H-4’/8’) and 7.34 (brt, 7.4, H-5'/7'), one aromatic proton at δ_H 7.30 (brt, 7.4, H-6’), four olefinic protons at δ_H 6.18 (dd, *J* = 15.0, 10.8, H-6), 5.85 (dd, *J* = 10.8, 1.2, H-5), 5.29 (dd, *J* = 6.7, 1.5, H-19), and 5.02 (dd, *J* = 15.0, 10.2, H-7), five downfield shifted aliphatic protons at δ_H 4.70 (q, *J* = 2.2, H-11), 3.78 (d, *J* = 9.0, H-3), 3.55 (m, H_α-1), 3.70 (dd, *J* = 10.6, 5.0, H_β-1), and 2.74 (m, H-15), suggesting their direct vicinity to an oxygen atom, three methylene groups at δ_H 1.54 (d, *J* = 14.0, H_α-13) and 1.65 (m, H_β-13), 1.27 (m, H_α-10) and 1.40 (m, H_β-10), and 3.62 (s, H_2-2’), five methine protons at δ_H 2.55 (d, *J* = 5.2, H-17), 2.18 (m, H-8), 1.91 (ddd, *J* = 14.0, 11.1, 3.0, H-14), 1.80 (m, H-2), and 1.55 (m, H-9), and six methyl groups at δ_H 1.69 (m, H_3-22), 1.67 (m, H_3-20), 1.61 (s, H_3-25), 1.21 (s, H_3-

24), 1.04 (s, H_3-23), and 0.78 (dd, J = 6.9, 3.2, H_3-21). The COSY spectrum revealed four spin systems, which consisted of H-21/H-2/H_3-21/H-3 (**A**), H_3-22/H-5/H-6/H-7/H-8/H-17/H-9/H_2-10/H-11/H_2-13/H-14 (**B**), H-19/H_3-20/H_3-25 (**C**), and H-4'/H-5'/H-6'/H-7'/H-8' (**D**) (Figure 3a). After assigning all protons to their attaching carbon atoms via analysis of the HSQC spectrum (Table 2), the aforementioned spin systems were connected through interpretation of the HMBC spectrum as follows: the first spin system (**A**) was connected to the second (**B**) by the correlation from H-3 to the quaternary carbon C-4 (δ_C 137.4) and CH_3-22 (δ_C 11.5), and by the J^3 correlation from H-5 to the oxygenated C-3 (δ_C 81.9). The third spin system (**C**) was elucidated as a but-2-ene moiety through a J^2 correlation from H_3-25 to the quaternary carbon C-18 (δ_C 134.7) and J^3 correlations to C-19 (δ_C 126.1). Moreover, the HMBC correlation from H_3-25 to C-17 and from H-8 to C-18 connected this moiety (**C**) to the decalin system at position C-17. By further HMBC evaluation and comparison with the known fusarielin B (**5**), which was also isolated in this study, the basic framework of **1** was found to be identical to that of **5**.[26]

The remaining signals (spin system **D** and H_2-2') were attributed to a phenyl acetate unit, as supported by the HMBC correlations from the aromatic protons H-4', H-5', H-7', and H-8' to C-3' (δ_C 135.7) and the methylene carbon C-2' (δ_C 42.7), and from H-6' to C-3'. Likewise, the methylene protons H_2-2' correlated with C-3' as well as with the aromatic carbons C-4'/8', C-5'/C7' (δ_C 129.9), and the carbonyl carbon C-1' (δ_C 171.8). The connection of the phenyl acetate function to the fusarielin B (**5**) framework was established through the HMBC correlation from H-11 to C-1' (Figure 3a), and was corroborated by the marked downfield shift (Δ 1.43 ppm) of this proton, as a result of deshielding effects from the resulting ester function. The relative configuration of **1** was deduced by the J-values extracted from the ^{1}H NMR spectrum (Table 2) and by interpretation of the 2D NOESY spectrum (Figure 3a) to be identical to that of fusarielin B (**5**). Thus, compound **1** was identified as a new natural product and was named fusarielin J.

Table 2 NMR spectroscopic data of 1 [CD_3OD] (^1H NMR in 600 MHz, ^{13}C in 150 MHz, δ in p.p.m.)

Position	δ_H	δ_C	*HMBC*	*NOESY*
1α	3.55 m	66.5	C-3, 21	21
β	3.70 (dd, 10.6, 5.0)		C-3, 21	21
2	1.80 m	39.1	3	
3	3.78 d (9.0)	81.9	C-1, 2, 4, 5, 22	5, 21, 22
4		137.4		
5	5.85 dd (10.8, 1.2)	128.0	C-3, 7, 22	3, 7
6	6.18 dd (15.0, 10.8)	127.7	C-8	8, 22
7	5.02 dd (15.0, 10.2)	136.7	C-5, 6	5, 9, 25, 10β
8	2.18 m	44.6	C-6, 18	6, 10α, 17
9	1.55 m	32.1		19
10	1.27 m	33.1		8, 14
	1.40 m			7
11	4.70 q (2.2)	76.7	C-9, 12, 13, 1’	23
12		71.2		
13α	1.54 d (14.0)	40.9	C-9, 12, 14, 15	
β	1.65 m		C-11, 12, 15	15
14	1.91 ddd (14.0, 11.1, 3.0)	37.5	C-15	10α
15	2.74 m	65.3	C-9, 13, 14, 16, 24	13β, 24
16		62.5		
17	2.55 d (5.2)	54.7	C-8, 9, 15, 16, 18, 19	8, 19, 24, 25
18		134.7		
19	5.29 dd (6.7, 1.5)	126.1	C-17, 20	9, 17, 20, 24
20	1.67 m	13.7	C-18, 19	19
21	0.78 dd (6.9, 3.2)	14.2	C-1, 2, 3	1, 2, 3
22	1.69 m	11.5	C-3, 4, 5	3, 6
23	1.04 s	27.2	C-11, 12, 13	11
24	1.21 s	22.4	C-15, 16, 17	15, 17, 19
25	1.61 s	18.6	C-17, 18, 19	7, 17
1’		171.8		
2’	3.62 s	42.7	C-1’, 3’, 4’/8’	4’/5’

3’		135.7	
4’/8’	7.26 brd (7.4)	129.9	C-2’, 6’
5’/7’	7.34 brt (7.4)	129.4	C-3’
6’	7.30 brt (7.4)	127.9	C-4’/8’

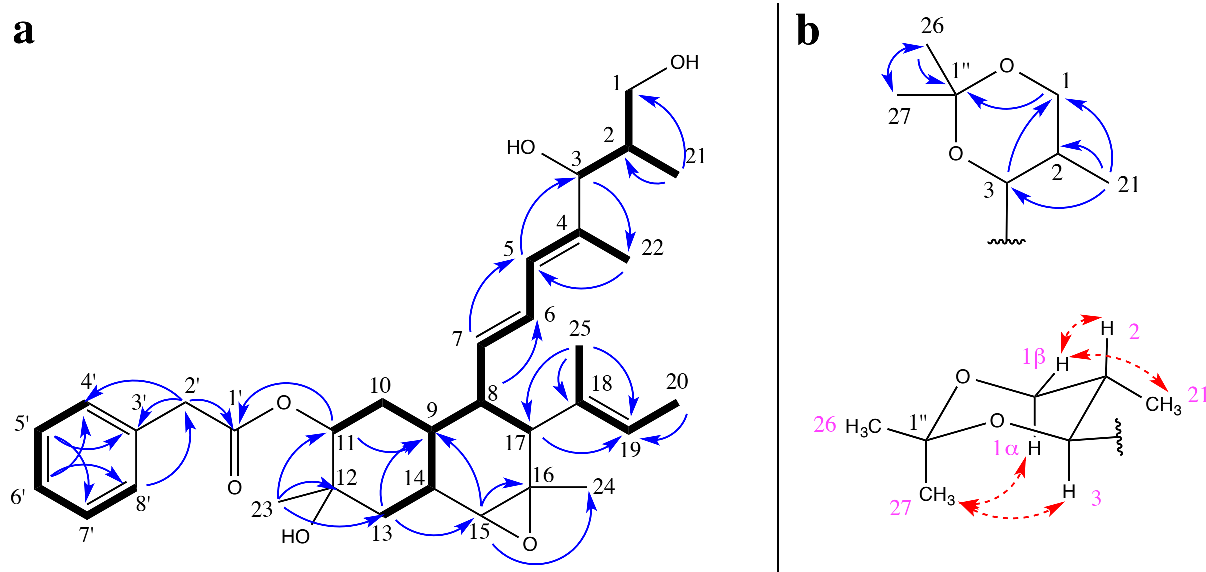

Figure 3 (**a**) Key COSY (bold lines) and HMBC (arrows) correlations of **1**; (**b**) Key HMBC (plain arrows) and NOE (dashed arrows) correlations of the 2,2,5-trimethyl-1,3-dioxane moiety of **2**.

Compound **2** was isolated as a white, amorphous solid and showed a similar UV/Vis spectrum as **1**. The molecular formula $C_{36}H_{50}O_6$ was deduced by the prominent ion peak at *m/z* 596.3944 $[M+NH_4]^+$ in the HRESIMS, suggesting an additional structural functionality of 40 amu compared to **1**. The ^1H NMR spectrum of **2** was very similar to that of **1**, indicating that both compounds shared the same fusarielin core structure. Additional signals were detected at δ_H 1.34 (s, H_3-26) and 1.48 (s, H_3-27), assigned to two methyl groups. In the HMBC spectrum of **2**, the correlations of both H_3-26 and H_3-27 with each other as well as with the remaining ketal quaternary carbon C-1’’ (δ_C 99.3) revealed the presence of an additional acetonide moiety. The HMBC correlation from H2-1 to C-1’’ confirmed the position of the acetonide group through ether linkages at C-1 and C-3 (Figure 3b), rationalizing the additional degree of unsaturation. The relative configuration of the fusarielin core structure of **2** was determined to be identical to that of **1** on the basis of the respective coupling constants and NOESY correlations (Table 3).

In addition, the six-membered dioxane ring adopted a chair conformation (Figure 3b), as suggested by the NOE correlations of H_3-27 with the axial protons H-1α and H-3, indicating their co-facial orientation. This was further corroborated by the ^{13}C chemical shifts of CH_3-26 and CH_3-27 (δ_C 29.9 and 19.4, respectively), which are characteristic of a *syn* 1,3-diol acetonide moiety. The acetonide function is known from other fungal natural products, *e.g.* ganodermacetal from the basidiomycete *Ganoderma amboinense*,[34] or cochliomycins A and B from the ascomycete *Cochliobolus lunatus*.[35] The isolation of **2** was performed in the absence of acetone, and the fact that no acetonide was formed, even after incubating 1 mg of **2** in 1 ml acetone for 24 hours, strongly suggested that **2** is a natural product and not an artefact arising from **1** during the isolation procedure. Therefore, compound **2** was identified as a new natural product and was given the name fusarielin K.

Table 3 NMR spectroscopic data of 2 [CD_3OD] (^1H NMR in 600 MHz, ^{13}C in 150 MHz, δ in p.p.m.)

Position	δ_H	δ_C	*HMBC*	*NOESY*
1α	3.55 m	66.6	C-3	21, 27
β	3.70 (dd, 10.6, 5.0)		C-2, 3, 1”	2, 21
2	1.80 m	32.2	C-3	1β
3	3.78 d (9.0)	83.5	C-4, 5, 22	5, 21, 22, 27
4		134.1		
5	5.85 dd (10.8, 1.2)	129.6	C-3, 7, 22	3, 7
6	6.18 dd (15.0, 10.8)	128.2	C-8	8, 22
7	5.02 dd (15.0, 10.2)	138.0	C-5	5, 9, 25, 10β, 17
8	2.18 m	44.6	C-6, 18	6, 17
9	1.50 m	32.0		19
10	1.28 m	32.7		8, 14
	1.40 m			7
11	4.70 q (2.2)	76.5	C-9, 12, 13, 1’	23
12		71.0		
13α	1.54 d (14.0)	40.7	C-9, 12, 14, 15	19
β	1.65 m		C-11, 12, 15	15
14	1.91 ddd (14.0, 11.1, 3.0)	37.4	C-15	10α

15	2.74 m	65.5	C-9, 13, 14, 16, 24	13β, 24
16		62.4		
17	2.55 d (5.2)	55.0	C-8, 9, 15, 16, 18, 19	8, 19, 24, 25
18		135.0		
19	5.29 dd (6.7, 1.5)	126.3	C-17, 20	9, 17, 20, 24
20	1.67 m	13.6	C-18, 19	19
21	0.78 dd (6.9, 3.2)	12.5	C-1, 2, 3	1, 2, 3
22	1.69 m	11.7	C-3, 4, 5	3, 6
23	1.04 s	27.1	C-11, 12, 13	11
24	1.21 s	22.2	C-15, 16, 17	15, 17, 19
25	1.61 s	18.5	C-17, 18, 19	7, 17
26	1.34 s	29.9	C-1, 27, 1''	
27	1.48 s	19.4	C-26, 1''	1β, 3
1'		171.8		
2'	3.62 s	42.5	C-1', 3', 4'/8'	4'/5'
3'		135.5		
4'/8'	7.27 m	129.8	C-2', 6'	
5'/7'	7.34 m	129.3	C-3'	
6'	7.31 m	128.1	C-4'/8'	
1''		99.3		

Compound **3** was isolated as a colorless, amorphous solid and displayed a similar UV spectrum as **1** and **2.** The molecular formula of **3** was identified as $C_{28}H_{42}O_4$ based on its prominent ion peaks detected at *m/z* 465.2978 $[M+Na]^+$, revealing a 40 amu increase in the molecular weight compared to fusarielin A (**4**). The ^1H and ^{13}C NMR data of **3** were similar to those of **4**, apart from the presence of two additional methyl signals at δ_H 1.33 (s, H3, H_3-26) and 1.46 (s, H3, H_3-27), thus suggesting the presence of an additional acetonide moiety as found in **2**. This assumption was further corroborated by careful analysis of the HMBC spectrum of **3**, in which the cross peaks from H_β-1 (δ_H 3.72), H_3-26, and H_3-27 to C-1' (δ_C 99.4) were discerned. The relative configuration of **3** was established on the basis of NOE correlations, coupling constants, and ^{13}C NMR data (Table 5), by analogy with **1** and **2.** Therefore **3** was identified as the 1,3-diol acetonide derivative of **4** and was given the name fusarielin L.

Table 4 NMR spectroscopic data of 3 [CD_3OD] (^1H NMR in 600 MHz, ^{13}C in 150 MHz, δ in p.p.m.)

Position	δ_H	δ_C	*HMBC*	*NOESY*
1 α	3.61 t (11.5)	66.7		27
β	3.72 dd (11.5, 5.2)		C-2, 3, 1''	2
2	1.85 m	32.3	C-1	1 β
3	3.91 d (10.3)	83.3	C-5, 22	1 α , 5, 27
4		134.4		
5	5.97 d (10.7)	129.7	C-3, 7, 22	3, 7
6	6.26 dd (15.0, 9.8)	127.8		8, 22
7	5.25 dd (15.0, 10.4)	137.3	C-5	5
8	2.16 d (11.9)	44.5		17, 6
9	1.33 m	[1]		
10 α	1.83 m	31.4		11
β	1.10 m			
11	2.99 d (5.6)	61.2	C-10	10 β , 10 α , 23
12		59.7		
13 α	1.69 m	36.5	C-14	13 β , 15
β	2.15 brd (11.8)			
14	1.62 m	35.0		
15	2.78 s	64.6	C-13, 14	13 β , 24
16		62.7		
17	2.54 d (5.2)	54.7		8, 19
18		[1]		
19	5.27 m	126.3		9, 17, 20
20	1.65 br s	13.3	C-19	
21	0.65 d (6.7)	12.3	C-1, 2, 3	1, 2, 3
22	1.72 d (1.4)	11.7	C-3, 4, 5	3, 6
23	1.34 s	22.8	C-12, 13	11
24	1.23 s	22.0	C-15, 16	17, 15
25	1.62 s	[1]		
26	1.33 s	29.8	C-1'', 27	

27	1.46 s	19.3	C-26, 1''	1α, 3
1'		99.4		

[1]not observed

The known compounds fusarielins A (**4**) and B (**5**) were identified by comparison of their spectroscopic data with those in the literature.[26] In addition, the absolute configurations of **4** and **5** (Figure 1) had previously been deduced on the basis of CD spectroscopy and chemical derivatization experiments.[26] Therefore, based on the similar NMR data and specific optical rotation values of **1**, **2**, and **3** ($[\alpha]_D^{20}$ -76, -53, and -33, respectively) compared with those of **4** and **5** ($[\alpha]_D^{20}$ -132 and -38, respectively) as well as on their close biogenetic relationship, it is assumed that these compounds (**1** – **5**) share the same absolute configuration (Figure 1).

