

# Sponge-associated fungi and their bioactive compounds: the *Suberites* case

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## Abstract

Specimens of *Suberites domuncula* that had been cultured in aquaria for 4 weeks were analyzed for their associated fungi. A total of 81 fungal strains belonging to 20 different genera was isolated and identified by morphological and molecular methods. The most frequently isolated taxa were *Cladosporium* spp., *Penicillium* spp., *Petriella* sp., *Phialophora* spp. and *Engyodontium album*. Based on chromatographic and mass spectrometric analysis of fungal extracts, as well as on bioassay results, *Aspergillus ustus*, *Penicillium* sp., *Petriella* sp. and *Scopulariopsis* sp. were selected for in-depth analysis of their natural products. A total of 19 different fungal metabolites, including three new natural products, was isolated and structurally identified. *A. ustus* yielded two sesquiterpenes, a drimane derivative and deoxyuvidin, as well as a sesterterpene ophiobolin H. The drimane derivative had an ED<sub>50</sub> value against L5178Y cells of 1.9 µg ml<sup>-1</sup> *in vitro*. The crude extract of *Petriella* sp. was also strongly cytotoxic against the L5178Y cell line. The cyclic tetrapeptide WF-3161 was primarily responsible for the activity; the ED<sub>50</sub> value was <0.1 µg ml<sup>-1</sup>. It was identical to the known compound WF-3161 and had been previously isolated from *Petriella guttulata*. In addition to WF-3161, three further natural products were obtained and unequivocally identified as new derivatives of infectopyrone by one- and two-dimensional NMR spectroscopy and by

mass spectroscopy. Of the new compounds, only dihydroinfectopyrone was active against L5178Y cells; the ED<sub>50</sub> value was 0.2 µg ml<sup>-1</sup>. *Penicillium* sp. yielded the largest number of metabolites. Viridicatin, viridicatol, cyclopenin and cyclophenol suppressed larval growth of the polyphagous pest insect *Spodoptera littoralis* when incorporated into an artificial diet at an arbitrarily chosen concentration of 237 ppm. Viridicatol was the most active compound and had an ED<sub>50</sub> value of ca. 50 ppm. *Scopulariopsis* sp. yielded three metabolites, including the known acetylcholinesterase inhibitors quinolactacin A1 and A2.

**Keywords:** marine natural products; sponge-associated fungal metabolites; *Suberites domuncula*.

## Introduction

In the last decade, marine microorganisms, such as bacteria, microalgae and fungi, have become increasingly important as sources for new bioactive natural products (Ebel 2006, König et al. 2006, Laatsch 2006, Ramaswamy et al. 2006, Shimizu and Li 2006). Fungi have proven to be particularly prolific sources of new compounds when compared to other microbial sources isolated from the sea. The first report of a bioactive natural product from a marine-derived fungus dates back to the 1940s when the fungus *Acremonium chrysogenum* Gams 1971 was isolated from a sewage outlet in the Mediterranean Sea close to the island of Sardinia. The fungus was the source of cephalosporin C, the parent compound of modern cephalosporin antibiotics that are indispensable for the treatment of numerous bacterial infections (Abraham and Loder 1972). Initially, progress with rigorous evaluation of marine fungal metabolites was slow. This situation changed dramatically in the 1990s when there was a sharp rise in interest in marine microbial metabolites that continues until today. Up to the year 2002, 272 new natural products had been isolated from marine-derived fungi (Bugni and Ireland 2004). A dramatic increase in the number of elucidated marine fungal structures began afterwards, illustrated by the fact that between 2002 and 2004, 240 additional compounds were described (Ebel 2006).

Even though the value of marine-derived fungi as a source of new bioactive metabolites is now commonly accepted, there is still much debate on the nature of fungi that are isolated from various marine substrates, such as drifting wood, algae or invertebrates. Due to the fact that numerous, if not most, of these fungi belong to genera already well known from the terrestrial environment, such as *Aspergillus*, *Penicillium*, *Cladosporium*, *Phoma*, and *Fusarium*, a true marine origin of these fungal strains is frequently doubted (Höller et al. 2000, Kohlmeier and

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Volkman-Kohlmeier 2003). It is possible that several, if not many, marine-derived fungi thus far investigated originated from terrestrial habitats (e.g., soil) from which they were washed to the sea and survived (as spores) until they were recovered by a marine chemist looking for new compounds from the sea. On the other hand, in the last few years more and more evidence has accumulated indicating an adaptation of these “ubiquitous” fungi to the marine environment (Geiser et al. 1998a,b, Alker et al. 2001, Duncan et al. 2002, Zuccaro et al. 2004). Nevertheless, the fact remains that regardless of their true origin, marine-derived fungi have developed into an important source of new and structurally unprecedented metabolites (Ebel 2006).

Interestingly, sponges continue to be one of the most important sources for the isolation of metabolite-producing marine-derived fungi (Höller et al. 2000, Jensen and Fenical 2002, Bugni and Ireland 2004, Ebel 2006), even though the presence of fungal mycelia growing in sponges has not yet been proven. In contrast to sponge-derived natural products, such as jaspaklinolide L, theopederin C, or mycalamide A, that bear obvious structural resemblances to known bacterial metabolites (Proksch et al. 2002, Butzke and Piel 2006) and are assumed to originate from bacterial sponge symbionts, there are no examples of known fungal metabolites that have been isolated from sponges. On the other hand, the sponge *Suberitis domuncula* (Olivi 1792) has receptor proteins for fungal cell wall components, e.g., (1 → 3)- $\beta$ -D-glucan, providing evidence that at some time in its life cycle (or in its evolutionary history) the sponge, if not colonized by, at least deals (or dealt) with fungi (Perović-Ottstadt et al. 2004). The presence of the (1 → 3)- $\beta$ -D-glucan receptor protein in *S. domuncula*, as well as the fact that sponges of this species can be easily maintained in aquaria over several months to a year were decisive in choosing *S. domuncula* as a model organism for our study.

