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Heptemerones A～G, Seven Novel Diterpenoids from *Coprinus heptemerus*: Producing Organism, Fermentation, Isolation and Biological Activities

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Abstract Seven novel diterpenoids, named heptemerones A～G, were isolated from the broth of submerged cultures of *Coprinus heptemerus*, a basidiomycete which previously had not been known to produce secondary metabolites. The compounds were purified by solid phase extraction and silica gel chromatography followed by preparative HPLC. Among the biological activities the inhibition of fungal germination was the most potent, and depended highly on the composition of the assay medium. In water, inhibition occurred at 5～10 fold lower concentrations as compared to complex media. Heptemerone G was the most active compound with MICs starting at 1 μg/ml. Four of the antifungal compounds exhibited plant protective activity in a leaf segment assay using *Magnaporthe grisea* as the pathogen. Growth of yeasts and bacteria was hardly affected. Cytotoxic activities were moderate and only heptemerone D was phytotoxic.

Keywords *Coprinus heptemerus*, heptemerones, diterpenes, inhibition of conidial germination, *Magnaporthe grisea*

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Introduction

In the course of our screening for novel plant protectants, *Coprinus heptemerus* was found to produce compounds which inhibited germination of conidia without interfering with mycelial growth of *Magnaporthe grisea*. The rationale behind this approach was to inhibit selectively infection-related differentiation in phytopathogenic fungi as described before [1～5]. So far no secondary metabolites have been described from *C. heptemerus*, a basidiomycete growing on rabbit dung [6]. Coprophilic fungi have been shown to be a good source for bioactive compounds in the past [7～10], therefore this fungus was selected for the isolation and characterisation of the active principles. In this paper we describe the producing organism, its cultivation, the isolation of seven structurally related diterpenoids and their biological activities, while the structure elucidation and physico-chemical properties of the compounds are reported in a separate paper [11].

Material and Methods

Microorganisms

Coprinus heptemerus, strain D99052, was grown and kept on YMG-medium consisting of glucose 1%, malt extract 1% and yeast extract 0.4% in tap water at 22°C. For solid media 1.5% agar was added.

Magnaporthe grisea strain 70-15 was obtained from the Fungal Genetics Stock Centre, Kansas. The strain was grown on CM medium as described by Talbot *et al*. [12].

Conidia were harvested from plates incubated at 28°C in a 16-hour-light and 8-hour-dark cycle. During the dark period the temperature was lowered to 24°C.

Fermentation

Ten pieces cut from agar slants were transferred into a 2000 ml Erlenmeyer flask containing 1000 ml YMG medium. The flasks were incubated on a rotary shaker at 120 rpm and 22°C. Fermentation on a larger scale was carried out in a Biostat D-300 (Braun, Melsungen, Germany) containing 200 liters of YMG medium with stirring (120 rpm) and aeration (30 liters air per minute) at 22°C. To prevent foaming, silicone antifoam (Merck, Darmstadt) was added. The fermentor was inoculated with 5 liters of a well grown culture in the same medium. Daily samples were withdrawn and assayed for pH, glucose and maltose content as well as for biological activity towards germination of *M. grisea* conidia. The culture broth was separated by filtration. Mycelia contained no active compounds were discarded. The culture broth from shake flasks was extracted with EtOAc, the organic phase dried with Na_2SO_4, concentrated *in vacuo* and the residue dissolved in MeOH to a concentration of 10 mg/ml.

Isolation of the Compounds

The broth was passed through a column of DIAION HP21 resin in water (Mitsubishi Chemical Industry LTD, Dusseldorf, column size: 100×440 mm). The eluate was discarded. After washing with water (6 liters), the compounds were eluted with MeOH (8 liters), followed by Me_2CO (6 liters). Upon concentration two crude products, CP I (20.4 g from the MeOH fraction) and CP II (19.4 g from the Me_2CO fraction) were obtained. Chromatography on silica gel in cyclohexane - EtOAc (column size: 95×220 mm; silica gel 60, 63~200 μm particle size; Merck, Darmstadt, Germany) and elution with cyclohexane - EtOAc with increasing amounts of EtOAc (90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50 and 40 : 60, 2.5 liters each) yielded three fractions with antifungal activity from each of the crude products. Final purification was achieved by preparative HPLC with a Jasco modular HPLC system (Gross-Umstadt, Germany) consisting of two binary pumps (PU-1586) and the multi-wavelength detector UV-1570M. The HPLC was fitted with a LiChrosorb RP-18 column (250×25 mm, 7 μm particle size; Merck) and run at a flow rate of 7.5 ml/minute and a linear gradient from water to MeOH in 90 minutes. Each run was performed with 120 mg aliquots of the active mixtures.