In a previous study on *F. tricinctum*, the influence of different carbon and nitrogen sources, as well as variation of pH, cultivation time, and temperature had been analyzed.[25] Interestingly, the use of complex carbon sources showed the strongest enhancement of fusarielin A (**4**) production.[25] As fruit juices are naturally high in carbohydrates (6-14 g per 100 ml), our experiments clearly underpin these findings. Moreover, our juice experiments led to an additional enhancement and/or induction of more and new fusarielin derivatives (**1**-**3**). However, the carbohydrate content (banana>apple>carrot) of each juice was not coherent with the yield of fusarielins per flask (apple>carrot>banana) (Table 1). Therefore, other juice components/factors may play an additional role in fusarielin induction and enhancement. In order to pinpoint which physical characteristic(s) of the juices (aka OSMAC effector) comports linear to the induction and enhancement of fusarielins, we compared several properties of the juices and media, including pH, osmolarity and juice contents, however, no interrelations were found (Table 5). This is not surprising, as previous OSMAC studies, *e.g.* using tap water *versus* distilled water, revealed that even slight changes in the cultivation medium, *e.g.* differences in metal ion concentrations, trigger the production of new bioactive secondary metabolites in endophytic fungi.[36]

Table 5 Nutritional and physical characterization of liquids and rice medium used for cultivation

	Liquids			
Properties	*Water*	*Banana*[1]	*Apple*[2]	*Carrot*[2]
pH[3,4]	6.5	5.0	4.5	6.0
pH	7.4[3]	3.2-3.5	2.9-4.0	4.9-5.4
osmolarity (mOsmol/kg)[3]	100	683	746	482
carbohydrates (g/100 ml)	0	14.4	11	6.2
proteins (g/100 ml)	0	0.4	0.001	1
calcium (mg/100 ml)	0	8	5	24
citric acid/ lemon juice		✓		✓
ascorbic acid		✓		✓

[1]Pfanner; [2]Naturkind; [3]measured at HHU Duesseldorf; [4]rice medium

All fusarielins (**1** – **5**) were submitted to a cytotoxicity assay against the human ovarian cancer cell line A2780. Compound **1** exhibited moderate cytotoxicity with an IC_{50} value of 12.5 μM against the A2780 cells. All other fusarielins (**2** – **5**) showed very weak activity (> 36 μM) in the same assay. For the known fusarielins A (**4**) and B (**5**) this was in accordance with previously published data.[26] Interestingly, the acetonide function in fusarielin K (**2**) and L (**3**) led to a 3- and 2-fold decreased activity, compared to the precursors fusarielins J (**1**) and A (**4**) (Table 6). The phenyl acetic acid function seems to play an important role in the cytotoxic activity of fusarielin J (**1**) and K (**2**). Compound **1** is 4-fold more cytotoxic than phenyl acetic acid lacking fusarielin A (**4**) and B (**5**), and **2** is about 2-fold more cytotoxic than fusarielin L (**3**).

Table 6 IC_{50} values of compounds 1-5, analyzed in an MTT assay against A2780 cells

	Compounds				
Bioactivity	***1***	***2***	***3***	***4***	***5***
Cytotoxicity, IC_{50} (μM)[1]	12.5	36.5	84.6	47.5	48.3

[1]positive control: cisplatin, IC_{50} 2.0 μM

To the best of our knowledge, this is the first report using fruit juice for an OSMAC approach and from the fusarielin derivatives identified in this study so far only fusarielin A (**4**) had been isolated from cultures of *F. tricinctum* before.[24,25,37] In addition, we were able to induce the production of the known fusarielins A (**4**) and B (**5**), and of two new fusarielins K (**2**) an L (**3**), along with the 80-fold enhancement of the new fusarielin J (**1**). In conclusion, employing juice as an addition to the fermentation media of fungi proves to be a promising OSMAC approach for the discovery of new bioactive secondary metabolites in the ongoing quest of natural product research to provide model structures for the development of potential future drugs.

Experimental Section

General Experimental Procedures

For a measurement of optical rotation (OR) of chiral compounds, the latter were dissolved in optically pure solvents (Uvasol®, Merck Chemicals GmbH, Darmstadt, Germany) and left to equilibrate for half an hour. The specific OR was then measured thrice and averaged for each sample, using a PerkinElmer 241MC polarimeter (Perkin-Elmer, Waltham, MA, USA). All NMR data (1D and 2D) were measured in deuterated methanol on Bruker Avance III 300, Bruker Avance DRX 500 or Bruker Avance III 600 MHz NMR spectrometers at 25 °C (Bruker BioSpin GmbH, Rheinstetten, Germany), and calibrated with to the deuterated methanol peak (δ_H 3.310 p.p.m. and δ_C 49.000 p.p.m.). The molecular weight of each compound was determined using an HP110 Agilent Finnigan LCQDecaXP Thermoquest ESI mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany), and the molecular formula was assigned with the help of HRESIMS spectra measured on a UHR-TOF maxis 4G mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany). All fractions and pure compounds were monitored via analytical HPLC DIONEX p580 UVD340S PDA DAD3000RS (Dionex Softron GmbH, Munich, Germany).[38] Compounds were purified via semipreparative HPLC Merck Hitachi HPLC, I-7100pump and I-7400detector (Merck KGaA, Darmstadt, Germany)[38] or preparative TLC polygram®SIL G/UV_{254}, pre-coated (Macherey-Nagel, Düren, Germany), using HPLC grade eluents (Merck Chemicals GmbH, Darmstadt, Germany) in each case. Initial and subsequent fractionation of crude extracts was performed using column chromatography with stationary phase either silica gel 60M (Macherey-Nagel)[38] or

Sephadex LH-20 25-100 µm bead size (Sigma-Aldrich, Munich, Germany). TLC plates were used to monitor the collected fractions by spotting them at 254 and 366 nm. The osmolarity of the fruit juices was measured with an osmomat3000 osmometer (Gonotec GmbH, Berlin, Germany). The pH values of the rice media were determined using pH-indicator strips (Merck Chemicals GmbH, Darmstadt, Germany).

Microbial Material

F. tricinctum had previously been isolated as an endophyte from rhizomes of *Aristolochia paucinervis* collected in Beni-Mellal (Morocco).[33] Fresh mycelia of *F. tricinctum* were grown on agar plates[39] and used for all cultivation experiments.

Culture Conditions

Aqueous solid rice culture control medium was prepared following our SOP.[39] For the OSMAC study, the demineralized water in the mentioned protocol was replaced with banana juice (Pfanner, St. Peter, Germany), apple juice (Naturkind, Kaiser's Tengelmann GmbH, Mülheim an der Ruhr, Germany), and carrot juice (Naturkind) by volume measurement (110 ml per 100 g rice, autoclavation at 121 °C for 20 min). Each medium was prepared in duplicate, resulting in eight Erlenmeyer flasks in total per experiment, which was performed three times. Per flask, half an agar plate mycelium was added under sterile conditions, using four fully overgrown plates per experiment in total. The cultures were left to grow at room temperature (21°C) and in the dark. After 14 days the mycelia of *F. tricinctum* had completely covered and grown through each medium, and thus were harvested at this time. This experiment was repeated twice.

Extraction and Isolation

Each flask was infused with 600 ml EtOAc, the solid medium was chopped up inside the flask and after covering each flask up, they were extracted on a horizontal shaker (140 rpm) for 8 hours. Subsequently the flasks were left in a static state overnight, before being filtered and dried over a rotary evaporator to generate the EtOAc crude extract per flask. The mean dry weight yield of the aqueous cultures amounted to ca. 180 mg/flask, that of the banana juice cultures to ca. 275 mg/flask, of the apple juice cultures to ca. 307 mg/flask, and that of the carrot juice cultures to ca. 353 mg/flask. For initial characterization, each extract was solved in 50 ml MeOH (HPLC grade, VWR) and injected into the above-mentioned analytical HPLC with 20 µl per extract. The extracts of

the apple and carrot juice cultures were combined and then submitted to VLC separation. As these yielded a sufficient quantity, extracts from banana juice were not included. The fraction obtained with the eluent n-hexane/EtOAc (1:4) amounted to 290 mg and was further separated over a Sephadex column with methanol. Five of the so generated sub-fractions were further worked on: Fraction 3 (96 mg) was purified via preparative TLC (methanol/DCM, 1:4) yielding compounds **1** (18.8 mg), **4** (5.2 mg), **2** (3.3 mg), **5** (7.2 mg), and **3** (1.4 mg).

Compounds **7**-**14** were identified from the crude extracts via LC-MS analysis with the corresponding known compounds previously isolated in our group.[33] An experimental calibration curve (concentration vs. area units; $R^2 > 0.9$) was produced for each purified compound by HPLC measurements of several concentrations, so that the yields per flasks for each compound could be calculated from the resulting curve function.

Fusarielin J (**1**): white, amorphous, hygroscopic solid; $[\alpha]_D^{20}$ -76° (*c* 0.135, MeOH); UV λ_{max} (PDA): 241 nm; ^1H, ^{13}C, and 2D NMR data Table 2; HRESIMS *m/z* 561.3182 $[M+Na]^+$ (calcd. for $C_{33}H_{46}O_6$, 561.3184).

Fusarielin K (**2**): white, amorphous, hygroscopic solid; $[\alpha]_D^{20}$ -53° (*c* 0.135, MeOH); UV λ_{max} (PDA): 241 nm; ^1H, ^{13}C, and 2D NMR data Table 3; HRESIMS *m/z* 596.3944 $[M+NH_4]^+$ (calcd. for $C_{36}H_{54}NO_6$, 596.3946).

Fusarielin L (**3**): colourless solid; $[\alpha]_D^{20}$ -33° (*c* 0.1125, MeOH); UV λ_{max} (PDA): 241 nm; ^1H, ^{13}C, and 2D NMR data Table 4; HRESIMS *m/z* 465.2978 $[M+Na]^+$ (calcd. for $C_{28}H_{42}O_4Na$, 465.2975).

Biological Assays

All fusarielins (**1** – **5**) isolated in this study were investigated in an MTT assay against the cisplatin sensitive A2780 human ovarian cancer cell line, following previous protocols.[40,41]

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Supporting information

OSMAC Approach leads to New Fusarielin Metabolites from *Fusarium tricinctum*

Catalina F. Pérez Hemphill[1], Parichat Sureechatchaiyan[2], Matthias U. Kassack[2], Raha S. Orfali[3], Wenhan Lin[4], Georgios Daletos[1], and Peter Proksch[1]

[1] Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Duesseldorf, Germany; [2]Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University, Duesseldorf, Germany; [3]Pharmacognosy Department, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia; [4]State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, People's Republic of China.

Correspondence: Dr G Daletos or Professor Proksch, Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University Duesseldorf, Universitaetsstrasse 1, D-40225, Germany.
E-mail: georgios.daletos@hhu.de or proksch@hhu.de.

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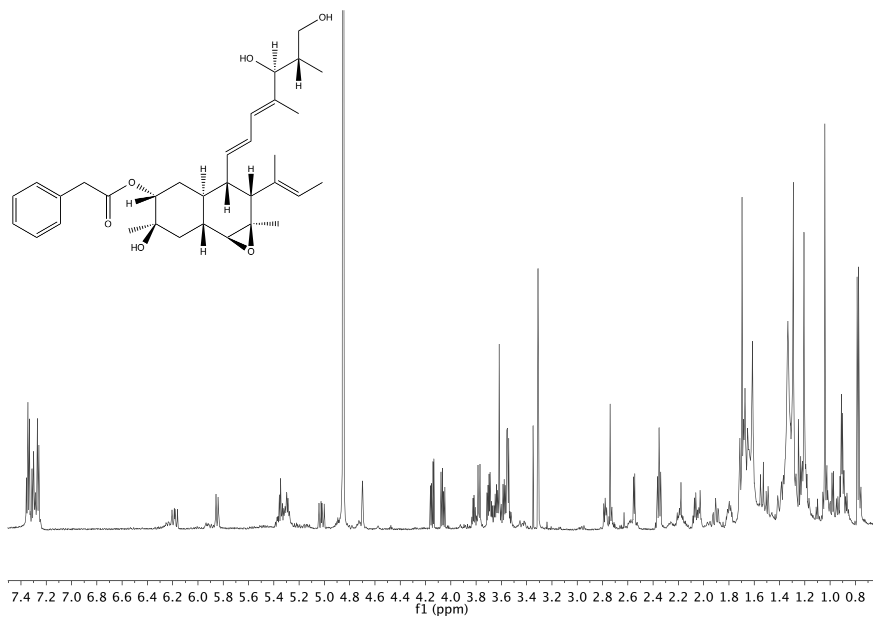

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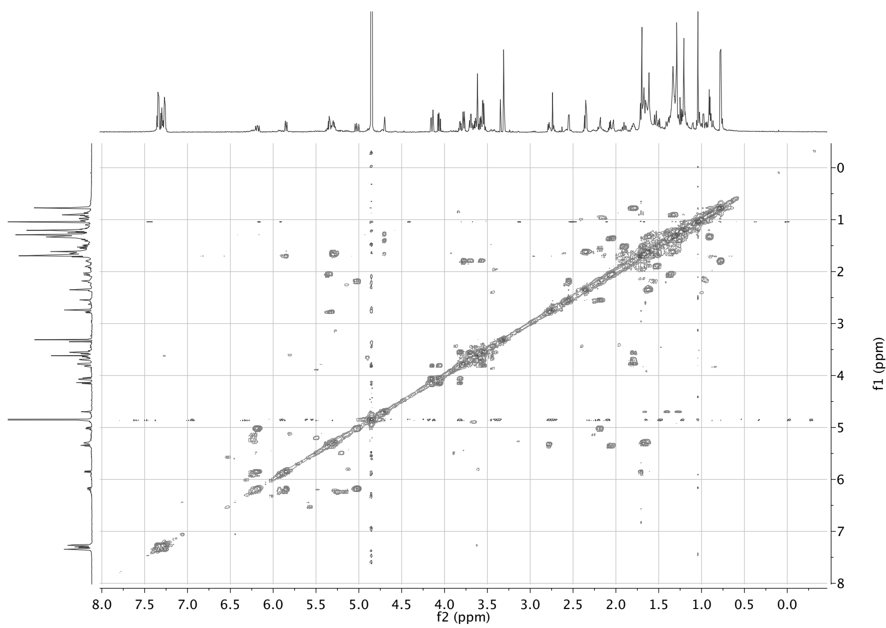

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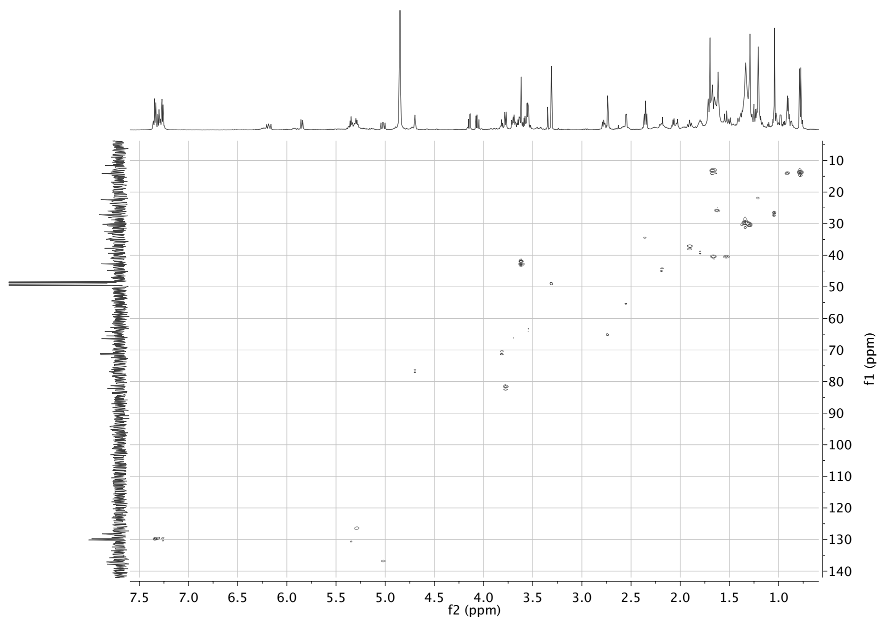

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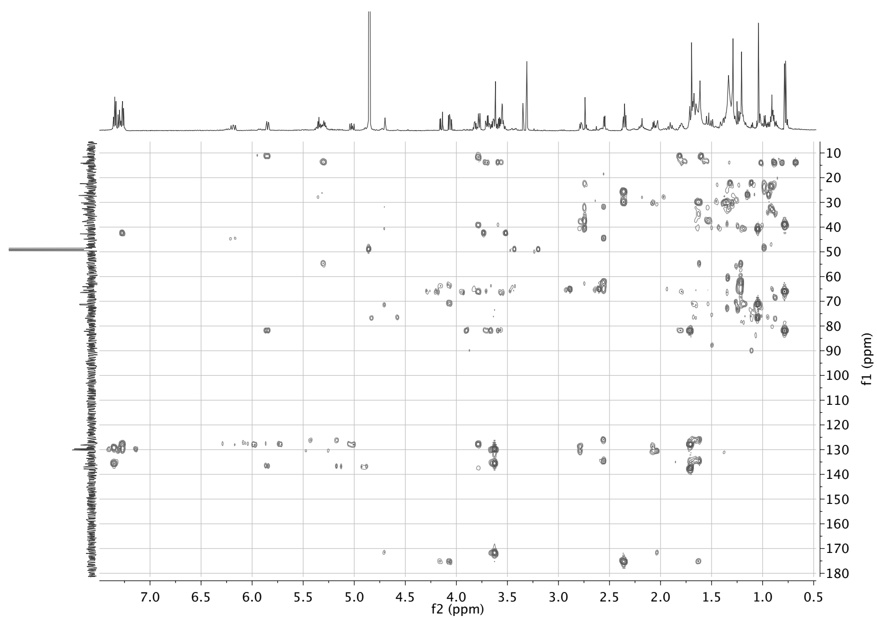

