

Flavipucine and Brunnescin, Two Antibiotics from Cultures of the Mycophilic Fungus *Cladobotryum rubrobrunnescens*

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Two antimicrobial metabolites were isolated from submerged cultures of *Cladobotryum rubrobrunnescens*, a mycophilic fungus growing on a *Inocybe* species. One of the compounds proved to be identical to flavipucine (**2**), an antibiotic previously isolated from *Aspergillus flavipes* (Casinovi *et al.*, 1968) and from a *Macrophoma* species (Sassa T. and Onuma Y. (1983), Agric. Biol. Chem. **47**, 1155–1157). The other metabolite, brunnescin (**1**), is a new tetrasubstituted furan derivative which exhibits antibacterial, antifungal and cytotoxic effects.

Introduction

High losses during crop production due to fungal infections make a search for new fungicides necessary. Rising consciousness of man's responsibility for the environment demands for more specific pesticides to be developed. Natural compounds obtained from mycophilic fungi seem to be highly suitable for these purposes (Barnett and Binder, 1973; Cooke, 1977). In a first classification of mycophilic fungi, Barnett (1963) divided them into biotrophic and necrotrophic organisms. Necrotrophic fungi are destructive parasites which kill their hosts by means of enzymes or toxins. Biotrophic organisms are characterized by a narrow host range and depend on nutrients from living host cells. Boosalis (1964) recommended the use of mycophilic fungi for control of phytopathogenic fungi and pointed out the effectiveness of the principle of antibiosis.

The principles effective in biological control are parasitism, competition and/or antibiosis. In plant protection the latter is considered to be the most important one (Papavizas, 1985). A mutant of *Trichoderma viride* which no longer produced the antibiotic gliovirin had also lost the ability for mycoparasitism. Therefore mycophilic fungi espe-

cially those of the necrotrophic type seem to be a good source for natural fungicides.

During a screening of fungal strains isolated from fruit bodies of Basidiomycetes, *Cladobotryum rubrobrunnescens* showed high activities against several fungi. *C. rubrobrunnescens* has been described to be a new species of the genus *Cladobotryum* (Helfer, 1991) and so far no secondary metabolites have been described from this organism. Therefore the antifungal metabolites were isolated and characterized.

In the following we wish to describe the fermentation, isolation, identification and biological properties of two metabolites from *C. rubrobrunnescens*.

Experimental

General

IR and UV spectra were measured with a Bruker ISF 48 and a Perkin-Elmer Lambda 16 UV/VIS spectrometer respectively. For analytical HPLC a Hewlett-Packard 1090 series II instrument was used. The NMR spectra were recorded with a Bruker ARX500 spectrometer, in acetone-D₆ with the solvent signals (2.05 ppm in the ¹H NMR spectra and 29.8 ppm in the ¹³C NMR spectra) as reference, and the mass spectra with a Jeol SX102 spectrometer. Melting points were

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determined with a Büchi model 510 “melting point” and are uncorrected.

Producing organism and its cultivation

Cladobotryum rubrobrunnescens W. Helfer was isolated from a fruit body of an *Inocybe* species collected in Regensburg, Germany (Helfer, 1991). The strain is deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on YMG medium (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, pH 5.5).

Fermentations were carried out at 22 °C in a Biolafitte C6 or Biostat U (Braun Melsungen) fermentor containing 20 l of YMG medium with aeration (3.4 l air/min) and agitation (150 rpm). 200 ml of a well grown culture (5 days) in the same medium were used as inoculum.

Oxygen consumption was measured using a Magnos 4 G magnopneumatic oxygen analyser (Hartmann & Braun, Frankfurt), carbon dioxide production was followed using an ADC carbon dioxide analyser type SG-305 and oxygen saturation of the culture broth was measured with an Ingold oxygen electrode. The data were registered on line, using the Micro MFCS process control system software equipment.

During fermentation the production of brunnescin and flavipucine was followed with the agar diffusion assay. Quantitative measurements were achieved by analytical HPLC.

The mycelial dry weight, pH, glucose concentration (hexokinase method) and the content of **1** and **2** were measured daily.

