Phylogenetic Identification of Fungi Isolated from the Marine Sponge *Tethya aurantium* and Identification of Their Secondary Metabolites

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**Abstract:** Fungi associated with the marine sponge *Tethya aurantium* were isolated and identified by morphological criteria and phylogenetic analyses based on internal transcribed spacer (ITS) regions. They were evaluated with regard to their secondary metabolite profiles. Among the 81 isolates which were characterized, members of 21 genera were identified. Some genera like *Acremonium*, *Aspergillus*, *Fusarium*, *Penicillium*, *Phoma*, and *Trichoderma* are quite common, but we also isolated strains belonging to genera like *Botryosphaeria*, *Epicoccum*, *Parasphaeosphaeria*, and *Tritirachium* which have rarely been reported from sponges. Members affiliated to the genera *Bartalinia* and *Volutella* as well as to a presumably new *Phoma* species were first isolated from a sponge in this study. On the basis of their classification, strains were selected for analysis of their ability to produce natural products. In addition to a number of known compounds, several new natural products were identified. The scopularides and sorbifuranones have been described elsewhere. We have isolated four additional substances which have not been described so far. The new metabolite *cillifuranone* (1) was isolated from *Penicillium chrysogenum* strain LF066. The structure of cillifuranone (1) was elucidated based on 1D and 2D NMR analysis and turned out to be a previously postulated intermediate in sorbifuranone biosynthesis. Only minor antibiotic bioactivities of this compound were found so far.
Keywords: Tethya aurantium; sponge-associated fungi; phylogenetic analysis; natural products; cillifuranone

1. Introduction

Natural products are of considerable importance in the discovery of new therapeutic agents [1]. Apart from plants, bacteria and fungi are the most important producers of such compounds [2]. For a long time neglected as a group of producers of natural products, marine microorganisms have more recently been isolated from a variety of marine habitats such as sea water, sediments, algae and different animals to discover new natural products [3,4]. In particular, sponges which are filter feeders and accumulate high numbers of microorganisms have attracted attention [5,6]. Though the focus of most of these investigations was concerned with the bacteria, a series of investigations identified marine sponges also as a good source of fungi [7–18]. Due to the accumulation of microorganisms, it is no surprise that sponges account for the majority of fungal species isolated from the marine realm [19]. However, the type of association and a presumable ecological function of accumulated fungi in sponges remain unclear and little evidence is available on fungi specifically adapted to live within sponges. One example is represented by fungi of the genus Koralionastes, which are known to form fruiting bodies only in close association with crustaceous sponges associated with corals [20].

Consistently, fungi isolated from sponges account for the highest number (28%) of novel compounds reported from marine isolates of fungi [19]. Marine isolates of fungi evidently are a rich source of chemically diverse natural products which has not been consequently exploited so far. Among a number of metabolites from sponge-associated fungi with promising biological activities are the cytotoxic gymnastatins and the p56\textsuperscript{Lck} tyrosine kinase inhibitor uloclloyd [21,11]. In view of these exciting data and our own previous work on the bacterial community associated with Tethya aurantium [22], we have now isolated and identified a larger number of fungi from this sponge. In European waters, Tethya aurantium is commonly found in the Atlantic Ocean, the English Channel, the North Sea as well as the Mediterranean Sea, where our specimens originated from [23]. Except for a single report from Indriani [24], fungi associated with Tethya sp. have not been investigated so far. The formation of natural products by these fungi and their biotechnological potential has not been evaluated yet.

For the identification of the fungal isolates from Tethya aurantium, we combined morphological criteria and phylogenetic analyses based on the sequence of the internal transcribed spacer (ITS) regions 1 and 2. On the basis of their classification, strains were selected for analysis of their ability to produce natural products. In addition to a number of known compounds, the new cyclodepsipeptides scopularide A and B were produced by a Scopulariopsis brevicaulis isolate [25]. Because of their antiproliferative activities against several tumor cell lines, these peptides and their activities have been patented [26]. During the present study, we have isolated four so far undescribed substances. Structure and properties of the new cillifuranone, a secondary metabolite from Penicillium chrysogenum strain LF066 are reported here.
2. Results and Discussion

2.1. Identification of the Fungal Strains Isolated from T. aurantium

In most studies on fungi associated with sponges the taxonomic classification of the fungi was based exclusively on morphological characteristics and in many cases identification was possible only at the genus level [17]. This can be attributed to the fact that taxonomic identification of fungi at the species level is not always easy. It is impaired by the fact that under laboratory conditions many fungi do not express reproductive features like conidia or ascomata, which represent important traits for identification. These fungi are classified as “mycelia sterilia”.

Therefore, morphological criteria as well as sequence information and the determination of phylogenetic relationships are considered to be necessary for the identification of fungi. Consequently, we have combined the morphological characterization with a PCR-based analysis using ITS1-5.8S-rRNA-ITS2 gene sequences to identify 81 fungi isolated from *Tethya aurantium*. Based on these criteria the strains could be identified to the species level (Figure 1, Table 1).

**Figure 1.** Scanning electron micrographs of *Fusarium* sp. strain LF236. (A) Multicellular, curved conidiospore; (B) Exudates in the surface layer of a liquid culture; (C) Intercalary chlamydospores in the mycelium.