For the time being, the true nature of sponge-fungal associations remains obscure and far more research needs to be devoted to this field. The study presented here is part of a larger project aimed at obtaining a broader picture and a better understanding of the fungal association with sponges using molecular, biochemical and culturing techniques. After screening 81 fungal strains isolated from the sponge *Suberitis domuncula* (Porifera, Demospongiae, Hadromerida), four isolates were selected for the characterization of active secondary metabolites that may play important roles *in situ*.

## Materials and methods

### General experimental procedures

$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR, COSY, HMQC and HMBC spectra (chemical shifts in ppm, referenced to residual solvent signals) were recorded on a Bruker ARX-400, DRX 500 or DMX 600 spectrometer (Bruker Daltonik GmbH, Bremen, Germany). For spectral simulation, the MestReC software package (version 4.9.9.9) was used (Mestrelab Research, Santiago de Compostela, Spain). Mass spectra were obtained on Thermofinnigan LCQ Deca (ESI) and

Micromass Q-ToF 2 (HRESIMS) mass spectrometers. For HPLC analysis, samples were injected into a HPLC system coupled to a photodiode-array detector (Dionex, Munich, Germany). Routine detection was at 254 nm in aqueous MeOH. The separation column (125 × 4 mm, i.d.) was a Eurospher-100-C18 reverse phase column (Knauer, Berlin, Germany). Optical rotations were recorded on a Perkin-Elmer-241 MC polarimeter. Semi-preparative HPLC was performed on a Eurospher-100-C18 reverse phase column utilizing a Merck-Hitachi L-7100 pump and L-7400 UV detector.

Solvents were distilled before use and spectral grade solvents were used for spectroscopic measurements. Thin layer chromatography (TLC) was performed on plates precoated with Si 60 F254 (Merck, Darmstadt, Germany). Compounds were detected from their UV absorbance ( $\lambda_{\text{max}}$  235–340 nm) and also by spraying the TLC plates with anisaldehyde reagent.

### Isolation and identification of the fungal strains

Specimens of the marine sponge *Suberitis domuncula* (Porifera, Demospongiae, Hadromerida) were collected in the Northern Adriatic Sea near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) for 4 weeks at 17°C as previously described (LePennec et al. 2003). In brief, the pH of artificial seawater (Tropic Marine Centre Ltd., Rickmansworth, UK) was adjusted to 8.0. The sponges, maintained in 200 l aquaria, were fed every second day with 5 ml of Coraliquid, a fluid plankton-based nutrition (Sera; Heinsberg-Dohse Aquaristik, Bonn, Germany) and with 20 ml of “Mainvit-plus”, containing strontium, molybdate, lithium, rubidium and iodine (Sera, Heinsberg, Germany). Every other day, 5 g of deep-frozen *Artemia salina* (Hundt, Wuppertal, Germany) were added for the hermit crab.

Because stringent surface sterilization procedures had previously proven to be too rigorous for delicate sponge tissues (Höller et al. 2000), sponges were either briefly sterilized for 30 s in 70% v/v ethanol followed by three subsequent washings in sterile commercial seawater or merely washed three times with sterile seawater. The sponge tissue was then cut into approximately 1.5 × 1.5 mm segments and plated on carrot-potato agar growth medium (Höller et al. 2000) with and without artificial sea salts and cultivated at 22°C. Emerging fungal mycelia were isolated and taken into culture. The identities of sporulating isolates were determined to at least the genus level according to morphology. In cases in which morphological identification was uncertain, molecular identification was undertaken.

### DNA extraction and PCR amplification

DNA was isolated from strains 8009, 7961, 7979 and 8017 using the ultra clean soil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) or using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Prior to lysis of the cells, samples were treated with 200 U of Lyticase (Sigma-Aldrich, Taufkirchen, Germany) for 40 min at 30°C. PCR amplification of the LSU was performed using the fungal specific primers NL209-NL912, as previously described (Zuccaro et al. 2003). ITS regions

were amplified using the forward primer ITS1f (Gardes and Bruns 1993) and the reverse primers NL912 or ITS4 (White et al. 1990). For the 18S rDNA, the primers used were nu-SSU-0817 and nu-SSU-1536 (Borneman and Hartin 2000). Additionally, part of the  $\beta$ -tubulin gene of strain 8009 was amplified using primers BenA1 and BenA2 (Geiser et al. 1998a,b) using standard parameters. PCR products were purified with ultra clean PCR clean-up (Mo Bio Laboratories Inc.) and sequenced by AGOWA (LGC group, Berlin, Germany) or using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and cloned into *pGEM-T* vector (Promega, Madison, WI, USA). In this case, plasmid clones were isolated by the NucleoSpin Plasmid Kit (Macherey-Nagel, Düren, Germany) and sequenced with the SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre, Madison, WI, USA) on a automated LiCor 4200 laser sequencer (LI-COR Inc., Lincoln, NE, USA).