The analytical HPLC system consisted of a Merck LiChrospher RP-18 column (5 µm, 125×4 mm) eluted with 1.5 ml/min, 40 °C, H₂O–acetonitrile linear gradient 0–70% acetonitrile in 20 min. The compounds were detected using Diode Array Detection (Hewlett Packard 1090 Series II). The retention time for brunnescin was 8.10 min, for flavipucine 8.60 min.

Isolation of flavipucine and brunnescin

After 120 h (brunnescin) or 240 h (flavipucine) of fermentation, the mycelia were separated from the culture fluid by filtration. The antimicrobial compounds were removed from the culture fluid (16.5 l) by adsorption onto HP 21 resin (Mitsubi-

shi) and subsequently eluted with methanol. The eluate was concentrated. The crude extract (2.1 g) was applied onto a column (28×3.5 cm) with silica gel (Merck 60, 0.063–0.2 mesh) and eluted with cyclohexane–ethyl acetate (1:1). Further purification was achieved by preparative HPLC on Merck LiChroGel PS I (10 µm, 250×23 mm) and elution with isopropanol (3 ml/min). Flavipucine eluted at 46 min and brunnescin at 93 min. Bioactivity-guided isolation yielded 0.55 mg/l of brunnescin and 2.34 mg/l of flavipucine.

Brunnescin (**1**) was obtained as a yellowish oil (6 mg), soluble in methanol, ethyl acetate and acetone. $R_f = 0.33$ (toluene:acetone 7:3, silica gel). UV (methanol) λ_{max} (ε): 236 (21,500), 340 (12,200). IR (KBr): 3420, 2955, 1725, 1695, 1635, 1595, 1565, 1440, 1350, 1305, 1265, 1205, 1175 and 1005 cm⁻¹. ¹H NMR (acetone-D₆) δ (ppm), m, *J* (Hz): 10.19, s, 9-H; 8.14, d, *J*₂₋₃ = 15.7, 3-H; 6.82, d, *J*₂₋₃ = 15.7, 2-H; 4.60, s, 8-H₂; 4.30, s, 12-H₃; 3.81, s, 11-H₃. ¹³C NMR (acetone-D₆) δ: 189.0 C-9, 168.2 C-10, 166.2 C-1, 162.1 C-7, 159.2 C-4, 132.7 C-3, 129.3 C-2, 115.5 C-5, 114.3 C-6, 63.5 C-12, 54.8 C-8, 52.5 C-11. EIMS (*m/z*) 70 eV: 237.0398 (7%, M⁺–CH₃O, C₁₁H₉O₆ requires 237.0399), 209.0440 (199%, M⁺–CH₃OOC, C₁₀H₉O₅ requires 209.0450), 191 (8%), 181 (10%), 179 (12%), 177 (10%), 113 (13%), 46 (13%). CIMS (*m/z*) 70 eV, NH₃: 286 (100%, M+NH₄⁺), 269 (27%, M+H⁺). CIMS (*m/z*) 70 eV, CH₄: 269.0649 (67%, M+H⁺, C₁₂H₁₃O₇ requires 269.0661), 251.0546 (100%, M+H⁺–H₂O, C₁₂H₁₁O₆ requires 251.0556).

Flavipucine (**2**) was obtained as yellow needles, soluble in methanol and ethyl acetate, $R_f = 0.42$ [toluene–acetone (7:3), silica gel], m.p. 134 °C. The spectral data were identical to those reported in the literature (Sassa and Onuma, 1983).

Biological assays

The antifungal and antibacterial activities during fermentation and isolation were monitored by the agar plate diffusion assay with *Nematospora coryli* and *Bacillus brevis* as test organisms. Tests for phytotoxicity were carried out as described before (Anke *et al.*, 1988). The tests for cytotoxicity and hemolytic activity were carried out as described previously (Zapf *et al.* 1994).

The incorporation of [2-¹⁴C]thymidine, [2-¹⁴C]uridine and [1-¹⁴C]leucine into macro-

molecules of HL 60 (ATCC CCL 240) and L 1210 (ATCC CCL 219) cells was carried out as described by Zapf *et al.* (1994). The reactivity of the compounds with L-cysteine as a model thiole was measured as reported by Kupchan *et al.* (1970).