Biological Assays

Germination assay: Conidia from 10-day-old cultures were harvested. After centrifugation at 1000 *g* for 10 minutes the conidia were resuspended in distilled water to a concentration of 5×10^5 per ml. The test was carried out in 24-well microtiter plates (Sarstedt, Nuremberg) with 2.5×10^4 conidia in 1 ml of distilled water and incubation for 16 hours at 28°C. Germinated conidia were counted with an inverted microscope (Leica DM IRB). Tests were conducted in triplicates and 100 conidia were counted 3 times.

Leaf segment assay: Leaves from 16-day-old rice (*Oryza sativa* var. Tainong 67) or barley (*Hordeum sativum* var. Scarlett) seedlings were cut into 5 cm long segments and placed on water agar plates (1.5% agar in tap water). *M. grisea* condia (2.5×10^4/ml) and the test compounds were suspended in a 0.2% gelatine solution. Four droplets (40 μl for barley and 20 μl for rice) of this solution were placed on each segment and the plates were incubated for 96 hours at 28°C. The number and the size of the lesions were compared with the control without test compounds.

Antimicrobial activity was determined in the serial dilution test and the inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described before [13]. Bacteria were tested in nutrient broth (Difco), yeasts and fungi in YMG medium.

Cytotoxic activity was assayed as described previously [14] with slight modifications. Jurkat cells (DSMZ ACC 282) and Mono-Mac-6 cells (DSMZ ACC 124) were grown in RPMI 1640 medium (GIBCO, BRL), HeLa S3 (ATCC CCL 2.2) and Hep G2 (DSMZ ACC 180) cells in D-MEM (GIBCO, BRL), supplemented with 10% fetal calf serum (GIBCO, BRL), 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulphate. The assays contained 1×10^5 cells/ml medium. The concentration at which cell proliferation was reduced by 50% is given as IC_{50} values.

Results

Producing Organism

The producing organism was isolated from fruiting bodies growing on rabbit dung. The features of the fruiting bodies and basidiospores corresponded with the description of *Coprinus heptemerus* M Lange & AH Smith given by Orton and Watling [15]. On agar plates, especially upon addition of rabbit dung the culture produced tiny fruiting bodies with stipes 1.5~3 cm long and basidiocarps 0.3~0.5 cm in diameter.

