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Eurotium (Aspergillus) repens Metabolites and Their Biological Activity

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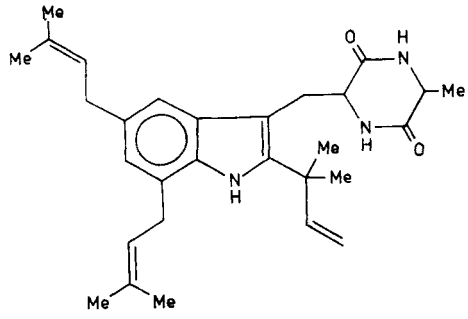
ABSTRACT. *Eurotium repens* mycelium cultivated under static conditions was used to isolate and identify metabolities—echinulin, physcion, erythroglaucin, flavoglaucin and asperentin; the filtrate of the culture yielded asperentin 8-methylether. The broadest biological activity spectrum was displayed by asperentin which had antibacterial and antifungal effects and, at a concentration of 86 μg/ml, caused 50 % mor7 tality in *Artemia saline* larvae. The highest cytotoxicity towards HeLa cells was found in physcion which caused 50 % growth inhibition at a concentration of 0.1 μg/ml.

The conidial form of *Eurotium repens* (*Aspergillus repens*) is taxonomically classified in the *Aspergillus glaucus* group (Rapper and Fennell 1965). Representatives of this group, including *A. repens*, produce metabolites of the type of oligoketides and dioxopiperazines (Miller 1961; Shibata *et al.* 1964). Some of these substances have an antibiotic activity.

From contaminated candy, we isolated a toxigenic fungus identified as *A. repens*. Apart from antibacterial and antifungal activity, crude mycelial extracts of this fungus were toxic to *Artemia salina* larvae. To identify the active substance, we tested six metabolites isolated from the mycelium and from the culture filtrate of the fungus.

Metabolite $C_{29}H_{39}N_3O_2$, isolated from the mycelium, corresponded to echinulin (*1*) and its mass spectrum was in keeping with that given in the literature (Cardillo *et al.* 1974). The ^{1}H-NMR spectrum confirmed the presence of one secondary methyl group, two tertiary aliphatic methyl groups, four olefinic methyl groups and all olefinic, aromatic and NH—protons required for the structure. Double resonance experiments confirmed the relative arrangement of substituents on double bonds of side chains. Chromatographic properties and the UV-spectrum were also identical with those known from the literature (Westley *et al.* 1968).

Orange-coloured crystalline substance $C_{16}H_{12}O_5$ exhibited in a mass spectrum fragmentation typical of hydroxyanthraquinones. Its ^{1}H-NMR spectrum contained signals of an aromatic methyl group, aromatic methoxyl, and two pairs of aromatic protons situated mutually in *meta*-position. A double resonance of the established long-range couplings showed that the methyl group is situated in *ortho*-position towards both protons of one of the pairs. Nuclear Overhouser effect, detected between the signal of the methoxyl and the two protons of the other pair, defined analogously



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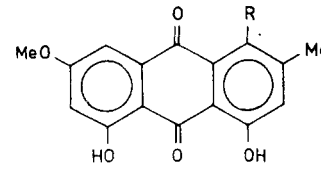
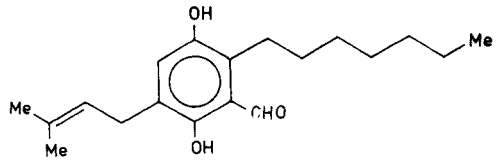


2a: R=H 2b: R=OH



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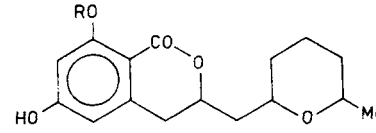

4a: R=H 4b: R=Me

the position of the methoxyl. These structural conditions are fulfilled by physcion (*2a*) (Steglich and Lösel 1969). The UV and IR spectra of the isolate were also in agreement with the literature (Thomson 1971).

The mass spectrum of the red metabolite $C_{16}H_{12}O_6$, possessing one more oxygen atom than physcion, had a similar character. The ^{1}H-NMR spectrum contained the signals of one aromatic methyl group, aromatic methoxyl, and three aromatic protons. Two of these are in meta-positions on the same ring, the remaining one is isolated. Procedure analogous to that used with physcion showed the presence of aromatic methyl neighbouring with isolated proton and methoxyl adjacent to the other two protons; the UV and IR spectra of the compound coincided with literature data (Ashley *et al.* 1939; Thomson 1971). Consequently, the metabolite was identified as erythroglaucin (*2b*).