Results and Discussion

Production and structural elucidation of the active compounds

A typical fermentation diagram of *Cladobotryum rubrobrunnescens* is shown in Fig. 1. The production of brunnescin (**1**) as determined by analytical HPLC started early and increased significantly after 50 h. After 120 h of fermentation, the antifungal activity had reached a first maximum,

which correlated with the highest content of brunnescin (**1**) and the consumption of glucose. At this time, both compounds were detected in the culture broth. The second maximum of activity was reached after 240 h, when the maltose was completely used up. At this stage of the fermentation **1** was no longer detectable.

Brunnescin (**1**) was isolated from fermentations harvested at the first activity peak and flavipucine (**2**) from fermentations harvested after 240 h. The isolation procedure is given in the experimental section.

The structures of the compounds are shown in Fig. 2. EI and CI high resolution mass spectrometry of brunnescin (**1**) clearly demonstrated that its elemental composition is $C_{12}H_{12}O_7$ (see the exper-

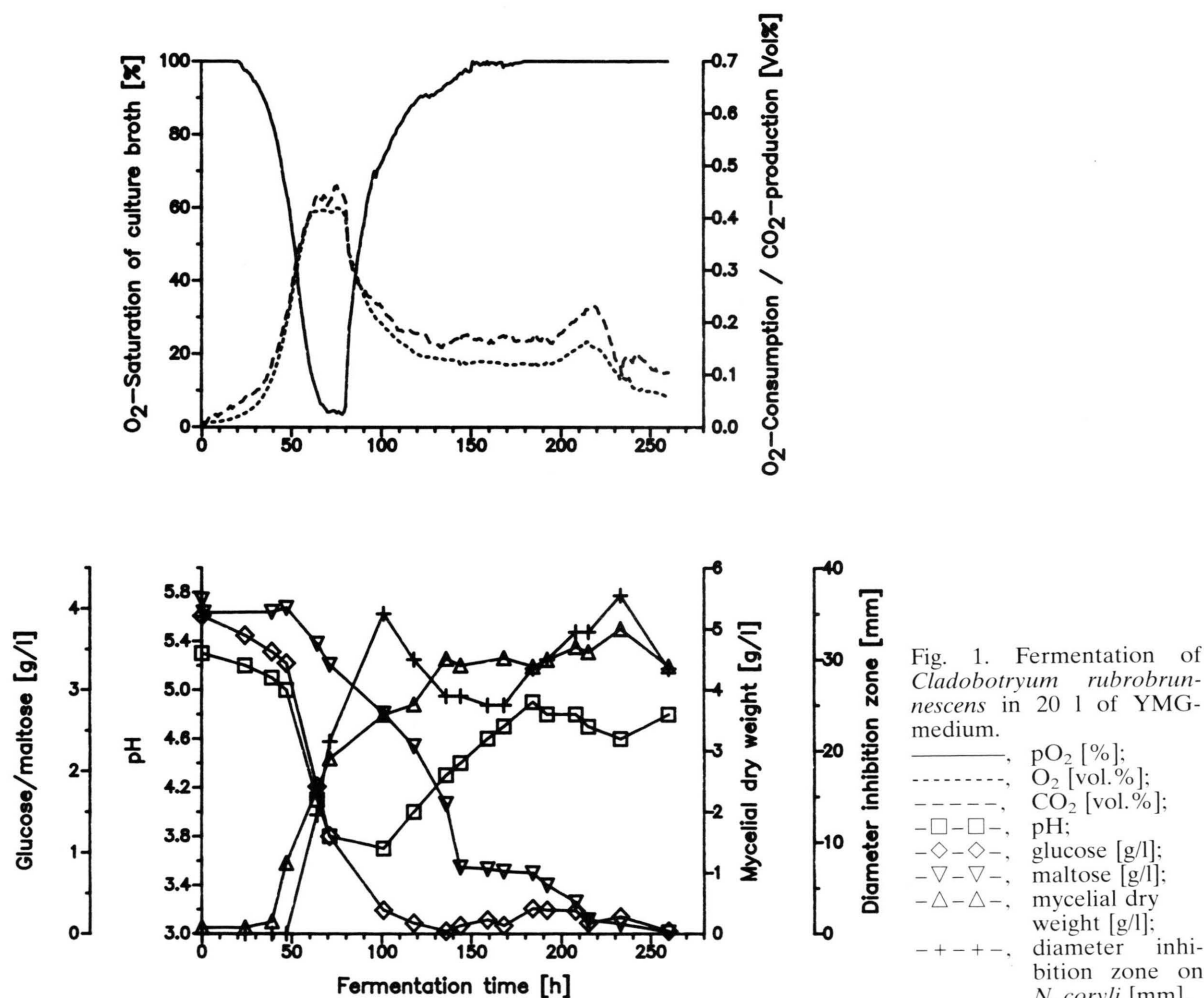


Fig. 1. Fermentation of *Cladobotryum rubrobrunnescens* in 20 l of YMG-medium.

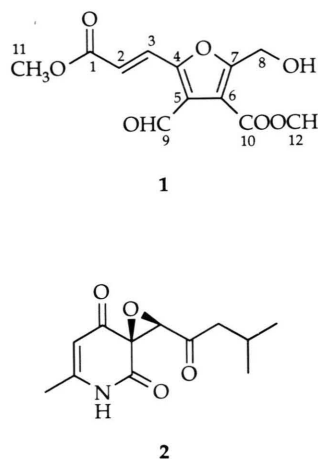


Fig. 2. The structures of brunnescin (**1**) and flavipucine (**2**).

imental part for details). The presence of a hydroxyl group was indicated by the $M+H^+$ peak at m/z 341 (corresponding to the protonated mono-TMS ether of brunnescin) observed in a CI- NH_3 mass spectrum of the product obtained after treatment of brunnescin (**1**) with TMSi-Cl in pyridine (the product was not isolated), in