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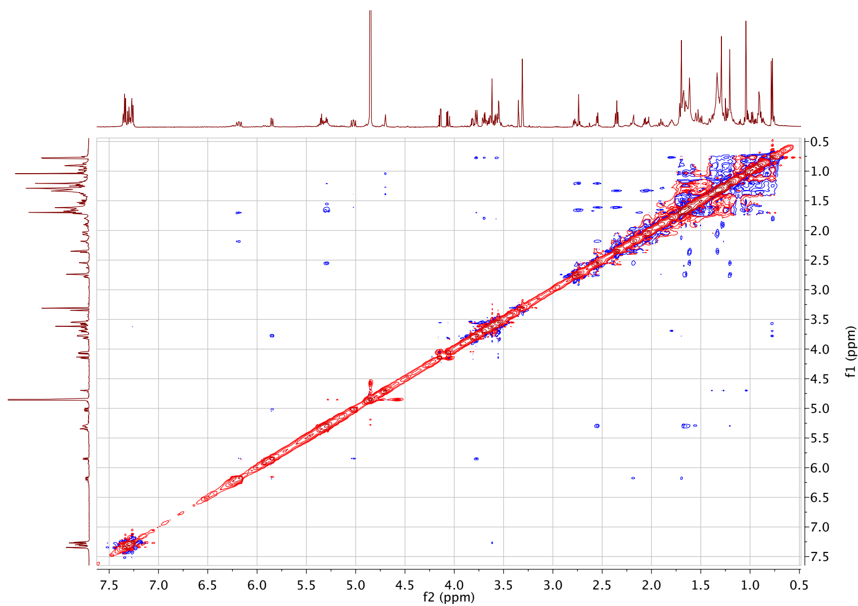

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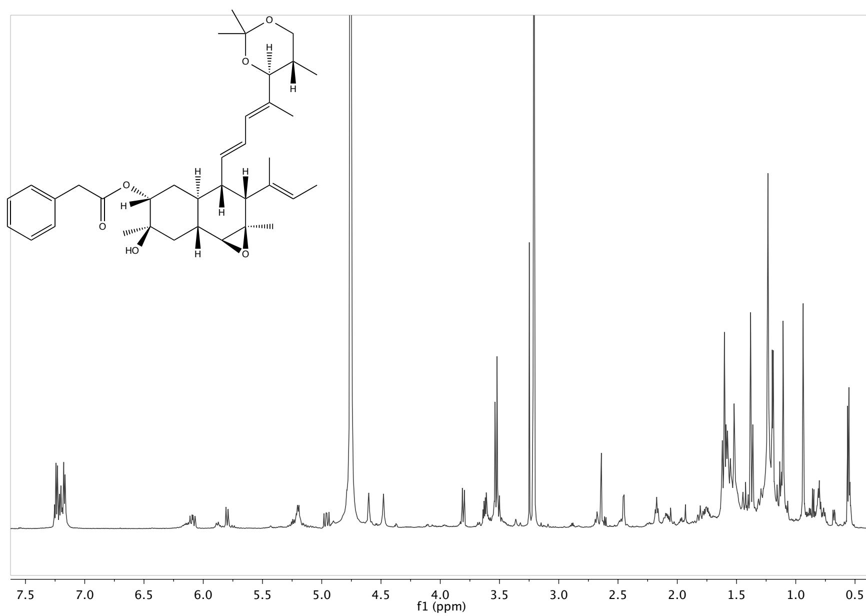

Figure S2.1. ^1H NMR spectrum of **2** (CD_3OD, 600 MHz).

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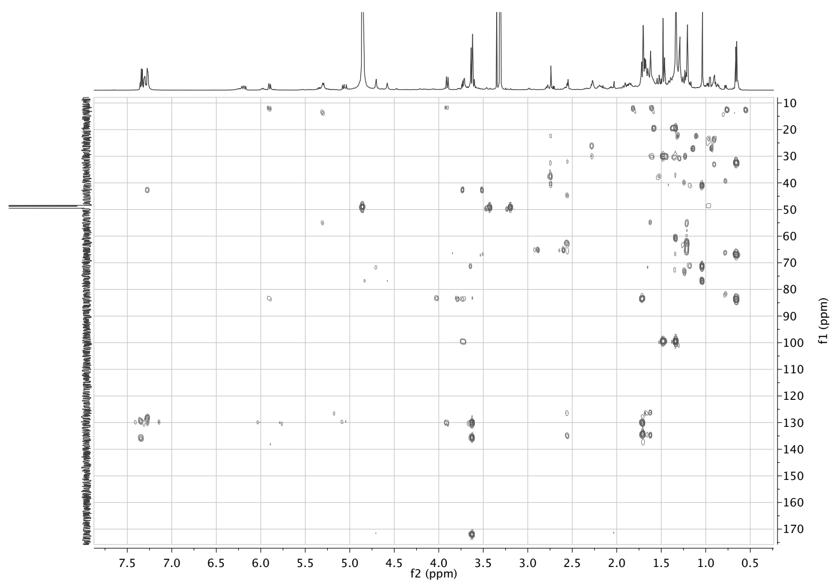

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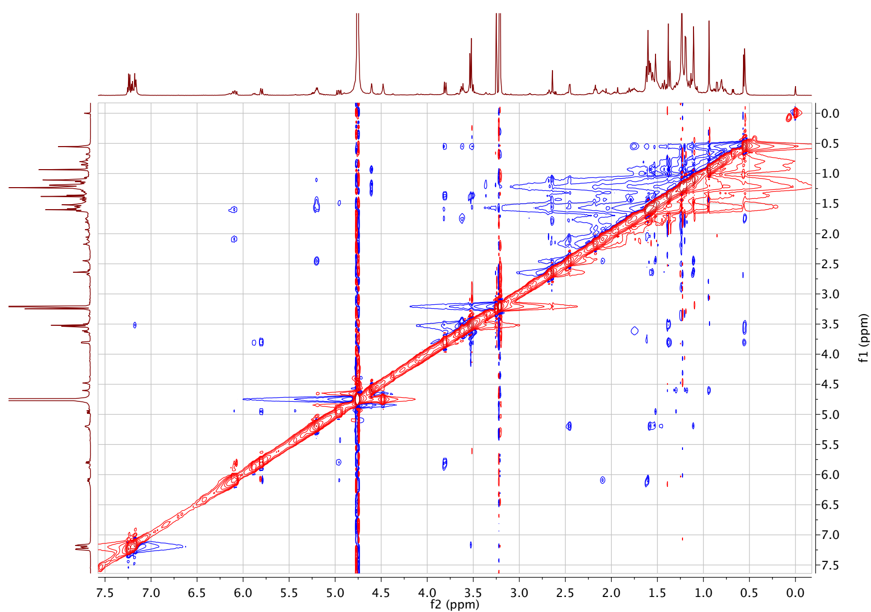

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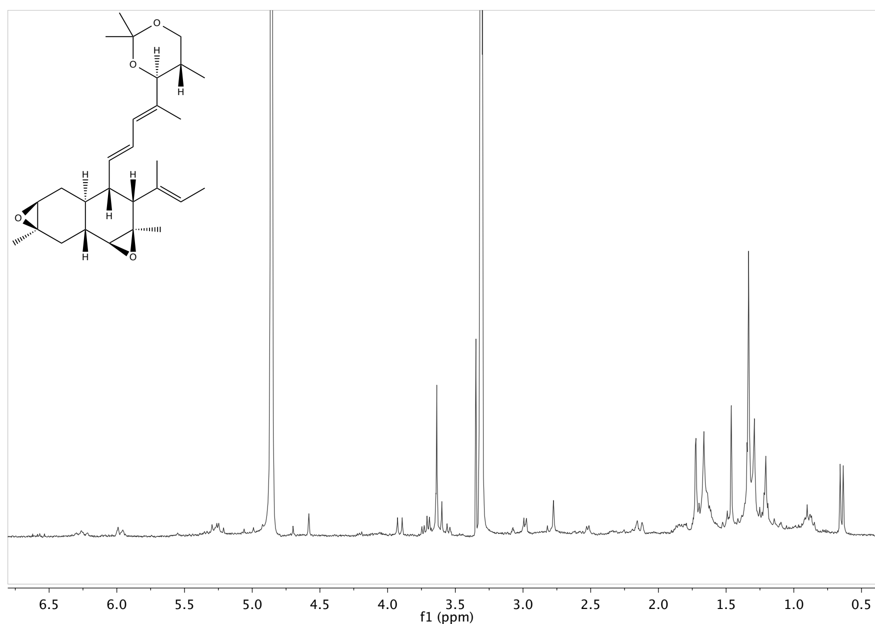

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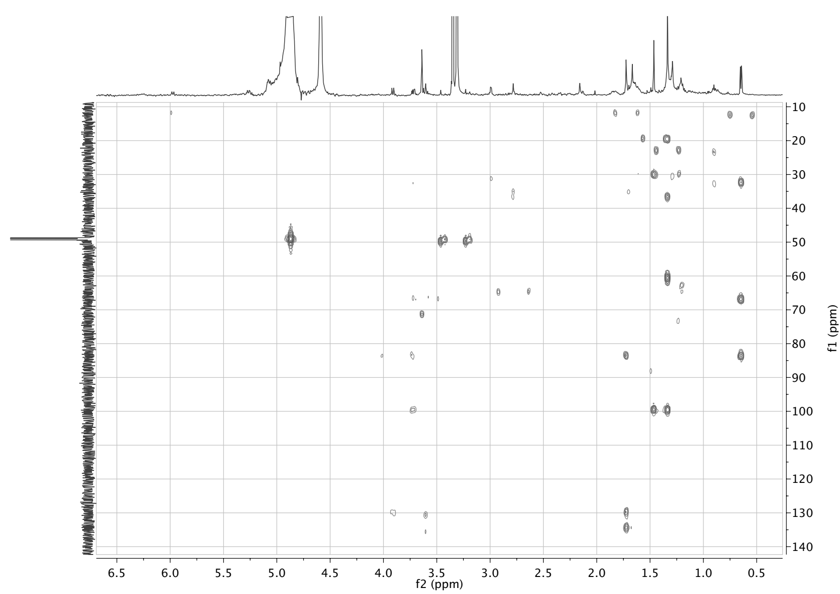

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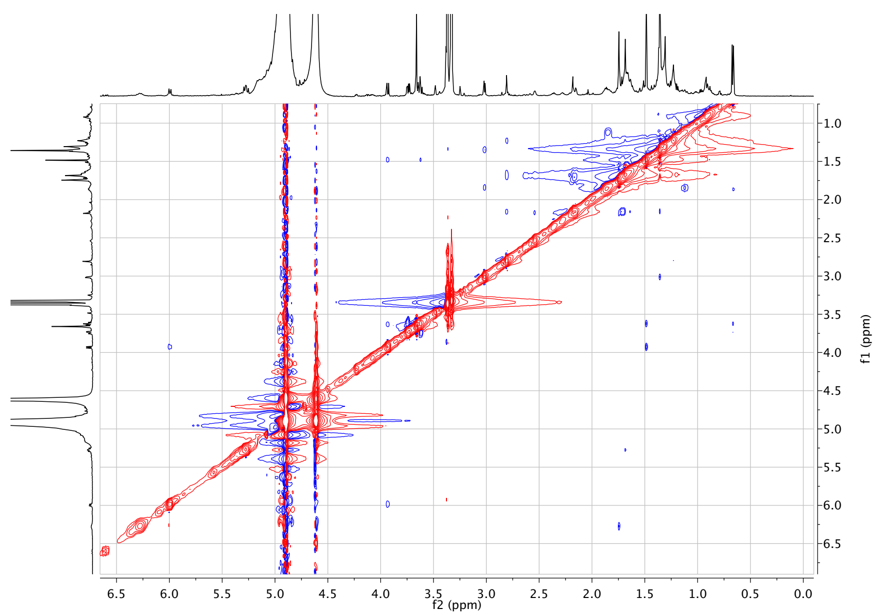

Figure S3.3. NOESY spectrum of **3** (CD_3OD, 600 MHz).

Chapter II - *Induction and enhancement of secondary metabolites with OSMAC*

Section 2

Optimization of Enniatin Production by Solid-Phase Fermentation of *Fusarium tricinctum*

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Optimization of Enniatin Production by Solid-Phase Fermentation of *Fusarium tricinctum*

Jian-ping Wang[a,b], Abdessamad Debbab[a], Catalina Francis Pérez Hemphill[a], and Peter Proksch[a,*]

[a] Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, Geb.26.23, D-40225 Düsseldorf, Germany. Fax: +49 211 8111923. E-mail: proksch@uni-duesseldorf.de

[b] Key Laboratory of Natural Medicinal Chemistry and Resources Evaluation of Hubei Province, College of Pharmacy, Huazhong University of Science and Technology, Wuhan, P. R. China

* Author for correspondence and reprint requests

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Enniatins are cyclic depsipeptides produced by fungi of the genus *Fusarium* that are of interest due to their pronounced biological activities; especially enniatins A, A_1, B, and B_1 possess anticarcinogenic and anti-HIV properties. In the present study, *F. tricinctum* was grown on seven solid media and in one liquid growth medium with or without addition of peptone or of amino acid precursors in order to identify favourable media with simple cultivation conditions for maximum enniatin production. Additionally, the optimal duration of growth was investigated for the highest yields of enniatins. From the different media analysed, white beans (*Phaseolus vulgaris*, solid medium) induced the highest accumulation of enniatins A, A_1, B, and B_1, that reached a maximum of 1,365 mg total enniatins in 1 L growth medium after 18 days of fermentation. Fermentation of *F. tricinctum* on white beans gave the highest yield of enniatins compared to all other media analysed in this study.

Key words: Enniatin, *Fusarium tricinctum*, Production Optimization

Introduction

Enniatins are cyclic hexadepsipeptides and consist of three D-2-hydroxyisovaleric acid (D-Hiv) residues linked alternatively to *N*-methyl-L-amino acid residues (*N*-methyl-L-valine, *N*-methyl-L-isoleucine, and *N*-methyl-L-leucine) (Strongman *et al.*, 1988). They are fungal metabolites first isolated by Gäumann *et al.* (1947) from *Fusarium orthoceras* var. *enniatinium* but have since then been reported from other *Fusarium* species such as *F. tricinctum* that was isolated as an endophyte from the host plant *Aristolochia paucinervis* (Debbab, 2007).

Due to ionophoric properties, enniatins have been shown to have insecticidal activity as well as antimycobacterial activity, one of the most potent being enniatin B (Ovchinnikov *et al.*, 1974; Lifson *et al.*, 1984; Visconti *et al.*, 1992; Doebler, 2000; Firáková *et al.*, 2007). They inhibit various enzymes, *e.g.* acyl-CoA-cholesterol-acyl transferase and cyclic nucleotide phosphodiesterase (Tomoda *et al.*, 1992). Additionally, Kamyar *et al.* (2004) showed that enniatins are easily incorporated into cellular membranes where they may form cation-selective pores. Furthermore, enniatins, especially enniatins A, A_1, B, and B_1, possess anticarcinogenic properties by induction of apoptosis and disruption of ERK signaling (Dornetshuber *et al.*, 2007; Lee *et al.*, 2008; Hyun *et al.*, 2009; Wätjen *et al.*, 2009). Interestingly, it has been shown that enniatins possess anti-HIV activity, whereby a mixture of enniatins B, B_1, and A exhibit the highest activity compared to all other enniatins isolated so far (Firáková *et al.*, 2007; Shin *et al.*, 2009).

Hence, enniatins are of considerable interest both as possible lead structures for the development of new anticancer or anti-HIV drugs and as molecular probes for the investigation of intracellular signal transduction pathways. An improved production of enniatins through a simple fermentation method of *Fusarium* sp. is therefore desirable. In this study, we investigated seven solid media and one liquid medium with or without the addition of peptone or of amino acid precursors for the optimization of the production of enniatins by *F. tricinctum*.

