# Rubrobramide, a Cytotoxic and Phytotoxic Metabolite from *Cladobotryum rubrobrunnescens*

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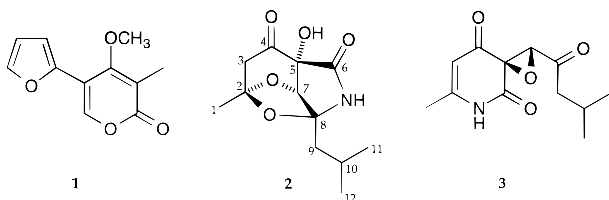
Cladobotrin (**1**) and rubrobramide (**2**) were isolated from the culture filtrate of *Cladobotryum rubrobrunnescens*, a mycophilic deuteriomycete. Rubrobramide (**2**), possessing a unique oxidized tricyclic system, is a new compound structurally related to flavipucine (**3**), which also is produced by the fungus. The structure of **2** was determined by spectroscopic methods. Cladobotrin (**1**) exhibited weak nematicidal activity toward *Meloidogyne incognita*, while **2** showed weak cytotoxic and phytotoxic activities.

The secondary metabolism of mycophilic fungi, fungi living on other fungi, is a rich source of bioactive natural products[1] and may serve to protect the mycophilic fungus from antifungal defensive compounds produced by the host.[2] In our continuing screening for bioactive metabolites from fungal cultures, extracts of the mycophilic deuteriomycete *Cladobotryum rubrobrunnescens* W. Helfer (Hyphomycetes) showed antifungal, cytotoxic, and nematicidal activities. Bioactivity-guided fractionation yielded four secondary metabolites from the culture broth of *C. rubrobrunnescens*, of which the antimicrobial and cytotoxic brunnescin and flavipucine (**3**) have been described in a preceding paper.[3] The two remaining metabolites have now been identified as cladobotrin (**1**), previously reported from *Cladobotryum varium*,[4] and a new compound for which we suggest the name rubrobramide (**2**). In this paper the isolation and structure determination of **2** is reported, and the biological activities of cladobotrin (**1**) and rubrobramide (**2**) are described.




The concentration of **1** and **2** in extracts of the culture broth of *C. rubrobrunnescens* reached a peak after 10 days of fermentation, and the metabolites were isolated as described in the Experimental Section. The structure of **1** was established by the comparison of its spectroscopic data with those previously published,[4] while that of **2** was determined by NMR spectroscopy and mass spectrometry. The molecular ion observed in the EIMS of **2** is very weak, but CIMS showed that the molecular weight is 255. High-resolution measurements suggested that the elemental composition was $C_{12}H_{17}NO_5$,

**Table 1.** $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR Data for Rubrobramide (**2**) in $CDCl_3$ and $(CD_3)_2SO$[a]

| | $CDCl_3$ | | $(CD_3)_2SO$ | |
|---|---|---|---|---|
| position | $\delta_H$, mult., $J$ | $\delta_C$, mult. | $\delta_H$, mult., $J$ | $\delta_C$, mult. |
| 1 | 1.61, s | 25.2 q | 1.50 s | 24.7 q |
| 2 | | 109.0 s | | 107.9 s |
| 3a | 2.98 d, 18.4 | 48.6 t | 2.85 d, 18.6 | 48.8 t |
| 3b | 2.83 d, 18.4 | | 2.74 d, 18.6 | |
| 4 | | 199.4 s | | 200.5 s |
| 5 | | 82.6 s | | 83.1 s |
| 6 | | 169.2 s | | 168.7 s |
| 7 | 4.87 s | 86.4 d | 4.77 s | 86.4 d |
| 8 | | 93.1 s | | 93.0 s |
| 9a | 1.84 m | 45.3 t | 1.74 dd; 5.8, 14.4 | 44.5 t |
| 9b | 1.84 m | | 1.71 dd; 6.8, 14.4 | |
| 10 | 1.87 m | 24.2 d | 1.80 qq; 6.6, 6.6 | 23.6 d |
| 11 | 1.01 d, 6.3 | 23.4 q | 0.94 d, 6.6 | 23.2 q |
| 12 | 1.01 d, 6.3 | 23.8 q | 0.92 d, 6.6 | 23.7 q |
| 5-OH | 4.42 brs | | 6.53 s | |
| 6-NH | 7.38 brs | | 9.36 s | |

[a] The chemical shifts are given in ppm relative to the solvent signals, and the coupling constants ($J$) are in Hz.

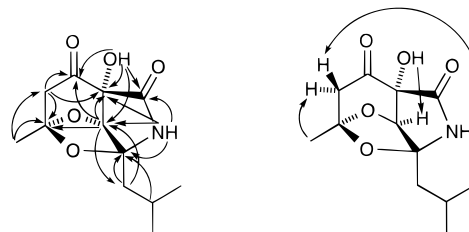



**Figure 1.** Pertinent HMBS (left) and NOESY (right) correlations observed with 2 in DMSO-$d_6$

which is in agreement with the 1D NMR data (presented in Table 1). The structure was then determined by analysis of COSY, NOESY, HMQC, and HMBC 2D NMR spectra, of which the pertinent correlations observed in the HMBC and NOESY spectra of **2** recorded in DMSO-$d_6$ are summarized in Figure 1. The 1-$H_3$ protons correlate to C-2, to which C-1 must be attached, and C-3, while the 3-$H_2$ protons correlate to C-2, C-4, and C-5. The HMBC correlations between the exchangeable proton at $\delta$ 6.53 and the two carbonyl carbons C-4 and C-6, as well as C-5 and C-7, and the correlation between 7-H and C-2 show that C-2 and C-7 are linked by an ether bridge and that the proton signal at $\delta$ 6.53