### Cultivation and extraction

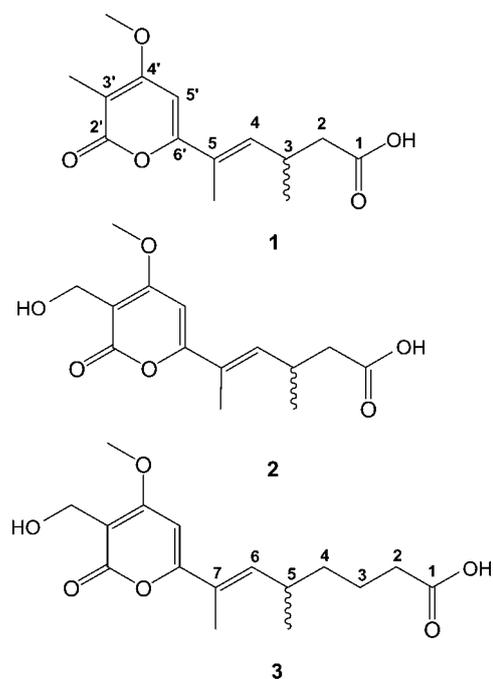
For production of secondary metabolites, the fungi were cultivated at 22°C for 21 days on both biomalt agar (Höller et al. 2000) and barley-spelt solid-substrate media (200 g barley, 200 g spelt whole grain flakes, 2 g soy peptone, 2 mg MnCl<sub>2</sub>, 250 ml distilled H<sub>2</sub>O). For initial analyses of the natural products, the cultures were lyophilized and extracted with ethyl acetate and the dried residues were defatted by petroleum extraction.

### Isolation of new infectopyrone derivatives from *Petriella* sp. (TUBS 7961)

The methanolic extract (1024 mg) of *Petriella* sp. was chromatographed over a silica gel column using CH<sub>2</sub>Cl<sub>2</sub>:MeOH (95:5) as eluting solvent, from which 11 fractions were obtained. Fraction 7 contained dihydroinfectopyrone (Figure 1, **1**, 13 mg). Compound **3** (Figure 1, **3**, 3 mg) was purified from fraction 11 (117 mg) over a Sephadex LH-20 column using CH<sub>2</sub>Cl<sub>2</sub>:MeOH (1:1) as solvent system. The second congener (Figure 1, **2**) was isolated from a large-scale methanolic extract (8 g) that was first chromatographed by vacuum liquid chromatography over a silica gel column by gradient elution utilizing decreasing ratios of CH<sub>2</sub>Cl<sub>2</sub> versus MeOH as the solvent system. Fraction 8 yielded compound **2** (76 mg) which was eluted with CH<sub>2</sub>Cl<sub>2</sub>:MeOH at a solvent ratio of 30:70. Purity of the compounds was monitored by reversed-phase HPLC utilizing a 35 min gradient program of 10–100% MeOH in H<sub>2</sub>O.

### Biological activities

**Cytotoxicity assay** Antiproliferative activity was examined against the cell line L1578Y and was determined by an MTT assay as described earlier (Kreuter et al. 1992). L1578Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplemented with 10% horse serum in roller tube culture. The cell culture media contained 100 U ml<sup>-1</sup> penicillin and 100  $\mu$ g ml<sup>-1</sup>



**Figure 1** New infectopyrone derivatives **1**, **2** and **3** from *Petriella* sp.

streptomycin and were changed twice per week. The cells were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

L1578Y was plated on a 96-multiwell plate with 50,000 cells/well. The cells were allowed to attach for 24 h and were then treated with different concentrations of the test samples. After this treatment, the medium was changed and the cells were incubated for 3 h under cell culture conditions with 20  $\mu$ g ml<sup>-1</sup> MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]. The conversion of the tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenases was determined as a marker of cell viability according to Mosmann (1983). After this incubation, the cells were fixed on the plate with an aqueous solution containing 1% formaldehyde and 1% CaCl<sub>2</sub> and then lysed with 95% isopropanol-5% HCO<sub>2</sub>H. The concentration of reduced MTT as a marker for cell viability was measured photometrically at 560 nm. Fractions and pure compounds dissolved in dimethyl sulfoxide (DMSO) were screened at a concentration of 10  $\mu$ g/well.

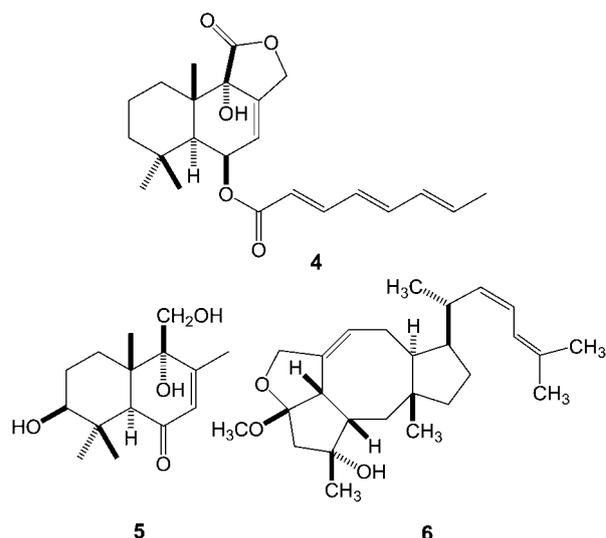
**Experiments with insects** Larvae of *Spodoptera littoralis* (Boisduval 1833) (Noctuidae) were from a laboratory colony reared on an artificial diet under controlled conditions as described previously (Srivastava and Proksch 1991, Nugroho et al. 1999). Feeding studies were conducted with neonate larvae (n=20) that were kept on an artificial diet treated with various concentrations of the compounds under study. After 6 days, survival of the larvae and weight of the surviving larvae were recorded and compared to controls. ED<sub>50</sub> values were calculated from the dose-response curves by probit analysis.