Another substance from the mycelium, $C_{19}H_{28}O_3$, crystallizes in yellow needles. Its ^{1}H-NMR spectrum implies that the compound is a pentasubstituted benzene derivative, with substituents formed by a seven-carbon unbranched aliphatic chain, 1-propene-2-yl radical, formyl, and two hydroxyl groups. This structure complies with that of flavoglaucin (*3*) (Quilico *et al.* 1949).

White crystalline substance $C_{16}H_{20}O_5$ was identified by its mass spectrum as asperentin (*4a*) (Grove 1972) or the identical cladosporine (Scott *et al.* 1971). Its ^{1}H-NMR contained the characteristic signals of a secondary methyl group, three oxymethine protons, two *meta*-substituted aromatic protons, and one chelated phenolic hydroxyl. The multiplicity of signals, magnitude of interaction constants, UV and IR spectrum and CD were in accordance with the literature (Scott *et al.* 1971; Grove 1972).

The culture filtrate yielded a white crystalline substance $C_{17}H_{22}O_5$. The *m/z* 193, 165 and 164 fragments in its mass spectrum indicate that it belonged to monomethylated dihydroxydihydroisocoumarins. Ions *m/z* 99 and 81 are typical of the methyltetrahydropyranyl residue (Grove 1972). The ^{1}H-NMR spectrum exhibited a secondary methyl group, aromatic methoxyl, benzyl methylene group, three oxy-

TABLE I. Antimicrobial and toxic properties of *E. repens* metabolites

Metabolite	Amount μg[a]	Antimicrobial activity[b] mm[c]	Toxicity ID_{50}^{e}
Echinulin (*1*)	125	13.2	25
Physcion (*2a*)	125	16.6	0.1
Erythroglaucin (*2b*)	100	0	—
Flavoglaucin (*3*)	125	13.5	50
Asperentin[f] (*4a*)	100	12.2	50
Asperentin 8-methylether (*4b*)	100	12.5	100

[a]Per disc. [b]Against *B. subtilis* on minimal agar. [c]Diameter of inhibition zones. [d]Against HeLa cells. [e]Concentration (μg/ml) causing 50 % growth inhibition (Horáková and Betina 1977). [f]Aspertin is effective-also against other fungi (*see* text). [g]34.6 against *N. sitophila* on malt extract agar. [h]LC_{90}^{i} 86 aganist *A. salina*. [i]Concentration (μg/ml) causing 50 % mortality of larvae (Ďuračková *et al.* 1977).

methine protons and two *meta*-substituted aromatic protons. These structural features coincide with the structure of 8-O-methylasperentin (*4b*) (Grove 1972); this coincidence was also confirmed by the UV and IR spectra and by OD.

Of these metabolites, echinulin, physcion, erythroglaucin and flavoglaucin have been described to occur in *A. repens* and other species of the group *A. glaucus*. They were found in different commonly produced combinations, *viz.* physcion, erythroglaucin and flavoglaucin (Gould and Raistrick 1934; Ashley *et al.* 1939), physcion and erythroglaucin (Carbone and Johnson 1964), echinulin (Quilico and Cardani 1951) or a mixture of echinulins (Cardillo *et al.* 1974; Dossena *et al.* 1974), echinulin and flavoglaucin (Quilico *et al.* 1949). The simultaneous production of echinulin and flavoglaucin, which is often found in fungi, reflects evidently a common pathway of biosynthesis of the isoprenoid subunit, with its isopropyl moiety being formed from leucine (Cardillo *et al.* 1977).

No asperentin and its 8-methyl ester have so far been found in strains of the group *A. glaucus*. These substances, along with other asperentin derivatives (6-methylasperentin, 4′-hydroxyasperentin, 5′-hydroxyasperentin, 5′-hydroxy-8-methylasperentin) are typical of *A. flavus* (Grove 1972, 1973) or *Cladosporium cladosporioides* (Scott *et al.* 1971).

Production of the above six metabolites by a single strain has not yet been described; it may serve as an example of a concomitant biosynthesis of metabolites from acetate or malonate units (phenol, anthraquinones and dihydroisocoumarins) (Turner 1971; Cattel *et al.* 1973) and a metabolite from amino acids (dioxopiperazine) (Birch *et al.* 1961).

