NEW NEMATICIDAL AND ANTIMICROBIAL COMPOUNDS FROM THE BASIDIOMYCETE *Cheimonophyllum candidissimum* (Berk & Curt.) SING

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION, AND BIOLOGICAL ACTIVITIES

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Six new bisabolane type sesquiterpenoids, cheimonophyllons A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), and cheimonophyllal (**6**) were isolated from the culture fluid of the basidiomycete, *Cheimonophyllum candidissimum*. The compounds exhibited nematicidal, weak antifungal and antibacterial as well as cytotoxic activities. The main product, cheimonophyllon A (**1**) ($C_{15}H_{22}O_4$), was also active in the Ames mutagenicity test.

During a screening of higher fungi for nematicidal activities[1], *Cheimonophyllum candidissimum*, TA 8644, was found to produce toxins active towards *Caenorhabditis elegans* in solid as well as in liquid cultures. Six new bisabolane type sesquiterpenoids were isolated from submerged cultures by an activity-guided isolation procedure. In this report we wish to describe the producing organism, its fermentation, and the isolation and biological activities of the new metabolites. Their structure elucidation will be published separately[2].

Materials and Methods

General

Materials for chromatography, solvents, and other chemicals (p. A. quality) were purchased from Merck, Darmstadt, FRG, except the following materials: yeast extract (Hartge, Hamburg, FRG), malt extract (Dr. FRÄNKLE and M. ECK, Fellbach, FRG). Analytical HPLC was carried out on LiChroSpher RP18 (5 μm; column size: 125 × 4 mm; flow rate 1.5 ml/minute; eluant system H_2O - MeOH).

Cheimonophyllum candidissimum TA 8644

The producing organism was isolated in 1986 by Dr. TIMM ANKE from the spore print of fruiting bodies collected near Durham, NC, USA, which showed the characteristics of the genus and species[3]. Both voucher specimen and the mycelial culture are deposited at the Lehrbereich Biotechnologie, University of Kaiserslautern, FRG.

Strain TA 8644 was cultivated and maintained in YMG medium composed of: glucose 0.4%, yeast extract 0.4%, malt extract 1%. pH was adjusted to 5.5 before sterilization. For solid cultures, 1.5% of agar-agar were added.

Screening and Assays for Determination of Biological Activities

For the screening, mycelial cultures of higher fungi were incubated with water agar cultures of *Caenorhabditis elegans* Maupas, following the method described by BARRON and THORN[4]. Strains which killed nematodes in this assay were grown in submerged cultures in YMG medium and extracts were investigated for nematicidal activities in a microtiter plate assay towards *C. elegans*[1].

The biological activities of compounds **1**~**6** were evaluated following procedures which have been previously described: antimicrobial activities[5]; cytotoxic activities and inhibition of macromolecular syntheses in mammalian cells[6]; nematicidal activities[1]; phytotoxic activities[5]. The Ames test[7] was carried out as a pour-plate assay (diameter of plates 5 cm, 1 ml top layer and 5 ml bottom layer) without S9 mix, using *Salmonella typhimurium* strains TA 97, TA 98, TA 100, TA 102, and TA 1535. Revertants were counted after 48~60 hours of incubation. Ethyl methanesulfonate and daunomycin (Sigma, St. Louis) were used as control mutagens.