## Results and discussion

A total of 81 fungal strains from eight sponge samples was isolated and identified according to morphology as belonging to 20 different genera (Table 1). The most frequently isolated taxa were *Cladosporium* spp., *Penicillium* spp., *Petriella* sp., *Phialophora* spp., and *Engyodontium album* [(Limber) de Hoog 1978]. *E. album* has routinely been isolated from seaweeds, suggesting a direct association with the marine environment (Zuccaro et al. unpublished data). Isolates belonging to *Phialophora* spp., in contrast, are frequent fungal endophytes of terrestrial plants (Schulz et al. 2006). *Cladosporium* and *Penicillium* are ubiquitous genera that are soil inhabitants, plant pathogens, and endophytes, but there are also marine isolates (e.g., Zuccaro and Mitchell 2005). *Petriella* spp. have, to our knowledge, not been previously reported from the marine environment.

Based on the chromatographic and mass spectrometric on-line evaluation of the fungal extracts and/or on the bioassay results, four fungi were chosen for a first detailed analysis of their natural products. These fungi were molecularly identified by BLAST analysis as *Aspergillus ustus* [(Bainier) Thom et Church 1926] (100%), *Penicillium* sp. [highest BLAST match for *Penicillium freii* (Frisvad et Samson 1994) with 99%] *Petriella* sp. higher BLAST match for *Petriella sordida* [(Zukal) Barron et Gilman 1961] with 99% and *Scopulariopsis* sp.

From *Aspergillus ustus*, TUBS 8009, two sesquiterpenes and a sesterterpene were isolated. The sesquiterpenes (Figure 2, **4** and **5**) were identified as drimane derivatives based on their spectral characteristics (<sup>1</sup>H and <sup>13</sup>C NMR and MS), as well as by comparison with previously published data (Ayer and Peña-Rodriguez 1987, Hayes et al. 1996), whereas the sesterterpene (Figure 2, **6**) was an ophiobolane derivative as shown by its spectral data (Cutler et al. 1984). Compound **4** is known as a



**Figure 2** Terpenoid metabolites **4**, **5** and **6** from *Aspergillus ustus*.

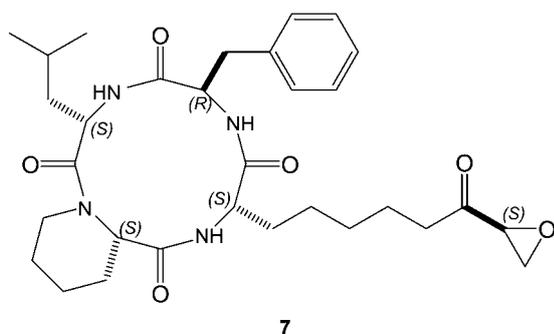
metabolite from *A. ustus* var. *pseudorelectus* Kozak 1989 isolated from a soil sample (Hayes et al. 1996). Compound **5**, also known as deoxyuvidin, was reported previously from *Alternaria brassicae* [(Berk.) Sacc. 1880], which is a pathogen on canola (Ayer and Peña-Rodriguez 1987). The ophiobolane derivative **6** that is known under the trivial name ophiobolin H was previously isolated from a terrestrial isolate of *A. ustus* obtained from seeds of *Pisum sativum* L. (Cutler et al. 1984). Finding some of the same metabolites in isolates of a given taxon from completely divergent habitats is a frequent phenomenon, e.g., coniothyromycins were produced by endophytic isolates of *Coniothyrium palmarum* (Corda 1840) from Lower Saxony, but also from *Coniothyrium* sp. isolated from Borneo soil samples (Krohn et al. 1994a,b).

The crude organic extract obtained from *Aspergillus ustus* analyzed in this study had strong cytotoxic activity when tested *in vitro* against the murine cancer cell line L5178Y at a concentration of 10 µg ml<sup>-1</sup>. This activity was traced to the drimane derivative **4**, which exhibited an ED<sub>50</sub> value of 1.9 µg ml<sup>-1</sup> against the L5178Y cell line. When tested against other cell lines, such as PC12 or HeLa, it proved inactive up to a concentration of 10 µg ml<sup>-1</sup>, thus indicating selectivity against lymphoma cell lines. Compounds **5** and **6** showed no activity against any of the cell lines tested at least up to a dose of 10 µg ml<sup>-1</sup>. Ongoing investigation of the extract derived from *A. ustus* suggests the presence of further terpenoid derivatives that are presently under investigation.

Similar to the extract of *Aspergillus ustus*, the extract of the second sponge-associated fungus, *Petriella* sp., exhibited strong cytotoxicity when tested against the L5178Y cell line and was therefore chosen for a detailed analysis of its secondary metabolites. Bioassay-guided isolation yielded a cyclic tetrapeptide (Figure 3, **7**) with a molecular weight of 568 as a prominent and strongly active component of the fungal extract. Spectroscopic analysis proved that compound **7** was the known WF-3161 previously reported as a component of the culture filtrate of *Petriella guttulata* (Barron et Cain 1961) isolated from a soil sample collected in Japan (Umehara et al.

**Table 1** Fungal taxa isolated from healthy (h) and diseased (d) *Suberites domuncula*; h/d indicates the ratio of isolates from healthy and diseased sponge tissue.

