6-Methylpurine, 6-Methyl-9-β-D-ribofuranosylpurine, and 6-Hydroxymethyl-9-β-D-ribofuranosylpurine as Antiviral Metabolites of *Collybia maculata* (Basidiomycetes) [1]

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Collybia maculata, Basidiomycetes, Nucleosides, 6-Methylpurine, Antiviral Activity

6-Methylpurine (**1**), 6-methyl-9-β-D-ribofuranosylpurine (**2**) and 6-hydroxymethyl-9-β-D-ribofuranosylpurine (**3**) were isolated from mycelial cultures of *Collybia maculata* and their antifungal, cytotoxic and antiviral activities investigated. This is the first report on the natural occurrence of these compounds.

Introduction

Basidiomycetes provide a good source for secondary metabolites with unusual structures and biological activities [2]. Reports on compounds with antiviral activities produced by these fungi have been scarce, however. When we included a test for inhibition of the multiplication of vesicular stomatitis virus (VSV) in baby hamster kidney (BHK) cells in our screening we detected that mycelial cultures of *Collybia maculata* produced three compounds with antifungal, cytotoxic, and antiviral (VSV) but no antibacterial activities. In the following we describe the production of these metabolites, the elucidation of their structures, and the investigation of their biological activities.

Experimental

Fermentation and isolation

Collybia maculata strain 79111 was derived from a fruiting body collected near Tübingen. For the maintenance on agar slants and for submerged cultivation the fungus was grown in a medium composed of 0.4% yeast extract, 1% malt extract, and 0.4% glucose. The pH was adjusted to 5.5. Fermentations were carried out in a tank containing 100 l of culture (Deutsche Metrohm) with aeration (10 l air/min) and agitation (100 rpm) at 22 °C. The inoculum was 15%. The production of the antibiotics could be followed by plate diffusion assay with *Nematospora coryli* as test organism. After 9–11 days of fermentation the mycelia were separated from the culture fluid by filtration. Compounds **1** and **2** were extracted from the culture fluid with *n*-butanol and purified by column chromatography on silica gel (Merck 60, eluant: dichloromethane/methanol 92:8) and Sephadex LH 20 (eluant: methanol). From the fractions containing **1** and **2**, **2** could be crystallized from methanol yielding 1.3 mg/l of culture. **1** was purified from the mother liquor by preparative TLC on silica gel (Merck 5554) developed in *n*-BuOH/EtOH/H_2O (4:1:2). Yield: 0.3 mg/l of culture. **3** was obtained by extraction of the mycelia with methanol and chromatography on silica gel and Sephadex LH 20 as above. Crystallization from methanol yielded 0.6 mg/l of culture.

Physical and spectroscopic data

NMR spectra were recorded on a Bruker WM-400 spectrometer in [D_6]DMSO with tetramethylsilane as the internal standard. The high-resolution mass spectra were determined with an AEI MS-50 spectrometer. Analytical thin-layer chromatography (TLC) was performed with Merck silica gel 60 F-254 aluminium-backed plates; solvent system: *n*-BuOH/EtOH/H_2O (4:1:5) unless otherwise stated. Melting

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points were obtained on a Reichert hot plate microscope and are uncorrected.

6-Methylpurine (**1**): m.p. 235 °C (Lit. [3] m.p. 235–236 °C), R_f 0.48; ^{1}H NMR: δ 2.77 (s, 3H), 8.58 (s, 1H), 8.81 (s, 1H); ^{13}C NMR: δ 19.6 (Q, J = 128 Hz, CH_3), 144.8 (D, J = 210 Hz, C-8), 151.6 (D, J = 202 Hz, C-2); MS (180 °C): m/z 134.0599 (M^+, 100%, calc. for $C_6H_6N_4$ 134.0593).