which 341 was the largest peak. However, when preparative acetylation of brunnescin (**1**) with acetic anhydride in pyridine was attempted, the compound degraded. The 1H NMR spectrum showed the presence of an aldehyde function, a trans substituted double bond, two methoxy groups and an isolated CH_2 group (containing the hydroxyl group according to its ^{13}C NMR shift), accounting for the remaining 11 hydrogens. Besides the ^{13}C signals that in a HMQC experiment were correlated to these proton signals, the signals for six additional carbons could be observed in the ^{13}C spectrum. Two of these are methyl ester carbonyl carbons, to which long-range correlations from the methoxy protons were observed in a HMBC experiment (results summarised in Fig. 3). The shifts of the remaining four carbons suggest that they make up a tetrasubstituted furan ring, and the HMBC and NOESY correlations observed (see Fig. 3) determine the structure of brunnescin (**1**).

Biological activities

In the serial dilution assay **1** and **2** inhibited the growth of bacteria and fungi (Table I). The effects

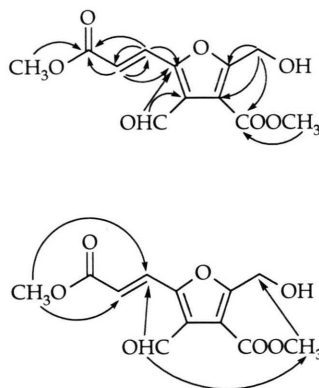


Fig. 3. 1H - ^{13}C long-range (top) and NOESY (bottom) correlations for brunnescin (**1**).

of **2** on bacteria were slightly higher than those of **1**. As shown in Table II, *Penicillium notatum* and *Paecilomyces varioti* were only sensitive to **1** in the plate diffusion assay.

The phytotoxic activities towards germinating seeds of *Lepidium sativum* were 600 $\mu g/ml$ for **1** and 180 $\mu g/ml$ for **2**. All of the control seeds were germinated.

Table I. Antimicrobial activities of brunnescin (**1**) and flavipucine (**2**).

