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Detection of pharmacologically active natural products using ecology. Selected examples from Indopacific marine invertebrates and sponge-derived fungi*,†

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Abstract: This review article presents our group's recent research findings with regard to bioactive natural products from marine sponges and tunicates, as well as from sponge-derived fungi. The organisms discussed originate in the Indopacific region, which has an exceptionally rich marine biodiversity. Major topics that are covered in our review include the chemical ecology of sponges, focusing on defense against fishes, as well as the isolation and identification of new bioactive constituents from sponges and tunicates. Sponge-derived fungi are introduced as an emerging source for new bioactive metabolites, reflecting the currently growing interest in natural products from marine microorganisms.

INTRODUCTION

Nature has continuously provided mankind with a broad and structurally diverse array of pharmacologically active compounds that have proved to be indispensable for the cure of deadly diseases or as lead structures for novel pharmaceuticals [1]. At least until the arrival of antibiotics such as penicillin, streptomycin, and others, higher terrestrial plants had certainly the strongest impact on drug discovery from natural sources. Oddly enough, even though the oceans cover over 70 % of the earth's surface, they have only comparatively recently attracted the serious attention of drug prospectors, which is in sharp contrast to the important and long-standing impact of the sea on human nutrition. Marine natural products chemistry began to focus on the discovery of new potential drugs in 1951 when Bergmann and Feeney [2] reported on the isolation of the unusual nucleosides spongouridin and spongothymidin from the sponge *Cryptotethya crypta*, which served as lead structures for antiviral drugs such as Ara-A. More than a decade later, the discovery of prostaglandins in the Caribbean gorgonian *Plexaura homomalla*

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†Dedicated to the memory of our late coworker Ms. Cho Cho Minh from Myanmar.

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[3] right at a time when prostaglandins had just been discovered as important mediators in the human body, served as a further stimulus in the search for new drugs from the sea.

Since these early times, well over 10 000 different natural products have been isolated from marine sources ([4] and preceding reports), reflecting both advances in spectroscopic structure elucidation methods, as well as in diving technology, which made marine organisms more easy to access. In contrast to the terrestrial environment where plants are the most prolific sources of natural products, in the sea this leading position is taken by invertebrates such as sponges, tunicates, bryozoans, or mollusks, with sponges being the most prolific sources of natural products ([4] and preceding reports). From the 13 or so marine natural products (or analogs derived from them) that are currently in clinical trials as candidates for new drugs, 12 are derived from invertebrates [5]. One compound (squalamine lactate) is isolated from sharks, whereas none so far is derived from algae or from marine microorganisms [5]. The two currently most promising marine natural products ziconotide and ecteinascidin 743 (ET 743) are derived from the cone shell *Conus magus* or from the tunicate *Ecteinascidia turbinata*, respectively [5]. While ziconotide is a new analgesic mainly helpful to alleviate pain associated with malignant diseases (cancer and AIDS) and against nonmalignant neuropathic pain [6], ET 743 is a new anticancer drug with a broad-spectrum antitumor activity, especially against solid tumors such as sarcomas and breast cancer [7].

The frequent occurrence of biologically active natural products in sessile or slow-moving marine invertebrates such as sponges, tunicates, or mollusks that are furthermore morphologically undefended, reflects an ecological adaptation that has been shaped during evolution, allowing these delicate organisms to survive and flourish in spite of usually heavy predation by fish and other predators [8–11]. Owing to the frequent parallelism between ecological function and pharmacological activity of natural products as exemplified by the success story of ziconotide, which is used as a hunting venom by *Conus* snails, ecological observations may be also valuable for drug screening in providing a rationale for marine bioprospecting that goes beyond the undirected "shot gun" approach still widely practiced.