| Strain | Morphological identification | Seq. length (nt) | Next related cultivated strain (BLAST) | Acc. No. | Similarity (%) | Overlap (nt) |
|--------|------------------------------|-----------------|----------------------------------------|----------|----------------|--------------|
| LF063  | *Cladosporium* sp.           | 483             | Fungal sp. ARIZ AZ0920                 | HM123596.1 | 99             | 482          |
|        |                              |                 | *Cladosporium sphaerospermum* isolate  | GU017501.1 | 99             | 482          |
| LF064  | *Scopulariopsis murina*      | 473             | Ascomycota sp. 840                    | GU934604.1 | 92             | 360          |
|        |                              |                 | *Phialemonium obovatum* strain CBS 279.76 | AB278187.1 | 89             | 340          |
| LF065  | *Penicillium* sp.            | 546             | *Penicillium glabrum* strain 4AC2K    | GU372904.1 | 99             | 545          |
| LF066  | *Penicillium* sp.            | 551             | *Penicillium chrysogenum* strain JCM 22826 | AB479305.1 | 99             | 549          |
| LF073  | Aspergillus sp. | 533 | Aspergillus versicolor isolate UOA/HCPF 8709 | FJ878627.1 | 100 | 532 |
| LF177  | Alternaria sp. | 568 | Lewia infectoria strain IA310 | AY154718 | 99 | 561 |
| LF178  | Cladosporium sp. | 479 | Fungal endophyte sp. g6 Cladosporium cladosporioides strain CC1 | HM537022.1 | 100 | 479 |
|        |                 |     |                                             | HM210839.1 | 100 | 479 |
| LF179  | Mycelia sterilia | 559 | Fungal endophyte isolate 9137 Paraphaeosphaeria sp. LF6 | EF419991.1 | 100 | 555 |
|        |                 |     |                                             | GU985234.1 | 99  | 557 |
| LF183  | Cladosporium sp. | 523 | Dothideomycetes sp. 11366 Cladosporium cladosporioides isolate SLP001 | GQ153254.1 | 99  | 522 |
|        |                 |     |                                             | FJ932747.1 | 99  | 521 |
| LF184  | Cladosporium sp. | 475 | Fungal endophyte sp. g6 Cladosporium cladosporioides strain CC1 | HM537022.1 | 100 | 475 |
|        |                 |     |                                             | HM210839.1 | 100 | 475 |
| LF236  | Fusarium sp. | 512 | Fusarium sp. CPK3469 Gibberella intricans strain ATCC MYA-3861 | FJ827615.1 | 99  | 511 |
|        |                 |     |                                             | GU291255.1 | 99  | 511 |
| LF237  | Fusarium sp. | 503 | Fusarium sp. CPK3337 Fusarium equiseti strain NRRL 36478 | FJ827616.1 | 100 | 503 |
|        |                 |     |                                             | GQ505743.1 | 100 | 503 |
| LF238  | Fusarium sp. | 513 | Fusarium sp. CPK3469 Fusarium equiseti strain NRRL 36478 | FJ827615.1 | 100 | 513 |
|        |                 |     |                                             | GQ505743.1 | 100 | 513 |
| LF239  | Fusarium sp. | 509 | Fusarium sp. NRRL 45997 Fusarium equiseti strain NRRL 36478 | GQ505761.1 | 99  | 503 |
|        |                 |     |                                             | GQ505743.1 | 99  | 503 |
| LF240  | Mycelia sterilia | 528 | Lewia sp. B32C Lewia infectoria strain IA241 | EF432279.1 | 99  | 525 |
|        |                 |     |                                             | AY154692.1 | 99  | 525 |
| LF241  | Mycelia sterilia | 513 | Botryosphaeria sp. GU071005 Sphaeropsis sapinea strain CBS109943 | AB472081.1 | 100 | 512 |
|        |                 |     |                                             | DQ458898.1 | 100 | 512 |
| LF242  | Penicillium sp. | 538 | Penicillium brevicompactum isolate H66s1 | EF634441.1 | 99  | 537 |
| LF243  | Penicillium sp. | 532 | Penicillium virgatum strain IHB F 536 | HM461858.1 | 100 | 530 |
| LF244  | Cladosporium sp. | 516 | Fungal endophyte sp. g2 Davidiella tassiana strain BLE25 | HM537019.1 | 99  | 516 |
|        |                 |     |                                             | FN868485.1 | 99  | 516 |
| LF245  | Fusarium sp. | 494 | Fusarium sp. CPK3514 Fusarium equiseti strain NRRL 13402 | FJ840530.1 | 100 | 494 |
|        |                 |     |                                             | GQ505681.1 | 100 | 494 |
| LF246  | Volutella sp. | 548 | Volutella ciliata strain BBA 70047 | AJ301966.1 | 99  | 547 |
| LF247  | Fusarium sp. | 520 | Fusarium sp. LD-135 Fusarium equiseti strain NRRL 13402 | EU336989.1 | 99  | 509 |
|        |                 |     |                                             | GQ505681.1 | 100 | 504 |
| LF248  | Botrytis sp. | 504 | Fungal endophyte sp. g18 Botryotinia fuckeliana strain OnionBC-1 | HM537028.1 | 100 | 504 |
|        |                 |     |                                             | FJ169667.2 | 100 | 504 |
| LF249  | Penicillium sp. | 552 | Penicillium sp. BM Penicillium commune isolate HF1 | GU566211.1 | 99  | 551 |
|        |                 |     |                                             | GU183165.1 | 99  | 551 |
| LF250  | Penicillium sp. | 564 | Penicillium chrysogenum strain ACBF 003-2 | GQ241341.1 | 97  | 547 |
| LF251  | Penicillium sp. | 550 | Penicillium sp. F6 Penicillium chrysogenum strain ACBF 003-2 | GU566250.1 | 100 | 550 |
|        |                 |     |                                             | GQ241341.1 | 100 | 550 |
| LF252  | Fusarium sp. | 513 | Fusarium sp. NRRL 45997 Fusarium equiseti strain NRRL 36478 | GQ505761.1 | 99  | 511 |
|        |                 |     |                                             | GQ505743.1 | 99  | 511 |
| LF253  | Trichoderma sp. | 543 | Hypocreia lixii strain OY3207 | FJ571487.1 | 100 | 540 |
| LF254  | Clonostachys sp. | 525 | Biomecia ochroleuca strain G11 | GU566253.1 | 100 | 524 |
| LF255  | Alternaria sp. | 518 | Fungal endophyte sp. g76 Alternaria alternata strain 786949 | HM537053.1 | 100 | 518 |
|        |                 |     |                                             | GU594741.1 | 100 | 518 |

Table 1. Cont.
| Code | Species/Strain | Genus/Species | Accession Number | Similarity | Length |
|------|---------------|---------------|-----------------|------------|--------|
| LF256 | Botrytis sp. | Beauveria bassiana strain G61 | GU566276.1 | 99 | 533 |
| LF257 | Cladosporium sp. | Davidiella tassiana strain G20 | GU566258.1 | 100 | 495 |
| LF258 | Phoma sp. nov. | Fungal sp. GFI 146 | A608980.1 | 93 | 470 |
| LF259 | Penicillium sp. | Septoria arundinacea isolate BJDC06 | GU361965.1 | 91 | 460 |
| LF260 | Sphaeropsisidales | Pyrenochaeta cava isolate olrin63 | AY354263.1 | 100 | 508 |
| LF491 | Aspergillus sp. | Penrzymes alliaceus isolate NRRL 4181 | EF661556.1 | 99 | 555 |
| LF494 | Fusarium sp. | Fusarium sp. CB-3 | GU932675.1 | 100 | 469 |
| LF496 | Mycelia sterilia | Verticillium sp. TF17TTW | FJ948142.1 | 99 | 529 |
| LF501 | Aspergillus sp. | Aspergillus granulosus isolate NRRL 1932 | EF652430.1 | 100 | 514 |
| LF508 | Not identified | Phoma sp. W21 | GU045305.1 | 99 | 497 |
| LF509 | Fusarium sp. | Fusarium sp. CPK3514 | FJ840530.1 | 99 | 503 |
| LF510 | Fusarium sp. | Fusarium sp. FL-2010c isolate UASWS0396 | HQ166535.1 | 99 | 514 |
| LF514 | Trichoderma sp. | Trichoderma sp. TM9 | AB369508.1 | 100 | 546 |
| LF526 | Eurotium sp. | Eurotium sp. FZ | HQ148160.1 | 100 | 486 |
| LF530 | Alternaria sp. | Alternaria sp. 7 HF-2010 | HQ380788.1 | 100 | 522 |
| LF534 | Penicillium sp. | Penicillium roseopurpureum strain E2 | GU566239.1 | 99 | 536 |
| LF535 | Acremonium sp. | Acremonium sp. FSU2858 | AY633563.1 | 99 | 523 |
| LF537 | Cladosporium sp. | Lecanicillium lecanii strain V56 | DQ007047.1 | 99 | 510 |
| LF538 | Mucor hiemalis | Mucor hiemalis isolate UASWS0442 | HQ166553.1 | 99 | 598 |
| LF540 | Not identified | Hypocre a lixis isolate FZ1302 | HQ259308.1 | 99 | 575 |
| LF542 | Mycelia sterilia | Peyronellaea glomerata isolate NMG_27 | HM776432 | 99 | 457 |
| LF543 | Alternaria sp. | Phoma pomorum var. pomorum strain CBS 539.66 | FJ427056.1 | 99 | 457 |
| LF547 | Aspergillus sp. | Aspergillus minutus isolate NRRL 4876 | EF652481.1 | 98 | 529 |
| LF550 | Mycelia sterilia | Bartalini a robillardoides CBS:122686 | EU552102.1 | 99 | 522 |
| LF552 | Epicoccum nigrum | Ellurema sp. 42-3 | AY148442.1 | 99 | 514 |
| LF555 | Penicillium sp. | Epicoccum nigrum strain GrS7 | FJ904918.1 | 99 | 503 |
| LF557 | Mycelia sterilia | Aspergillus sp. Da91 | HM991778.1 | 100 | 528 |
| LF558 | Not identified | Aspergillus sp. Da91 | HM991778.1 | 100 | 525 |
| LF559 | Mycelia sterilia | Fusarium sp. FL-2010f | HQ166539.1 | 99 | 522 |
| LF560 | Not identified | Fusarium oxysporum strain TS08-137-1-1 | AB470850.1 | 99 | 522 |
| LF562 | Trichirachium sp. | Phoma sp. W21 | GU045305.1 | 100 | 498 |
| LF563 | Trichoderma sp. | Hypocre a lixis isolate DLEN2008014 | HQ149778.1 | 100 | 537 |
| LF576 | Clonostachys sp. | Bionectria cf. ochroleuca CBS 113336 | EU552110.1 | 99 | 503 |
| LF577 | Penicillium sp. | Penicillium brevicompactum isolated isolate NMG_25 | HM776430.1 | 99 | 534 |
| LF580 | Scopulariopsis brevicaulis | Scopulariopsis brevicaulis strain NCPF 2177 | AY083220.1 | 99 | 686 |
| LF581 | Fusarium sp. | Fusarium sp. NRRL 45996 | GQ505760.1 | 99 | 502 |
Table 1. Cont.