Antimicrobial spectrum, toxicity and cytotoxicity of metabolites from *E. repens* are shown in Table I. The substances were effective against *Bacillus subtilis* only on minimal agar; on enriched agar, they had no effect even against *Escherichia coli* and *Pseudomonas ovalis*. An antifungal effect was exerted only by asperentin, inhibiting the growth of *Candida albicans*, *C. utilis*, *C. pseudotropicalis*, *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Neurospora sitophila*, *Alternaria tenuis*, *Monilia fructigena*, *Trichothecium roseum*, and *Paecilomyces viridis*. A similar antimicrobial spectrum was observed by Scott *et al.* (1971) with cladosporin. Toxic effect on *Artemia salina* larvae was exerted also by asperentin only. Entomogenic strains of *Aspergillus flavus* producing asperentins were described by Grove (1972).

The cytotoxicity of the four metabolites under study was low, only physcion having a relatively powerful effect on HeLa cells.

MATERIALS AND METHODS

Strain and cultivation. Eurotium (*Aspergillus*) *repens* S-669, isolated from contaminated candy, was maintained on malt extract agar (MEA). The strain is deposited in the Czechoslovak Collection of Microorganisms, Brno, under the number F-505.

The culture on agar slope was used to prepare spore suspension which surved to inoculate sporulation medium (MEA) in three 1200 ml Roux flasks. The flasks were incubated for 5 d at 28 °C; the sporulated culture yielded 600 ml spore suspension. Forty 2000 ml Roux flasks, each containing 200 ml YES medium (Davis *et al.* 1966) were inoculated with 15 ml spore suspension and cultivated under static conditions at 28 °C. After 8 d of cultivation, the activity of the culture against *Neurospora sitophila* was tested by the plate method. The cultivation was terminated after 21 d when the antibiotic activity reached a maximum.

Metabolite isolation. Wet mycelium after the filtration of 8 litres of cultures was extracted with chloroform and dried. Dried mycelium was extracted with diethyl ether in Soxhlet apparatus. The residue (25g) after evaporation of diethyl ether treated with 60 ml of acetone–benzene mixture (1 : 1). Filtration yielded a white in soluble residue which was crystallized from chloroform and recrystallized from 1-butanol to yield 1.2 g echinulin (*1*): m.p. 231–235 °C; λ_{max}(MeOH): 227, 280 and 296 nm; CD, λ(MeOH): 252, 274 nm ($\Delta\varepsilon$ 0, +1.46); IRν_{max}: 1660, 1650 cm^{-1}; mass spectrum (*m/z*, relative intensity, composition) 461 (3, $C_{29}H_{39}N_3O_2$, M^+), 334 (100, $C_{24}H_{32}N$), 319 (7, $C_{23}H_{29}N$), 278 (32, $C_{20}H_{24}N$), 266 (17, $C_{19}H_{24}N$), 69 (32, C_5H_9); ^{1}H NMR (100 MHz, $(CD_3)_2SO$, δ): 1.39 d (J = 7 Hz, 3H, CH_3CH), 1.54 s (6H, 2×CH_3–), 1.62 s, 1.67 br s (each 6H, 4×olefinic CH_3), 3.56 d (J = 8 Hz, 4H, 2×=C–CH_2–CH=), 3.84 mt (1H, CH–CH_3), 3.99 mt (1H), 4.90 dd (J = = 18 and 1 Hz, olefinic), 5.15 d (J = 11 and 1 Hz), 1H, olefinic), 5.37 mt (2H, 2×CH=C$(CH_3)_2$), 6.29 q (J = 18 and 11 Hz, 1H, –CH=CH_2), 6.65 br s (1H, aromatic), 7.58 br s (1H, aromatic), 7.18 br s and 8.15 br s (2×NH), 9.48 br s (tryptophan NH).

The acetone–benzene extract after separation of echinulin was evaporated, the evaporate (20 g) was dissolved in benzene and chromatographed on a silica gel column in benzene (60×800 mm; 1 kg sorbent). The column was eluted with a sequence of benzene, benzene–chloroform 9 : 1, benzene–chloroform 4 : 1, chloroform, chloroform–methanol 9 : 1, chloroform–methanol 4 : 1 and methanol. The metabolites were eluted from the column in the sequence: physcion, erythroglaucin, flavoglaucin, and asperentin. Collected fractions (25 ml each) were tested for their activity against *Bacillus subtilis* and *Neurospora sitophila* on a minimal agar medium (Betina *et al.* 1975).