Even though the sea has yielded numerous promising drug candidates, the development of real drugs from this source has been extraordinarily slow mainly due to the pressing supply problem. The natural concentrations of many pharmacologically active compounds from marine organisms are often minute and sometimes account for less than 10–6 % of the respective wet weight. For example, in order to obtain approximately 1 g of the promising anticancer drug ET 743, close to 1 metric tonne (wet weight) of its natural source *E. turbinata* has to be harvested and extracted [12].

Chemical synthesis is often not an economically feasible alternative to harvesting from wild stocks, as the most interesting compounds, such as ET 743, are in many cases structurally highly complex featuring numerous chiral centers. Mariculture of sponges or tunicates with the aim of securing a sustainable supply of marine natural products is currently being developed [12,13]. Even though both the tunicate *E. turbinata* (the natural source of ET 743), as well as the bryozoan *Bugula neritina* (the source of the well-known bryostatins) are already accessible through mariculture [12], the available biomass yields are still far below those required if either compound was to be introduced into the drug market.

A further biotechnological approach aiming at the solution of the pressing supply problem of marine-derived drugs addresses the possible involvement of associated microorganisms ("endosymbionts") in the biosynthesis of natural products from marine invertebrates [5]. Most marine invertebrates analyzed so far have been shown to harbor microorganisms such as cyanobacteria, bacteria, unicellular algae, and fungi. These microorganisms (sometimes called endosymbionts) may reside in the extra- and intracellular space of their hosts [14–16]. Sometimes they account for copious amounts of the biomass of the respective invertebrate, as is the case of the Mediterranean sponge *Aplysina aerophoba* where up to 40 % of the total biomass may be made up of microorganisms. Based on obvious structural similarities between compounds isolated, for example, from sponges and compounds known from bacteria, it has often been speculated that microorganisms that either reside in or on invertebrates or that are taken up through filter-feeding may be the true producers of natural products recovered from their hosts [5].

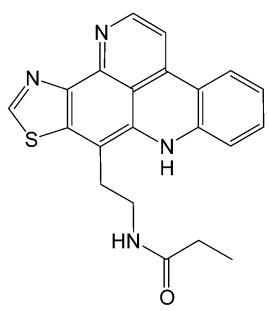
In most cases, these speculations are drawn from the fact that numerous compounds isolated, for example, from sponges, tunicates, and other marine invertebrates show striking structural similarities or are even identical to natural products of microbial origin. For example, a close inspection of the structural features of the promising new anticancer drug ET 743, derived from the tunicate *E. turbinata*, reveals striking similarities to safracin B, a metabolite of *Pseudomonas fluorescens* [17]. In fact, the bacterial metabolite is now being used as a template for the partial synthesis of ET 743. Other examples corroborate the striking structural similarities of compounds from marine invertebrates and from bacteria and hence call for detailed investigations regarding the role of microbial symbionts in sponges, tunicates, and other marine organisms rich in bioactive natural products.

This paper highlights some of our recent research regarding the discovery of new bioactive metabolites from marine invertebrates, with emphasis on sponges as the most prolific producers of natural products in the sea. The examples shown originate in the Indopacific region, which has an exceptionally high biodiversity of sponges and other marine invertebrates, and cover ecological aspects of sponge-derived natural products, as well as the discovery of new bioactive marine natural products from sponges and tunicates. In addition, we provide an overview of our ongoing research on the chemistry of sponge-derived fungi, which, owing to their structurally unique metabolites, have recently attracted considerable interest of marine natural product chemists.

RESULTS AND DISCUSSION

Defensive pyridoacridine alkaloids from the tropical sponge *Oceanapia* sp.