| Code | Species/Strain | Species/Strain | Accession | Similarity | Length |
|------|----------------|----------------|-----------|------------|--------|
| LF584 | Aspergillus sp. | 543 | Aspergillus sp. N13 | GQ169453.1 | 99 | 542 |
| LF590 | Penicillium sp. | 522 | Penicillium citreonigrum strain Gr155 | FJ904848.1 | 100 | 522 |
| LF592 | Paecilomyces sp. | 544 | Fungal endophyte sp. P1201A Paecilomyces lilacinus strain CG 271 | EU977225.1 | 99 | 541 |
| LF594 | Mycelia sterilia | 531 | Fusarium sp. FL-2010c Fusarium acuminatum strain NRRL 54217 | HQ166535.1 | 99 | 527 |
| LF596 | Penicillium sp. | 529 | Penicillium sp. FF24 Penicillium canescens strain QLF83 | FJ379805.1 | 100 | 529 |
| LF607 | Penicillium sp. | 532 | Penicillium sp. 17-M-1 Penicillium sclerotiorum strain SK6RN3M | EU076929.1 | 99 | 523 |
| LF608 | Cladosporium sp. | 495 | Fungal sp. mh2981.6 Cladosporium cladosporioide isolates CC | GQ996077.1 | 100 | 495 |
| LF610 | Clonostachys sp. | 528 | Fungal sp. mh2053.3 Bionectria ochroleuca isolate Rd0801 | GQ996069.1 | 99 | 524 |
| LF626 | Mycelia sterilia | 588 | Trichoderma cerinum isolate C.P.K 3619 | GU111565.1 | 99 | 586 |
| LF627 | Aspergillus sp. | 509 | Aspergillus sp. 4-1 | HQ316558.1 | 100 | 509 |
| LF629 | Mycelia sterilia | 510 | Cladosporium cladosporioide isolates F12 | HQ380766.1 | 99 | 509 |
| LF630 | Penicillium sp. | 518 | Penicillium brevicompactum isolate H66s1 | EF634441.1 | 100 | 518 |
| LF631 | Epicoccum nigrum | 514 | Epicoccum nigrum strain AZ-1 | DQ981396.1 | 99 | 512 |
| LF634 | Aspergillus sp. | 581 | Aspergillus terreus isolate UOA/HCPF 10213 | GQ461911.1 | 99 | 579 |
| LF644 | Clonostachys sp | 528 | Bionectria rossmaniae strain CBS 211.93 | AF210665.1 | 99 | 521 |
| LF646 | Mycelia sterilia | 510 | Cladosporium cladosporioide isolates F12 | HQ380766.1 | 99 | 509 |

A = anamorph; T = teleomorph; Alternaria (A) = Lewia (T); Aspergillus (A) = Petromyces (T) and Eurotium (T); Beauveria (A) = Cordyceps (T); Botrytis (A) = Botryotinia (T); Cladosporium (A) = Davidiella (T); Fusarium (A) = Gibberella (T); Clonostachys (A) = Bionectria (T); Trichoderma (A) = Hypocrea (T); Phoma = Pleurophoma (synonym); nt = nucleotides; * phylogenetic data to LF580 are derived from the 18S rRNA gene sequence.

First of all, morphological criteria enabled the identification of most of the fungal isolates at the genus level (Table 1). However, under the culture conditions applied, 11 strains did not produce spores and were designed as “mycelia sterilia”. A morphological classification of these strains was not possible. Despite the formation of spores, another four strains could not be classified on the basis of morphological characteristics.

In order to verify the results of the morphological examination and identify the strains at the species level, they were subjected to ITS1-5.8S-ITS2 gene sequence analysis. The results obtained from this sequence analysis corresponded well with those from the morphological identification (Table 1, Figure 2) and in addition allowed identification of those strains not identified microscopically. In most cases, the sequence data and the phylogenetic relationships allowed the identification at the species level. Results from BLAST search are depicted in Table 1. Taking together morphological and genetic characteristics, most isolates belong to the Ascomycotina with representatives of the fungal classes Dothideomycetes (24 isolates), Eurotiomycetes, (25 isolates), Sordariomycetes (30 isolates) and Leotiomycetes (1 isolate). Only a single isolate (strain LF538) was classified as belonging to the Mucoromycotina.
**Figure 2.** Phylogenetic consensus tree based on ITS1-5.8S-ITS2 gene sequences calculated by Bayesian inference assuming the general time reversible (GTR) model (6 substitution rate parameters, gamma-shaped rate variation, proportion of invariable sites). Isolates from *Tethya aurantium* obtained during this study are printed in bold. Numbers on nodes indicate Bayesian posterior probability values. nt = nucleotides.
Figure 2. Cont.

