# METABOLITES FROM THE MARINE FUNGUS *Eurotium repens*

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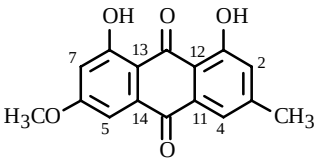
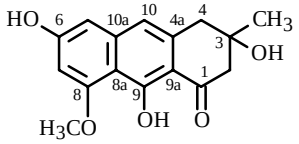
*1,8-Dihydroxy-6-methoxy-3-methyl-9,10-anthracenedione (physcion, **1**), 3,4-dihydro-3,6,9-trihydroxy-8-methoxy-3-methyl-1(2H)-anthraceneone (asperflavin, **2**), and 2,5-dihydroxy-3-(3-methyl-2-butenyl)-6-[(1*E*)-1-heptenyl]-benzaldehyde (tetrahydroauroglaucin, **3**) were shown to be the main pigments of the marine isolate of the fungus* Eurotium repens. *In addition to the pigments, the fungal metabolites included the diketopiperazine alkaloid echinulin (**4**). The structures of the compounds were identified using NMR spectroscopy and mass spectrometry. The cytotoxic activity of **1**-**3** toward sex cells of the sea urchin* Strongylocentrotus intermedius *was determined.*

**Key words:** marine fungus-micromycetes, *Eurotium repens*, pigments, echinulin, cytotoxic activity.

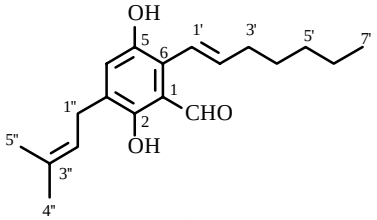
Metabolites from marine isolates of fungi-micromycetes belong to many classes of natural compounds including alkaloids, peptides, polyketides, shikimate-derivative metabolites, and compounds of mixed biogenesis [1-4]. In continuation of research on biologically active compounds in extracts of marine fungi, we discovered that the fungus *E. repens* isolated from the sponge *Suberites domuncula* (Zelenyi Island, Kuril Islands) synthesizes a series of pigments that differ in chromatographic mobility and color. Biologically active metabolites from *E. repens*, which is parasitic [5], have been reported. It seemed interesting to compare metabolites and pigments from two strains of the fungus isolated from different ecological habitats. The ethylacetate extract of the fungus, which contained a mixture of pigments, was cytotoxic against sex cells of the sea urchin *S. intermedius* and exhibited anti-staphylococcus activity. Herein we report the structures of pigments (**1**-**3**) and a diketopiperazine alkaloid (**4**) from the marine isolate of the fungus *E. repens*.



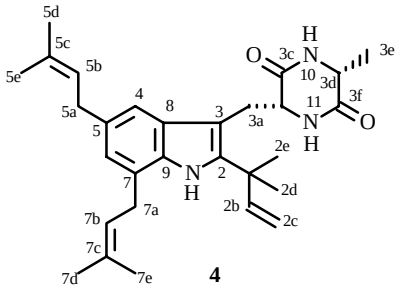

**1**




**2**




**3**




**4**

The total ethylacetate extract of the fungus produced hexane, $CHCl_3$, and butanol fractions. Separation of the $CHCl_3$ fraction over silica gel using a hexane:ethylacetate gradient with increasing polarity produced pure **1**-**4**.

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TABLE 1. NMR Spectra ($CDCl_3$, δ, ppm, J/Hz) of **1**

| C atom | $\delta_C$ | DEPT | $\delta_H$ | HMBC | C atom | $\delta_C$ | DEPT | $\delta_H$ | HMBC |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 162.5 | C | | | 9 | 190.8 | C | | |
| 2 | 124.5 | CH | 7.08 (br.s) | 4, 12, 15, 1 | 10 | 182.0 | C | | |
| 3 | 148.4 | C | | | 11 | 133.2 | C | | |
| 4 | 121.3 | CH | 7.62 (br.s) | 2, 10, 12, 15 | 12 | 113.7 | C | | |
| 5 | 108.2 | CH | 7.36 (d, 2.5) | 7, 10, 13, 6, 14 | 13 | 110.3 | C | | |
| 6 | 166.5 | C | | | 14 | 135.2 | C | | |
| 7 | 106.8 | CH | 6.69 (d, 2.5) | 5, 6, 13, 8 | 15 | 22.1 | $CH_3$ | 2.45 (s) | 2, 3, 4 |
| 8 | 165.2 | C | | | 16 | 56.1 | $OCH_3$ | 3.94 (s) | 6 |