Sponges of the genus *Oceanapia* occur frequently in the Indopacific as well as Pacific Oceans. The hitherto undescribed and conspicuously red-colored Micronesian sponge *Oceanapia* sp. was found in shallow sandy areas around the Micronesian island, Truk, at a depth of only a few meters [10]. In spite of its exposed growth habit (at least with reference to the parts protruding into the water column) and its conspicuous red color, which makes it easily detectable, the sponge appeared to be largely unharmed by potential predators such as fish, suggesting the presence of defensive sponge metabolites. This hypothesis was experimentally corroborated by field feeding experiments with co-occurring natural fish communities. A crude solvent extract derived from the fistulae of *Oceanapia* sp. was incorporated at its physiological concentration into artificial fish food. Treated vs. nontreated cubes of diet were subsequently exposed to the natural fish community. Whereas the fish readily consumed the control cubes, the treated cubes were largely left untouched. During bioassay-guided fractionations, pyridoacridine alkaloids of the kuanoniamine type (e.g., **1**), which are also responsible for the conspicuous red color of the sponges, proved to be the main deterrent constituents of *Oceanapia* sp. [10]. The alkaloids clearly deterred feeding by fish in field feeding experiments at their respective physiological concentrations. Laboratory experiments with fish that are known as sponge feeders such as *Pomacanthus imperator* [10] likewise demonstrated the deterrent activity of kuanoniamines. Sponge specialists are generally



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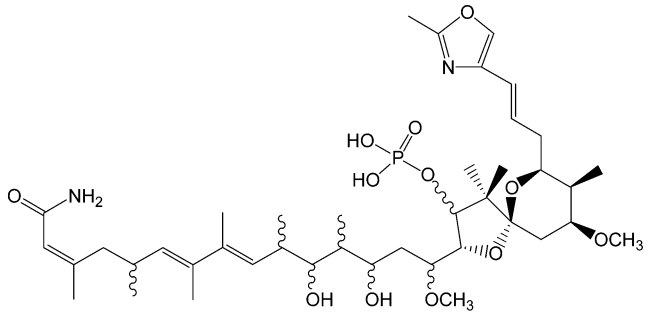
expected to be more tolerant toward secondary sponge metabolites than other fish species with a broader dietary range.

Oceanapia sp. has a peculiar growth form. Part of the sponge, the base, is buried in sand and therefore hidden from fish or other potential predators. The fistulae, on the other hand, protrude into the seawater and are hence vulnerable to attack. The same is true for a round-shaped structure, the so-called capitum, which is loosely attached to the fistulae and is believed to be involved in asexual propagation of the sponge. The same sponge individual, therefore, has exposed as well as cryptic parts that can be expected to be at a very different risk of predation by fish. The "optimal defense theory", as well as the "plant apparency model" (both of them originally developed for higher terrestrial plants [18–21]), predict a positive correlation between chemical defense and the risk of being discovered by herbivores (plant apparency model) or suggest that the most valuable plant parts (e.g., those needed for reproduction) should be preferentially defended (optimal defense theory). The distribution of defensive alkaloids in *Oceanapia* sp. follows both the plant apparency model, as well as the optimal defense theory. There is a clear pattern of alkaloid distribution in exposed vs. unexposed parts of *Oceanapia* sp. The highest alkaloid concentrations (up to 5 % of the dry weight of the sponges) were found in the fistulae and especially in the asexual propagation units (capitum). Significantly lower alkaloid concentrations (reaching only 0.8 % of the dry weight) were in comparison detected in the base of the sponges that is buried in sand and thus hidden from potential predators [10].

New bioactive natural products from the sponge *Theonella swinhoei* and the tunicate *Eudistoma toealensis*

The Indonesian archipelago, including the province Irian Jaya as well as the independent state Papua New Guinea, consists of more than 18 000 different islands with a total shore line exceeding 80 000 km and may be considered a hot spot of marine biodiversity. This holds especially true for marine invertebrates such as sponges that are among the most conspicuous inhabitants of the coral reefs that can still be found largely unspoiled, especially in the less populated eastern parts of the archipelago.