AY373880 *Aspergillus versicolor* strain CBS 583.65
LF073
LF627
LF553
LF554

EF652430 *Aspergillus granulosus* isolate NRRL 1932
LF501

EF652481 *Aspergillus minutus* isolate NRRL 4876
LF547

GQ169453 *Aspergillus sp.* N13
LF584

EF661556 *Petromyces alliaceus* isolate IBT 14317
LF491

AJ005673 *Petromyces albertensis* isolate IHB F 536
HM152566 *Eurotium chevalieri* isolate UPM A11
LF526

HQ148160 *Eurotium* sp. FZ

AY373915 *Penicillium glabrum* strain FRR 835
AY373933 *Penicillium spinulosum* strain FRR 1750
LF065

EU807940 *Penicillium sclerotiorum* strain SK6RN3M

GU566239 *Penicillium roseopurpureum* strain E2

GU388431 *Penicillium citroviride* isolate D5
FJ904848 *Penicillium citrinum* strain Gr155
LF590

FJ025212 *Penicillium canescens* strain QLF83
LF259

LF242
LF577
LF630

AY484912 *Penicillium brevicompactum* strain NRRL 2011
HM776430 *Penicillium brevicompactum* isolate NMG_25

LF243
AF455527 *Penicillium commune* isolate wb193
AY325984 *Penicillium expansum* strain VIC
LF249

AY373896 *Penicillium aethiopicum* strain FRR 2007
AY213669 *Penicillium chrysogenum* strain CBS 306.48
AB479305 *Penicillium chrysogenum* strain JCM 22826
LF066

AY373917 *Penicillium griseofulvum* strain FRR 3571
LF251

GQ241341 *Penicillium chrysogenum* strain ACBF 003-2
LF250
Figure 2. Cont.

AY904056 Cladosporium tenuissimum isolate 21L-211-Mexico
LF244
FN868485 Davidiella tassiana strain BLE25
AJ300332 Cladosporium oxysporium strain CBS 125.80
LF184
LF178
LF183
HM210839 Cladosporium cladosporioides strain CC1
LF608
AF393691 Cladosporium cladosporioides strain ATCC 200941
LF629
LF646
LF537
LF257
GU566258 Davidiella tassiana strain G20
AF455517 Cladosporium herbarum isolate wb221
AJ300335 Cladosporium cladosporioides strain CBS 169.54
GU017501 Cladosporium sphaerospermum isolate KH00280
LF643

FJ427056 Phoma pomorum var. pomorum strain CBS 539.66
LF542
HM776432 Peyronellea glomerata isolate NMG 27
FJ427057 Phoma pomorum var. pomorum strain PD 81/592
LF552
LF631
DQ981396 Epicoccum nigrum strain AZ-1
LF508
LF558
GU062248 Pyrenochaeta cava isolate I170
LF260

AJ608980 Fungal sp. GFI 146
FJ426987 Phoma chrysantemicola strain PD 92/468
AJ496632 Phaeosphaeria pontiformis strain CBS 589.86
LF177

AY154695 Alternaria tritici strain IA245
AY762947 Alternaria orygenensis strain EGS 29-194
AM176663 Lewia infectoria clone (25)5
LF240
FM958526 Alternaria infectoria strain CBS 210.86
AY762946 Alternaria metachromatica strain EGS 38-132
AY154705 Alternaria citri strain IA265
LF530
LF543
LF594741 Alternaria alternata strain 786949

LF255

AY642531 Paraconiothyrium brasiliense strain CBS 100299
LF179
GU985234 Paraphaeosphaeria sp. LF6
EF094552 Microsphaeropsis arundinis strain 04-359-2614

AB472081 Botryosphaeria sp. GU071005
DQ458898 Sphaeropsis sapinea strain CBS 109943
LF241

FJ433878 Saccharomyces boulardii
strain UOA/HCPF EM 10049
As shown in the phylogenetic tree (Figure 2), 10 isolates of the Dothideomycetes closely affiliated to fungal species of the order Capnodiales (Cladosporium spp. and its teleomorph Davidiella). Within the order Pleosporales (13 isolates) 5 isolates were closely related to Alternaria sp., including Lewia injectoria as teleomorph. From the same order we also isolated 2 strains of the species Epicocum nigrum and 1 Paraphaeosphaeria strain. Furthermore, 5 isolates of the genus Phoma were found, of which one isolate (strain LF258) shows only 91% sequence similarity to Septoria arundinacea as next relative according to BLAST results (Table 1) and presumably represents a new species in the Phoma lineage. The low similarity of this isolate to known sequences is also reflected in the position in the phylogenetic tree, clustering distinct from other Phoma species. A single isolate (strain LF241) was assigned to Sphaeropsis sapinea/Botryosphaeria sp. within the order Botryosphaeriales in the class Dothideomycetes.

All 25 isolates assigned to the class Eurotiomycetes were affiliated to the order Eurotiales and are represented by 14 isolates closely affiliating to Penicillium species (P. glabrum, P. virgatum/ P. brevicipactum, P. griseofulvum/P. commune, P. chrysogenum, P. sclerotiorum/P. citreonigrum, P. citreoviride/P. roseopurpureum, P. canescens), 10 representatives of the genus Aspergillus (including 2 of its teleomorphs, Petromyces alliaceus and Eurotium chevalieri) and 1 isolate of Paecilomyces.

The 30 isolates affiliated to the class Sordariomycetes were grouped into the orders Hypocreales (26 isolates), Microascales (2 isolates) and Xylariales (1 isolate). Within the Hypocreales, one isolate each was closely related to Beauveria bassiana (strain LF256), Tritirachium album (strain LF562), Verticillium sp. (strain LF496), and Volutella ciliata (strain LF246). The most frequent genera within this order were Fusarium (13 isolates), Trichoderma (5 isolates, including Hypocrea teleomorphs) and Clonostachys (4 isolates, including teleomorphs of Bionectria). The Microascales were represented by two isolates of the genus Scopulariopsis. One of these (strain LF064), morphologically classified as Scopulariopsis marina, was distantly related to the next cultured relative (89% according to BLAST search) and appears as a sister group to the Scopulariopsis lineage in the phylogenetic tree. The order Xylariales was represented by only a single isolate (strain LF550), Bartalinia robillardoides. As the only representatives of the order Helotiales within the class Leotiomycetes strain LF248 was closely related to Botryotinia fuckeliana.

The combination of microscopic and genetic analyses has proven to be a reliable method for the identification of fungal isolates and results from both approaches corresponded quite well (Table 1, Figure 2). The reliable identification of isolates is a fundamental prerequisite in order to characterize the producers of marine natural products [3], to determine the occurrence of fungal species in different habitats, and to correlate distinct secondary metabolite patterns to fungal species. Therefore, we highly recommend that morphological identification of fungal isolates consequently should be verified by molecular means and thus raise the number of reliably identified species in public databases.

A number of investigations, mainly with the aim of finding novel natural products, showed that most marine sponges harbor a plethora of cultivable fungi within their tissue [10–15,18]. Despite the large number of fungi isolated from sponges, a selective accumulation of specific taxa within sponges and a truly marine nature of these fungi is doubted, which is why they are commonly referred to as “marine-derived” [27,28]. In fact, it could be shown that the phylogenetic diversity of fungi isolated from different sponges varies [17]. It has also been stated that the taxa frequently isolated from
sponges resemble those described from terrestrial habitats [11,16,28]. This is in good accordance with our results, showing representatives of Acremonium, Aspergillus, Fusarium, Penicillium, Phoma, and Trichoderma to be abundant in Tethya aurantium and with those of Wang (2006) demonstrating that they also are widely distributed among different sponges from various locations [29]. Although it appears that the marine environment indeed provides various habitats for fungi, this has rarely been demonstrated. Nonetheless, due to the accumulation of fungi within sponges a large number of strains can be isolated which increases the probability to find representatives of less common taxa which might produce unprecedented secondary metabolites. For example, fungi belonging to the genera Beauveria, Botryosphaeria, Epicoccum, Tritirachium, and Paraphaeosphaeria have rarely been obtained from marine sponges [11,18,24] and, to the best of our knowledge, we have isolated Bartalinia sp. and Volutella sp. from a marine sponge for the first time.