Taxon	Number of isolates	h/d
<i>Cladosporium</i> sp.	12	7/5
<i>Penicillium</i> sp.	10	6/4
<i>Engyodontium album</i>	9	4/5
<i>Phialophora</i> sp.	9	5/4
<i>Petriella</i> sp.	7	3/4
<i>Exophiala</i> sp.	5	2/2
Unidentified	4	3/1
<i>Acremonium</i> sp.	3	2/1
<i>Paecilomyces</i> sp.	2	1/1
<i>Sporobolomyces</i> sp.	2	1/1
<i>Trichoderma</i> sp.	2	1/1
<i>Aspergillus ustus</i>	1	h
<i>Chaetomium</i> sp.	1	h
<i>Fusarium</i> sp.	1	h
<i>Gliomastix</i> sp.	1	h
<i>Nodulisporium</i> sp.	1	h
<i>Phoma</i> sp.	1	h
<i>Scopulariopsis</i> sp.	1	d
<i>Stemphylium</i> sp.	1	d
<i>Stilbella</i> sp.	1	h
<i>Tolyposcladium</i> sp.	1	h
<i>Mycelia sterila</i>	6	4/2



**Figure 3** Cyclic tetrapeptide **7** isolated from *Petriella* sp.

1983). The amino acid sequence of the peptide is *cyclo*(L-Leu-L-Pip-L-Aoe-D-Phe), where Pip represents pipercolic acid and Aoe represents the unusual amino acid 2-amino-8-oxo-9,10-eoxydecanoic acid, which is in congruence with the published data (Kawai et al. 1986). Compound **7** exhibited exceptionally strong activity against the L5178Y cell line, with an  $ED_{50}$  value  $<0.1 \mu\text{g ml}^{-1}$ . This is again in congruence with previous studies that demonstrated *in vivo* activity of this peptide. When mice that had been inoculated intraperitoneally with lymphocytic leukemia P-388 cells were treated 24 h later by intraperitoneal administration of compound **7**, a prolongation of the survival time occurred (Umehara et al. 1983). The treated over control (T/C) (%) value increased (dose dependent) from 133 (dose of  $12.5 \text{ mg kg}^{-1} \text{ d}^{-1}$ ) to 175 (dose of  $100 \text{ mg kg}^{-1} \text{ d}^{-1}$ ).

In addition to the tetrapeptide **7**, further compounds exhibiting cytotoxicity were isolated from the extract of *Petriella* sp. The compound showing the strongest activity after peptide **7** proved to be a new natural product (**1**). In the HR-ESIMS, **1** exhibited a pseudomolecular ion at  $m/z$  267.1220, which was consistent with a molecular formula of  $\text{C}_{14}\text{H}_{19}\text{O}_5$  for its  $[\text{M}+\text{H}]^+$  (calc, 267.1227) ( $[\alpha]_D^{20} = -3.2$ ,  $c=1.0$ , MeOH).

From the multiplicities observed in the  $^1\text{H}$  NMR spectrum of **1** (Table 2), as well as corresponding cross signals in the COSY spectrum, the presence of the side chain comprising  $\text{H}_2-2$  ( $\delta$  2.42 and 2.35),  $\text{H}-3$  ( $\delta$  3.13),  $3-\text{CH}_3$  ( $\delta$  1.12), as well as  $\text{H}-4$  ( $\delta$  6.38), could easily be derived. The HMBC correlations from  $\text{H}_2-2$  and  $\text{H}-3$  to a carbon resonating at  $\delta$  175.5 (C-1) suggested that the terminus of this side chain was a free carboxylic acid. On the other hand, HMBC correlations from  $\text{H}-4$  to C-5 ( $\delta$  127.1), as well as  $5-\text{CH}_3$  ( $\delta$  12.4), established the presence of a methyl-bearing carbon as part of a double bond with the protonated C-4 ( $\delta$  139.5) at the other end of the side chain in **1**. The 4-methoxy- $\alpha$ -pyrone moiety in **1** was evident from the HMBC correlations observed for the two methyl groups  $4'-\text{OCH}_3$ , ( $\delta$  3.98, to C-4',  $\delta$  168.7) and  $3'-\text{CH}_3$  ( $\delta$  1.88, to C-4', C-2', and C-3',  $\delta$  167.1 and 102.0, respectively), as well as  $\text{H}-5'$  ( $\delta$  6.49, to C-3', C-4', and C-6',  $\delta$  161.2). Finally, the connection of the two substructures at C-5 and C-6' was established through HMBC correlations of  $\text{H}-5'$  to C-5, as well as  $\text{H}-4$  to C-6'. Thus, **1** was identified as the 2,3-dihydro derivative of infectopyrone, which had been isolated previously from *Alternaria infectoria* Simmons 1986, a plant pathogenic fungal strain frequently causing infec-

tions of cereals and other food products (Larsen et al. 2003). Dihydroinfectopyrone (**1**) exhibited an  $ED_{50}$  value of  $0.2 \mu\text{g ml}^{-1}$  when tested against the L5178Y cell line.

Compound **2** had a molecular weight 16 amu higher than that of dihydroinfectopyrone (**1**), suggesting that it is an oxygenated congener. This was corroborated by the HR-ESIMS, showing a pseudomolecular ion at  $m/z$  283.1170, which was consistent with a molecular formula of  $\text{C}_{14}\text{H}_{19}\text{O}_6$  for its  $[\text{M}+\text{H}]^+$  (calc, 283.1176) ( $[\alpha]_D^{20} = -60.6$ ,  $c=0.5$ , MeOH).

Correspondingly, in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data obtained for **2** (Table 2), the signal attributed to  $3'-\text{Me}$  in **1** was missing, and instead, chemical shifts characteristic of a hydroxymethyl functionality were observed ( $\delta_{\text{H}}$  4.44, 2H, and  $\delta_{\text{C}}$  53.8), while the remaining signals and correlations observed in the HMBC spectrum of **2** were almost identical to the corresponding ones obtained for **1**. Thus, **2** was identified as the 3'-demethyl-3'-hydroxymethyl derivative of dihydroinfectopyrone.