TABLE 2. $^{13}C$ NMR and PMR Spectra ($CDCl_3$, δ, ppm , J/Hz) of **3**

| C atom | $\delta_C$ | DEPT | $\delta_H$ | HMBC[a] | C atom | $\delta_C$ | DEPT | $\delta_H$ | HMBC[a] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 117.2 | C | | | 4′ | 28.7 | $CH_2$ | 1.52 (m) | 2′, 3′, 5′, 6′ |
| 2 | 155.1 | C | | | 5′ | 31.4 | $CH_2$ | 1.37 (m) | |
| 3 | 130.4 | C | | | 6′ | 22.4 | $CH_2$ | 1.37 (m) | |
| 4 | 125.1 | CH | 7.02 (s) | 1″, 2, 5, 6 | 7′ | 14.0 | $CH_3$ | 0.92 (t, 6.8) | 5′, 6′ |
| 5 | 144.8 | C | | | 1″ | 27.2 | $CH_2$ | 3.31 (d, 7.4) | 2, 2′, 3, 3″, 4 |
| 6 | 124.0 | C | | | 2″ | 121.0 | CH | 5.30 (br.t, 7.4) | 1″, 4″, 5″ |
| 7 | 196.2 | CH | 10.10 (s) | 1, 2, 3, 6 | 3″ | 133.9 | C | | |
| 1′ | 120.1 | CH | 6.47 (br.d, 16.2) | 3′, 5, 6 | 4″ | 25.8 | $CH_3$ | 1.76 (s) | 2″, 3″, 5″ |
| 2′ | 142.7 | CH | 5.98 (dt, 16.1, 6.9) | 3′, 4′, 6 | 5″ | 17.7 | $CH_3$ | 1.70 (s) | 2″, 3″, 4″ |
| 3′ | 33.4 | $CH_2$ | 2.30 (br.q, 7.1) | 1′, 2′, 4′, 5′ | | | | | |

The electron-impact mass spectrum (EIMS) of **1** gave a peak at 284 Da. The UV spectrum of **1** had absorption maxima at 226, 253, and 435 nm and confirmed that anthraquinone was the main fragment [6]. The $^{13}C$ NMR spectrum contained resonances at 190.8 (C-9) and 182.0 (C-10) ppm, which indicated a quinone was present, and at 165.2, 166.5, and 162.5 ppm for three oxygenated aromatic C atoms. The PMR spectrum confirmed that **1** contained two pairs of protons situated in *meta*-positions to each other in two tetrasubstituted benzene rings (Table 1). Mass spectrometry and NMR spectroscopy showed that **1** was identical to physcion, an anthraquinone pigment isolated from various terrestrial macroorganisms and micromycetes [5, 7, 8]. Physcion is known to be a potent antibacterial [7]. According to our data, physcion exhibits cytotoxic activity against sex cells of the sea urchin *Strongylocentrotus intermedius* at a concentration of 25 μg/mL. The observation of physcion in the extract of the marine isolate of a fungus is indicative of the important ecological role of anthraquinone pigments, which protect host organisms from external bacterial infections.