Although evidence is presented that some bacterial symbionts of sponges are the producers of metabolites originally assumed to be produced by the sponge [4], equivalent evidence for fungi is lacking. There is actually little evidence for sponge-specific fungal associations and the only reports on this matter deals with the above mentioned Koralionastes species and a yeast living in symbiosis with the sponge Chondrilla sp. [30]. In fact, most of the studies had the biotechnological potential of sponge-derived fungi in mind but not the ecological role. Our culture-dependent approach was not considered to approach the aspect of specificity of the association with the sponge and molecular-based studies would be more suited to identify specifically sponge-associated fungi.

2.2. Secondary Metabolite Analyses

With the cultivation-based approach used in this study, we obtained a variety of strains from a surprisingly broad range of phylogenetic groups of fungi. A selection of the isolated and identified fungi was subjected to analysis of their secondary metabolite profiles. Strains were selected in order to represent a wide spectrum of genera, representatives of a variety of different genera, some known to include strains and species known for the production of secondary metabolites others from less common taxa. Some of the strains selected according to systematic criteria did not produce detectable amounts of secondary metabolites under the applied culture conditions. Unraveling their potential of secondary metabolite production will require more intense studies. Those extracts which did contain at least one compound in significant amounts were analyzed by HPLC with DAD (UV) and MS-detection and the metabolites which could be identified are listed in Table 2. A high percentage of the substances identified this way could be verified by $^1$H NMR spectroscopy (see Table 2). The majority of these metabolites have been reported from fungi before. Two of our previous reports on metabolites from fungi isolated from Tethya aurantium deal with the antiproliferative scopularides A–B [25,26] and the sorbifuranones A–C [31].
Table 2. Secondary metabolites identified in extracts of fungi isolated from the sponge *Tethya aurantium*.

| Genus                  | Strain | Compound                                      | Reported from                                                                 | Bioactivity | Method of dereplication |
|------------------------|--------|-----------------------------------------------|-------------------------------------------------------------------------------|-------------|-------------------------|
| *Alternaria*           | LF177  | infectopyrone                                 | Alternaria infectoria, Leptosphaeria maculans/Phoma lingum                   |             | UV, MS, NMR             |
|                        |        | phomenin A & B                                | Phoma tracheiphila, Leptosphaeria maculans/Phoma lingum, Ercolaria funera    | phytotoxin  | UV, MS, NMR             |
| *Aspergillus*          | LF627  | sterigmatocystin                              | Aspergillus versicolor, Chaetomium                                             | mycotoxin [32]| UV, MS                  |
|                        |        | totoamid D                                    | Aspergillus sp.                                                                |             | UV, MS                  |
|                        |        | steptacidin A                                 | Aspergillus ochraceus                                                          | cytotoxic   | UV, MS, NMR             |
|                        | LF547  | cinerein                                      | Botrytis cinerea                                                              | plant growth regulator, phytotoxin | UV, MS, NMR |
|                        |        | (2′E,4′E,6′E)-6-(1′-carboxyocta-2′,4′,6′-trien)-9-hydroxydrim-7-ene-11,12-olide | *Aspergillus usus*                                                           |             | UV, MS, NMR             |
|                        |        | (2′E,4′E,6′E)-6-(1′-carboxyocta-2′,4′,6′-trien)-9-hydroxydrim-7-ene-11-al | *Aspergillus usus*                                                           |             | UV, MS, NMR             |
|                        | LF553  | compound A                                    | hit in Scifinder [33], but no publication available                          |             | UV, MS, NMR             |
|                        |        | compound B                                    | no hit in database                                                            |             | UV, MS, NMR             |
|                        | LF584  | sydonic acid                                  | Aspergillus sydowii                                                           | weakly antibacterial [34]| UV, MS, NMR |
|                        |        | hydroxysydonic acid                           | Aspergillus sydowii                                                           |             | UV, MS                  |
|                        |        | WIN-6 6306                                    | *Aspergillus flavipes*                                                        | substance P antagonist, inhibition of HIV-1 integrase [35]| UV, MS       |
|                        |        | aspochalasines                                | *Aspergillus flavipes* and other *Aspergillus* sp.                            | antibiotic, moderately cytotoxic [36] | UV, MS, NMR |
| *Aspergillus*          | LF491  | isokotanin A–C                                | *Aspergillus alliaceus* and *Petromyces alliaceus*                            | moderate antiinsectan activities [37] | UV, MS, NMR |
| *Petromyces*           |        | 14-(N,N-Dimethyl-l-leucinyloxy)paspalinine     | *Aspergillus alliaceus*                                                       | potassium channel antagonist [38] | UV, MS, NMR |
|                        |        | nominine or a similar indoloditerpene         | *Aspergillus nomius, Aspergillus flavus, Petromyces alliaceus                 | insecticidal properties | UV, MS, NMR |

Note: *Method of dereplication* includes UV, MS, or NMR.
| Phoma          | LF258 | monocerin | *Helminthosporium monoceras, Fusarium larvarum, Dreschlera ravenelii, Exserohilum rostratum, Readeriella mirabilis* | antifungal, insecticidal and phytotoxic properties | UV, MS |
|---------------|-------|------------|-------------------------------------------------------------------------------------------------------------|--------------------------------------------------|--------|
|               |       | intermediate in the bio-synthesis of monocerin | *Dreschlera ravenelii*                                                                 |                                                  | UV, MS, NMR |
|               |       | evernin- or isoeverninaldehyde | *Guignardia laricina* | weak phytotoxin | UV, MS, NMR |
| Epicoccum     | LF552 | epicoccamide | *Epicoccum purpureascens and other Epicoccum sp.*, *Aurelia aurita* | binding inhibitor of HIV-1 rev protein to Rev response element (RRE) | UV, MS |
|               |       | orevactane | *Epicoccum nigrum* | | UV, MS |
| Eurotium      | LF526 | echinulin | *Eurotium repens, Aspergillus amstelodami, Aspergillus echinulatus, Aspergillus glaucus* | experimentally hepatic and pulmonary effects | UV, MS |
|               |       | neoechinulines | *Aspergillus amstelodami* | antioxidative activity | UV, MS |
|               |       | auroglaucines and flavoglaucine | *Aspergillus and Eurotium spp.* | mycotoxin, shows antineo-plastic properties [39] | UV, MS |
| Fusarium      | LF236 | equisetin | *Fusarium equiseti and Fusarium heterosporum* | antibacterial activity, inhibition of HIV-1 integrase | UV, MS, NMR |
|               | LF238 | equisetin | *Fusarium equiseti and Fusarium heterosporum* | antibacterial activity, inhibition of HIV-1 integrase | UV, MS, NMR |
|               |       | fusarins | *Fusarium moniliforme* | mutagenic [40] | UV, MS |
|               | LF594 | enniatine | various *Fusarium* sp. | ionophore, insecticidal, ACAT inhibition, GABA receptor binding | UV, MS |
| Paecilomyces  | LF592 | leucinostatins | *Paecilomyces lilacinus and other Paecilomyces* sp. | active against Gram-positive bacteria and fungi | UV, MS |
| Compound   | Description                                                                 | Properties                                                                 |
|------------|------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| compound C| no hit in database                                                           | UV, MS, NMR                                                                |
| meleagrin  | *Penicillium meleagrinum* and *Penicillium chrysogenum*                      | structurally similar to tremorgenic mycotoxins                            |
| roquefortin C | *Penicillium roquefortii* and other *Penicillium sp.*                       | neurotoxin                                                                |
| sorbifuranones A–C | *Penicillium chrysogenum* [31]                                               | UV, MS, NMR                                                                |
| 2′,3′-dihyrosorbcilllin | *Penicillium notatum, Verticillium intertextum*                           | weakly antibacterial [41]                                                  |
| bisvertonolone   | *Penicillium chrysogenum, Verticillium intertextum,* Acremonium strictum, Trichoderma longibrachiatum | β-1,6glucan biosynthesis inhibitor, antioxidative, inducer of hyphal malformation in fungi |
| ergochromes     | Aspergillus ochraceus, Claviceps purpurea, Aspergillus aculeatus, Gliocladium sp., Penicillium oxalicum, Phoma terrestris, Pyrenochaeta terrestris | teratogenic effects                                                       |
| mycophenolic acid | *Penicillium brevicompactum* and other *Penicillium sp.*                   | antineoplastic, antiviral immunosuppressant properties, useful in treating psoriasis and leishmaniasis, |
| citreoviridins | *Penicillium citreoviride, Penicillium toxicarium, Penicillium ochrosalmoneum, Aspergillus terreus | neurotoxic                                                                |
| territrem B    | *Penicillium sp. and Aspergillus terreus*                                    | inhibitor of acetylcholinesterase                                          |
| sclerotiorin   | *Penicillium sclerotiorum* and *Penicillium multicolor*                     | inhibits cholesterol ester transfer protein activity                      |
| sclerotioramine|                                                                              | UV, MS, NMR                                                                |
| compound D    | no hit in database                                                           | UV, MS, NMR                                                                |
### Table 2. Cont.