Fractions containing physcion (*2a*) were evaporated and physcion was crystallized from chloroform. The yield was 109 mg orange crystals, m.p. 207 °C; λ_{max}(MeOH): 214, 263 and 296 nm; IRν_{max}: 3350, 1670, 1625, 1610 cm^{-1}; mass spectrum (*m/z*, relative intensity, composition): 284 (100, $C_{16}H_{12}O_5$, M^+), 255 (13, $C_{15}H_{11}O_4$), 254 (9, $C_{15}H_{10}O_4$), 241 (8, $C_{14}H_9O_4$), 226 (6, $C_{14}H_{10}O_3$), 213 (6, $C_{13}H_9O_3$), 198 (5, $C_{13}H_{10}O_2$), 185 (5, $C_{12}H_9O_2$), 139 (4, $C_{11}H_7$), 128 (10, $C_{10}H_8$), 115 (3, C_9H_7); ^{1}H NMR (100 MHz, $CDCl_3$ + $(CD_3)_2SO$, δ): 2.43 s (3H, aromatic Me), 3.91 s (3H, aromatic OMe), 6.66 d, (J = 2.2 Hz, 1H, aromatic), 7.05 br s (1H, aromatic), 7.85 d, J = 2.5 Hz, 1H, aromatic) and 8.10 br s (1H, aromatic).

During elution from the column, flavoglaucin and erythroglaucin partially overlap and the evaporation residues of these eluates yield thus a mixture of long yellow needles of flavoglaucin and red erythroglaucin crystals. Flavoglaucin was separated

by dissolving in methanol, crystallization of the evaporation residue from a mixture of chloroform–diethyl ether (1 : 1) and a recrystallization from benzene. The procedure afforded 330 mg flavoglaucin (*3*): m.p. 100–105 °C; λ_{max}: 236 and 274 nm; IR ν_{max}: 3400, 1630, 1580 cm^{-1}; mass spectrum (*m/z*, relative intensity, composition): 304 (99, $C_{19}H_{28}O_3$, M^+), 249 (100, $C_{15}H_{21}O_3$), 230 (30, $C_{15}H_{18}O_2$), 215 (34, $C_{14}H_{15}O_2$); ^1H NMR: (100 MHz, $CDCl_3$, δ): 0.88 t (J = 6 Hz, CH_3CH_2-), 1.20–2.05 mt (10H), 1.60 br s, 1.65 br s (each 3H, $2\times CH_3-C=$), 2.89 t (J = 8 Hz, 2H, $=C-CH_2-CH_2-$), 3.28 d (J = 7 Hz, 2H, $=C-CH_2-CH=$), 5.29 mt (1H, olefinic), 6.89 s (1H, aromatic), 10.24 s (1H, $-CH=O$).

Erythroglaucin remaining after flavoglaucin separation was dissolved in chloroform and purified by preparative TLC on Silufol plates (200 × 200 mm) developed in benzene. The main red band yielded 10 mg erythroglaucin (*2b*): m.p. 204–208 °C; λ_{max} (MeOH): 231, 254, 273, and 306 nm; optically inactive; ν_{max}: 3450, 1650, 1620, 1603, 1548 cm^{-1}; mass spectrum (*m/z*, relative intensity, composition): 300 (100, $C_{16}H_{12}O_6$, M^+), 283 (7, $C_{16}H_{11}O_5$), 271 (12, $C_{15}H_{11}O_5$), 257 (11, $C_{14}H_9O_5$), 243 (4, $C_{14}H_{11}O_4$), 229 (7, $C_{13}H_9O_4$); 1H NMR (59.797 MHz, $CDCl_3$, δ): 2.37 s (3H, CH_3-), 3.94 s (3H, OMe), 6.70 d (J = 2.8 Hz, 1H, aromatic), 7.43 d (J = 2.8 Hz, 1H, aromatic).