The specimen of the sponge *Theonella swinhoei* that was analyzed in this study had been collected near the coast of Karkar Island, Papua New Guinea. The genus *Theonella*, which belongs to the order Lithistidae, has been well investigated in the past owing to its structurally unique metabolites that are often characterized by strong biological activities. Known secondary metabolites from these sponges, for example, include the macrocyclic swinholides [22], as well as cyclic peptides and peptide lactones [23,24]. The methanolic extract of *T. swinhoei* collected at Karkar Island aroused our interest owing to its exceptionally strong insecticidal activity. When incorporated into artificial diet and offered to neonate larvae of the polyphagous pest insect *Spodoptera littoralis*, complete mortality of the larvae was observed at an extract concentration equaling 2000 ppm. High-performance liquid chromatography (HPLC) analysis of the extract revealed only one major peak, which subsequently proved to be the active principle that we called swinhoeiamide A, **2** [25]. Swinhoeiamide A is a new natural product structurally related to the calyculins, which were described for the first time from the Japanese Lithistid sponge *Discodermia calyx* [26]. After the initial discovery of calyculin derivatives, these compounds were also described from the Epipolasid sponge *Lammelomorpha strongylata*, which, in addition to calyculins, yielded further structurally related compounds called calyculinamides [27]. Adding to the patchy reports on the occurrence of calyculin derivatives in obviously unrelated marine sponges was the discovery of calyculinamide congeners called clavosines, in the Astrophorid sponge *Myriastra clavosa* [28]. Apparently, the distribution of calyculin congeners in marine sponges follows no clear systematic pattern. Even though the secondary metabolites from *T. swinhoei* from a wide range of localities have been extensively studied in the past with regard to natural products [29], our study is the first to report on calyculin derivatives in this sponge. This raises questions with regard to the actual source of these structurally unusual secondary metabolites in our specimen of *T. swinhoei*.



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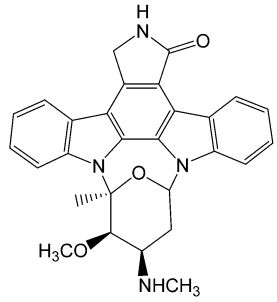
The structure of swinhoeamide A was elucidated on the basis of extensive NMR and mass spectrometric data [25]. Swinhoeiamide A (**2**) was shown to differ from the known calyculins, calyculinamides, or clavosines by two novel structural features: The complex side-chain attached to C29 in the oxazole ring system, which is present in the latter derivatives, is replaced by a methyl substituent in the structure of swinhoeiamide A, and the double bond usually found between C4 and C5 is hydrogenated. Additionally, C21 is methoxylated with the oxygen substituent being in an equatorial orientation as found in the clavosine series [28].

Swinhoeiamide A showed strong biological activities in several assays systems. It showed, for example, pronounced insecticidal activity against larvae of *S. littoralis* [25]. When incorporated into an artificial diet at a range of concentrations and offered to neonate larvae in a chronic feeding bioassay, the LC_{50} of swinhoeiamide A was 2.98 ppm and thus comparable to that of the well-known natural plant-derived insecticide azadirachtin. In addition to its insecticidal activity, swinhoeiamide A exhibited fungicidal activity against the human pathogenic fungi *Candida albicans* and *Aspergillus fumigatus*. The minimum inhibitory concentration (MIC) values of swinhoeiamide A against the two fungi amounted to 1.2 for the former and 1.0 μg/ml for the latter fungus [25]. The most interesting activity of swinhoeiamide A, however, was detected when tested against human cancer cell lines in vitro [30]. The compound exhibited a dose-dependent cytotoxicity against more than 50 cell lines obtained from different species (human, monkey, bovine, mouse, and insect) with IC_{50} values ranging from 20–90 ng/ml (0.025–0.113 nM) [30]. Interestingly, nondividing peripheral blood cells proved to be resistant to the cytotoxic action of swinhoeiamide A, suggesting that the compound acts preferentially on proliferating cells rather than on quiescent cells, which makes it an interesting candidate for further studies.