Fermentation of Strain TA 8644 and Isolation of Bioactive Metabolites

Fermentations were carried out in a 20 liter Braun fermentation apparatus with aeration (3 liters per minute) and agitation (150 rpm). On line data on oxygen saturation of the culture medium (pO_2; Ingold 405 electrode), CO_2 production (ADC carbon dioxide analyser SB-305, ADC England, London), and oxygen consumption (Magnos 4G oxygen analyser, Hartmann and Braun Frankfurt/Main, FRG) were recorded by the Braun MicroMFCS process control system. An antifoam alarm system was connected with automatic delivery of silicone antifoam (Merck). For inoculum 400 ml of a six days old shake culture (120 rpm) in the same medium were used. During fermentation, 100 ml samples were extracted with ethyl acetate. The organic extracts were filtered through 5 g anhydrous Na_2SO_4, dried *in vacuo* (40°C) and redissolved in 1 ml methanol. Antimicrobial activities were followed in the agar diffusion assay with *Nematospora coryli* and *Bacillus brevis*, using 10 μl of these extracts. Nematicidal activities were determined using different aliqouts of the extracts. Activities were calculated as multiples of the lethal dose contained in 1 ml of culture fluid extracts (LD_{100}/ml). After termination of the fermentation, the mycelium, containing no active compounds, was separated from the fluid by filtration and discarded. The culture fluid (18 liters) was applied onto Mitsubishi HP 21 resin. The column (30 × 7 cm) was washed with 3 liters of H_2O and 2 liters of water-acetone (8 : 2). The bioactive fractions were eluted with 2.5 liters of acetone. The acetone was evaporated *in vacuo* to an aqueous residue, which was extracted twice with EtOAc. After evaporation of the solvent from the combined EtOAc extracts, 1.4 g of an oily crude product were obtained. MPLC on silica gel 60 (25~40 μm; column size 250 × 25 mm; flow rate 5 ml/minute) in cyclohexane-EtOAc (3 : 1) yielded 189 mg of an intermediate product (in fraction 250~320 ml). From this intermediate product, 54 mg of **1**, 9 mg of **2**; 30 mg of **3**, 2 mg of **4**, 16 mg of **5**, and 7 mg of **6** were separated by HPLC (LiChroSorb Diol, 7 μm; column size 250 × 25 mm; flow rate 5 ml/minute) in cyclohexane-*tert*-butyl methyl ether (3 : 2). On analytical HPLC, the compounds were identified using the following gradient: 0~70% MeOH (0~20 minutes); 70~100% MeOH (20~30 minutes); detection at 210 nm (compounds **3** and **5**) and 230 nm (**1**, **2**, **4** and **6**). Retention times (minutes) for compounds **1**~**6** were: **1**: 17.7; **2**: 18.4; **3**: 20.2; **4**: 17.1; **5**: 18.1; **6**: 15.8.

Results and Discussion

Producing Organism

Cheimonophyllum candidissimum (Berk and Curt.) Sing. is a pleurotoid wood-inhabiting basidiomycete with colorless, globose spores[3]. Together with the related *Pleurocybella*, the genus *Cheimonophyllum* belongs to the *Collybiae* tribus within the *Tricholomataceae*[8]. On agar cultures, after two weeks of incubation, the fungus started to

Fig. 1. Primordia-forming mycelial culture of *C. candidissimum*, TA 8644, in solid YMG culture.

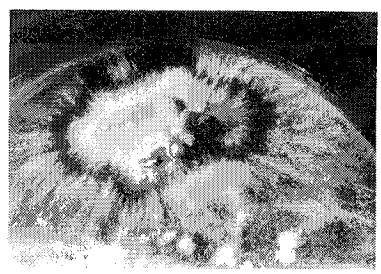


Fig. 2. Fermentation diagram of *C. candidissimum* in YMG medium.

(○) CO_2 production [vol %]; (—) O_2 consumption [vol %]; (▼) nematicidal activitiy [LD/ml culture fluid extract]; (◆) diameter of inhibition zone [mm] *Bacillus brevis*; (■) pH value; (●) mycelial weight [g/liter]; (▲) glucose [g/liter]; (—) O_2-saturation [%].