Asperentin-containing fractions were evaporated to dryness, the residue was washed with methanol and recrystallized from methanol. The yield of white asperentin crystals (*4a*) was 50 mg: m.p. 188–191 °C; TLC (Silica gel Merck, toluene–ethyl acetate–90 % formic acid, 6 : 3 : 1): spot with blue fluorescence R_F in UV light (265 nm); λ_{max} (MeOH): 216, 267, 301 nm (ε 20500, 13100, 6000); CD, λ (MeOH): 234, 244, 248, 254, 268, 284, 301 nm ($\Delta\varepsilon$ +2.52, 0, −0.42, 0, +1.96, 0, −0.85); ν_{max}: 3250, 1635, 1620, 1613, 1585 cm^{-1}; mass spectrum (*m/z*, relative intensity, composition): 292 (5, $C_{16}H_{20}O_5$, M^+), 274 (1, $C_{16}H_{18}O_4$), 256 (1, $C_{16}H_{16}O_3$), 179 (12, $C_9H_7O_4$), 176 (8, $C_{10}H_8O_3$), 151 ($C_8H_7O_3$), 150 (12, $C_8H_6O_3$), 125 (5, $C_8H_{13}O$), 99 (100, $C_6H_{11}O$), 81 (67, C_6H_9), 55 (18), 43 (12), 41 (8); ^1H NMR (100 MHz, $CDCl_3$, δ): 1.18 d (J = 6.5 Hz, 3H, CH_3CH), 1.24–1.82 mt (6H), 1.77 dt (J = 15 and 3 Hz, 1H), 2.07 dd J = 15 and 4 Hz, 1H), 2.96 d (J = 7 Hz, 2H), 2.70–4.20 mt (2H, O–CH), 4.77 mt (ΣJ = 28 Hz, 1H, CHOCO), 6.20 d (J = 2 Hz, 1H, aromatic), 6.26 d (J = 2 Hz, 1H, aromatic), 11.08 br s (1H, chelated phenolic OH).

The culture filtrate (7 litres) yielded asperentin 8-methylether (*4b*), which was isolated by the method of Grove (1972). Crude extract (5 g) gave 20 mg asperentin 8-methylether: m.p. 228–229 °C; TLC (silica gel Merck, chloroform–methanol 97 : 3) R_F 0.25; λ_{max} (MeOH): 230, 267 and 298 nm (ε 18450, 14000 and 7850); CD, λ (MeOH): 294, 284, 268, 256, 244, 232 nm ($\Delta\varepsilon$ +1.22, +1.08, +4.39, 0, −0.96, 0, +4.36); ν_{max} 3300, 1660, 1595, 1570 cm^{-1}; mass spectrum (*m/z*, relative intensity, composition): 306 (4, $C_{17}H_{22}O_5$, M^+), 288 (15, $C_{17}H_{20}O_4$), 270 (5, $C_{17}H_{18}O_3$), 235 (5, $C_{13}H_{15}O_4$), 208 (31, $C_{11}H_{12}O_4$), 203 (20, $C_{12}H_{11}O_3$), 193 (58, $C_{10}H_9O_4$), 125 (25, $C_8H_{13}O$), 99 (69, $C_6H_{11}O$), 81 (100, C_6H_9), 55 (41), 43 (34), 41 (26); ^1H NMR (100 MHz, $CDCl_3$ + $(CD_3)_2SO$, δ): 1.17 d (J = 6.5 Hz, 3H), 1.25–1.42 mt (2H), 1.50–1.88 mt (4H), 2.03 dd (J = 4 and 14 Hz, 1H), 2.90 d (J = 7 Hz, 2H), 3.80 to 4.15 mt (2H), 3.85 s (3H), 4.50 mt (1H), 6.26 d (J = 2.3 Hz, 1H), 6.48 d (J = 2.3 Hz, 1H).

Measurement of spectra. UV and visible spectra were measured on a Perkin-Elmer PE-356 spectrophotometer, IR spectra were measured in KBr pellets on a Unicam SP-200. Circular dichroism was measured on a JASCO spectrometer, mass spectra on a Varian MAT-311 spectrometer using a direct inlet system, ionization energy 11 aJ, high resolution measured by the peak-matching technique. ^1H-NMR spectra were measured at 100 MHz (Varian HA-100, CW) or at 59.797 MHz (Jeol FX-60, FT).

Antimicrobial activity and toxicity. Antimicrobial activity was assayed on minimal and enriched medium using *B. subtilis* ATCC 6633 (Betina *et al.* 1975) and on MEA plates using *N. sitophila.* The cytotoxicity to HeLa cell cultures and toxicity to *A. saline* larvae were determined according to Horáková and Betina (1977) and Ďuračková *et al.* (1977).

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