Initially, the structural elucidation of the remaining infectopyrone-derived congener **3** was hampered by the small amounts isolated and the fact that, due to the limited supply of the compound, it was not possible to achieve complete purification, rendering the quality of the NMR spectra rather poor in comparison to those measured for **1** or **2**. However, close comparison with the spectral data of the previous derivatives resulted in the deduction that **3** was a dihydro congener of **2**, differing by insertion of two additional methylene groups in the side chain next to the carboxylic acid function. This was in agreement with the HR-ESIMS, which confirmed the molecular formula as  $\text{C}_{16}\text{H}_{22}\text{O}_6$ , again derived by a corresponding pseudomolecular ion at  $m/z$  333.130 ( $[\text{M}+\text{Na}]^+$   $\text{C}_{16}\text{H}_{22}\text{NaO}_6$ , calc. 333.1309) ( $[\alpha]_D^{20} = -17$ ,  $c=0.1$ , MeOH). The position of the two additional  $\text{CH}_2$  units was established from the COSY spectrum, which revealed a common spin system comprising  $\text{H}_2-2$ ,  $\text{H}_2-3$ ,  $\text{H}_2-4$ ,  $\text{H}-5$ ,  $\text{H}-6$  and  $5-\text{CH}_3$ , as well as  $7-\text{CH}_3$ . Further evidence was provided by the HMBC spectrum, which in addition to supporting the said spin system also revealed the connection of C-2 to a free carboxylic acid function (C-1), and the attachment of the pyrone ring through C-6' and C-7. Key correlations included both  $\text{H}_2-2$  and  $\text{H}_2-3$  with C-1, as well as  $7-\text{CH}_3$  with C-6' (Table 2). When tested for cytotoxic properties under experimental conditions similar to those employed for **1**, both **2** and **3** showed almost no activity.

*Penicillium* sp., TUBS 7979, was selected for further analysis based on the marked growth inhibitory activity (when tested at a dose of 1000 ppm incorporated into artificial diet) of its crude organic extract against larvae of *Spodoptera littoralis*. In total, 10 different known compounds, including viridicatin (Figure 4, **8**) (Cunningham and Freeman 1953), its hydroxylated analog viridicatol (Figure 4, **9**) (Birkinshaw et al. 1963), cyclophenin (Figure 4, **10**) (Bracken et al. 1954), its hydroxyl derivative cyclophenol (Figure 4, **11**) (Hodge et al. 1988), emodin (Figure 4, **12**) (Cohen and Towers 1995), physcion (Figure 4, **13**) (Anke et al. 1980), the methylated chrysophanol derivative (Figure 4, **14**) (Thomson et al. 1987), the acetophenone derivative clavatul (Figure 4, **15**) (Gatenbeck and Brunsberg 1966) and citrinin (Figure 4, **16**) (Barber et al.

**Table 2** NMR data for dihydroinfectopyrone (**5**) and its 3'-hydroxymethyl derivative (**6**) in MeOH-*d*<sub>4</sub> (500 and 125 MHz, respectively), and for the new dihydro 3'-hydroxymethyl infectopyrone derivative (**7**) in MeOH-*d*<sub>4</sub> (500 and 125 MHz, respectively).

<b>5</b>			<b>6</b>			<b>7</b>			
	$\delta_{\text{H}}$ (mult, J Hz)	$\delta_{\text{C}}$	HMBC (H → C)	$\delta_{\text{H}}$ mult (J Hz)	$\delta_{\text{C}}$	HMBC (H → C)	$\delta_{\text{H}}$ (mult, J Hz)	$\delta_{\text{C}}$	HMBC (H → C)
1		175.5			175.5			177.2	
2	A 2.42 (dd, 15.3, 6.5) B 2.35 (dd, 15.3, 7.7)	41.8	1, 2, 3, 4, 3-Me 1, 2, 3, 4, 3-Me	A 2.43 (dd, 15.3, 6.4) B 2.36 (dd, 15.3, 7.8)	41.9	1, 2, 3, 4, 3-Me 1, 2, 3, 4, 3-Me	2.25–2.30 (2H, m)	34.7	1, 3, 4
3	3.13 (ddq, 7.7, 6.5, 6.7) <sup>b</sup>	31.2	1, 2, 3, 4, 5, 3-Me	3.14 (ddq, 7.8, 6.4, 6.7) <sup>b</sup>	31.3	1, 2, 3, 4, 5, 3-Me	1.55–1.61 (2H, m)	23.8	1, 2, 4
4	6.38 (dd, 9.9, 1.3)	139.5	2, 4, 5, 6', 3-Me, 5-Me	6.43 (dd, 9.9, 1.4)	140.7	2, 3, 4, 5, 6', 3-Me, 5-Me	A 1.47–1.52 (m) B 1.36–1.43 (m)	37.4	2, 3, 2, 3
5		127.1			127.3		2.60–2.72 (1H, m)	33.9	
2'		167.1			166.3		6.41 (dd, 10.0, 1.2)	142.7	6'
3'		102.0			105.0			126.8	
4'		168.7			170.5			166.3	
5'	6.49 (s)	94.1	5, 3', 4', 5', 6'	6.51 (s)	94.2	5, 3', 4', 5', 6'		104.7	
6'		161.2			163.5			170.6	
3-Me	1.12 (3H, d, 6.7)	20.2	2, 3, 4, 3-Me	1.12 (3H, d, 6.7)	20.1	2, 3, 4, 3-Me	6.50 (s)	93.7	7, 3', 4', 5', 6'
5-Me	2.00 (3H, d, 1.3)	12.4	4, 5, 6', 5-Me	2.01 (3H, d, 1.4)	12.4	4, 5, 6'		163.5	
3'-Me	1.88 (3H, s)	8.2	2', 3', 4', 3'-Me		53.8	2', 3', 4', 3'-CH <sub>2</sub>	1.06 (3H, d, 6.7)	20.3	4, 5, 6, 5-Me
3'-CH <sub>2</sub> OH				4.44 (2H, s)	57.3	4', 4'-OMe	<b>1.99 (3H, d, 1.2)</b>	<b>12.5<sup>b</sup></b>	<b>6, 6'</b>
<b>4'-OMe</b>	<b>3.98 (3H, s)</b>	<b>57.0</b>	<b>4', 4'-OMe</b>	4.02 (3H, s)			4.45 (2H, s)	53.8	2', 3', 4', 3'-CH <sub>2</sub>
				4.02 (3H, s)			<b>4.02 (3H, s)</b>	<b>57.2</b>	<b>4', 4'-OMe</b>