Another marine organism with interesting biological activities was the delicate tunicate *Eudistoma toealensis*, which was observed growing on submersed mangrove roots in a mangrove area at Truk Lagoon (Micronesia). In spite of the obvious lack of morphological defense structures, the tunicate was apparently avoided by the surrounding fish community. The only animal observed while feeding on the tunicate was the flatworm *Pseudoceros* sp. [31]. Like the tunicate, the flatworms were not eaten by fish, suggesting that both invertebrates were probably chemically defended from predators. This hypothesis was experimentally tested by incorporating crude solvent extracts of the respective invertebrates at natural concentrations into artificial fish food and offering the treated food cubes along with untreated control food to the natural fish community co-occurring with the tunicate. Whereas the fish readily consumed the control food, the food particles treated with the extract of *E. toealensis* or *Pseudoceros* sp. were barely touched [32].

Subsequent fractionation of the tunicate and flatworm extract yielded a series of staurosporin (**3**) derivatives, including several new compounds [31,33]. Staurosporine was initially discovered by Omura and coworkers (1977) in actinomycetes such as *Saccharothrix aerocolonigenes* subsp. *staurosporea* [34]. Subsequently, staurosporine derivatives were also isolated from several taxonomically unrelated marine invertebrates such as ascidians and a prosobranch mollusk [35–37], which, like the case of calyculin derivatives from sponges, raises questions with regard to the actual origin of the respective natu-



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ral products. The staurosporines that were found in the tunicate were also detected in the associated flatworm, thereby corroborating the initial observation that *Pseudoceros* sp. had been feeding on *E. toealensis*. The total staurosporine concentration in the flatworms, however, was not only several fold higher than that found in the tunicate, but the patterns of staurosporine derivatives in *E. toealensis* and *Pseudoceros* sp. were moreover found to differ to a certain degree, indicating preferential uptake of certain derivatives from the tunicate or biotransformation of the sequestered metabolites in the flatworms [31,32]. When the staurosporine-containing fractions obtained from the crude extracts of *E. toealensis* and *Pseudoceros* sp. were again incorporated into an artificial diet at their respective natural concentrations and subjected to the above-mentioned fish feeding experiment, the staurosporine fraction obtained from the flatworms deterred feeding by fish, whereas this was not the case for the staurosporine fraction from the tunicate [32]. Whereas these diverging results can probably be explained by the initially observed differences in staurosporine concentrations in tunicate and flatworms, the fact nevertheless remains that the crude extract of *E. toealensis* had been strongly deterrent [31], whereas the partially purified staurosporine fraction of this extract was not. Therefore, unidentified natural products with fish deterrency probably reside in the tunicate extract. These latter compounds still await their isolation and identification.

The staurosporines from *E. toealensis* and *Pseudoceros* sp. provide a vivid example for the repeatedly observed parallelism between ecological function and pharmacological activity of marine natural products as staurosporine and several of its congeners are well known for their strong inhibitory activity against protein kinases such as protein kinase C [38]. Staurosporine derivatives are of considerable interest for anticancer chemotherapy, and one of them, 7-hydroxystauroporine (UCN-01), is currently undergoing clinical studies as a potential new anticancer drug [39]. Of the staurosporine derivatives isolated from *E. toealensis* and *Pseudoceros* sp. 3-hydroxystaurosporine exhibited exceptionally strong activity against monocytic leukemia cells (MONO-MAC 6) with an IC_{50} of 13.3 ng/ml (27.6 nM) [40].

Bioactive natural products from sponge-derived fungi *Aspergillus versicolor* and *Penicillium* cf. *montanense*