6-Methyl-9-β-D-ribofuranosylpurine (**2**): m.p. 210 °C (Lit. [4, 5] m.p. 209–210 °C); R_f 0.42; $[\alpha]_D$: −47.5° (c = 0.12, in H_2O) (Lit. [5] $[\alpha]_D$ −52.0); ^{1}H NMR (+D_2O): δ = 2.74 (s, 3H), 3.58, 3.68 (ABX system, J = −12.0/4.0/3.5 Hz, 5′-CH_2OD), 3.98 (ddd, J = 4.0/3.5/3.5, 4′-H), 4.19 (dd, J = 5/3.5 Hz, 3′-H), 4.63 (dd, J = 5.7/5.0 Hz, 2′-H), 6.02 (d, J = 5.7 Hz, 1′-H), 8.77 (s, 8-H), 8.80 (s, 2-H); ^{13}C NMR: δ 19.1 (Q, J = 128 Hz, CH_3), 61.3 (T, 140, CH_2-5′), 70.3 (D, 149, C-3′), 73.6 (148, C-2′), 85.7 (D, 148, C-4′), 87.6 (D, 164, C-1′), 132.9 (dq, 10/6, C-5), 144.0 (Dd, 214/4.4, C-8), 150.1 (m, C-4), 151.6 (D, 203, C-2), 158.3 (m, C-6). The assignment of the signals was confirmed by the ^{1}H/^{1}H and ^{1}H/^{13}C shift correlated 2D NMR spectra. MS (180 °C): m/z = 266.1018 (M^+, 1%, calc. for $C_{11}H_{14}N_4O_4$ 266.1015), 235 (1.2), 177 (28.6), 163 (89.5), 135 (100, $C_6H_7N_4$), 107 (7.2), 73 (12.7), 57 (10.5), 55 (55.0).

6-Hydroxymethyl-9-β-D-ribofuranosylpurine (**3**): m.p. 152–156 °C; R_f 0.38 (*n*-BuOH/EtOH/H_2O 4:1:2); ^{1}H NMR (+D_2O): δ 3.57, 3.67 (ABX system, J = −12/4.0/3.5 Hz, 5′-CH_2OD), 3.98 (ddd, J = 4.0/3.5/3.5 Hz, 4′-H), 4.19 (dd, J = 5.0/3.5 Hz, 3′-H), 4.63 (dd, J = 5.7/5.0 Hz, 2′-H), 4.89 (s, 6-CH_2OH), 6.03 (d, J = 5.8 Hz, 1′-H), 8.73 (s, 8-H), 8.88 (s, 2-H); ^{13}C NMR: δ 59.9 (T, J = 140 Hz, 6-CH_2OH), 61.2 (T, 140, 5′-CH_2OH), 70.3 (D, 149, C-3′), 73.6 (D, 148, C-2′), 85.7 (D, 148, C-4′), 87.6 (D, 166, C-1′), 131.41 (br. d, 11, C-5), 144.4 (Dd, 214/4.4, C-8), 150.8 (m, 4-H), 151.7 (D, 203, 2-H), 159.7 (m, C-1); MS (200 °C): m/z = 282.0957 (M^+, 0.4%, calc. for $C_{11}H_{14}N_4O_5$ 282.0965), 265 (0.6), 251 (1.6), 193 (24), 151 (88, $C_6H_7N_4O$).

Antimicrobial activity

The antifungal activities were determined in the conventional plate diffusion assay as described previously [6]. All fungi were grown on YMG medium.

Cell culture

Ehrlich ascites tumor (ECA) cells were grown in suspension culture in F-12 medium containing 15% horse serum at 37 °C. Murine sarcoma virus transformed Balb/3T3 cells ATCC CCL 163.2 (M-MSV-Balb/3T3) were grown in Dulbecco's modified Eagle's medium containing 10% of fetal calf serum. Baby hamster kidney cells ATCC CCL 10 (BHK-21) were grown in a modified Eagle's BME medium containing 10% tryptose phosphate broth and 10% fetal calf serum.

All media contained 65 μg/ml of penicillin and 100 μg/ml of streptomycin. Except for the ECA cells the cultures were incubated in an humidified atmosphere containing 5% of CO_2. The temperature was kept at 37 °C. Cytotoxicity was tested in microtiter plates with 10^6 cells in 250 μl of medium containing the antibiotic. At suitable intervals the cells were examined under the microscope either directly or after staining with crystal violet or trypane blue.

Vesicular stomatitis virus (VSV)

VSV (ATCC VR 158, Indiana strain) was propagated in BHK-21 cells grown in microtiter plates. After removal of the medium from the cells 250 plaque forming units (PFU) of VSV in 25 μl of modified Eagle's BME medium with 2% of fetal calf serum were added and incubated for 1 h at 37 °C. Then 75 μl of the same medium containing the compounds to be tested were added and the cultures incubated until 75–90% of the cells in the controls containing no antibiotic were lysed. Cytotoxicity for BHK-21 cells was tested simultaneously in controls containing the compounds but no virus. The effect of the antibiotics on virus multiplication and lysis of cells was tested after staining the cells with crystal violet in 70% aqueous methanol according to [7]. Virus titers were assayed by withdrawing 10 μl aliquots in 6 h intervals and determining the plaques formed on BHK-21 cells after suitable dilution of the virus-containing samples [8].