Strain	MIC [$\mu g/ml$]	
	1	2
Bacteria:		
<i>Arthrobacter citreus</i>	> 100	50
<i>Bacillus brevis</i>	50	10
<i>Bacillus subtilis</i>	> 100	20
<i>Escherichia coli</i> K 12	> 100	> 100
<i>Micrococcus luteus</i>	> 100	50
<i>Mycobacterium phlei</i>	20	50
<i>Streptomyces spec.</i>	> 100	100
Fungi:		
<i>Nadsonia fulvescens</i>	> 100	> 100
<i>Nematospora coryli</i>	10	20
<i>Saccharomyces cerevisiae</i> S 288 c	> 100	> 100
<i>Saccharomyces cerevisiae</i> is 1	50	> 100
<i>Fusarium oxysporum</i>	n.t.	> 100
<i>Paecilomyces varioti</i>	> 100	> 100
<i>Penicillium notatum</i>	> 100	> 100
<i>Mucor miehei</i>	100	100
<i>Rhodotorula glutinis</i>	> 100	> 100
<i>Ustilago nuda</i>	20	10

Table II. Antimicrobial effects of brunnescin (**1**) and flavipucine (**2**); 50 µg/disk*.

Strain	Diameter inhibition zone [mm]	
	1	2
<i>Bacillus brevis</i>	0	29
<i>Bacillus subtilis</i>	n.t.	23
<i>Enterobacter dissolvens</i>	n.t.	10
<i>Nematospora coryli</i>	33	30
<i>Penicillium notatum</i>	10	0
<i>Paecilomyces varioti</i>	8	0
<i>Mucor miehei</i>	7	0

* Diameter: 6 mm.

n.t., not tested.

The cytotoxic activities are shown in Table III. The growth of all cells was strongly inhibited by flavipucine. Brunnescin was less cytotoxic. In RBL (rat leukemia) cells the difference between the two compounds was one order of magnitude.

As shown in Table IV and Fig. 4, both compounds exhibited strong effects on the biosynthesis of macromolecules in HL 60 and L 1210 cells. The effects of **1** and **2** in both cell lines were non-specific and the incorporation of all precursors was affected.

1 and **2** showed a fast reaction with cysteine; after 20 seconds two adducts were detectable on

Table III. Cytotoxic effects of brunnescin (**1**) and flavipucine (**2**).

Cells	IC ₅₀ [µg/ml]	
	1	2
L 1210	2	1
BHK 21	n.t.	5
HL 60	2	2
RBL	10	1

Table IV. Effects of brunnescin (**1**) and flavipucine (**2**) on the incorporation of radioactive labelled precursors into L 1210 cells (% incorporation of reference: thymidine 4020 cpm; uridine 13954 cpm; leucine 19394 cpm).

	Compound added [µg/ml]	Incorporation of radioactivity precursor [%]		
		Thymidine	Uridine	Leucine
Brunnescin	4	40	70	11
	20	0	1	1
Flavipucine	1	78	100	89
	4	5	100	7
	10	0	41	1
	100	0	10	0

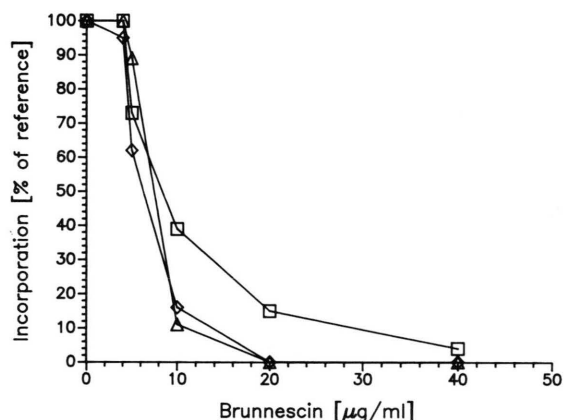
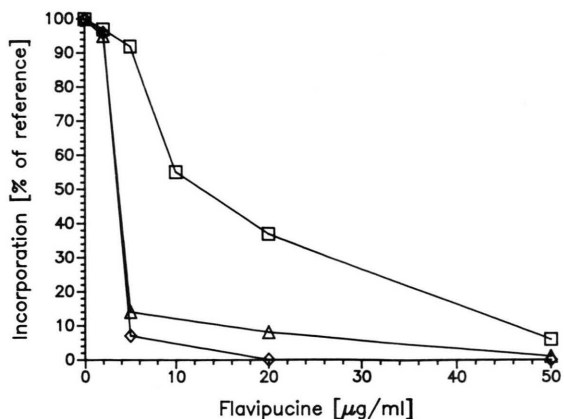