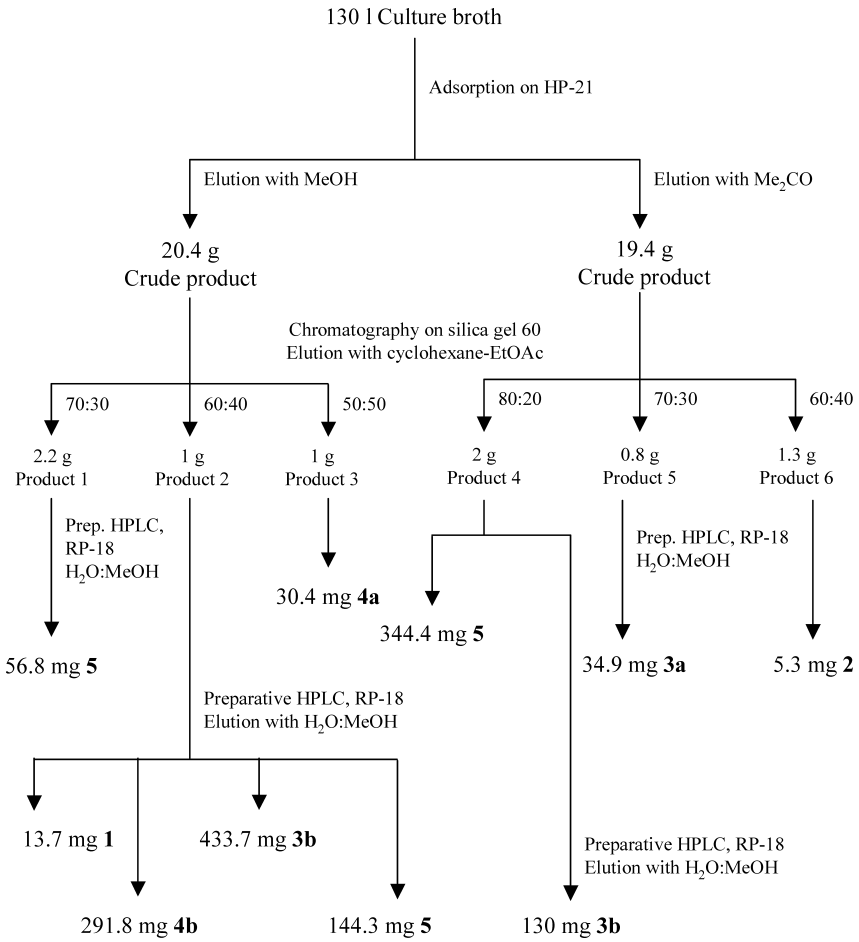

Fig. 1 Purification of heptemerones A (**1**)~G (**5**) from a culture broth of *C. heptemerus*.

Fermentation and Isolation of Compounds 1, 2, 3a, 3b, 4a, 4b, and 5

Eight different media were used for the evaluation of the production of the bioactive metabolites: malt medium (4% malt extract or 2% malt extract), potato - dextrose medium (2% glucose, 0.4% potato powder), CM medium, corn meal medium (corn meal 1%, glucose 1%, KCl 0.05%, $NaNO_3$ 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.15%), YMG medium, YMG medium with 0.4% glucose, and Czapek yeast medium [16]. Growth in corn meal and potato dextrose medium was rather slow and no bioactive compounds have been detected. Among the other media, YMG medium yielded the highest activity in the crude extracts. Therefore, this medium was chosen for the production and isolation of the compounds in a 200-liters fermentor. The fermentation was terminated after six days after exhaustion of the carbon sources. The active compounds were isolated from the broth as shown in Fig. 1. It is interesting to note that the composition of the heptemerone mixture was less complex when the fungus was grown in flasks. These crude extracts contained only compounds **3b**, **4a** and **4b**. The concentrations of the single compounds, however, were much higher: 17.8 mg/liter of **3b**, 15.6 mg/liter of **4a**, and 47.5 mg/liter **4b** *versus* 4.2 mg/liter of **1**, 2 mg/liter of **2**, 1.6 mg/liter of **3a**, 8.4 mg/liter of **3b**, 5.1 mg/liter of **4a**, 7.5 mg/liter of **4b**, and 9.1 mg/liter of **5** in the fermentor (for structures see Fig. 2).

Biological Activities of Compounds 1, 2, 3a, 3b, 4a, 4b, and 5

The antibacterial activities were very weak. *Escherichia coli*, *Mycobacterium phlei* and *Arthrobacter citreus* were not affected by any of the compounds. Compounds **3a**, **3b** and **5** were bacteriostatic towards *Pseudomonas fluorescens*, *Micrococcus luteus*, *Corynebacterium insidiosum*, *Bacillus brevis* and *B. subtilis* at 20 μg/ml. No activity towards *Candida albicans* and *Candida glabrata* up to 100 μg/ml was observed.

Mycelial growth of *M. grisea* was weakly affected at concentrations higher than 40 μg/ml of compounds **1**, **2**, **3a**,