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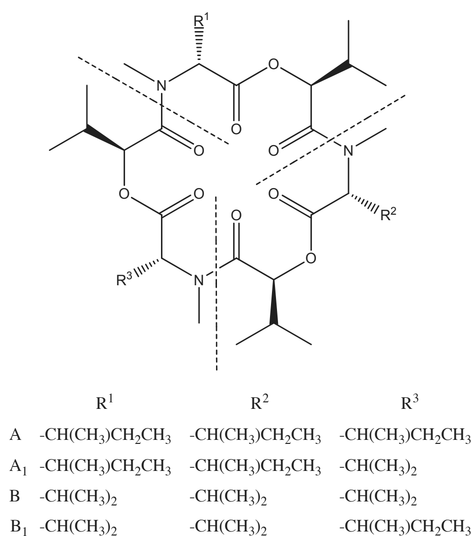
Results and Discussion

Effects of different media on enniatin production by F. tricinctum

F. tricinctum was cultivated on seven solid media and in a liquid modified Wickerham medium (static vs. shaking cultures). Cultures on solid media were harvested and extracted after 10 days, when the fungus had completely overgrown the various media. Cultures in liquid media were harvested after 24 days (static culture), due to slow growth, or after 7 days when cultures were shaken. Total enniatin concentrations (expressed in mg/L medium) and profiles of individual compounds (Fig. 1) were assessed by high-performance liquid chromatography (HPLC). Total enniatin concentrations varied considerably among the different growth media analysed (Fig. 2) and were highest (1,113 mg/L) when the fungus was grown on white beans (*Phaseolus vulgaris*). Soybeans proved to be the second best medium compared to white beans and resulted in a total enniatin concentration of 541 mg/L. All other media (solid or liquid) were clearly inferior and yielded total enniatin concentrations less than 100 mg/L. Shaking of the cultures of *F. tricinctum* in liquid modified Wickerham growth medium vs. static cultures reduced the total enniatin concentrations from 63 to 9.1 mg/L (Fig. 2).



	R^1	R^2	R^3
A	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$
A_1	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)_2$
B	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)_2$
B_1	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$

Fig. 1. Chemical structures of enniatins A, A_1, B, and B_1.

As a result of this comparative investigation, legume crops such as white beans or soybeans that are rich in proteins give rise to the highest enniatin concentrations and are hence preferable as growth media compared to other media rich in carbohydrates such as grains or potatoes. Liquid Wickerham medium (static or shaking cultures) gave likewise low yields of enniatins compared to white beans or soybeans and, therefore, offers no feasible alternative for production of the investigated compounds.

Enniatin patterns on the other hand proved to be remarkably homogenous between the various treatments analysed (data not shown). These findings are in accordance with the study of Pieper *et al.* (1992) who isolated enniatin synthetases from several *Fusarium* species and found that each synthetase preferably accepts certain nutrients resulting in a distinct production pattern of enniatins. In our study, enniatin B was consistently found to be the major constituent for all growth media analysed followed by enniatins B_1, A_1, and A. For cultures growing on white beans the average percentages of compounds were 43% for enniatin B, 38% for enniatin B_1, 12% for enniatin A_1, and 7% for enniatin A (Fig. 3).

Influence of peptone and of amino acid precursors on enniatin production on solid and in liquid media

Peptone and selected amino acid precursors (L-valine, L-leucine, and L-isoleucine) that constitute biogenetic building blocks of the investigated enniatins were studied for their effects on enniatin production by *F. tricinctum* on solid (white beans) as well as in liquid media (Fig. 4). Neither addition of peptone nor of amino acids had a significant effect on enniatin production on white beans when compared to controls. There was a slight increase of the yields of total enniatins in the presence of 2.5 g peptone/L and of 0.5 g of each of the three amino acids analysed/L. This was, however, not statistically significant (Fig. 4A). Apparently, white beans supply all necessary nutrients for the growth of *F. tricinctum* and precursors for the production of enniatins in sufficient quantities. Hence, supplementation by addition of peptone or by selected precursors does not provide a further stimulus for the production of these compounds.

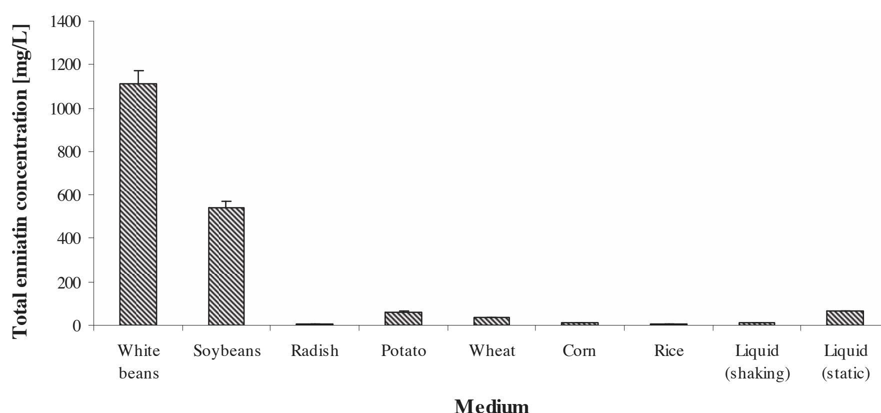


Fig. 2. Comparison of the enniatin production in the various tested media. Cultures were harvested after 10 days (solid media), 24 days (shaking liquid medium), and 7 days (static liquid medium).

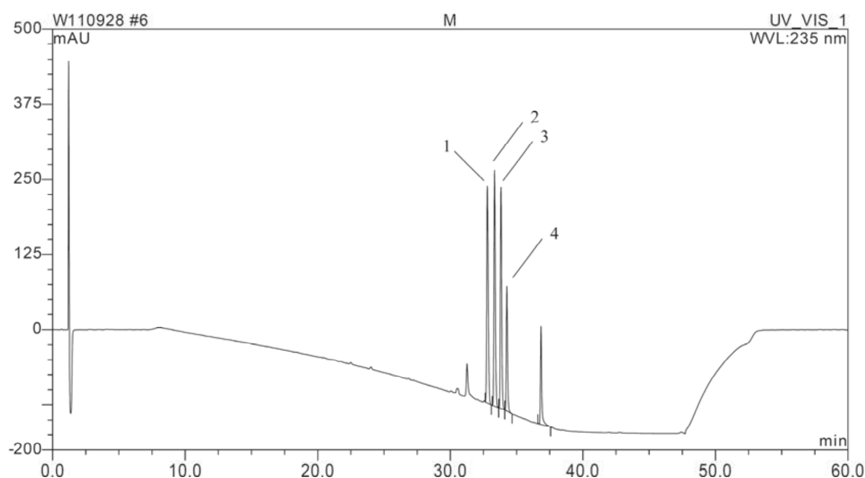


Fig. 3. HPLC chromatogram showing the composition of enniatins B with 43% (1), B_1 with 38% (2), A_1 with 12% (3), and A with 7% (4) in cultures of *F. tricinctum* grown on white beans extracted after 18 days. The retention times were detected at 235 nm: enniatin B with 32.797 min (1), B_1 with 33.337 min (2), A_1 with 33.823 min (3), and A with 34.267 min (4).

On the other hand, peptone and amino acids had a strong influence on enniatin production in cultures growing in liquid modified Wickerham medium. Addition of peptone (5.0 g/L) to static cultures of *F. tricinctum* raised the total enniatin concentrations from 63 mg/L in controls to 510 mg/L (Fig. 4B). Addition of amino acids (0.5 g of each amino acid/L) also caused a strong

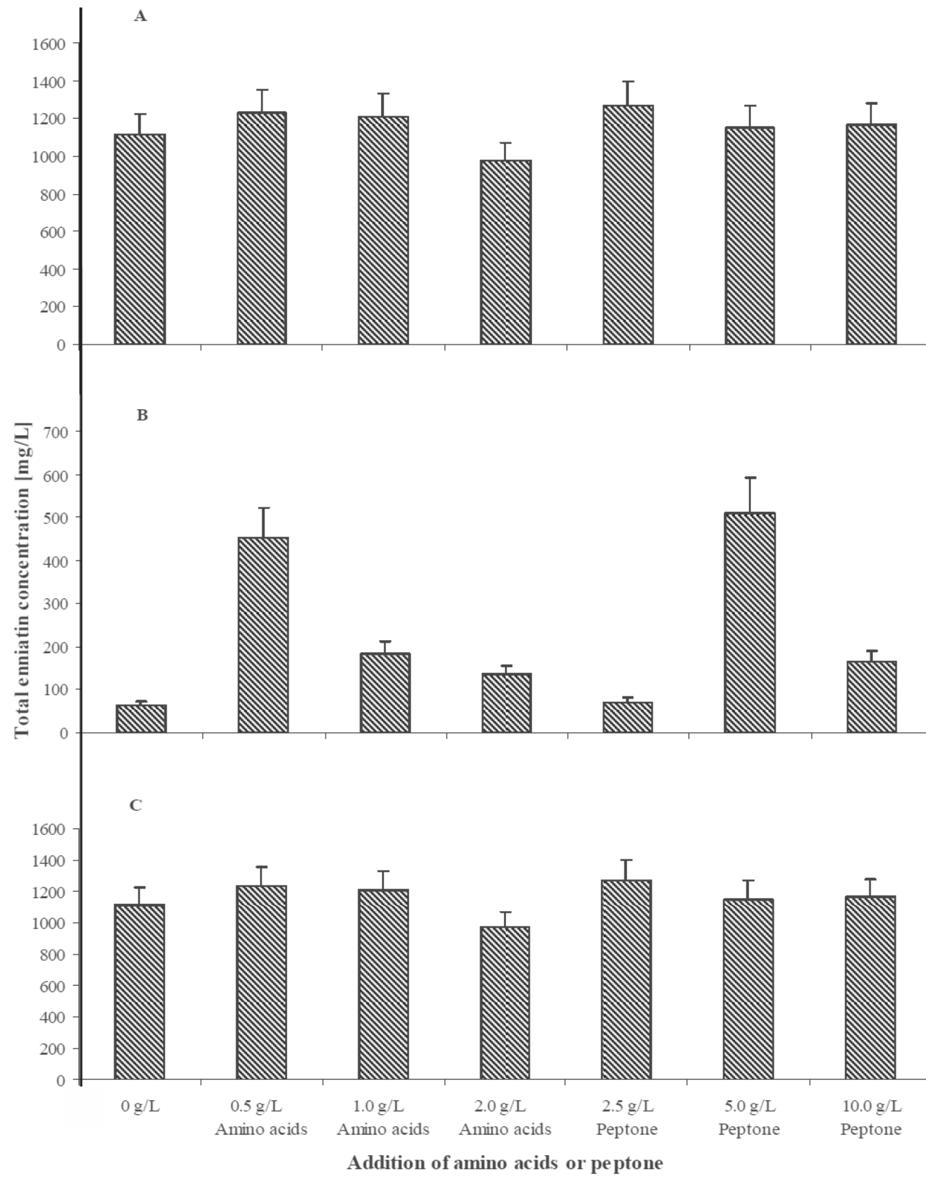


Fig. 4. Influence of peptone and amino acid mix (L-valine, L-leucine, and L-isoleucine) on total enniatin concentration in different media: (A) white beans; (B) liquid Wickerham medium (static culture); (C) liquid Wickerham medium (shaking at 150 rpm).

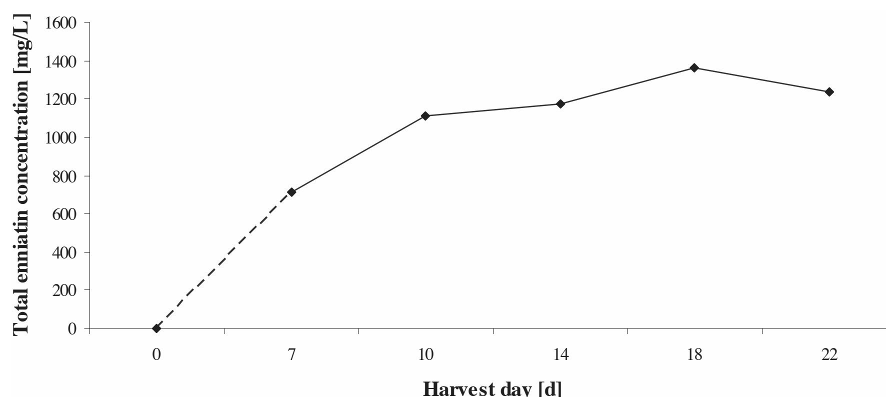


Fig. 5. Time course of enniatin production by *F. tricinctum* on white beans solid medium. The carry over is small and can be neglected. It is therefore shown only as a dashed line from day 0 until day 7.

increase in compound production and raised the total enniatin concentrations to 451 mg/L (Fig. 4B). Higher concentrations of amino acids resulted in a decrease in enniatin production which is probably due to toxicity of the amino acids. When peptone or the mix of amino acids were added to growing cultures of *F. tricinctum* maintained under shaking, an increase in enniatin production was likewise observed (Fig. 4C). Addition of 5.0 g peptone/L raised the total enniatin concentrations from 9 to 102 mg/L, whereas addition of 0.5 g of each of the three amino acids/L increased the total enniatin concentrations to 135 mg/L. Total concentrations of enniatins that resulted from addition of peptone or amino acids to liquid Wickerham medium were, however, less than those observed for static cultures of *F. tricinctum* on white beans or on soybeans.

Time course and peak production of enniatins on white beans solid medium

Production of enniatins by *F. tricinctum* on white beans was monitored over a period of 22 days (Fig. 5). After 10 days of growth (harvest time for the comparative experiments shown in

Table I. Comparison of reported studies with this current study concerning *Fusarium* species, growth medium, supplements, duration of cultivation in days, yield of enniatins A, A_1, B, B_1 (in mg/L), and type of enniatins.

Parameter	This study	Audhya and Russell (1973)	Audhya and Russell (1974)	Madry *et. al.* (1983)	Visconti *et al.* (1992)	Meca *et al.* (2010)
Fusarium sp.	*F. tricinctum*	*F. sambucinum* Fuckel HLX316	*F. sambucinum* Fuckel HLX316	*F. oxysporum* ETH 1536/9-C9/5/82	*F. acuminatum* MRC 3308	*F. tricinctum*
Growth medium	White beans	Liquid	Liquid	Liquid FDM	Liquid	Corn-derived
Supplements	–	Lactose, tryptone	Lactose, tryptone	Amino acids	–	–
Days of cultivation	18	28	34	4	7	30
Enniatin yield (mg/L)	1365	2000–3000	1724	5000	300	1320
Enniatin type	A, A_1, B, B_1	A, B, C	A, B, C (calculated as A)	A, A_1, B, B_1 (calculated as A)	A, A_1, B, B_1, B_2, B_3, B_4	A, A_1, B, B_1

Fig. 2), total enniatin concentrations amounted to 1,113 mg/L. Production of compounds peaked at day 18 with 1,365 mg/L followed by a gradual decline to 1,236 mg/L on day 22 (Fig. 5). The optimum harvest time for extraction of enniatins on white beans solid medium is thus between days 14 and 18 under the simple conditions chosen for our study. This compares favourably to reported yields of enniatins in other fermentation studies (Table I). For example, Audhya and Russell (1973, 1974) attained yields of 1,724 – 3,000 mg enniatins/L with liquid surface cultures of *F. sambucinum* Fuckel HLX316 which were supplemented with lactose and tryptone and grown for 28 and 34 days, respectively. Enniatins A, B, and C were determined as enniatin A with a photometric method rather than by HPLC detection of individual metabolites as done in our study. When considering the duration of cultivation (34 vs. 18 days) the yield in our study is superior. Another example is a yield of up to 5,000 mg enniatins/L liquid chemically defined production medium (*Fusarium*-defined medium, FDM) supplemented with amino acids and a submerged cultivation for 4 days of *F. oxysporum* ETH 1536/9-C9/5/82, a variant strain which was obtained by a duplicate treatment with nitrosoguanidine as described by Zocher *et al.* (1978, 1982) (Madry *et al.*, 1983). Whereas the yield of enniatins A, A_1, B, and B_1 reported in the latter study is superior to the yield obtained in our study, the quantification method employed lacks the selectivity of an HPLC-based method as employed in this study. Preparation methods of the media and of the strain are furthermore more complex and more time-consuming compared to our method. In a further study, Visconti *et al.* (1992) described a yield of an enniatin mixture (A, A_1, B, and B_{1-4}) of about 300 mg/L shaking liquid medium after 7 days of cultivation of *F. acuminatum* MRC 3308. Finally, Meca *et al.* (2010) obtained a yield of 1,320 mg enniatins (mixture of A, A_1, B, and B_1)/L corn-derived medium after cultivation of *F. tricinctum* for 30 days. Thus, the yield of enniatins produced on white beans as reported in our study compares well to other studies reported in the literature with regard to the simple cultivation methods and the short cultivation time needed until harvest of the cultures. This opens new opportunities for an easy and efficient production of enniatins A, A_1, B, and B_1. Nevertheless, it should be mentioned that other enniatins, such as enniatins H, I, and MK1688, attained through submerged culture of *F. oxysporum* KFCC 11363P, gave optimum results employing an FDM (maximum production of total enniatins, 2,399 mg/L after 8 days) (Lee *et al.*, 2011).

Experimental

Microorganism

Fusarium tricinctum (GenBank accession number AB470859.1) was isolated from fresh healthy rhizomes of *Aristolochia paucinervis* Pomel (Aristolochiaceae) and identified as described previously (Debbab, 2007).