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corresponds to 5-OH. The amide NH correlates to all carbons in the lactam ring, and the position of the isobutyl group on C-8 was proven by HMBC correlations between this carbon and both 9-$H_2$ and 10-H. It is reasonable to assume that **2** is formed from flavipucine (**3**). The carbon skeleton of the two compounds is the same, and the addition of water to the C-2/C-3 double bond of **3** produces a hypothetical compound in which the carbon oxidation pattern is the same as in **2**. The opening of the epoxide ring of **3** could be the driving force for its conversion to **2**, and the fact that no such transformation of **3** could be observed during its isolation suggests that the conversion is enzymatic. Although the two compounds are closely related, **2** possesses a unique oxidized tricyclic system that has never been reported previously.

Cladobotrin (**1**) exhibited weak nematicidal activity toward *Meloidogyne incognita* (Kofoid & White) Chitwood ($LD_{50}$ = 100 $\mu$g $mL^{-1}$), while **2** was inactive at concentrations up to 100 $\mu$g $mL^{-1}$. Compound **2** showed weak cytotoxic activity toward L-1210 cells, whereas HL-60 or HeLa S3 cells were not sensitive up to 100 $\mu$g $mL^{-1}$. At 50 $\mu$g $mL^{-1}$, L-1210 cell proliferation was reduced to 50%. In the plant germination assay with *Lepidium sativum* and *Setaria italica*, **2** inhibited the germination of 50% of the seedlings of *L. sativum* at 100 $\mu$g $mL^{-1}$. Compound **2** showed no antimicrobial activities, up to 100 $\mu$g $mL^{-1}$, toward several common bacteria, filamentous fungi, and yeasts. For **1**, antifungal activity against *Ganoderma lucida* has been reported.[4]

## Experimental Section

**General Experimental Procedures.** Preparative HPLC was carried out on a JASCO HPLC (PU 980, MD 910), analytical HPLC was carried out on a Hewlett-Packard 1090 Type II with LiChrosphere RP18 (10 $\mu$m; 125 × 4 mm) and a $H_2O$−MeOH gradient. $^1H$ NMR (500 MHz) and $^{13}C$ NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5-mm probehead equipped with shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals [$\delta_H$ 7.26 and $\delta_C$ 77.0 in $CDCl_3$, $\delta_H$ 2.50 and $\delta_C$ 39.5 in $(CD_3)_2SO$] were used as reference. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed, and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). MS were recorded with a JEOL SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin-Elmer $\lambda$ 16 and a Bruker IFS 48 spectrometer. The melting points (uncorrected) were determined with a Reichert microscope, and optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C.

**Producing Strain.** *C. rubrobrunnescens* W. Helfer was isolated from a fruit body of an *Inocybe* species collected in Regensburg, Germany.[1] 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 a medium consisting of: yeast extract 4 $gL^{-1}$, malt extract 10 $gL^{-1}$, glucose 4 $gL^{-1}$ (YMG), pH 5.5.

**Fermentation and Isolation.** Fermentations were carried out at 24 °C in a fermentor containing 20 L of YMG medium with aeration (3.4 L air/min) and agitation (120 rpm); 200 mL of a well-grown culture (5 days) in the same medium were used as inoculum. After 10 days the cultures were harvested. The mycelium containing none of the metabolites was separated from the culture fluid and discarded. The compounds were extracted from the culture fluid by adsorption onto HP 21 resin (Mitsubishi). After washing the resin with $H_2O$, the metabolites were eluted with MeOH. The crude extract (3.8 g) obtained by concentration was fractionated on Si gel (Merck 60, 63−200 $\mu$m) using cyclohexanes−EtOAc. Cladobotrin (**1**) (1 mg) was isolated by elution with cyclohexanes−EtOAc (4:1) followed by preparative HPLC (LiChrospher RP-18, 7 $\mu$m, column size 250 × 25 mm, flow rate 5 mL $min^{-1}$) with $H_2O$−MeCN (1:1). Compound **2** (95 mg) was isolated by elution with cyclohexanes−EtOAc (3:2), followed by preparative HPLC (same column as above) with $H_2O$−MeCN (3:2).

**Rubrobramide (2):** obtained as white crystals (EtOH), mp 131−133 °C; $[\alpha]_D$ +177 ° (*c* 1.0 in $CHCl_3$); UV (MeOH), $\lambda_{max}$ ($\epsilon$) no maxima above 210 nm; IR (KBr) 3483, 3435, 3280, 2956, 2877, 1752, 1722, 1435, 1388, 1311, 1232, 1190, 1122, 1054, 1008, 882, and 596 $cm^{-1}$; see Table 1 for NMR data; EIMS (70 eV), *m/z* (rel int) 255 (1%, $M^+$), 213 (100%), 171 (35%), 170 (31%), 154 (33%), 153 (24%), 128 (46%), 127 (53%), 112 (38%), 83 (41%), 43 (54%). CIMS ($CH_4$), *m/z* (rel int) 256.1179 (M + $H^+$, 100%, $C_{12}H_{18}NO_5$ requires 256.1185), 238 (M + $H^+$ − $H_2O$, 70%).

**Biological Assays.** The assays for nematicidal,[5] antimicrobial,[6] phytotoxic,[6] and cytotoxic[7] activities were carried out as described previously.

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