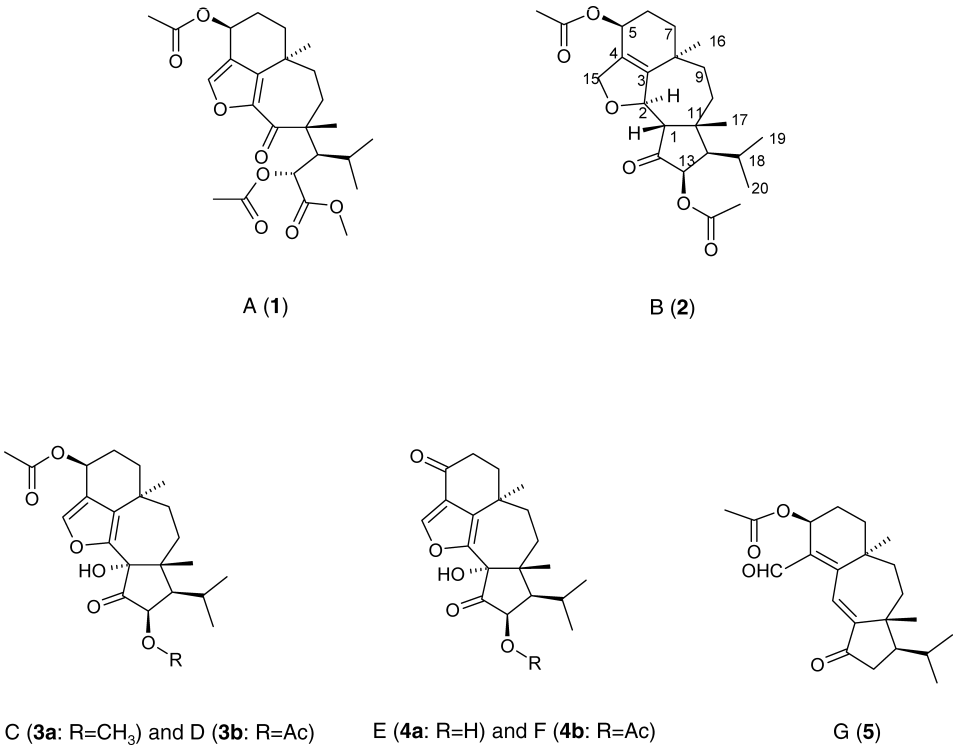

Fig. 2 Structures of heptemerones A (**1**)∼G (**5**)

3b, **4a**, and **4b**, whereas compound **5** inhibited the growth at 10 μg/ml.

The antifungal activities, measured as inhibition of spore germination, are given in Table 1. Activities in water were much higher as compared in YMG medium. Compound **5** was the most active one, followed by **3a**, **3b** and **4b**. *M. grisea* and *Colletotrichum graminicola* were the most susceptible fungi. These four compounds also showed protective activity in the leaf segment assay with *Oryza sativa* and *Hordeum sativum* (Table 2).

Phytotoxic activities were only recorded for compound **3b** towards *O. sativa* starting at 65 μg/ml. At this concentration the shoot length was reduced by 85%, while the root length was only weakly affected at 330 μg/ml (reduction by 50%). The other compounds were not phytotoxic up to 330 μg/ml; germination and growth of *Lepidium sativum* was not inhibited.

Cytotoxic activities, as shown in Table 3, varied according to the cell line used. Jurkat and Mono-Mac-6 cells were the most sensitive cells and HeLa S3 cells were hardly affected. Even so **5** showed the highest inhibition of proliferation, compound **1** was selectively cytotoxic.

No nematicidal activities towards *Caenorhabditis elegans* and *Meloidogyne incognita* were observed at concentrations up to 100 μg/ml.

Discussion

Coprinus heptemerus, a tiny, coprophilous basidiomycete that is often overlooked, belongs to a rather large genus which is currently under revision [17]. Some species of the 350 that comprise the genus have been reported to produce bioactive secondary metabolites [18]. Especially *C. quadrifidus*, a prolific producer of polyines [19], and *C. atramentarius* [20] have thoroughly been investigated. But more than 90% of the species, however, remain to be examined for their secondary metabolite spectrum. *C. heptemerus*, the species investigated here, produced seven structurally related diterpenes which were named heptemerones A∼G (**1**, **2**, **3a**, **3b**, **4a**, **4b** and **5**). These compounds are structurally not related to any of the metabolites reported from other *Coprinus* species. Compounds with the same carbon skeleton, the guanacastepenes, have been isolated from an endophyte of *Daphnopsis americana* [21, 22], which most probably belongs to the Basidiomycetes [23]. In contrast to Ascomycetes, Basidiomycetes are prolific producers of terpenoids including the dipterpenoids, heptemerones and guanacastepenes [24]. The latter were isolated due to their antibacterial activity whereas the antibacterial activity of