Fig. 4. Effects of compounds **1** and **2** on the incorporation of radioactive labelled precursors into macromolecules in HL 60 cells. —△—△—, incorporation of [2-¹⁴C]-thymidine, control (100%) = 1783 cpm; —□—□—, incorporation of [2-¹⁴C]-uridine, control (100%) = 4200 cpm; —◇—◇—, incorporation of [1-¹⁴C]-leucine, control (100%) = 2670 cpm.

TLC and the antifungal activity towards *N. coryli* was no longer detectable. Therefore we conclude that the various biological activities of the two compounds are due to their reactivity with nucleophiles.

Brunnescin (**1**) is a new fungal furan derivative. Biologically active furan derivatives have been isolated in a large number from different groups of organisms including fungi. Gregatins A–D produced by *Aspergillus panamensis* (Anke *et al.*, 1980) show antimicrobial and cytotoxic effects. The phytotoxins pyrenocine A and B were isolated from *Pyrenochaeta terrestris*, causal agent of

pink-rot of onion (Sato *et al.*, 1979). 3-(3-Carboxy-4-furanyl)alanine is an unusual amino acid isolated from the basidiocarps of two mushrooms, *Phyllotopsis nidulans* and *Tricholomopsis rutilans* (Doyle and Levenberg, 1974; Hatanaka and Niimura, 1975). 5-Hydroxymethyl-2-furancarboxylic acid has been isolated from several Ascomycetes and Deuteromycetes and shows antitumor properties (Munekata and Tamura, 1981; Munekata *et al.*, 1981). Wyerone acid and related polyacetylenic phytoalexins, produced by *Vicia faba*, exhibit antimicrobial and resistance-inducing activities (Letcher *et al.*, 1970; Hargreaves *et al.*, 1976; Thynn *et al.*, 1989; Wolff *et al.*, 1988). However, tetrasubstituted furans are rare in Nature, examples of furans with a similar skeleton are the F-acids (**3**) ($m=2$ or 4 ; $n=8$, 10 or 12) (see Fig. 5) isolated from fish oils (Glass *et al.*, 1974) and the urofuranic acids (**4**) ($m=2$ or 4) isolated from human urine and blood (Spiteller *et al.*, 1980). The F-acids (e.g. compound **3** with $m=4$ and $n=8$) are by mammals oxidised to urofuranic acid (**4**) ($m=4$) and the diacid **5** (Schödel *et al.*, 1986).

Flavipucine was isolated first from cultures of *Aspergillus flavipes* (Casinovi *et al.*, 1968; Findlay and Radics, 1972), its biosynthesis has been partially described (Casinovi *et al.*, 1987). It was also isolated as a fruit rot toxin in various apple races (Sassa and Onuma, 1983) from cultures of a phyto-

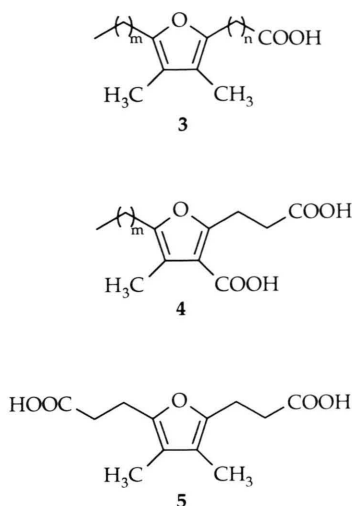


Fig. 5. Furans with similar carbon skeleton isolated from natural sources.

pathogenic *Macrophoma* species. *C. rubrobrunnescens* thus is a new producer of flavipucine, and there is no close relationship to the two other producers.

Acknowledgements

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