<sup>a</sup>Obtained from HMBC spectrum; <sup>b</sup>coupling pattern confirmed by simulation; bold font: <sup>1</sup>J correlation.

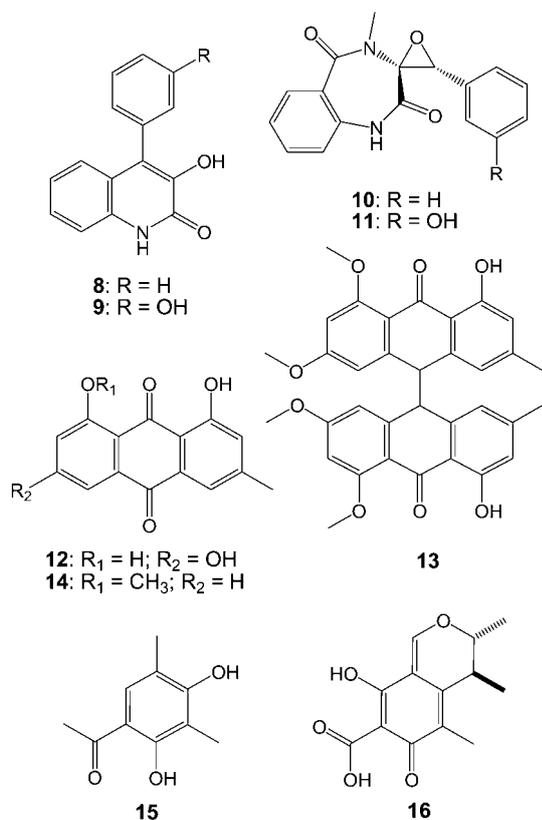


Figure 4 Metabolites 8–16 derived from *Penicillium* sp.

1987) were isolated and identified. The growth inhibitory activity of the extract against *S. littoralis* larvae was traced mainly to viridicatin (**8**), viridicatol (**9**), cyclophenin (**10**) and cyclophenol (**11**). All four compounds clearly suppressed larval growth at an arbitrarily chosen concentration of 237 ppm when compared to controls (Figure 5). Viridicatol (**9**), which differs from viridicatin (**8**) only by one hydroxyl substituent, was by far the most active compound encountered and had an EC<sub>50</sub> value of ca. 50 ppm (Figure 6). The larval growth inhibitory activities of compounds 8–11 are reported here for the first time.

The fourth fungal strain, TUBS 8017, selected for an investigation of the produced natural products, belonged to the genus *Scopulariopsis*. From this strain, three dif-

ferent natural products were isolated, which included the rather frequently occurring cladosporin (Figure 7, **17**) (Reese et al. 1988), and also the structurally unusual tetramic acid derivatives quinolactacin A1 (Figure 7, **18**) (Kim et al. 2001) and A2 (Figure 7, **19**) (Kim et al. 2001). The latter two compounds were previously isolated from the genus *Penicillium* and have recently aroused interest due to their inhibitory activity against TNF- $\alpha$  production by macrophages that had been stimulated by LPS (Kakinuma et al. 2000), as well as their inhibitory activity against acetylcholinesterase (Kim et al. 2001). While TNF- $\alpha$  is involved in inflammations and may even lead to septic shock, acetylcholinesterases are interesting drug targets for the treatment of neurodegenerative disorders, such as Alzheimer's disease. The naturally occurring ACE inhibitor galanthamin from *Galanthus* spp. is in clinical use for this indication. There is hope that the quinolactacins may prove to be further drug candidates for the treatment of this disease.

## Conclusion

These investigations are the initial report on a larger study (using molecular and classical cultivation methods) on the fungal diversity and chemical productivity of sponge-derived fungi associated with the Mediterranean sponge *Suberites domuncula*. As can be seen in Table 1, specimens of *S. domuncula* that were kept in aquaria yielded a relatively high diversity of fungi, including numerous ubiquitous taxa, e.g., *Aspergillus*, *Penicillium* and *Cladosporium*, which have repeatedly been isolated from sponges in the past (Höller et al. 2000, Ebel 2006), but also *Engyodontium album* and *Petriella* sp. that have not been previously reported from sponges. Fungi are probably not responsible for diseases of aquarium-grown *S. domuncula*. However, this should be verified with diseased and healthy wild samples.