Macromolecular syntheses in ECA cells

ECA cells were grown for 24 h as described above and suspended in phosphate buffered saline (10^6 cells/ml). The incorporation of labelled thymidine, uridine, and leucine into DNA, RNA, and protein was tested as described previously [6].

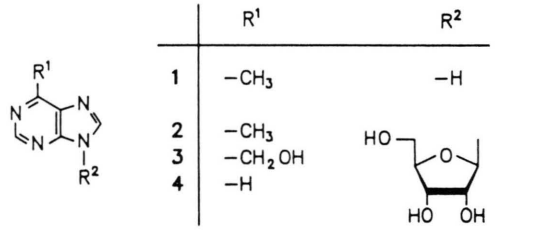
Adenosine deaminase (ADA)

ADA from bovine spleen (type IV, 63 U/mg) was purchased from Sigma. The enzymic activity was

tested according to [9]. The assays contained 0.1 M phosphate buffer (pH 7.5), 3.15×10^{-3} U of enzyme, 1.3×10^{-4} M adenosine and 1.3×10^{-4} to 1.3×10^{-5} M **1**, **2** or **3**.

Results and Discussion

During the fermentation the accumulation of the antibiotics started after 2 days and then paralleled growth. The highest yields were obtained after 10–11 days when the glucose of the medium was used up and growth ceded (Fig. 1). The three antifungal, cytotoxic and antiviral metabolites were isolated as described in the experimental section. From their spectra and melting points, given in the experimental section, **1** and **2** were identified as 6-methylpurine and 6-methyl-9-β-D-ribofuranosylpurine, respectively. The ^1H and ^{13}C NMR data of **2** are in excellent agreement with literature values reported for nebularine (**4**) [10, 11]. **1** and **2** have been obtained before by syntheses [3, 4], however, they have not been identified so far as natural products. Compound **3**, $C_{11}H_{14}N_4O_5$, closely resembles **2** in its ^1H and ^{13}C NMR spectra with the exception of the 6-methyl signals which are replaced by those of a hydroxymethyl group. **3** is therefore 6-hydroxymethyl-9-β-D-ribofuranosylpurine, a compound which has previously been obtained in impure form by dehydrogenation of the photoadduct resulting from irradiation of nebularin (**4**) in methanol [12].



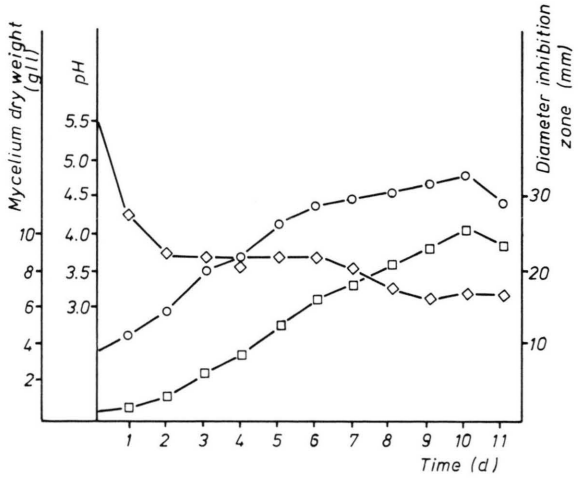


Fig. 1. Time course of a fermentation of *Collybia maculata*. Dry weight of the mycelium (−□−), pH (−◇−), antifungal activity in the plate diffusion assay on test plates of *Nematospora coryli* (diameter inhibition zone −○−).

Only a few nucleosides have been reported as secondary metabolites from fungi [13]. From basidiomycetes nebularin (**4**) [14] had been described as an antibacterial antibiotic produced by *Clitocybe nebularis* and lentinacin [15] as a hypocholesterolemic compound produced by *Lentinus edodes*. Recently two biologically active nucleosides clitidine [16, 17] and clitocine [18] have been isolated from fruit-bodies of *Clitocybe acromelalga* and *C. inversa*, respectively.