| Source     | Metabolite     | Compound                | Properties                                      | Spectra                  |
|------------|----------------|-------------------------|-------------------------------------------------|--------------------------|
| Penicillium | LF596          | griseofulvin            | Penicillium griseofulvin and other Penicillium sp. | antifungal, possible human carcinogen | UV, MS, NMR |
|            |                | tryptothiolin           | Aspergillus clavatus                            | tremorgenic toxin        | UV, MS       |
|            |                | nortryptothiolin        | Aspergillus clavatus and Aspergillus fumigatus  | tremorgenic toxin        | UV, MS       |
|            |                |                         | fimalins A and C                                | substance P inhibitor, neurokinin binding inhibitor | UV, MS, NMR |
| Scopulariopsis | LF580       | scopularide A and B     | Scopulariopsis brevicaulis                      | antiproliferative [25,26] | UV, MS, NMR |
| Clonostachys | LF254         | T-988B                  | Tilachlidium sp.                               | cytotoxic                | UV, MS, NMR |
|            |                |                         | bionectin B                                    | antibacterial (MRSA)     | UV, MS, NMR |
|            |                | verticillin C           | Verticillum sp.                                 | antibiotic               | UV, MS       |

* According to the Dictionary of Natural Products [42] if not stated otherwise; b blank cells indicate that no entry concerning bioactivity in the Dictionary of Natural Products was available and no report on bioactivity was found.

For four compounds, database searches [33,42–43] did not lead to a hit (B, C, D) or no publication was available (A). The structure elucidations of compounds A and B, metabolites with modified diketopiperazine substructures, and compound D, a new azaphilone derivative, are in progress. Compound C was identified as the new metabolite cillifuranone and its structure is described in the following.

*Penicillium* strain LF066, the producer of cillifuranone, was singled out for further investigations, because first surveys proved it to be a very potent producer of secondary metabolites. From the same strain, sorbifuranone B and C as well as 2′,3′-dihydroxosorbiticillin have already been described by Bringmann *et al.* [31] and it was obvious that the full potential of the strain had not been exploited, yet. Further analysis led to the detection of xanthocillines and sorbifuranones and the isolation of sorbifuranone B, meleagrin, roquefortin C, a couple of ergochromes as well as the new cillifuranone (1) whose structure was elucidated based on 1D and 2D NMR experiments.

The $^{13}$C NMR spectrum of 1 displayed 10 clearly distinguishable carbon signals which was in good agreement with the molecular formula C$_{10}$H$_{12}$O$_{4}$, deduced from the result of a HRESI-MS measurement (calculated for C$_{10}$H$_{12}$O$_{4}$Na 219.0628, measured 219.0627). The carbon signals included resonances belonging to three carbonyl or enol carbons ($\delta$C 170.9, 196.7 and 201.7), three sp$^3$ hybridized methylene carbons ($\delta$C 20.9, 31.6 and 76.3), one methyl group ($\delta$C 14.0), two olefinic methines ($\delta$C 118.5 and 133.0) and finally one quaternary olefinic carbon ($\delta$C 112.6). The structure of the molecule could be delineated from 1D ($^1$H, $^1$C and DEPT) and 2D NMR ($^1$H-$^1$C HSQC, $^1$H-$^1$H COSY and $^1$H-$^{13}$C HMBC) spectra. From the $^1$H-$^1$H COSY spectrum two separate spin systems could be identified. The first one consisted of the olefinic methine groups CH-9 ($\delta$C 133.0, $\delta$H 7.32) and CH-10 ($\delta$C 118.5, $\delta$H 6.83), forming an E-configured double bond as proven by their $^3$J coupling constant of 16 Hz. The corresponding protons H-9 and H-10 both showed $^1$H-$^{13}$C HMBC correlations to the carboxyl carbon C-11 ($\delta$C 170.9) as well as to the quaternary carbons of the furanone ring, C-3 ($\delta$C 201.7) to C-5 ($\delta$C 196.7). C-3 was the ketone carbonyl group included in the furanone substructure which was in accordance with its chemical shift. C-4 ($\delta$C 112.6) and C-5 were also part of the furanone and formed a tetrasubstituted double bond in which C-5 was located adjacent to an.
oxygen atom. Compared to an unsubstituted enol, the resonance of C-5 was shifted further downfield due to the conjugation of the double bond $\Delta^4,5$ with the carbonyl carbon C-3. Apart from C-9 of the double bond $\Delta^{9,10}$, the carbonyl carbon C-3 and the oxygen atom of the furanone ring, $\Delta^4,5$ was also connected to C-6 of the second spin system consisting of the methylene groups CH$_2$-6 ($\delta_C$ 31.6, $\delta_H$ 2.76) and CH$_2$-7 ($\delta_C$ 20.9, $\delta_H$ 1.76) as well as the methyl-group CH$_3$-8 ($\delta_C$ 14.0, $\delta_H$ 1.03). Thus, the second spin system evidently was an n-propyl-chain. The furanone ring was completed with the methylene group CH$_2$-2 ($\delta_C$ 76.3, $\delta_H$ 4.67). Its $^1$H and $^{13}$C shifts proved it to be linked to an oxygen atom, the $^1$H-$^{13}$C HMBC correlations to C-3 and C-5 secured its exact position. Thus, the structure of cillifuranone (1) could be unambiguously determined (Figure 3, Table 3).