Table 1 Antifungal activities of compounds **1**, **2**, **3a**, **3b**, **4a**, **4b**, and **5**. Inhibition of germination of conidia, MIC represents the concentration at which no germination of the conidia was observed

Test organism (medium)	MIC [μg/ml]						
	1	**2**	**3a**	**3b**	**4a**	**4b**	**5**
Bipolaris victoriae (H_2O)	>100	>100	>100	100	>100	>100	20
Bipolaris victoriae (YMG)	>100	>100	>100	50	>100	>100	50
Botrytis cinerea (H_2O)	>100	50	20	20	>100	50	5
Botrytis cinerea (YMG)	>100	>100	100	100	>100	100	20
Colletotrichum graminicola (H_2O)	>100	>100	10	20	>100	50	1
Colletotrichum graminicola (YMG)	>100	>100	50	100	>100	100	5
Drechslera oryzae (H_2O)	>100	>100	20	20	>100	20	10
Drechslera oryzae (YMG)	>100	>100	>100	>100	>100	>100	100
Fusarium solani (H_2O)	>100	—	100	100	>100	>100	20
Fusarium solani (YMG)	>100	>100	>100	100	>100	>100	100
Magnaporthe grisea (H_2O)	>100	>100	10	20	>100	20	1
Magnaporthe grisea (YMG)	>100	>100	50	50	>100	50	5
Ascochyta pisi (YMG)	>100	>100	>100	>100	>100	>100	10
Aspergillus ochraceus (YMG)	>100	>100	>100	>100	>100	>100	50
Cladosporium cladosporioides (YMG)	>100	>100	>100	>100	>100	>100	20
Paecilomyces varioti (YMG)	>100	>100	>100	>100	>100	>100	50
Penicillium notatum (YMG)	>100	>100	50	>100	>100	>100	20
Penicillium janczewskii (YMG)	>100	>100	>100	>100	>100	>100	50
Phytophthora infestans (YMG)	>100	>100	>100	>100	>100	>100	>100
Septoria tritici (YMG)	>100	>100	>100	>100	>100	100	20

Table 2 Antifungal activities of compounds **1**, **2**, **3a**, **3b**, **4a**, **4b**, and **5** in the leaf segment assay with *Oryza sativa* and *Hordeum sativum* and *M. grisea* as pathogen. MIC represents the concentration at which no lesions were observed after 96 hours

Test plant	MIC [μg/ml]						
	1	**2**	**3a**	**3b**	**4a**	**4b**	**5**
Oryza sativa	100	100	10	20	>100	10	1
Hordeum sativum	50	100	20	>50	>100	50	1

the heptemerones is negligible compared to their antifungal and cytotoxic properties. The antibacterial inhibition zones for guanacastepene A however, the most active compound of the series, is in the same range as for the most active heptemerone *i.e.* compound **5**. This compound, similar to guanacastepene A, possesses two reactive groups, an α,β-unsaturated aldehyde function and an α,β-unsaturated ketone group, which are likely to be responsible for the biological activities since in the presence of cysteine the activities were abolished. Upon incubation with an equimolar amount of cysteine, after one hour the compound was no longer detectable (data not shown). This is in agreement with the non-selective inhibition of all macromolecular syntheses in *E. coli* by guanacastepene A [23]. Similar results were obtained for heptemerone G (**5**) in *M. grisea* (data not shown). With the exception of a weak inhibition of *Candida albicans*, no antifungal or cytotoxic activities have been reported for the guanacastepenes, while

Table 3 Cytotoxicity of compounds **1**, **2**, **3a**, **3b**, **4a**, **4b**, and **5** towards Jurkat cells, Mono-Mac-6 cells, HeLa S3 cells, and HepG2 cells. IC_{50} represents the concentration at which cell proliferations was reduced to 50%

Compound	IC_{50} [μg/ml]			
	Jurkat	Mono-Mac-6	HeLa S3	HepG2
1	5~10	20	100	>100
2	50	>100	>100	>100
3a	5~10	10	20	20
3b	20~50	10	100	>100
4a	50	50	>100	50
4b	10	20	100	100
5	2	1	20	20

none of the heptemerones affected the two *Candida* strains tested in this investigation. The selective cytotoxic activity of compound **1** towards cells growing in suspension is noteworthy as **1** showed no activity in the other test systems.

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