Reagents

All chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany). All fungi culture reagents were purchased from PAA (Coelbe, Germany). The reference substances of enniatin A, A_1, B, and B_1 (Fig. 1) were isolated and purified as described previously (Debbab, 2007). All spectroscopic data obtained were in accordance with published reference data (supplementary data containing ^{13}C NMR spectra, HPLC chromatograms, and UV spectra can be obtained from the author for correspondence on demand).

Optimization strategies for enniatin production by F. tricinctum

Solid media for enniatin production by *F. tricinctum*

Erlenmeyer flasks (1 L each) containing 100 g of dry legumes [small white beans (Mueller's Muehle, Unna, Germany) or soybeans] or grains (wheat, corn or rice) and 100 mL of distilled water were autoclaved. Potatoes and radish were used fresh (140 g fresh weight after peeling) and sliced prior to autoclaving. Pieces of similar size from Petri dishes with the growing fungus were transferred under sterile conditions to the Erlenmeyer flasks containing the autoclaved medium. The fungus was grown on solid media at room temperature (25 °C) for 10 d followed by extraction. Four independent growth experiments were run separately, and all experiments were performed in triplicate.

Liquid medium for enniatin production by *F. tricinctum*

Liquid cultures of the fungus were kept in Erlenmeyer flasks (1 L each) containing 200 mL liquid Wickerham medium (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, distilled water added up to 1000 mL, pH adjusted to 7.2 – 7.4) at room temperature under static conditions for 24 d or under shaking (150 rpm) for 7 d.

Addition of branched-chain amino acids or peptone to solid or liquid media

L-Valine, L-leucine, and L-isoleucine were dissolved in 10 mL sterile water and added to the autoclaved media by sterile filtration (pore size, 0.45 μm) resulting in final concentrations of 0.5, 1.0, 2.0 g of each amino acid/L, respectively. Peptone was dissolved in 10 mL sterile water and added to the autoclaved media by sterile filtration resulting in final concentrations of 2.5, 5.0, 10.0 g/L, respectively. Fungi were allowed to grow for 10 d at room temperature on solid media until extraction. Fungi in liquid modified Wickerham medium (static culture) were kept for 24 d until extraction, whereas fungi kept under shaking (150 rpm) were harvested after 7 d.

Extraction and quantification of enniatins

For cultures on solid media, 250 mL ethyl acetate (EtOAc) were added to the cultures at the time of harvest and left overnight. Culture media containing the mycelia were then cut into pieces inside the flasks to allow complete extraction and were kept on a shaker (150 rpm) for 3 d. After filtration, fresh EtOAc (250 mL) was added to the cultures, and extraction was repeated as described above. The combined EtOAc phases were then washed with distilled water, and the solvents were removed by rotary evaporation. The dry residue obtained from the EtOAc extract was dissolved in 2 mL methanol (MeOH), and 5 μL were injected into an analytical HPLC instrument to determine the content of enniatins.

For liquid cultures, 250 mL of EtOAc were added at the time of harvest, and the content was thoroughly mixed with an Ultraturrax at 4000 rpm for cell destruction for 10 min followed by filtration under vacuum using a Buchner funnel. The liquid phase was transferred to a separation funnel, and EtOAc and H_2O phases were separated after vigorous shaking. Extraction of the aqueous phase was repeated twice by adding fresh EtOAc. The combined EtOAc phases were reduced to dryness by rotary evaporation, the residue dissolved in 2 mL MeOH, and 5 μL were subjected to HPLC analysis for quantification of the enniatins.

Individual enniatins were identified by HPLC in comparison to previously isolated reference compounds (Debbab, 2007) using a linear gradient starting with MeOH/nanopure H_2O (10:90), adjusted to pH 3.5 with phosphoric acid, and reaching 100% MeOH after 35 min. All peaks were detected by a UV-VIS photodiode array detector. The HPLC instrument consisted of a pump (Dionex P580A LPG; Morgan Hill, CA, USA), a detector (Dionex photodiode array detector UVD 340S), an injector, a separation column, and the reservoir of the mobile phase. The separation column (125 x 2 mm, ID) was pre-filled with Eurospher-100 C18 (5 μm), with an integrated pre-column (Knauer, Berlin, Germany).

The temperature of the column oven was set at 25 °C. The wavelength for detection of the enniatins was set at 235 nm. The flow rate was 1.0 mL/min. Calibration tests with previously isolated reference compounds (Debbab, 2007) were performed, and a suitable calibration curve was established. A correlation coefficient value higher than 0.9997 indicated linearity within the used concentration range.

For total enniatin quantification, clear separation of enniatins A, A_1, B_1, and B (given in the order of increasing retention times) was readily achieved with the procedure described above. Total enniatin contents were calculated based on the calibration curves.

Statistical analysis

Analysis of variance (one-way ANOVA) was performed to test the significance of differences between means obtained in each experiment at the 5% level of significance ($p < 0.05$).

Acknowledgements

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Supporting Information

Optimization of Enniatin Production by Solid Phase Fermentation of *Fusarium tricinctum*

Jian-ping Wang[1,2], Abdessamad Debbab[1], Catalina Francis Pérez Hemphill [1], Peter Proksch [1]

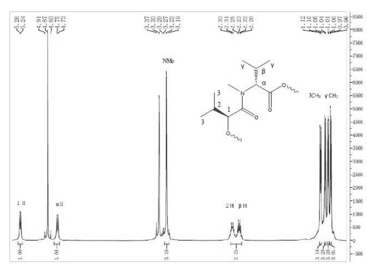
Affiliation

[1]Institut für Pharmazeutische Biologie und Biotechnologie,
Heinrich-Heine-Universität Düsseldorf, Germany

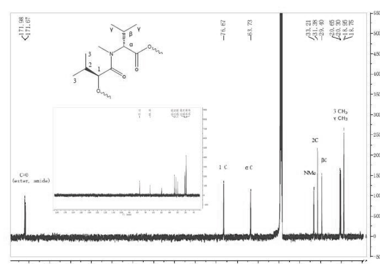
[2]Key Laboratory of Natural Medicinal Chemistry and Resources Evaluation of Hubei Province, College of Pharmacy, Huazhong University of Science and Technology, Wuhan, PR China

Correspondence:

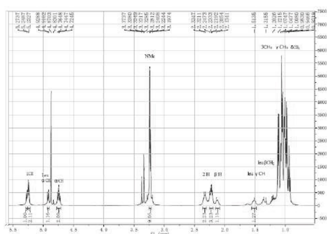
Peter Proksch, Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, Geb.26.23. D-40225 Düsseldorf, Germany. Tel.: +49 211 81 14163; Fax: +49 211 8111923. E-mail: proksch@uni-duesseldorf.de



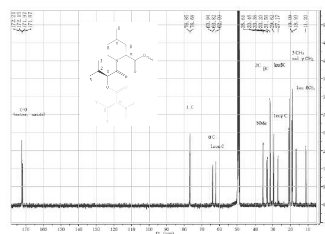
^{1}H NMR spectrum of Enniatin B



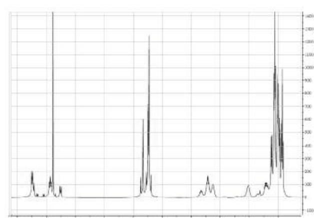
^{13}C NMR spectrum of Enniatin B



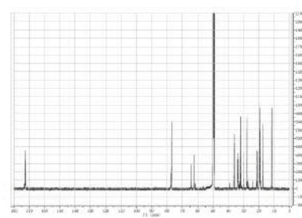
^{1}H NMR spectrum of Enniatin B1



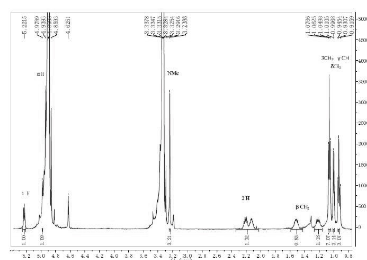
^{13}C NMR spectrum of Enniatin B1



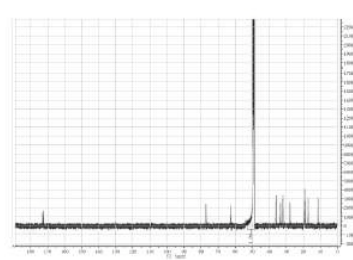
^{1}H NMR spectrum of Enniatin A1



^{13}C NMR spectrum of Enniatin A1



^{1}H NMR spectrum of Enniatin A



^{13}C NMR spectrum of Enniatin A

Fig. 1S. ^{1}H and ^{13}C NMR (ARX 500 NMR spectrometer) for enniatin B, B1, A1, A

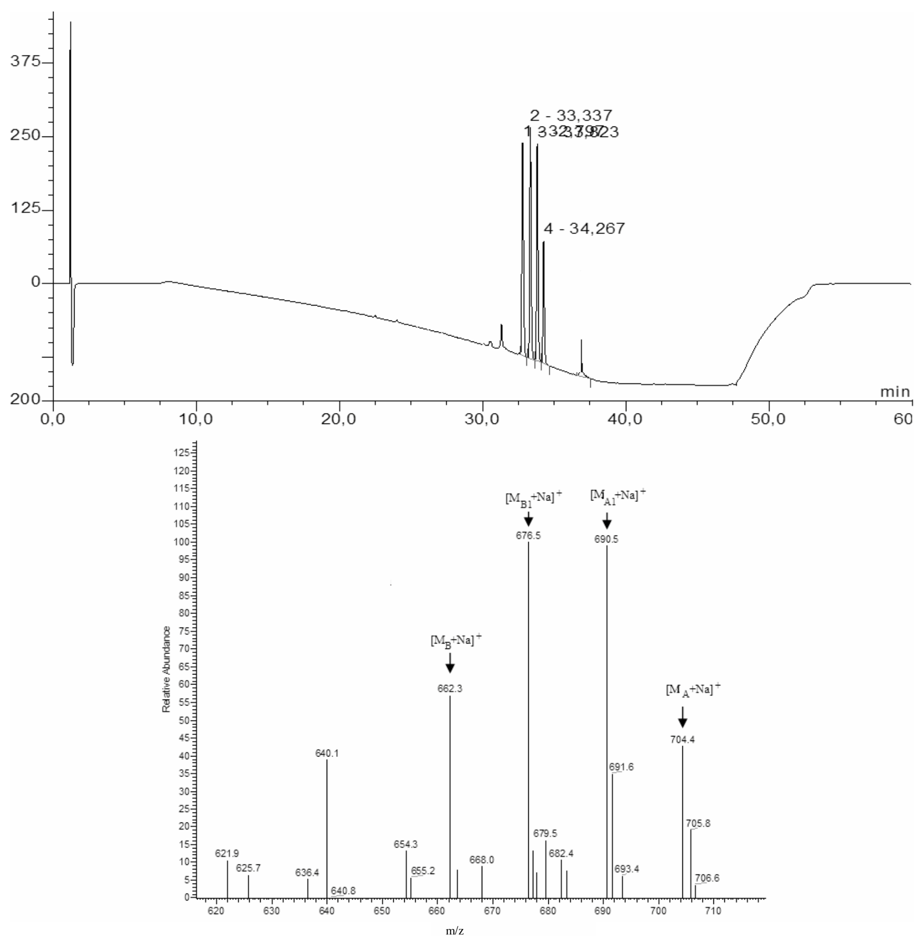


Fig. 2S. HPLC/ESI-MS (Finnigan LC Q-Deca mass spectrometer) of enniatin B, B1, A1, and A.

Discussion

In the field of drug discovery, natural products play an imminent role, contributing today to 41% of applied medicinal drugs worldwide, and to 51% of those used in anti-cancer therapy [Newman and Cragg, 2016], which is especially amazing, as less than 1% of all organic compounds known to man are in fact natural products [von Nussbaum *et al.*, 2006].
Of the natural products derived antineoplastic agents approved by the FDA, 53% stem from microorganisms [Trendowski, 2015]. It is therefore argued, that microbes, especially from extreme habitats, *e.g.* located in or around saltwater (marine habitat), are promising sources for new drug leads and should be investigated more (cf. Introduction) [Trendowski, 2015; Newman and Cragg, 2016]. This is underpinned and stressed in the sense, that further research on marine microorganisms has led to the development of the antineoplastic agent plinabulin in the last 10 years [Nicholson *et al.*, 2006], which is currently in clinical phase III studies, and was derived from a natural product isolated from a marine endophytic fungus [Kanoh *et al.*, 1997; Kim, 2012]. Furthermore, the most recent review on natural products with marine origin records an increased isolation rate of new and bioactive metabolites from Mangrove-derived fungi, with most of the compounds being produced by endophytes [Blunt *et al.*, 2016]. Ongoing investigation of secondary metabolites from endophytic fungi isolated from Mangroves is therefore highly promising and fermenting these microorganisms under so-called standard laboratory conditions in the course of this dissertation has led to the discovery of new and bioactive natural products and has added to further the structural and biological elucidation of known compounds:

The cultivation of *Pestalotiopsis clavispora* on solid rice medium (Chapter I, Section 1) yielded a total of 14 secondary metabolites, including seven new polyketides. Of these fungal compounds, the new natural product pestalpolyol I (**1**, Figure 6) exhibited strong cytotoxic activity (IC_{50} 4.1 μM) against a murine lymphoma cell line (L5178Y). The remaining six new natural products and the seven known compounds isolated, were also tested against this cell line, but showed no considerable growth inhibition [Pérez Hemphill *et al.*, 2016].

Pestalotiopsis clavispora was isolated from petioles of the Mangrove *Rhizophora harrisonii*, collected in Port Harcourt (Nigeria). It is a plant which hasn't been used medicinally or alimentary, but is generally described as tannin, charcoal and wood resource and extracts of the flowers and leaves are used in skin care products in Japan [Hatani *et al.*, 2003]. Members of the fungal genus *Pestalotiopsis* are most prominently known as common plant pathogens and for the ability to produce taxol [Strobel *et al.*, 1996; Maharachchikumbura *et al.*, 2014]. The species *Pestalotiopsis clavispora* itself is known as a plant pathogen in various plants, such as in persimmon, loquat, and strawberry crops [Palou *et al.*, 2015; Palou *et al.*, 2016; Zhao *et al.*, 2016], but has also caused keratitis in humans [Monden *et al.*, 2013]. It was previously isolated, likewise as an endophyte, from the Mangrove *Bruguiera sexangula*, and cultivation experiments with that particular strain yielded new and known triterpenoids and thymidine [Luo *et al.*, 2011; Deng *et al.*, 2011].

Interestingly, contrary to this, all 14 compounds isolated from this same fungal species, which was likewise obtained as a Mangrove-derived endophyte in the course of this dissertation, but from another Mangrove, were polyketides. This is another nice example, of how an endophyte chemically adapts to its environment, as the concerning Mangroves not only belong to different genera, but also dwell in different geographical loci, which is a known factor to effect also the endophytes residing in the same plant species [Wanderley Costa *et al.*, 2012].

The biogenetic background of the compounds isolated from *Pestalotiopsis clavispora* in this current study, resides in the polyketide pathway, and consists of a long chain branched aldehyde (**1**), five isocoumarins (**2**-**6**), a xanthone (**7**), four phthalides (**8**-**11**), a θ-lactone (**12**), and two depsidones (**13**, **14**), (Figure 6). This plethora of different polyketide groups suggests that the polyketide synthases (PKS) involved are plentiful and have different functional enzymes, which in turn means that the genes coding these PKS are multifarious, which is typical for *Pestalotiopsis* species [Wang *et al.*, 2015], and increases the chance of these clusters being easily modulated, *e.g.* directly by epigenetic modulators or indirectly by the cultivation conditions of the fungus.