The antifungal activity of **1**, **2**, and **3** in the plate diffusion assay is shown in Table I. The antibiotics exhibit some selectivity against the sensitive fungi. Most of the fungi tested were not inhibited in the same assay. Up to concentrations of 100 μg/ml no inhibition of bacterial growth could be observed in the serial dilution assay performed as described earlier [6]. The cytotoxic activities of **1**, **2**, and **3** are quite high (Table II), but could be observed only after prolonged incubation (24–36 h) with the antibiotics. The cytotoxic effects of **1** and **2** are in agreement with the data described in the literature [12]. The effect of the three antibiotics on the short time incorporation (30 min) of thymidine, uridine, and leucine into TCA-precipitable material was investigated with ECA cells. DNA, RNA, and protein syntheses were inhibited almost simultaneously and only at high concentrations. A 50% inhibition was

Table I. Antifungal spectra of **1**, **2**, and **3** in the plate diffusions assay.

Testorganism	Diameter inhibition zone [mm] 100 μg/disc		
	1	**2**	**3**
Alternaria solani	30	–	–
Ascochyta pisi	30	30	30
Nematospora coryli	–	30	22
Neurospora crassa	20	12	18
Paecilomyces varioti	15	–	10
Pythium debaryanum	–	–	12

Table II. Cytotoxic activities of **1**, **2**, and **3**.

Testorganism	Concentration with 90% lysed cells [μmol/l]		
	1	**2**	**3**
ECA-cells	30	9	141
BHK-21	3.72	5.6	10.6
Balb/3T3	1.8	1.5	1.4
SV-T2	1.8	0.9	0.84
M-MSV-Balb/3T3	3	1.5	1.4

Table III. Inhibition of adenosine deaminase in percent of the controls without antibiotics.

Compound	Inhibition [%] Concentration [μM]	
	13	130
1	0	7
2	0	31.5
3	36	83.2

observed at concentrations of 100 μM or above. In Fig. 2 the effect of **1**, **2**, and **3** on the propagation of VSV in BHK cells is compared with ara A (9-β-D-arabinofuranosyladenine). The inhibitory action of **1** and **3** compares favourably with ara A. **1**, **2**, and especially **3** are inhibitors of adenosine deaminase (Table III), which is in good agreement with previously published results [12].

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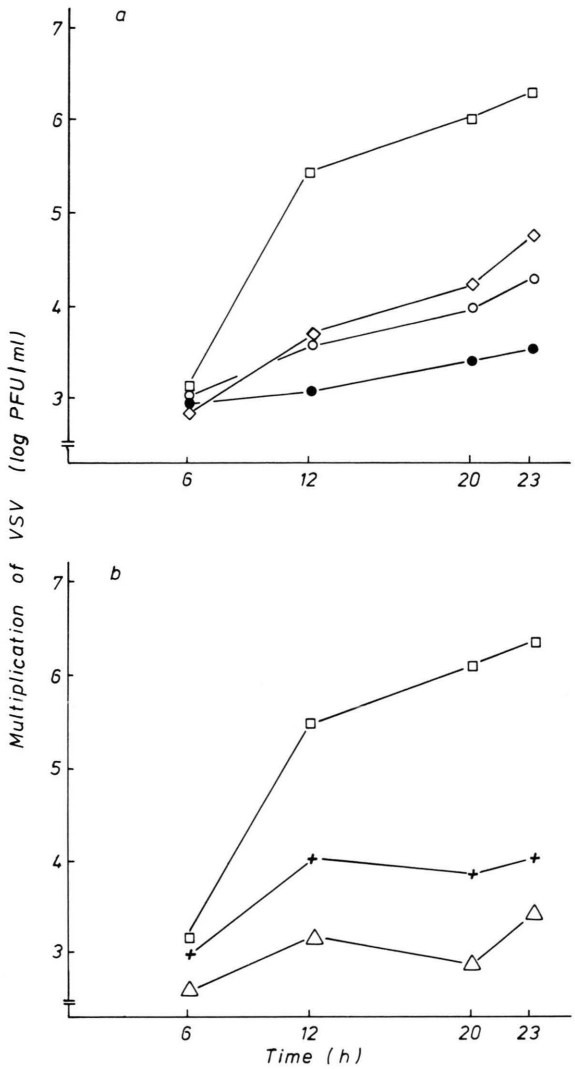

Fig. 2. Effect of **1**, **2**, **3** and ara A on the multiplication of VSV in BHK-21-cells. a) Control, no additions −□−; 150 μM ara A −◇−; 9 μM **2** −○−; 90 μM **2** −●−. b) Control, no additions −□−; 90 μM **1** −△−; 140 μM **3** −+−.

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