The EIMS of **2** gave a peak at 288 Da. The chemical shifts of resonances and the spin—spin coupling constants of two protons (H-5 and H-7) in the PMR spectrum of **2** indicated that the protons were located on a single aromatic ring in the *meta*-position to each other ($\delta_H$ 6.50, d, J = 2.2 Hz; $\delta_H$ 6.40, d, J = 2.2 Hz). The position of the third aromatic proton on C-10 was established from the HMBC spectrum of **2**, which revealed correlations of this proton with C-4, C-5, C-8a, C-9a, and C-10a. The molecular weight and the presence of nine quaternary C atoms in its $^{13}C$ NMR spectrum indicated that **2** contained three fused six-membered rings, two of which were aromatic and one of which was saturated and disubstituted. The substituents in this polycyclic system were located on C-1 ($\delta_C$ 201.9, carbonyl), C-3 ($\delta_C$ 70.4, $CH_3$- and OH-), C-6 and C-9 ($\delta_C$ 159.9, 165.4, OH-), and C-8 ($\delta_C$ 161.5, methoxyl). The physicochemical properties of **2** suggested that it was identical to asperflavin, which was isolated from terrestrial fungi *Microascus tardifaciens* [9] and *Aspergillus flavus* [10]. The difference in the specific rotation of asperflavin isolated by us and that reported in the literature [9, 10] indicates that we isolated **2** as a racemic mixture. Compound **2** exhibited cytotoxic activity against sex cells of the sea urchin *S. intermedius* at a concentration of 10 μg/mL.

The EIMS of **3** gave a molecular weight of 302 Da. The UV and $^{13}C$ NMR spectra of **3** (Table 2) were consistent with the presence of a pentasubstituted benzene ring (C-1, 117.2; C-2, 155.1; C-3, 130.4; C-4, 125.1; C-5, 144.8; C-6, 124.0). The presence of an isopentenyl substituent on C-3 was confirmed by the PMR ($CH_3$-4″, 1.70, s; $CH_3$-5″, 1.76, s; H-2″, 5.30, t; H-1″, 3.31, d) and HMBC spectra. The presence of a singlet for an aldehyde proton at weak field in the PMR spectrum of **3** (10.10 ppm, H-7) and the mass spectrum indicated that **3** was an auroglaucin analog, which are metabolites of the fungus *Aspergillus rubber* [11] that often parasitizes food products. The mass spectrum and NMR spectra determined that **3** was identical to tetrahydroauroglaucin [11-13]. Compound **3** exhibited cytotoxic activity against sex cells of the sea urchin *S. intermedius* at a concentration of 0.5 μg/mL, i.e., it was a spermicide.

The EIMS of **4** showed a molecular weight of 461 Da. The molecular weight suggested that **4** should have an uneven number of N atoms. The PMR spectrum contained two broad singletss for protons of two CONH groups ($\delta_H$ 5.65 and 6.06). The presence of these groups was confirmed by the presence in the $^{13}C$ NMR spectrum of two amide carbonyl C atoms at 168.4 (C-3c) and 167.5 (C-3f). Another proton with HMBC correlations with C-2 ($\delta_C$ 141.4), C-3 ($\delta_C$ 104.1), C-8 ($\delta_C$ 128.9), and C-9 ($\delta_C$ 132.2) was located on a N atom in an indole ring. These data and the presence of a $\alpha,\alpha$-dimethylallyl group ($\delta_H$ 1.51, 6H, $\delta_C$ 27.8, 27.9; $\delta_H$ 5.15, 2H, m, $\delta_C$ 112.3; $\delta_H$ 6.10, 1H, dd, $\delta_C$ 145.7) and two isopentenyl substituents indicated that **4** belonged to the echinulin series of metabolites, which are characteristic of certain higher plants and fungi [14-18]. The mass spectrum, NMR spectra, and specific rotation were in excellent agreement with those of echinulin isolated from higher plants [14].

Thus, we observed a difference in the metabolite composition of the marine isolate of *E. repens* and this fungus species isolated from terrestrial sources [5]. The biosynthesis of physcion (**1**) and echinulin (**4**) is apparently a signature for *E. repens* regardless of the habitat. Asperflavin (**2**) and tetrahydroauroglaucin (**3**) are synthesized only by the marine isolate of the fungus and are not observed among the metabolites of terrestrial *E. repens*.