**Figure 3.** Spin systems deduced from the $^1$H-$^1$H COSY spectrum (bold) and selected $^1$H-$^{13}$C HBMC correlations (arrows) relevant to the structure elucidation of cillifuranone (1).

![Image of chemical structure](image)

Table 3. NMR spectroscopic data of cillifuranone (1) in methanol-d4 (500 MHz).

| Position | $\delta_C$, mult. | $\delta_H$, (J in Hz) | COSY | HMBC |
|----------|------------------|-----------------------|------|------|
| 1        |                  |                       |      |      |
| 2        | 76.3, CH$_2$     | 4.67, s               | 6    | 3, 5, 6, 7 |
| 3        | 201.7, C         |                       |      |      |
| 4        | 112.6, C         |                       |      |      |
| 5        | 196.7, C         |                       |      |      |
| 6        | 31.6, CH$_2$     | 2.76, t (7.5)         | 2, 7 | 4, 5, 7, 8 |
| 7        | 20.9, CH$_2$     | 1.76, sext. (7.5)     | 6, 8 | 5, 6, 8 |
| 8        | 14.0, CH$_3$     | 1.03, t (7.5)         | 7    | 6, 7 |
| 9        | 133.0, CH        | 7.32, d (16.0)        | 10   | 2, 3, 4, 5, 10, 11 |
| 10       | 118.5, CH        | 6.83, d (16.0)        | 9    | 3, 4, 5, 9, 11 |
| 11       | 170.9, C         |                       |      |      |

Cillifuranone (1) was tested in a panel of bioassays evaluating the compound with respect to cytotoxic, antimicrobial and enzyme inhibitory activity. Very low activity was only found against *Xanthomonas campestris* (24% growth inhibition) and *Septoria tritici* (20% growth inhibition) at a concentration of 100 µM.

Strain LF066 was identified as *Penicillium chrysogenum*, a species that in our experience often produces metabolites deriving from sorbicillinol as a biosynthetic precursor (sorbicillinoids). The detection of bisvertinolone and the sorbifuranones in culture extracts of the fungus was consistent with this experience. Furanone substructures are abundant in natural products and can be found in
metabolites from bacteria, fungi and plants [42] and presumably are products from different biosynthetic pathways [44–46]. From the genus *Penicillium* a number of furanone containing compounds has been described, including simple small molecules like penicillic acid, but also more complex ring structures such as the rotiorins [47] or rugulovasines [48]. In most cases the furanone ring is a furan-2(5*H*)-one, whereas in cillifuranone (1) we have a furan-3(2*H*)-one. With that differentiation being made the number of related compounds gets fewer, furan-3(2*H*)-ones do not seem to be as ubiquitous as the furan-2(5*H*)-ones. From *Penicillium* strains, apart from the above mentioned sorbitifuranones, berkeleamide D [49] and trachyspic acid [50], both spiro compounds like sorbitifuranone C, are examples. The new cillifuranone represents a substructure of the sorbitifuranones, albeit with a different configuration of the exocyclic double bond, and represents the substructure which makes the sorbitifuranones unique in the compound class of the sorbicillinoids. Just recently Bringmann *et al.* [31] published the structures of the sorbitifuranones and postulated 1 to be an intermediate in their biosynthesis (Figure 4) which makes the isolation of 1 a very interesting result. The only difference between the postulated intermediate and our structure is, as stated above, the configuration of the double bond. However, in the crude extract of the strain, we detected two isomers with the same molecular weight and a very similar UV-spectrum, so that we assume that both isomers were present, but after the isolation process only the *E*-isomer was obtained, so that it might be the favoured configuration under the applied conditions.

**Figure 4.** Biosynthesis of sorbitifuranone A via Michael reaction of an isomer of cillifuranone (1) and sorbicillinol as postulated by Bringmann *et al.* (modified from [31]).
3. Experimental Section

3.1. Sampling Sites

The Limsky kanal (Canal di Lemme or Limsky channel) is a semi-closed fjord-like bay in the Adriatic Sea nearby Rovinj (Istrian Peninsula, Croatia). It is situated along an east-west axis, with an approximate length of 1 km and a maximum width of about 650 m and reaches a maximum depth of 32 m [51]. The sampling site was located at N45°7972’ and E13°43,734’.

3.2. Sponge Collection

Several specimens (13) of the Mediterranean sponge *Tethya aurantium* were collected by scuba diving. They were obtained in April 2003, June 2004, May 2005 and August 2006 in a depth of 5–15 m. The sponges were collected into sterile plastic bags, cooled on ice and transported immediately to the laboratory, where they were washed three times with sterile filtered seawater (0.2 µm). The sponge tissue was then cut into small pieces of approximately 0.1 cm³ each, which were either placed directly onto GPY agar plates (LF236 to LF255) or homogenized and diluted with membrane-filtered seawater (all other isolates).

3.3. Isolation, Cultivation and Storage of Fungi

Fungi were isolated on a low nutrient GPY agar, based on natural seawater of 30 PSU, containing 0.1% glucose, 0.05% peptone, 0.01% yeast extract and 1.5% agar. Small pieces of sponge tissue or 50 µL of the homogenate (undiluted or 1:10 or 1:100 diluted with sterile seawater) were used as inoculum. The agar plates were incubated for periods of 3 days to 4 weeks and were checked regularly for fungal colonies, which were then transferred to GPY agar plates. Pure cultures were used for morphological identification by light microscopy and for scanning electron microscopy. Fungal isolates were stored as agar slant cultures at 5 °C, and additionally were conserved at –80 °C using Cryobank vials (Mast Diagnostica).

3.4. Morphological Identification of Fungal Isolates

The morphology of GPY agar grown fungal isolates was studied using a stereo microscope (10–80× magnification) and a phase-contrast microscope (300–500× magnification). By this method, the majority of spore-producing isolates could be identified up to the generic level using the tables of Barnett and Hunter [52] and, for more detailed descriptions, the site of MycoBank [53]. Morphological identification of the selected pure cultures was supported by light microscopy and scanning electron microscopy.

3.5. Scanning Electron Microscopy

For electron microscopy, young GPY agar colonies were cut in 1 cm² samples, transferred through an ethanol series (30, 50, 70, 90, 3 × 100%; each 15 min) and subsequently critical-point-dried in liquid carbon dioxide (Balzers CPD030). Samples were sputter-coated with gold-palladium (Balzers SCD004) and analyzed with a ZEISS DSM 940 scanning electron microscope.
3.6. Genetic Identification of Fungal Isolates and Phylogenetic Analysis

DNA-extraction was performed using the Precellys 24 system (Bertin Technologies). To one vial of a Precellys grinding kit with a glass beads matrix (diameter 0.5 mm, peqlab Biotechnologies GmbH) 400 µL DNase-free water (Fluka) were added. Cell material from the fungal culture was then transferred to this vial and homogenized two times for 45 s at a shaker frequency of 6500. The suspension was centrifuged at 6000 g for 10 min and 15 °C. The supernatant was stored at −20 °C until further use in PCR.