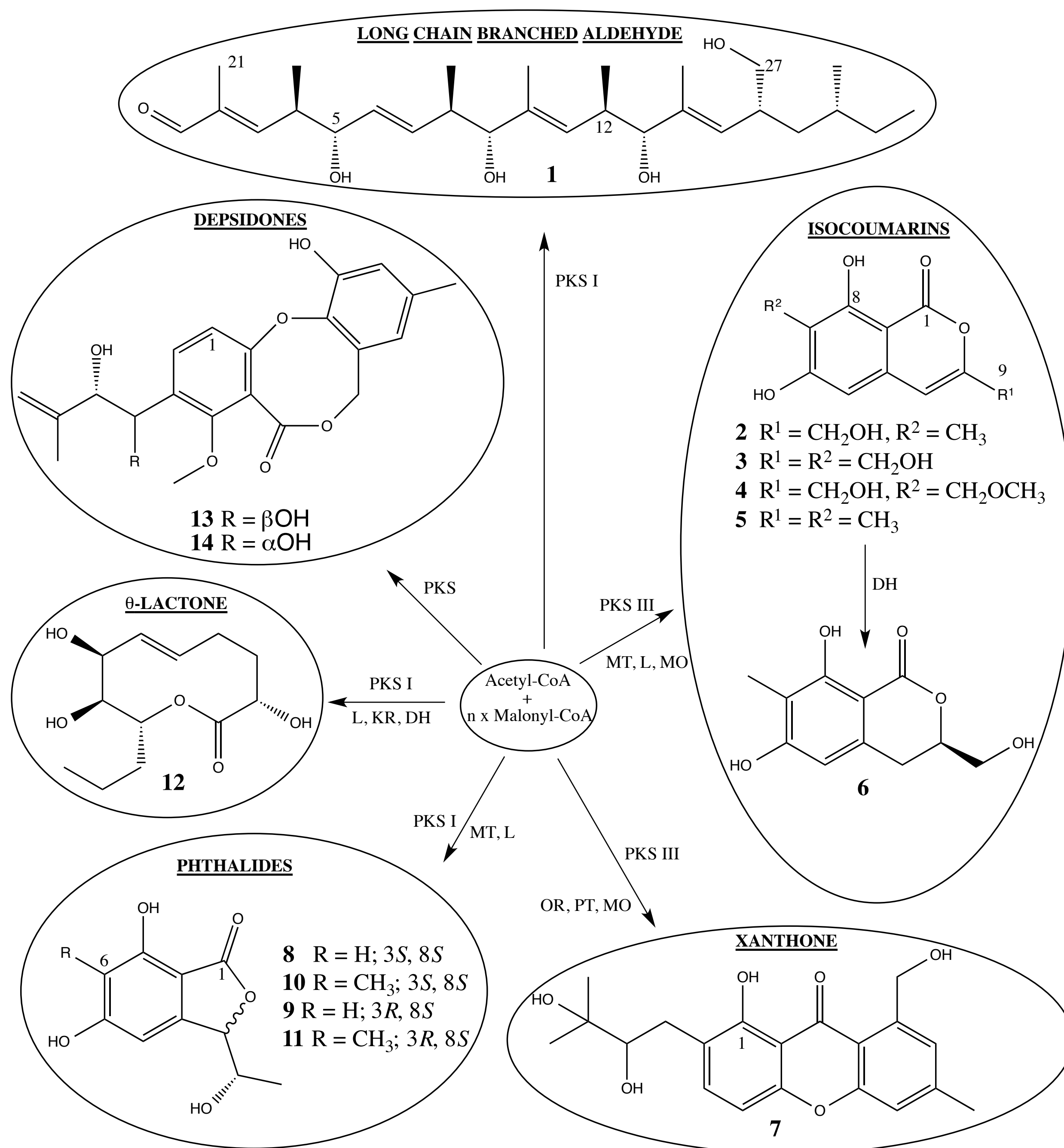


Figure 6. Proposed biosynthesis of polyketides isolated from *Pestalotiopsis clavispora*. Pestalpolyol I (**1**), pestapyrones A (**2**), B (**3**), C (**4**), similanpyrone B (**5**), (*R*)-(-)-periplanetin D (**6**), pestaxanthone (**7**), norpestaphthalides A (**8**), B (**9**), pestaphthalides A (**10**), B (**11**), 2-*epi*-herbarumin II (**12**), pestalotiollides A (**13**), B (**14**). DH dehydrogenases, KR keto-reductases, L lactonization, MO monooxigenases, MT methyl-transferases, OR oxidoreductase, PKS polyketide synthases, PT prenyl transferase.

The typical starter and extender units of each polyketide usually consist of one acetyl-CoA and a number of malonyl-CoA molecules, depending on the specificity of the PKS, initially forming long chain branched polyketides of different lengths, as a consequence of a so called head-to-tail reaction of these building blocks, with final decarboxylation and cyclisation reactions by dehydrating enolisation [Dewick, 2009]. PKS are divided into three types: type I (PKS I, fungi and bacteria) are described as multifunctional and large, type II (PKS II, bacteria) are monofunctional conglomerates, and type III (PKS III, fungi, bacteria, and plants) have only one active site, as opposed to several domains. In the following only type I and III PKS will be discussed.
A PKS contains several modules, which each consist of at least a β-ketoacyl-synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP), the latter not being present in type III PKS, to form a continuous chain of C_2 building blocks. Each of these modules can contain additional enzymes for subsequent structural modifications, *e.g.* keto-reductases (KR), dehydrogenases (DH), enoyl-reductases (ER), and/or methyl-transferases (MT). Further enzymatic reactions, *e.g.* oxidative cyclizations or dimerizations, usually occur post-PKS, though some may also be catalyzed by/within a PKS, the mechanism of which is not yet known [Dewick, 2009].

The new natural product pestalpolyol I (**1**), presents a long chain branched polyketide, which is typically found at the biosynthetic dawn of pretty much all polyketides, before many structural modifications are implemented. It shows a close structural resemblance to the fungal metabolite cubensic acid, which was previously isolated from *Xylaria cubensis*, and for which the PKS (type I) involved has been described in detail [Leeper and Vederas, 2000]. Pestalpolyol I bares a C_2 shorter backbone and an additional methylation in position C10, revealing one less KS/AT/ACP functionality in the final module 5 and an additional MT in module 3 of this PKS, compared to that of cubensic acid [Leeper and Vederas, 2000]. Presumably, after pestalpolyol I is released from module 5, the resulting initial carboxylic end is subsequently reduced by a post-PKS enzyme, to generate the existing C1 aldehyde function (Figure 6). Pestalpolyols have so far only been isolated from *Pestalotiopsis* species [Lie *et al.*, 2015; Xie *et al.*, 2015]. Nothing is described about the biological activities of cubensic acid, but similar derivatives, including pestalpolyol A, have also exhibited cytotoxic activity against several cancer cell lines [Li *et al.*, 2015], likewise to pestalpolyol I, which may therefore present a promising lead-structure for the development of further anti-tumor entities.

The isocoumarins isolated in this study have a 6-hydroxymellein core-structure, which differs from similanpyrone B (**5**) by a hydrogen substituent in position 7 and a reduced C3/C4 bond. The biosynthesis of 6-hydroxymellein is known to be performed by a type III PKS, forming the lactone ring by a reaction of the final thioester function with the hydroxy group of a starter unit [Dewick, 2009]. In the case of isocoumarins **2**-**5**, typical post-PKS enzymes may catalyze the reduction (DH), methylation (MT), and hydroxylation (MO) reactions necessary, resulting in the structural differences observed. 6-hydroxymellein is a common fungal product, isolated from various genera, *e.g. Phomopsis* and *Aspergillus* species [Haroon *et al.*, 2013; Yang *et al.*, 2015], and is known to have antifungal properties [Dewick, 2009], and structurally similar fungal isocoumarins, *e.g.* orthosporine, isolated from *Pleospora* sp., exhibit weak to no cytotoxic effects against cancer cell lines [Marmann, 2014], likewise to the isocoumarins isolated in the course of this dissertation.
One of the new isocoumarins, (*R*)-periplanetin D (**6**), is the enantiomer of the natural product (*S*)-periplanetin, previously isolated from the American cockroach *Periplaneta americana*, and is described as an insect neuropeptide, with hyperglycemic hormonal functions [Konopinska *et al.*, 1989; Luo *et al.*, 2014]. The production of such similar metabolites by fungi and insects alike may have resulted from an earlier co-evolution of the fungus and the insect, which may have been underlined by environmental factors [Beemelmanns *et al.*, 2016]. But this similarity in secondary metabolite products may have also occurred from a horizontal gene transfer of the same polyketide synthase genes much earlier in evolution [Kusari *et al.*, 2009]. Such horizontal gene transfers from fungi to insects are investigated and have been described in the literature, but for genes encoding other enzymes [Nakabachi, 2015]. At the same time, filamentous fungi are known to reside in the gut of *Periplaneta americana*, *e.g.* playing an eminent symbiotic role in the production of essential amino acids [Ayayee *et al.*, 2016]. It may well be, that (*S*)-periplanetin, previously isolated from this insect, was in fact produced by fungi residing inside of this insect or living within it in symbiosis [Beemelmanns *et al.*, 2016]. Though it is known, that enantiomeric production of natural products occurs in a single species, as well as amongst different kingdoms, not much is known about the enantioselective biogenesis of polyketides in different organisms [Finefield *et al.*, 2012]. Therefore using *Pestalotiopsis clavispora* and *Periplaneta americana* for further comparative studies on polyketide biosynthesis enzymes or even PKS gene clusters, appears to be a fruitful approach.

Pestaxanthone (**7**) is a prenylated xanthone with a high structural similarity to paeciloxanthone, isolated from an *Aspergillus* sp. strain, and differs in the positioning of the slightly modulated prenyl side chain in position C1 of the xanthone core structure, instead of C4.[Simpson, 2012] The biosynthesis of paeciloxanthone is well investigated and stems from an emodin moiety, an anthraquinone produced by a type III PKS. (The biosynthesis of emodin is discussed in more detail on page 105ff.) To form a xanthone structure, emodin is converted into a benzophenone through an oxidative cleavage by a post-PKS CYP-450 dependent oxidoreductase, and subsequent dehydrative recyclization involving phenolic functional groups [Dewick, 2009; Simpson *et al.*, 2012]. The resulting xanthone is then further manipulated by a prenyl transferase (PT), adding an isoprene unit, which in the case of **7** is further oxidized, to give the methylbutanediol moiety. Coherent with other prenylated xanthone derivatives, which differ only in the modulation pattern of the prenyl side chain (prenxanthone, isolated from *Penicillium* sp.) [Gao *et al.*, 2013], pestaxanthone (**7**) exhibited no cytotoxic activity towards cancer cells. But interestingly enough, in the case of paeciloxanthone, in which the prenyl side chain is in another position in the xanthone moiety, this compound exhibits strong anti-tumor activity [Wen *et al.*, 2008].

Similar to the previously discussed isocoumarins, the biosynthesis of phthalides is generally described with the lactonization of a polyketide chain, which is preceded by an aldol condensation along with the aromatization of the resulting six membered ring [Dewick, 2009]. This explains the formation of **8** and **9**, whereas again PKS MTs, as well as post-PKS methylations may have led to the 6-methyl derivatives **10** and **11** (Figure 6). These simple phthalide structures show no pronounced bioactivity in the assay mentioned above. This lack of bioactivity is in accordance with similar compounds, in fact diastereoisomers described in the literature and isolated from another *Pestalotiopsis* species, *P. foedan*, and from the lichen *Graphis proserpens* [Ding *et al*., 2008a; Takenaka *et al.*, 2011], also show no bioactivity.

The structure of the known θ-lactone 2-*epi*-herbarumin II **12** is very similar to that of macrolides, which is why its biosynthesis is proposed in a similar manner, through lactonization of a highly reduced polyketide chain, produced by a type I PKS [Dewick, 2009]. **12** showed no activity in the anti-tumor assay used in this study, comparable to other herbarumin II type compounds, which were isolated from the fungus *Paraphaeosphaeria recurvifoliae*, and are described as phytotoxins in the literature [Fürstner *et al.*, 2002; Seo *et al.*, 2007].

Finally, the PKS involving biosynthetic pathway of the two known depsidones **13** and **14** is not yet elucidated. Feeding experiments with *Penicillium purpurogeneum* have provided evidence of the polyketide nature for purpactin A, which differs from the depsidones in this study only in the substitution pattern of the prenyl side chain attached to C3 [Nishida *et al.*, 1992; Nakagawa and Omura, 1996]. Coherent with highly similar depsidones penicillides and purpactins, which are produced by *Penicillium* species, these structures exhibited no anti-tumor activity, but interestingly, a new class of cholesteryl ester transfer protein inhibitors, the dibenzodioxocinones, was developed from them by structural example [Niu *et al.*, 2015].
What is striking about the discussed polyketides, is that their bioactivity, with regard to their cytotoxic effects towards cancer cell lines, is overall weak, with exception of the long chain branched aldehyde pestalpolyol I, an observation which is comparable to similar structures described in the literature.
Interestingly, all of the polyketides isolated from *Pestalotiopsis clavispora* in the course of this dissertation are comparable to polyketides previously isolated from other *Pestalotiopsis* species, in the sense that they are fairly structurally and biogenetically simple entities, involving only few additional enzymes to the minimal modules of PKS (KS+AT±ACP), and paltry post-PKS enzymes [Xu *et al.*, 2011; Wang *et al.*, 2014; Niu *et al.*, 2015; Li *et al.*, 2015; Xie *et al.*, 2015]. Dimeric or conjugated polyketides produced by *Pestalotiopsis* species are only described in experiments involving genetic modulations [Wu *et al.*, 2016], which may explain why none such were isolated in this study.

Yet given the fungal diversity inside plants, it is not always necessary to perform modulative experiments, to obtain structurally complex and bioactively potent polyketidic natural products. In effect, cultivating another Mangrove-derived endophyte, *Acremonium* sp., on solid rice medium in the course of this dissertation (Chapter I, Section 2), resulted in the production of the known anthracene derivative torrubiellin B (Figure 7), which after isolation could be conclusively elucidated via ECD calculations as (5'*R*,10'*S*,10a'*R*)-torrubiellin B, and continuously investigated for its cytotoxic potential towards human carcinoma cell lines.

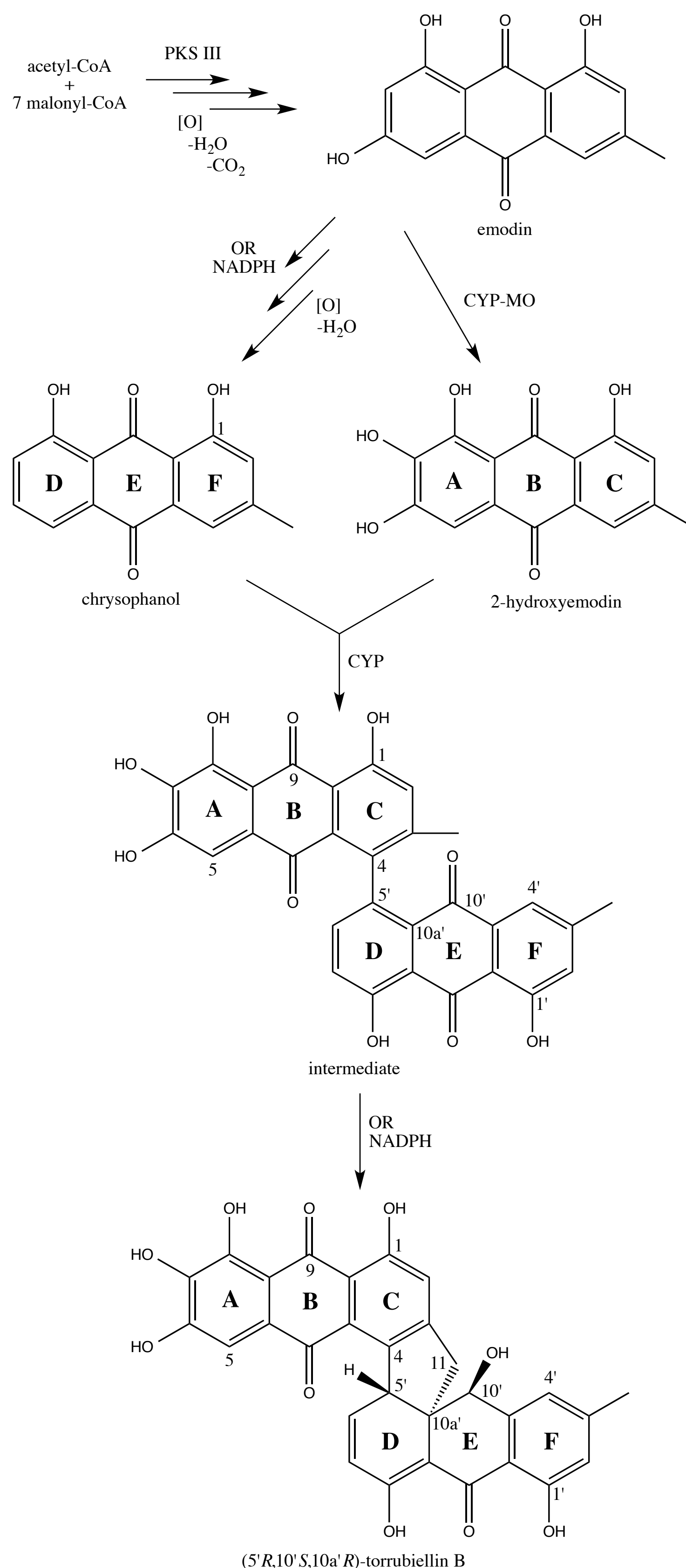


Figure 7. Proposed biosynthetic pathway of torrubiellin B. CYP CYP dependent enzyme, CYP-MO CYP dependent monooxygenase, OR oxidoreductase, PKS polyketide synthase. All oxidations ([O]), dehydrations and decarboxylations probably occurred through spontaneous chemical reaction.