## EXPERIMENTAL

PMR and $^{13}C$ NMR spectra in $CDCl_3$ were recorded on Bruker DRX-500 and Bruker DPX-300 spectrometers; mass spectra, in Varian MAT 371 (70 eV) and Varian 311A (70 eV) spectrometers; UV spectra, on a Shimadzu (Japan) UV-1601 PC spectrophotometer. IR spectra of solutions in $CHCl_3$ were obtained on a Specord M82 (Carl Zeiss, Jena) instrument. Melting points were measured on a Leica Galen III instrument; optical rotation, on a Perkin—Elmer 141 polarimeter. TLC was performed on Sorbfil CTX-1A (ZAO Sorbpolimer) silica-gel plates using toluene:isopropanol (6:1); column chromatography, over silica gel L (40/100 μm, Czechoslovakia) with elution by hexane:ethylacetate (20:1-2:1).

**Fungus Cultivation.** The strain was isolated from the sponge *Suberites domuncula* collected at a depth of 3 m near Zelenyi Island (Kuril Islands). Fungus was cultivated for 20 d at 22°C in four 1-L flasks, each of which contained medium of the following composition: unhopped beer wort (50 mL), washed agar (5 g), and seawater (200 mL).

**Isolation of 1-4.** Fungus mycelium with medium was extracted twice with ethylacetate. The extract was evaorated. The residue was dissolved in ethanol:water (1:4). The resulting solution was extracted successively with hexane, $CHCl_3$, and butanol (2 times). The $CHCl_3$ extract was evaporated in vacuo. The dry solid (700 mg) was chromatographed over a column (25 × 2 cm) of $SiO_2$. Compounds **1** (5 mg) and **2** (12 mg) were eluted by hexane:ethylacetate (20:1); **3** (6 mg), hexane:ethylacetate (4:1); **4** (10 mg), hexane:ethylacetate (2:1).

**Physcion (1)**, $C_{16}H_{12}O_5$, red crystals, mp 207°C (5% ethylacetate in hexane). IR spectrum ($CHCl_3$, ν, $cm^{-1}$): 2929, 2854, 1626, 1568, 1483, 1140. UV spectrum (EtOH, $\lambda_{max}$, nm): 226, 253, 265, 283, 435 (log ε 3.73, 3.46, 3.40, 3.35, 3.18). Mass spectrum (EI, 70 eV, *m/z*, $I_{rel}$, %): 284 (100) $[M]^+$, 241 (9), 213 (7), 185 (8), 128 (11). Table 1 gives the NMR spectra.

**Asperflavin (2)**, $C_{16}H_{16}O_5$, yellowish-green oil. UV spectrum (EtOH, $\lambda_{max}$, nm): 229, 267, 316, 331, 391 (log ε 3.46, 3.52, 2.89, 2.80, 3.19). Mass spectrum (EI, 70 eV, *m/z*, $I_{rel}$, %): 288 (90) $[M]^+$, 270 (25), 255 (25), 241 (15), 230 (60), 212 (12), 180 (12), 151 (50).

**Tetrahydroauroglaucin (3)**, $C_{19}H_{26}O_3$, yellow crystals, mp 60°C (5% ethylacetate in hexane). IR spectrum ($CHCl_3$, ν, $cm^{-1}$): 3538, 3014, 2930, 1644, 1613, 1443, 1306, 1203, 1172. UV spectrum (EtOH, $\lambda_{max}$, nm): 230, 274, 394 (log ε 4.38, 3.94, 3.73). Mass spectrum (EI, 70 eV, *m/z*, $I_{rel}$, %): 302 (80) $[M]^+$, 287 (3), 284 (3), 269 (15), 247 (20), 231 (100), 228 (24), 203 (10), 189 (20), 175 (90). Table 2 gives the NMR spectra.

**Echinulin (4)**, $C_{29}H_{39}N_3O_2$, white crystals, mp 228°C (hexane:ethylacetate), $[\alpha]_D^{20}$ -38.4° (*c* 0.21, $CHCl_3$). IR spectrum ($CHCl_3$, ν, $cm^{-1}$): 3459, 3391, 3006, 2975, 2930, 1681, 1441, 1317. UV spectrum (EtOH, $\lambda_{max}$, nm): 279, 285, 295 (log ε 4.14, 4.13, 4.03). Mass spectrum (EI, 70 eV, *m*/*z*, $I_{rel}$, %): 461 (15) $[M]^+$, 334 (100), 319 (3), 278 (12), 194 (3), 69 (5).


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