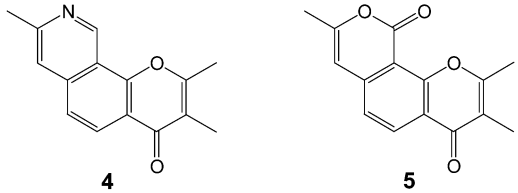
As already outlined in the introduction, current marine natural products research is characterized by an increased awareness of the biosynthetic potential of marine microorganisms [41,42]. This shift of interest after decades of research focusing mainly on invertebrates and algae has surfaced mainly within the last 10 years and with a multitude of new compounds discovered has considerably broadened our knowledge of the chemical diversity present in the sea [43]. One reason for the current interest in metabolites from marine microorganisms lies clearly in the already outlined chemical similarity between natural products from invertebrates and those from bacteria [5]. The initial hope of gaining access to a sustainable production of rare bioactive metabolites, for example, from sponges by isolating and culturing microorganisms that inhabit the respective invertebrates, however, soon proved to be too

simplistic. It is now known that only a tiny fraction of the real microbial diversity found, for example, in sponges can be cultured using currently available media and fermentation technologies. If natural products from sponges and other invertebrates are indeed biosynthesized by associated microorganisms, as suggested by the numerous striking chemical similarities observed, it is unlikely that these respective microbes can be grown employing standard media, as the latter can be expected to differ widely from the physiological conditions present in any marine invertebrate. With the availability of gene technology, a transfer of natural product encoding genes (e.g., genes encoding for polyketide synthases), for example, from highly specialized microbial sponge symbionts into manageable systems such as *E. coli* and others, offers perhaps new opportunities to trace the origin of natural products found in marine invertebrates and to establish a sustainable production of these compounds in the future.

Another more pragmatic reason for the new interest of marine natural products researchers in microbial metabolites is probably due to the fact that after three decades of studying invertebrates and algae it is now becoming increasingly difficult to isolate new compounds from these respective sources. Marine bacteria, cyanobacteria, and fungi, on the other hand, are often prolific sources of hitherto undescribed compounds [43–45] and hence offer new opportunities for chemists and drug prospectors alike. This second approach has been the guideline of our own research on marine microbial metabolites, which focuses on sponge-derived fungi as demonstrated in this paper for two fungi isolated from the tropical sponge *Xestospongia exigua* collected at Bali, Indonesia.

One of the fungi isolated from the inner core of *X. exigua* proved to be *Aspergillus versicolor*, which is well known from the terrestrial environment. In this context, it is perhaps of interest to briefly comment on the criteria that apply for the definition of "marine fungi" within the framework of marine natural products research. There is a continuous debate about the definitions a sea-derived fungus has to meet in order to truly qualify as a "marine" species. Obligate marine fungi are usually considered to include those species that grow and sporulate exclusively under saltwater conditions [46]. However, when looking at lists of fungi recently isolated from various marine sources (containing, e.g., *Aspergillus* and *Penicillium* spp.) [43], the question arises whether all of these taxa can truly be defined as "marine fungi" or whether their presence in the sea is merely accidental, resulting, for example, from wind or rain wash. The latter may carry fungal spores into the sea where they can remain dormant over some time and are reawakened by marine natural products chemists who isolate fungal cultures from the sea. Whereas this discussion is certainly interesting from an ecological point of view, the more pragmatic approach held by many marine natural products chemists, including ourselves, is certainly also valid. Any fungus that can be isolated from a marine source, that can furthermore be cultivated using seawater-based media, and that, last but not least, yield interesting new secondary metabolites is considered worthy of inclusion in a marine-oriented drug discovery program regardless of its true origin. This approach has in the past certainly been met with success regarding the isolation of numerous new bioactive fungal constituents, whereas the actual origin of the analyzed fungi remains debatable.