Acremonium sp. was isolated from leaves of the Mangrove *Sonneratia caseolaris*, which were collected in Hainan (China), and are consumed as food in some south east Asian countries, along with the fruits, that appear as apples [Kathiresan *et al.*, 2010], and are traditionally used to treat bleeding wounds or sprained joints. Investigation of the leaves has shown antidiabetic and cholesterol-lowering effects, when applied as powder in rats, and antifungal effects towards *Fusarium oxysporum*, when extracted with ethanol. So far only antioxidative flavonoids have been isolated from leaves of *Sonneratia caseolaris* [Simlai and Roy, 2013].
The fungal genus *Acremonium* comprises about 100 species, some of which rate among the most simplest anamorphic filamentous fungi [Summerbell *et al*., 2011], and all of which are mostly saprophytes and non pathogenic for humans. Most famously, *Acremonium* sp. is known for the discovery of cephalosporins, which were first isolated in 1945 from a representative of this genus, then still called *Cephalosporium* [Abraham, 1987]. Since then, *Acremonium* species have proven to be fruitful sources for new and bioactive metabolites, which is highlighted by a recent review, counting 317 natural products of divers biosynthetic and structural backgrounds isolated from this genus so far [Tian *et al.*, 2016].
By analysis of the crude ethyl acetate extract of *Acremonium* sp. rice cultures, the biogenetic precursors of torrubiellin B were narrowed down to emodin monomers, as these were the only detectable anthraquinones, compared to previous options, which included chrysophanol and aloe-emodin [Isaka *et al.*, 2012]. Therefore, the biogenetic formation of torrubiellin B by the endophyte *Acremonium* sp. was proposed by an oxidative coupling of two emodin moieties [Pérez Hemphill *et al.*, 2015].
As emodin itself is an ubiquitous natural product [Izhaki, 2002], emodin dimers are expectantly vast and occur as homo- and as heterodimers, *e.g.* cladofulvin and skyrin, or torrubiellin B, respectively [Griffiths *et al.*, 2016].
The biosynthesis of emodin is well understood and involves many enzymes, *e.g.* ketosynthase and acyltransferase, all grouped as a type III polyketide synthase, to form this oxygenated anthracene metabolite from one acetyl-CoA and seven malonyl-CoA starting blocks [Couch and Gaucher, 2004; Dewick, 2009; Chiang *et al*., 2010]. Yet this biosynthesis of emodin also includes several steps of spontaneous chemical dehydration or oxygenation reactions, *e.g.* the dehydration of atrochrysone to emodin anthrone, and the oxidation of the latter to emodin (Figure 7) [Chiang *et al*., 2010]. The biological transformation of emodin to 2-hydroxyemodin via a cytochrome P450 dependent

monooxygenase has also been well understood for a while (Figure 7) [Mueller *et al.*, 1998]. Furthermore, the biosynthetical formation of chrysophanol from emodin was recently shown to occur under involvement of an NADPH-dependent oxidoreductase in combination with spontaneous chemical oxidation and dehydration reactions (Figure 7) [Griffiths *et al.*, 2016]. These recent investigations have also shown, that in the case of emodin dimers, enzymatic and chemical modification of emodin precursors occur in the same way as described above, and prior to dimerization [Griffiths *et al.*, 2016]. The oxidative dimerization of emodin derivatives in fungi involves cytochrome P450-dependent enzymes, similar to those identified in bacteria, but the exact mechanism is not yet fully understood [Funa *et al.*, 2005; Griffiths *et al.*, 2016].
Congruent with these recent findings, and in contrary to previous assumptions [Pérez Hemphill *et al.*, 2015], the emodin precursors of torrubiellin B were very likely modified as described above to 2-hydroxyemodin (ABC) and chrysophanol (DEF) prior to an oxidative C4-C5'-coupling of these two monomers, which parallel to that described for cladofulvin, may involve a cytochrome P450-dependent enzyme [Griffiths *et al.*, 2016]. Also in line with the respective previous study, the sole occurrence of emodin in the extracts, that yielded torrubiellin B, hint at this reduced monomer to be the substrate of the enzyme catalyzing the dimerization, and that this enzyme is capable of modifying it to the monomers contained in the product. Therefore these were probably biosynthesized by type III polyketide synthases, where all described enzymatic reactions occur in one and the same binding site [Dewick, 2009].
The fixation of the biaryl axis over the methylene linkage from $CH_3$11 of ring C to C10a of the DEF-moiety (Figure 7), was probably catalyzed by an NADPH dependent oxidoreductase [Griffiths *et al.*, 2016], as the neighboring C-bond 5'/10a' and the carbonyl function at C10' resulted in their reduced form in torrubiellin B.
Not much is known about torrubiellins, and so far only the 6,7-didesoxy analogue, torrubiellin A, a chrysophanol homodimer, has been isolated from another fungus, *Torrubiella* sp., together with torrubiellin B. Interestingly, the monomers chrysophanol and aloe-emodin were isolated along with emodin in the respective study, suggesting chrysophanol may also be a substrate for the above-mentioned enzyme catalyzing the dimerization. The occurrence of aloe-emodin (11-hydroxy-chrysophanol) has no apparent relation with the discussed biogenesis, but may suggest a stronger oxidative activity of the fungus it was isolated from.

Very similar anthraquinone dimers, uredinorubellins I and II, were previously isolated from the fungus *Ramularia uredinicola* [Miethbauer *et al.*, 2008]. These constitute of a chrysophanol homodimer (uredinorubellin II) and a helminthosporin (5-hydroxy-chrysophanol)-chrysophanol heterodimer (uredinorubellin I). In each case the monomers are connected in the same position as the torrubiellins, via an oxidative coupling and a fixation of the biaryl axis with a methylene linker, leading to a reduced form of the lower monomers, as in torrubiellin B. But the lower monomers in the uredinorubellins apparently undergo an additional hydration, resulting in a hydroxyl group in position 8a'. Likewise to the above mentioned torrubiellin study, the monomeric precursors of these uredinorubellins, helminthosporin and chrysophanol, were also isolated.
The co-occurrence of the monomers in these two studies underpin the biogenetic findings described above, that stated any chemical or enzymatic modification of the basic monomeric precursors probably occurs post-PKS, but prior to dimerization. Uredinorubellins furthermore are classified as precursors of rubellins, which differ by the formation of a seven-membered lactone ring, formed from the respective E ring of an uredinorubellin [Miethbauer *et al.*, 2008]. In turn, torrubiellins may present the precursors of uredinorubellins, yet this can only be suggested once torrubiellins are isolated along with uredinorubellins from the same fungal organism.
As mentioned above, torrubiellins have so far only been isolated from a *Torrubiella* and an *Acremonium* species [Isaka *et al.*, 2012; Pérez Hemphill *et al.*, 2015], whereas uredinorubellins were only isolated from the fungus *Ramularia uredinicola* [Miethbauer *et al.*, 2008]. Rubellins in turn have been isolated from the fungi *Mycosphaerella rubella*, *R. uredinicola* and *R. collo-cygni* [Arnone *et al.*, 1989; Miethbauer *et al.*, 2008], but also from the plant *Urginea rubella* [Steyn *et al.*, 1986]. The fact that compared to its precursors uredinorubellins and potentially torrubiellins, the supposed end-product rubellin is found in more species and across kingdoms, may also be an indication for the hypothesis made in the preceding paragraph, regarding rubellins as biosynthetic end-products.
Nonetheless, the mentioned organisms are not known to produce other anthraquinone dimers, not even the discussed dimers lacking the methylene linkage. Additionally, no anthraquinone dimers are known so far, that are connected via the C and D ring of two emodin derivatives (Figure 7).

As concluded in previous studies, whether an organism biosynthesizes one dimer or the other, may strongly depend on the selectivity of the dimerization enzymes towards their substrates [Griffiths *et al.*, 2016].
Therefore each of these organisms present highly interesting gateways for analyzing the responsible and seemingly unique dimerization enzymes, to help further understand the biosynthesis and possibly utilize this knowledge to biotechnologically induce the production of further such bioactively potent dimers.
The isolation of sufficient amounts of torrubiellin B from *Acremonium* sp., facilitated its application in MTT assays with five different human cancer cell lines, each with a cisplatin sensitive variant and a resistant mutant, including the tongue cell line Cal27, the esophageal cell line Kyse510, the breast cell line HCC38, the ovarian cell line A2780, and the breast cell line MDA-MB-231. In these anti-tumor assays (5'*R*,10'*S*,10a'*R*)-torrubiellin B exhibited significantly cytotoxic IC_{50} values of 0.2 - 2.6 µM. Currently, further investigations on the mechanism of action and antineoplastic potential of this fungal metabolite are being carried out, additional to the development of a total chemical synthesis of (5'*R*,10'*S*,10a'*R*)-torrubiellin B.
Torrubiellin A showed fewer biological activities [Isaka *et al.*, 2012], indicating that the 6,7-dihydroxy functional group is vital herefore.
Interestingly, the additional hydroxy function in the A ring of uredinorubellin I, which stemmed from the helmithosporin precursor, led to an increased effect on the cell viability in human (HT29), murine (J774A.1), and rabbit (HIG-82) cancer cell lines at a concentration of 10 µM, whereas the dehydroxy analogue, uredinorubellin II, only showed effects on the mouse and the rabbit cell lines, indicating that the additional hydroxyl function enables an impact on human cells, which was likewise observed for the corresponding torrubiellin metabolites [Isaka *et al.*, 2012; Pérez Hemphill *et al.*, 2015].
Furthermore, the monomers of all dimers described show comparably less biological activities than the dimers [Miethbauer *et al.*, 2008; Isaka *et al.*, 2012], which may present a defensive functionality of the dimers for the producing fungus.
In any case, especially if torrubiellin B is to be further developed towards a medical agent for clinical application in humans, the complete elucidation of the absolute configuration of its chemical structure is highly important, since only one of the stereoisomers may be the one with the desired pharmacological activity. More than half of all drugs currently on the market are chiral and the importance of elucidating their

3D-structure is vital [Rentsch, 2002]. As known from past examples in drug discovery, *e.g.* thalidomide (cf. Introduction), different stereoisomers of the same compound can exhibit lethal differences in their pharmacological activities, which makes it obligatory to investigate these for each stereoisomer separately.
The formation of such a complex and tumor cytotoxic polyketide from a filamentous fungus thus presents another example for the importance and promising prospect of further investigating secondary metabolite production of endophytic fungi grown on solid rice medium under standard laboratory conditions.
The exact function of polyketides in fungi is not well known [Finefield *et al.*, 2012]. But given the abundance of polyketides yielded in countless studies from fungi, these secondary metabolites appear to be easily formed and have basic functionalities [Gaffoor *et al.*, 2005]. Along with the many biological activities described for polyketides, including antibiotic and cytotoxic activities [Agarwal and Moore, 2014], these natural products may be produced as a quick and initial chemical defense mechanism against changing or even offensive external conditions [Gaffoor *et al.*, 2005]. But even so, many polyketide gene clusters remain silent under standard laboratory conditions [Sørensen *et al*., 2012].

Concededly, along with all the described positive aspects of investigating endophytes under standard laboratory conditions, the diversity loss of secondary metabolite production, or the production of too little amounts to be detected [Chiang *et al.*, 2010], is a problem and along with this, the rediscovery rate of known natural products is quite high. This problematic of yielding more or less the same secondary metabolites under standard laboratory conditions has been thoroughly discussed [Scherlach and Hertweck, 2009; Brakhage and Schroeckh, 2011; Ramos and Said, 2011; Ochi and Hosaka, 2013; Hewage *et al.,* 2014; Reen *et al.*, 2015], and even in the experiments performed and presented in Chapter I of this dissertation the share of known compounds isolated was above 50%, which is why the investigation of further cultivation options, including the alteration of cultivation medium, were also explored in this dissertation.
Consequently, finding external triggers for the activation of so-called 'silent' biosynthetic pathways is thought to enhance the chemical diversity of natural products isolated from fungi [Scherlach and Hertweck, 2009; Brakhage, 2013; Rutledge and Challis, 2015]. The investigation of fungi brings along the advantage of easily influenceable cultivation conditions, combined with a relatively fast growth of organic tissue and the possibility to

produce large-scale cultures, additional to the ability of taking an impact on metabolic biosynthesis pathways of secondary metabolite production, by implying external cues, elaborated as the OSMAC approach (cf. Introduction) [Bode *et al.*, 2002; Williams, 2012]. Heeding the conclusive methods against this backdrop, Chapter II of this dissertation implements several experimental approaches for the activation of silent biosynthetic pathways for secondary metabolite production in endophytic fungi, by a variation of the culture medium for the endophyte *Fusarium tricinctum*.

This endophyte was isolated from the rhizome of *Aristolochia paucinervis*, which was previously collected from extremely hot and dry plains in Morocco [Wätjen *et al.*, 2009; Ola *et al.*, 2013]. *Aristolochia paucinervis* is distributed mainly in the western Mediterranean region, including the Iberian peninsula and Macronesia, and in traditional medicine the dried roots are used to treat various health issues, over vessel and intestinal disorders, to dermal mycosis [Rankou *et al.*, 2015]. Extracts from rhizomes and leaves from this plant have exhibited bactericidal and antifungal activities [Gadhi *et al.*, 2001a,b].
Fusarium tricinctum also occurs as a saprophyte and as a plant pathogen causing head blight [Sugita-Konishi and Kumagai, 2005]. It can furthermore produce mycotoxins, such as enniatins, which were on the pharmaceutical market as an OTC local antibiotic and are currently being investigated for their anti-cancer properties [Wätjen *et al.*, 2009; Dornetshuber-Fleiss *et al.*, 2015].
Cultivating *F. tricinctum* on solid rice medium with fruit juices instead of water (Chapter II, Section 1), led to the 80-fold enhanced production of novel fusarielin J (Figure 8), which exhibited a strong cytotoxic activity against the human cancer cell line A2780sens (IC_{50} 12.5 μM). Additionally, this change in the culture medium induced the production of the new fusarielins K and L (Figure 8), and the known metabolites fusarielin A and B, which revealed very weak cytotoxic activities in the assay against the above mentioned cell line.

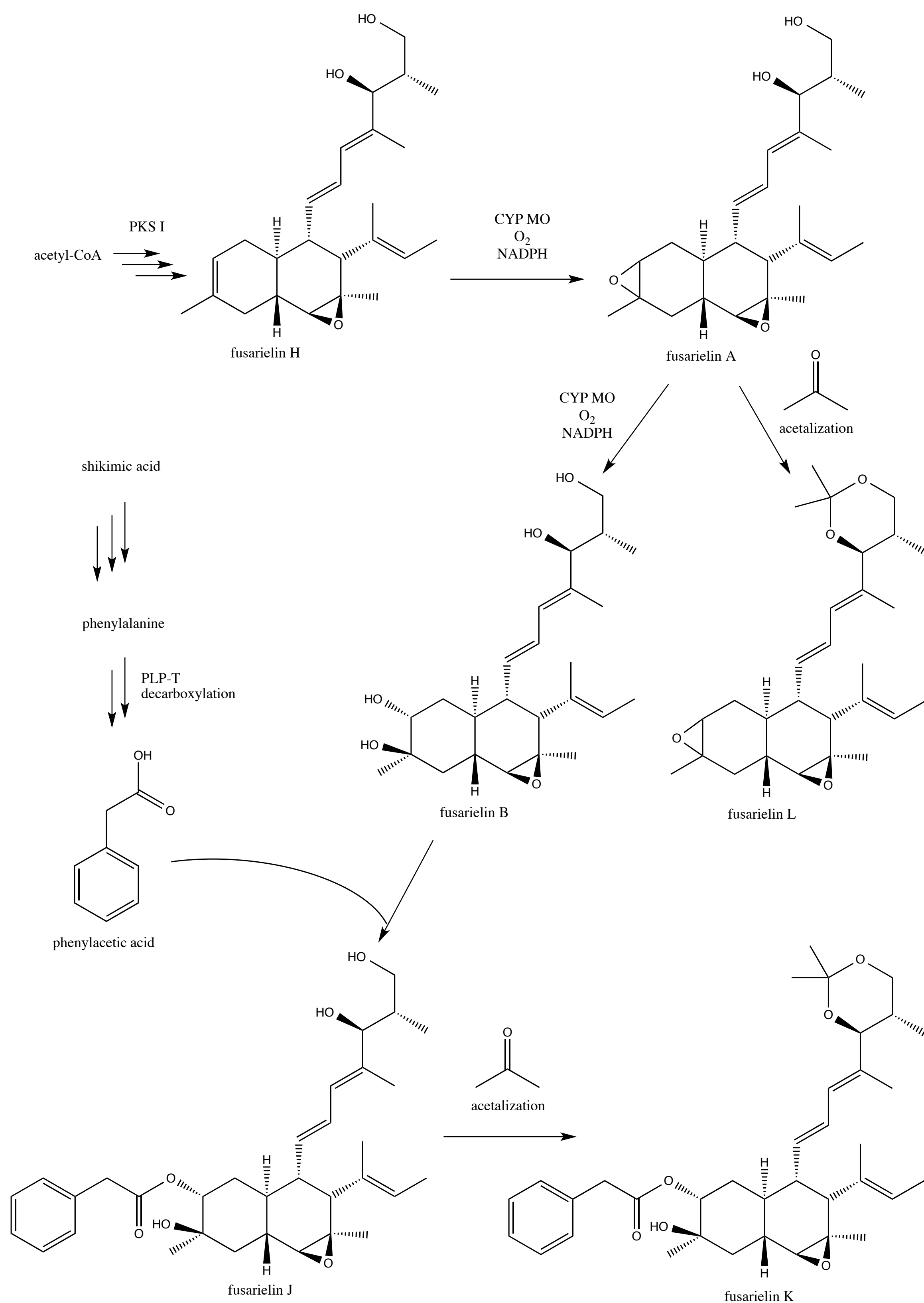


Figure 8. Proposed biosynthetic pathway of fusarielins A, B, J, K, and L. CYP-MO CYP dependent monooxygenase, PKS polyketide synthase, PLP-T PLP dependent transaminase.