Fungal specific PCR by amplifying the ITS1-5.8S rRNA-ITS2 fragment was carried out using puReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare) with the ITS1 (5’-TCC GTA GGT GAA CCT GCG G-3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) primers according to White et al. [54]. PCR was conducted as follows: initial denaturation (2 min at 94 °C), 30 cycles of primer denaturation (40 s at 94 °C), annealing (40 s at 55 °C), and elongation (1 min at 72 °C) followed by a final elongation step (10 min at 72 °C). PCR products were checked for correct length (complete ITS1, 5.8S rRNA and ITS2 fragment length of *Penicillium brevicompactum* strain SCCM 10-I3 (EMBL-acc. No. EU587339 is 494 nucleotides), a 1% agarose gel in 1x TBE buffer (8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA).

PCR products were sequenced using the ABI PRISM® BigDye™ Terminator Ready Reaction Kit (Applied Biosystems) on an ABI PRISM® 310 Genetic Analyzer (Perkin Elmer Applied Biosystems). The ITS1 primer was used for sequencing. Sequence data were edited with ChromasPro Version 1.15 (Technelysium Pty Ltd.). Sequences from fungal strains obtained during this study were submitted to the EMBL database and were assigned accession numbers (FR822769-FR822849). Closest relatives were identified by sequence comparison with the NCBI Genbank database using BLAST (Basic Local Alignment Search Tool) [55]. Sequences were aligned using the ClustalX version 2.0 software [56] and the alignment was refined manually using BioEdit (version 7.0.9.0) [57]. For alignment construction, ITS1-5.8S-ITS2 gene fragments from closest cultured relatives according to BLAST as well as type strains were used, whenever possible. However, not from all fungal species, ITS sequences from type strains were available in NCBI/Genbank. The ITS1-5.8S-ITS2 gene sequence of *Saccharomyces boulardii* strain UOA/HCPF EM10049 (acc. No. FJ433878) was used as outgroup sequence for phylogenetic calculations. Phylogenetic calculations were performed with all closest relatives according to BLAST results (data not shown). For clarity, not all of these sequences were included in Figure 2. Phylogeny was inferred using MrBayes version 3.1 [58,59], assuming the GTR (general time reversible) phylogenetic model with 6 substitution rate parameters, a gamma-shaped rate variation with a proportion of invariable sites and default priors of the program. 1,000,000 generations were calculated and sampled every 1000th generation. Burn-in frequency was set to 25% of the samples. The consensus tree was edited in Treeview 1.3 [60].

3.7. Fermentation and Production of Extracts

The fungi were inoculated in 2 L Erlenmeyer flasks containing 750 mL modified Wickerham-medium [61], which consisted of 1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 3% sodium chloride (pH = 6.8). After incubation for 11–20 days at 28 °C in the
dark as static cultures, extracts of the fungi were obtained. The mycelium was separated from the culture medium and extracted with ethanol (150 mL). The fermentation broth was extracted with ethyl acetate (400 mL). Both extracts were combined. Alternatively, cells and mycelium were not separated and the culture broth was extracted together with the cells of some cultures using ethyl acetate. After evaporation of the solvents, the powdery residue was reextracted with ethyl acetate (100 mL). The resulting residues were dissolved in 20 mL methanol and subjected to analytical HPLC-DAD-MS.

3.8. Chemical Analysis

UV-spectra of the identified metabolites were obtained on a NanoVue (GE Healthcare). NMR spectra were recorded on a Bruker DRX500 spectrometer (500 and 125 MHz for $^1$H and $^{13}$C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references ($\delta_\text{H}$ 3.31 ppm and $\delta_\text{C}$ 49.0 ppm for methanol-d4). High-resolution mass spectra were acquired on a benchtop time-of-flight spectrometer (MicrOTOF, Bruker Daltonics) with positive electrospray ionization. Analytical reversed phase HPLC-DAD-MS experiments were performed using a C$_{18}$ column (Phenomenex Onyx Monolithic C18, 100 × 3.00 mm) applying an H$_2$O (A)/MeCN (B) gradient with 0.1% HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL/min) on a VWR Hitachi Elite LaChrom system coupled to an ESI-ion trap detector (Esquire 4000, Bruker Daltonics).

Preparative HPLC was carried out using a VWR system consisting of a P110 pump, a P311 UV detector, a smartline 3900 autosampler and a Phenomenex Gemini-NX C18 110A, 100 × 50 mm, column or a Merck Hitachi system consisting of an L-7150 pump, an L-2200 autosampler and an L-2450 diode array detector and a Phenomenex Gemini C18 110A AXIA, 100 × 21.20 mm, column.

For the preparation of cillifuranone (1), the same solvents were used as for the analytical HPLC, with a gradient from 10% B, increasing to 60% B in 17 min, to 100% B from 17 to 22 min. 1 eluted with a retention time of 6.8 min and the amount of 23.2 mg of 1 could be obtained from a culture volume of 10 L.

Properties of cillifuranone (1): pale yellow needles; UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 278 (4.36); for 1D and 2D NMR data see Table 3 and SI; HRESIMS m/z 219.0627 (calcd for C$_{10}$H$_{12}$O$_3$Na 219.0628).

3.9. Bioassays

Possible antimicrobial effects of cillifuranone (100 µM) were tested against Bacillus subtilis (DSM 347), Brevibacterium epidermidis (DSM 20660), Dermabacter hominis (DSM 7083), Erwinia amylovora (DSM 50901), Escherichia coli K12 (DSM 498), Pseudomonas fluorescens (NCIMB 10586), Propionibacterium acnes (DSM 18977), Pseudomonas aeruginosa (DSM 50071), Pseudomonas syringae pv. aptata (DSM 50252), Staphylococcus epidermidis (DSM 20044), Staphylococcus lentus (DSM 6672), Xanthomonas campestris (DSM 2405), Candida albicans (DSM 1386), Phytophthora infestans, and Septoria tritici according to Schneemann et al. [62]. In addition, cytotoxic activity of cillifuranone (50 µM) towards the human hepatocellular carcinoma cell line HepG2 and the human colon adenocarcinom cell line HT29 were performed according to Schneemann et al. [62]. Inhibitory activities of cillifuranone (10 µM) against the enzymes
acetylcholinesterase, phosphodiesterase (PDE-4B2), glycogen synthase kinase 3β, protein tyrosin phosphatase 1B, and HIV-1 reverse transcriptase were tested according to Helaly et al. [63].

4. Conclusion

The marine sponge *T. aurantium* was found to be a valuable source of secondary metabolite producing fungi. In addition to a variety of known substances, several new natural products were found and it is likely that additional ones can be identified during further studies. The antiproliferative active scopularides [25,26] were the first new metabolites from a *Scopulariopsis* species isolated from *T. aurantium*. The new cillifuranone (I) is a second example of a natural product produced by a fungal isolate from *T. aurantium*. Additional compounds were detected of which the chemical structures are not yet described. The application of alternative cultivation methods, which have not been used so far, are expected to further increase the spectrum of produced metabolites of our isolates obtained from *T. aurantium*.

The combination of morphological criteria and the results of the ITS1-5.8S-ITS2 fragment sequencing have been proven to be a valuable tool for the identification of fungal isolates. Apart from representatives of genera, which are widely distributed in terrestrial samples and in addition also reported from different sponges, we also identified members of taxa which so far have not been described to be associated with sponges. These strains distantly affiliated to *Bartalinia* sp. and *Votutella* sp. and one strain most likely is a new *Phoma* species.

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