Chemical investigations of cultivable fungi are the second goal of this project. Even though to date only four fungal taxa obtained from *Suberites domuncula* have been chemically investigated, the number of secondary metabolites obtained (19) and the chemical diversity of these compounds, which include terpenoids (**4–6**), alka-

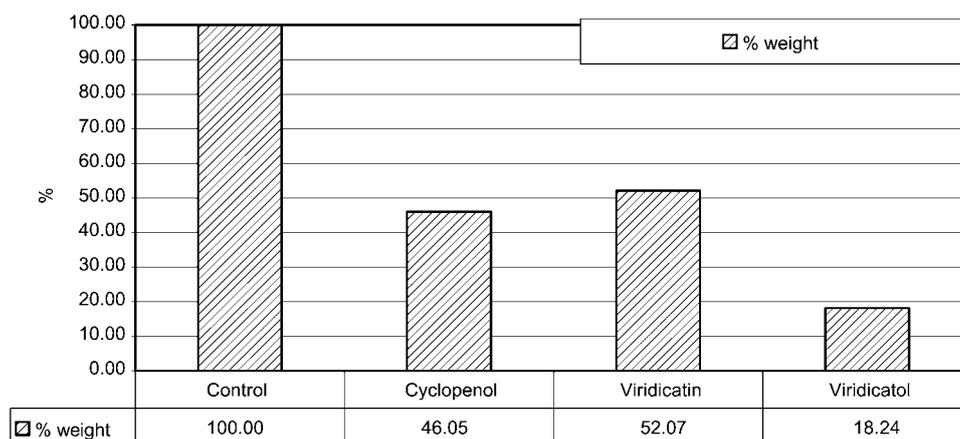
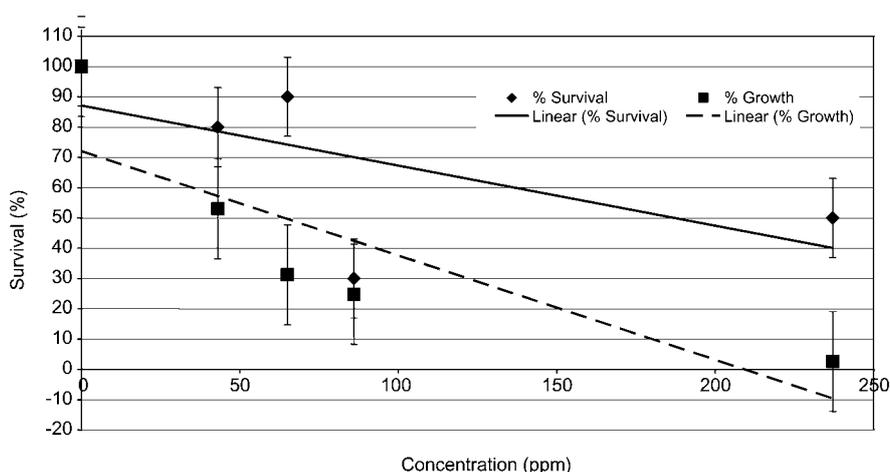
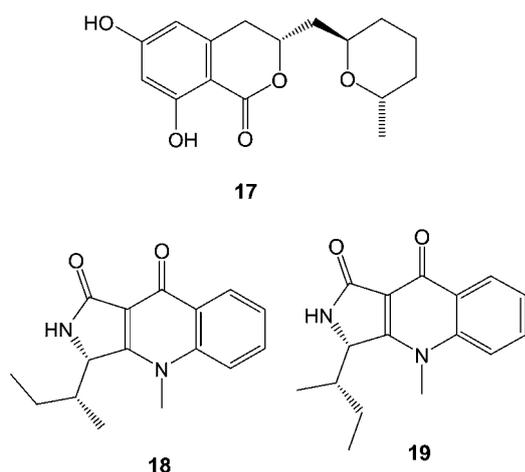


Figure 5 Growth inhibitory activities of cyclophenol **11**, viridicatin **8** and viridicatol **9** against *Spodoptera littoralis* at an arbitrarily chosen concentration of 237 ppm.



**Figure 6** Viridicatol in the feeding assay of *Spodoptera littoralis*.



**Figure 7** Compounds **17**, **18** and **19** obtained from *Scopulariopsis* sp.

loids (**8–11**, **18**, **19**), polyketides (**1–3**, **12–17**) and peptides (**7**) are impressive, especially when viewed against their host *S. domuncula*. While sponges in general are the most prolific marine invertebrates in terms of natural products (Blunt et al. 2007), *S. domuncula* has so far yielded only three secondary metabolites (MarinLit 2006) and is thus among the chemically poorer sponges. Fungal isolates clearly surpass their host in terms of chemical diversity, as shown in this study for only four taxa out of more than 20 fungal strains isolated so far. It is tempting to speculate that the diversity of fungi isolated from *S. domuncula*, as well as their metabolic productivity, is largely a result of the paucity of active metabolites produced by the host. However, we must first determine which of the fungi are actually associated and are not simply spores trapped by sponge filtration. Only molecular analyses will answer this question.

Based on these first results, we expect in the near future to increase the list of structurally unusual bioactive metabolites when the remaining fungi have been analyzed (Table 1). We intend to identify and compare fungal strains that are amenable to culturing conditions to those that are likewise present in the sponge, but can only be identified using molecular methods, such as ITS sequences (Zuccaro et al. 2003, Bugni and Ireland 2004,

Ebel 2006), in order to obtain a clearer picture of the fungal diversity in relation to various factors, such as locality within the Mediterranean Sea, season and the influence of controlled conditions (in aquaria vs. the wild). Additionally, we will study the secondary metabolites that these fungi produce, as they may play important roles *in situ*.

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