Even though fungi of the genus *Aspergillus*, including *A. versicolor*, have been the subject of numerous chemical investigations in the past (for a compilation of references, see [47]), the strain of *A. versicolor* isolated from the sponge *X. exigua* proved to be highly interesting with regard to its natural product profile. Following chromatographic workup of a crude EtOAc extract obtained from the mycelia and culture broth of *A. versicolor*, seven compounds (e.g., **4** and **5**) were isolated [47]. The fungal metabolites identified turned out to be novel chromones containing an additional annealed heterocyclic ring system, which was either a pyridine ring or a dihydropyran ring. These new metabolites of *A. versicolor* are structurally unusual in several respects. Firstly, 2,3-methylated chromones are only rarely encountered as natural products. Known examples include chaetomin D, a bis(naphtho-γ-pyrone) derivative from the fungus *Chaetomium gracile* [48] or 6-acetyl-7-hydroxy-2,3-dimethylchromone from the plant *Graphis scripta* [49]. Moreover, compound **4** features an additional annealed pyridine ring that is replaced by a dihydropyran ring in the remaining compounds, yielding an angular tricyclic chromone ring system, which, to the best of our knowledge, is unprecedented in nature, proving that chemical analysis even of taxonomically well-known fungi such as *A. versicolor* following isolation



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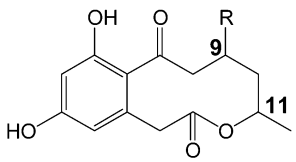
from a marine sponge and subsequent cultivation in seawater-based media can yield new and unexpected metabolites. Analysis of the biological activity of the new metabolites from *A. versicolor* is presently being carried out.

A further fungal strain isolated from *X. exigua* proved to be *Penicillium* cf. *montanense*, which is likewise known from the terrestrial habitat. Like *A. versicolor* that was isolated from the same sponge, *P*. cf. *montanense* yielded new metabolites hitherto unreported from nature [50]. Using HPLC-electrospray ionization (ESI)-mass spectrometry (MS), the pseudomolecular ions of the major secondary constituents **6–8** were identified, which, together with on-line obtained NMR data (using HPLC-NMR), indicated the presence of a macrocyclic, as well as a phenolic ring system in the analyzed compounds and allowed preliminary assignment of their gross structures. The proposed structures could be unequivocally corroborated following isolation of the compounds from the combined EtOAc extracts of mycelia and liquid culture broth. Using one- and two-dimensional NMR techniques, compounds **6–8** were identified as new 10-membered macrolides (decalactones) containing a fused 1,3-dihydrobenzene ring [50]. They differed from each other by the presence or absence and by the stereochemical orientation of a hydroxyl substituent at C9 of the macrocyclic ring system. In spite of detailed chirooptical investigations by means of quantum mechanical calculations that had in the past proved to be extremely helpful for the assignment of the absolute configuration of natural products likewise isolated from sponge-derived fungi [51], it was impossible to assign the absolute configuration at the stereocenter(s) of the new compounds. Owing to the high flexibility of the macrocyclic ring systems of the analyzed new compounds and the energetic similarity of conformers with enantiomeric chromophores, no unequivocal circular dichroism (CD) spectra could be calculated for the new decalactones [50]. From the new decalactones, only compound **7** proved to be active against *Candida albicans* when tested in the agar plate diffusion assay at a concentration of 20 μmol per filter disk [52]. The other compounds, including the stereoisomer, showed no fungicidal activity in this assay, whereas all three were inactive when tested against bacteria such as *B. subtilis*, *S. aureus*, or *E. coli*.



6 R = H
7 R = OH; 9, 11-*cis*
8 R = OH; 9, 11-trans

The new decalactones from *P*. cf. *montananse* bear structural similarities, for example, to sporostatin isolated from the fungus *Sporomiella* sp. M5032, which is an inhibitor of cyclic adenosine phosphodiesterase [53], or to the curvularins, which are 12-membered macrolides isolated from terrestrial strains of *Curvularia*, *Penicillium*, *Alternaria*, or *Cochliobus* [54–57]. Like the above-mentioned chromones isolated from a sponge-derived strain of *A. versicolor*, the isolation of new decalactones from *P*. cf. *montanense* serves as a further proof of the striking chemical uniqueness of many sponge-derived fungi (in this context, see also [51,52,58,59] and further references cited therein), regardless of whether they qualify as true obligate marine fungi or whether their presence in the sea is more of an accidental nature as suspected for both fungi analyzed in this study.

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