Judging from the highly reduced fusarielin decalin moiety, one immediately is reminded of the fungal product lovastatin, when questioning about the biosynthetic origin of fusarielins. Lovastatin is produced by several species, *e.g. Aspergillus* and *Penicillium* species [Javed *et al.*, 2014], and its decalin backbone is derived from the acetate pathway and formed via a Diels-Alder cyclization, catalyzed by a polyketide synthase [Dewick, 2009]. A handful of years ago, the involvement of a fully reducing type I PKS in the biosynthesis of fusarielins was proven, of which the domains are also listed. Herein fusarielin H is suggested to be the end-product of the fusarielin biosynthesis in *F. graminearum* [Sørensen *et al*., 2012].

Fusarielin H differs from fusarielin A in positions C11 and C12, where it bears a double bond, which is probably epoxidized by a post-PKS CYP-450-dependent enzyme [Dewick, 2009], forming fusarielin A. The latter is then further converted to fusarielin L by acetalization with endogenous acetone. The biosynthetic nature of such reactions is not described, but they are assumed to occur in a similar mechanism observed in chemical organic acetalization reactions [Vollhardt, 2011], as acetone has been described to be a natural volatile product of fungi, including the *Fusarium* genus [Goepfert, 1941; Micheluz *et al.*, 2016]. Such acetone-derived acetals are rare, but also known from other fungal natural products [Shao *et al.*, 2011; Yang *et al.*, 2012].

Through further oxidation of the epoxide function in fusarielin A, a C11/C12 vicinal dihydroxy function, and thus fusarielin B is formed. The conjugation of fusarielin B at OH-11 with phenylacetic acid results in fusarielin J. Finally, fusarielin K is then formed to an acetonide, via acetalization of fusarielin J with acetone in positions 1-OH and 3-OH, similar to the above described fusarielin L.

Phenylacetic acid is a product of the shikimic acid pathway, emerged from the amino acid phenylalanine by a transaminase and subsequent decarboxylation of the phenylpyruvic acid intermediate (Figure 8). Phenylacetic acid conjugates are not so common in natural products, but one prominent example is benzylpenicillin. It is found more as a plant hormone [Muir, 1967; Wightman and Lighty, 1982], and as a secondary metabolite of fungi for itself, *e.g.* occurring in the genera *Fusarium*, *Rhizoctonia*, and *Cladosporium* [Ding *et al.*, 2008b; Kettout and Rahmania, 2010; Bartz, 2012; Marmann 2014].

Though acetonide functions are easily hydrolyzed, the co-occurrence of fusarielin L and the fact that sufficient amounts were isolated under aqueous conditions with elevated temperatures, indicates that these acetonide products are stable and remain stable in

the viable fungus. Thus fusarielin J can be determined as the precursor of fusarielin K (Figure 8).
Fusarielin B exhibited very weak cytotoxicity, which comes to show, that the additional phenylacetic acid moiety in fusarielin J is responsible for the strong increase in biological activity, compared to its precursor. The observed stark bioactive differences between fusarielin J and K originate only from the acetonide forming dimethylmethylene bridge of the latter (Figure 8).
Fusarielins themselves have been isolated from fungal species of the genera *Fusarium, Aspergillus*, and *Penicillium*.
Phenylacetic acid is produced by various fungi, *e.g. Rhizoctonia* species, and interestingly, it showed slight antibiotic effects [Marmann, 2014], growth-inhibiting effects towards tomato and beans seeds [Iacobellis and DeVay, 1987], and was able to completely inhibit the growth of the mouse lymphoma cell line L5178Y at a concentration of 10 µg/ml in previous investigations [Marmann, 2014], which again proves the significance of this phytohormone in the enhancement of the anti-tumor activity of fusarielins.
Conclusively it may be interesting to semi-synthetically produce natural product conjugates with phenylacetic acid and investigate the cytotoxic activity of these derivatives.
Furthermore, it may be of interest to investigate the inhibitory potential of fusarielins against the HMG-CoA reductase in the cholesterol biosynthesis, as they show a high structural similarity with statins. These drugs bind in the same binding sight as the natural substrate, HMG-CoA, and their pharmacophore is described as the dihydroxyheptoic acid and an apolar substituent [Roche, 2005]. The decalin moiety in fusarielins is located in a similar distance to the dihydroxy end of a heptane chain, and even though the fusarielins isolated in this dissertation bare no carboxylic nozzle, which is present in statins, others exist with such functional group, *e.g.* fusarielin I [Sørensen *et al.*, 2012; Chen *et al.*, 2015]. An additional conjugation of fusarielin I with phenylacetic acid may advantage the binding affinity to the HMG-CoA reductase, providing an enlarged apolar substituent with another carboxylic function. The latter may very well have a high chance of interacting with the same Arg^{568} enzymatic side chain, as does rosuvastatin, the newest statin developed for the treatment of hypercholesterolemia, with the additional indication of primary prevention of cardiovascular events [Istvan and Deisenhofer, 2001].

As it is not uncommon to find, that polyketides are induced and enhanced in OSMAC experiments with fungi [Hewage *et al.*, 2014; Yuan *et al.*, 2015; Yu *et al.*, 2015], this may also attribute to the above set hypothesis, that these secondary metabolites may constitute to an initial metabolic reaction to nutritional changes of the fungus being investigated.
The fact that fusarielins J and K are conjugation products of phenylacetic acid, is a hint at an anabolic cultivation condition, that is further corroborated by an increase of total extract yield from the regarding juice-cultivars, compared to the aqueous ones. Furthermore, other *Fusarium* species, that cause wilting diseases in date palms, have been found to produce phenylacetic acid [Kettout and Rahmania, 2010], which indeed has exhibited phytotoxic properties [Morooka *et al.*, 1980]. Therefore, the function of an up-regulated phenylacetic acid production may exhibit a reaction towards the present fruit juices.
Though this is the first report of cultivating a fungal strain on fruit juice supplemented solid rice medium, the described results are explainable with the OSMAC term, and can be traced back to an activation of secondary metabolite production, effected by changes in the cultivation medium [Bode *et al.*, 2002]. Studies have shown, that even the slightest medial change, such as using distilled instead of tap water, can effect the metabolic profile of fungi and even induce the production of new and bioactive metabolites [Paranagama *et al.*, 2007]. Pinpointing the observed effects on one single ingredient, or even a handful of factors in the present juice experiment is tricky: though several physical components of the media and the juices were compared, *e.g.* the osmolarity, pH, and sugar content, none appeared coherent with the observed enhancement and induction of fusarielins. Furthermore, as this juice experiment was performed in the same way using white beans, instead of rice as solid medium, but showed no effect whatsoever on the production of fusarielins, the combination of rice and fruit juice may be vital herefore.

Even though polyketides belong to one of the three main classes of fungal secondary metabolites [Hoffmeister and Keller, 2007], the predominant production of polyketide metabolites (Chapter I and II-1) suggests that 'laboratory fungi' are not highly stimulated to engage greatly in the production of any other group of secondary metabolites [Hewage *et al.,* 2014].

But, as *F. tricinctum* is also known as a reliable producer of cyclohexadepsipeptides, a second OSMAC investigation was performed in the cause of this dissertation (Chapter II, Section 2), analyzing the effect of several different solid and liquid media on its metabolic profile, with special regard to enniatins A, A1, B, and B1, as these are constitutively produced by this endophyte. Though enniatins are mycotoxins and can have negative health effects, they have also shown to be promising natural products for the medicinal application in humans, with a mixture of enniatins A, B, and C marketed as a local antibiotic against nasopharyngeal infections until recently, when too many allergic incidences led to the retraction of fusafungin, respectively. But with 29 different enniatins discovered so far [Sy-Cordero *et al.*, 2012], they exhibit various biological activities, so that enniatin A1, B, and B1 in particular are currently being investigated for their anti-cancer potential [Wätjen *et al.*, 2009; Dornetshuber-Fleiss 2015].
Enniatins are produced by various fungi, including *Verticillium* and *Halosarpheia* species, but are mainly distributed in the *Fusarium* genus [Sy-Cordero *et al.*, 2012]. The biosynthesis is well understood and is performed by a non-ribosomal peptide synthase (NRPS), in the case of enniatins A, A1, B, and B1 connecting D-2-hydroxyisovaleric acids with *N*-methyl-L-amino acids, including valine, isoleucine, and leucine [Sy-Cordero *et al.*, 2012]. This results in the stereospecific biosynthesis of highly complex cyclodepsipeptides, usually bearing at least six stereo centers. Conclusively, the chemical total synthesis of these natural products is quite difficult and has so far only succeeded for enniatin B [Hu *et al.*, 2012]. Therefore, the biotechnological production of enniatins by fermentation of filamentous fungi is still direly needed, to continue the biological investigation of these potent metabolites.
Submitting *F. tricinctum* to different solid media, including rice, soybeans, radish potato, wheat, corn, and beans medium, and to liquid Wickerham medium with or without the addition of either peptone or an amino acid mixture containing L-valine, L-leucine, and L-isoleucine, each in different concentrations, resulted in the highest enniatin accumulation on solid beans medium [Wang *et al.*, 2013].
This experiment represents a further advantage of cultivating one fungal strain on various media: though in this example no new secondary metabolites were induced, finding a cultivation medium, that significantly enhances enniatin production is valuable to produce larger quantities of these natural products, as they are currently being investigated for their antineoplastic properties [Wätjen *et al.*, 2009; Dornetshuber-Fleiss *et al.*, 2015]. Though in this study media were also used with addition of peptone or

amino acids, apparently the protein composition of beans was most favorable for the proliferation of the endophyte and the enhancement of enniatin production, which comes to show that sometimes the easiest medium variations may give the best results. Furthermore, *F. tricinctum* proves to have a very reliable production of the discussed cyclodepsipeptides, hinting at stable NRPS, which in turn are translated from probably equally stable gene clusters. This trait makes *F. tricinctum* a promising candidate for NRPS gene modulative research, which is currently being performed with other fungal species, by genetically or chemically toying with gene regulators or with the chromatin structures [Soukup *et al.*, 2016], but also specifically with other enniatin producing species, by reprogramming the NRPS through hybridization of two different enzymes to fuse a new and synthetic NRPS [Zobel *et al.*, 2016]. Apparently, enniatin producing NRPS are particularly promising in these approaches for natural product induction or design, because they are well controllable, have a high substrate tolerance and are able to biosynthesize bioactive secondary metabolites with complex molecular structures [Zobel *et al.*, 2016].

Conclusion and Prospect

The research results of this dissertation show, that it is still rewarding to investigate Mangrove-derived endophytic fungi on solid rice medium, as to say under so-called standard laboratory conditions, to yield new and bioactive natural products, that may serve as future drug leads. Furthermore, the highly influential effect of the culture medium composition on the metabolic profile of endophytic fungi towards the production of new and chemically divers secondary metabolites, in other words implying an OSMAC method upon these microorganisms, was likewise demonstrated in this thesis.

As the medium composition is variable in vast different ways, and the resulting effects on the fungal secondary metabolite production are multifold, further experiments in this realm of natural product research are promising and highly advisable.

Against the backdrop of the development of a clinical phase III anti-cancer drug (plinabulin), developed from an endophytic fungal natural product, surely the research and results at hand demonstrate the right path to succeeding in this ongoing quest for new natural product derived medicinal agents.

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Abbreviations

A2780	human ovarian cancer cell line
ACP	acyl carrier protein
Arg	arginine
AT	acyltransferase
Cal27	human head-neck cancer cell line
CD	circular dichroism
CisR	cisplatin resistant
CYP	cytochrome P450 cofactor
DH	dehydrogenase
ECD	electronic circular dichroism
ER	enoyl-reductase
FDA	(US) food and drug administration
HCC38	human breast cancer cell line
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IC_{50}	half maximal inhibitory concentration
KR	keto-reductase
KS	β-ketoacyl-synthase
Kyse510	human esophageal squamous cancer cell line
L	lactonization
L5178Y	mouse lymphoma cell line
MDA-MB-231	human breast cancer cell line
Me	methyl group (CH_3)
MO	monooxigenase
MS	mass spectrometry
MT	methyl-transferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
µM	micromolar (10^{-6}mol/L)
NADPH	nicotinamide adenine dinucleotide phosphates
NMR	nuclear magnetic resonance spectroscopy
NRPS	non-ribosomal peptide synthases
OR	oxidoreductase
ORT	optical rotation
OSMAC	one strain – many compounds
OTC	over-the-counter drug
PKS	polyketide synthases
PLP	pyridoxal 5'-phosphate
PLP-T	PLP dependent transaminase
PT	prenyl-transferase
SAHA	suberanilohydroxamic acid

Research contribution

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Declaration of Academic Honesty (English)

I produced the present dissertation autonomously and without impermissible help. The author herself performed most of the methods necessary for the production of this work. The following experimental methods were performed in other institutions:

HR-ESI mass spectrometry
The measurements were performed by Dr. Peter Tommes and Mr. Ralf Bürgel in the Division of Mass Spectrometry in the Scientific Institute of Chemistry of the Heinrich-Heine University, Düsseldorf, an a UHR-QTQF maXis G4 machine from Bruker Daltonics.

NMR spectroscopy
All measurements were performed in the NMR division of the Scientific Institute of Chemistry at the Heinrich-Heine University, Düsseldorf. Measurements on the machines Bruker Avance III-300 and -600 wee performed by Mrs. Beuer and those on Bruker Avance III-500 by Mrs. Rau.

CD spectroscopy
Measurements were performed by Prof. Dr. Tibor Kurtán in the Department of Organic Chemistry of the University Debrecen, Hungary, on a Jasco-810 Spectropolarimeter.

Antimicrobial assays
The antibiotic assays were performed in the group of Prof. Dr. Rainer Kalscheuer in the Institute of Pharmaceutical Biology and Biotechnology of the Heinrich-Heine University, Düsseldorf.

Cytotoxicity assays
The investigation of cytotoxic activity against human carcinoma cell lines was performed in the group of Prof. Dr. Matthias U. Kassack in the Institute for Pharmaceutical and Medicinal Chemistry at the Heinrich-Heine University, Düsseldorf. Assays with murine cancer cell lines were performed in the group of Prof. Dr. Werner E. G. Müller in the Institute of Physiological Chemistry in the Johannes Gutenberg-University, Mainz.

The authorization to release the publications within this dissertation was obtained from the publishers.

I hereby declare, on my word of honor, that I autonomously produced the dissertation at hand, which is entitled

Bioactive fungal natural products from extremophilic endophytes

No further references or resources were used, apart from those declared. This dissertation was neither in the same, nor in a modified form presented in another examination procedure. Furthermore I declare that I have hitherto not undertaken an unsuccessful doctorate procedure.

Düsseldorf,

Catalina Francis Pérez Hemphill

Declaration of Academic Honesty (German)

Die vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die meisten der für die Anfertigung dieser Arbeit erforderlichen Methoden wurden vom Autor selbstständig durchgeführt. Folgende Methoden wurden in anderen Institutionen durchgeführt:

HR-ESI-Massenspektrometrie
Die Messungen wurden durch Herrn Dr. Peter Tommes und Herrn Ralf Bürgel in der Abteilung der Massenspektrometrie der Wissenschaftlichen Einrichtung Chemie in der Heinrich-Heine Universität Düsseldorf an einem UHR-QTQF maXis G4 Gerät von Bruker Daltonics durchgeführt.

NMR-Spektroskopie
Die Messungen wurden alle in der NMR-Abteilung der Wissenschaftlichen Einrichtung Chemie, an der Heinrich-Heine Universität Düsseldorf durchgeführt. Messungen an den Geräten Bruker Avance III-600 und -300 wurden durch Frau Beuer ausgeführt. Messungen an dem Gerät Bruker Avance III-500 wurden durch Frau Rau ausgeführt.

CD-Spektroskopie
Die Messungen wurden an einem Jasco-810 Spektropolarimeter durch Prof. Dr. Tibor Kurtán der Abteilung für Organische Chemie der Universität Debrecen in Ungarn durchgeführt.

Antimikrobielle Assays
Die Untersuchungen auf antibakterielle und antimykotische Aktivitäten wurden durch den Arbeitskreis (AK) Prof. Dr. Rainer Kalscheuer am Institut für Pharmazeutische Biologie und Biotechnology der Heinrich-Heine Universität Düsseldorf vorgenommen.

Zytotoxizität
Die Untersuchungen auf zytotoxische Aktivitäten gegenüber humanen Krebs-Zelllinien wurden durch den AK von Prof. Dr. Matthias U. Kassack am Institut für Pharmazeutische Chemie der Heinrich-Heine Universität Düsseldorf und solche gegenüber murinen

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Die Genehmigungen zur Veröffentlichung der Publikationen im Rahmen dieser Dissertation wurden von den entsprechenden Verlagen eingeholt.

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel

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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. Außerdem erkläre ich, dass ich bisher keine erfolglosen Promotionsverfahren unternommen habe.

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