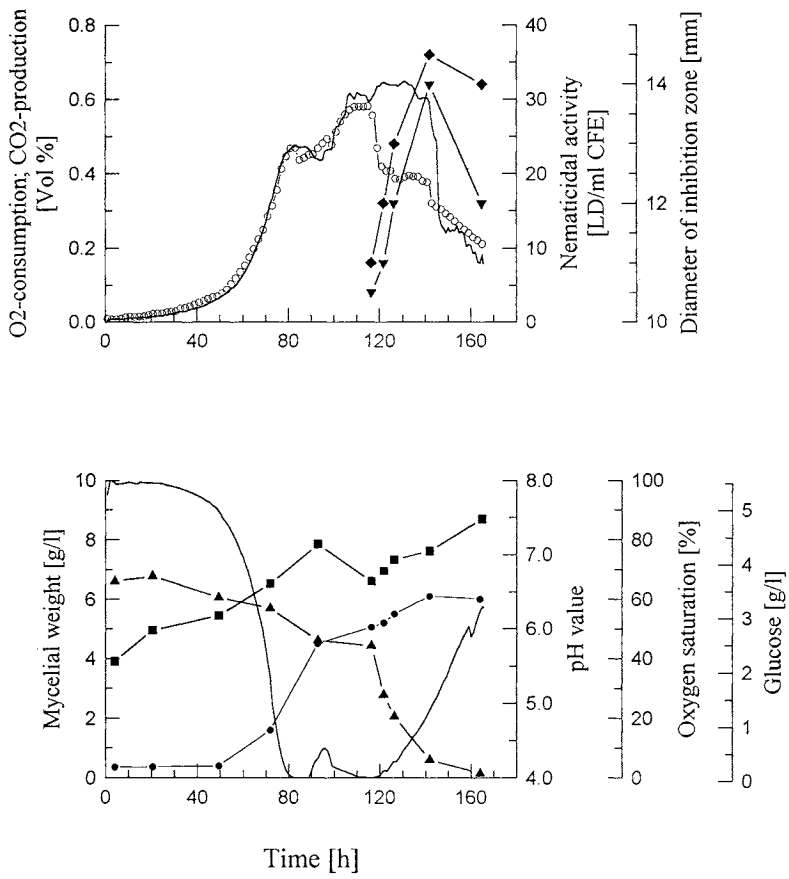
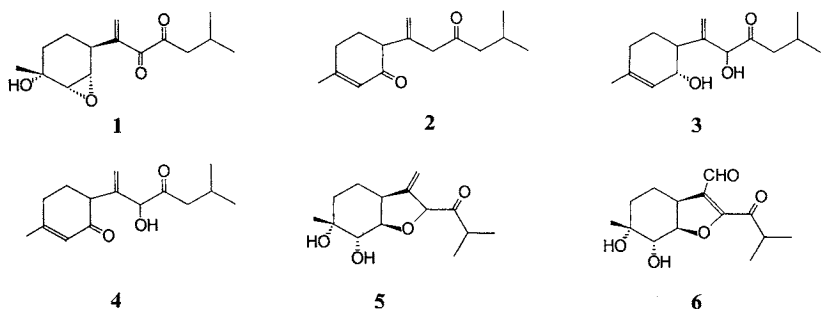


Fig. 3. Structures of compounds **1**~**6** from *C. candidissimum*.

1; Cheimonophyllon A, **2**; Cheimonophyllon B, **3**; Cheimonophyllon C, **4**; Cheimonophyllon D, **5**; Cheimonophyllon E, **6**; Cheimonophyllal.



produce characteristic primordia, which are shown in Fig. 1.

Fermentation and Production of Cheimonophyllons

A typical fermentation diagram in YMG medium is shown in Fig. 2. The production of bioactive metabolites started after the oxygen saturation of the culture had gone through a minimum. It was preceded by a slight decrease of pH value and an increase of pO_2, accompanied by heavy foam production. The fermentation was terminated after eight days as the antimicrobial activities showed no further increase and the nematicidal activity started to decrease. If the aeration rate during fermentation was raised from 3 liters to 4 liters per minute, and the speed of the stirrer from 150 to 180 rpm, biological activities were lower. Compound **3** was still present in the extracts, but compounds **1**, **2**, and **4**~**6** were detected only in minute amounts, suggesting that these metabolites are preferentially produced under oxygen-limited conditions. The isolation of the active compounds was conducted as described in the methods section, and the structures of compounds **1**~**6** are shown in Fig. 3.

Biological Activities

The biological activities of compounds **1**~**6** are summarized in Tables 1~4. **1**, **2**, **4**, and **6** exhibited

Table 1. Nematicidal activities of compounds **1**~**6** towards *Caenorhabditis elegans* after 18 hours. Concentrations tested: 5, 10, 25, 50, and 100 μg/ml.

Compound	LD_{50} (μg/ml)	LD_{90} (μg/ml)
1	10	25
2	25	50
3	50	100
4	10	25
5	>100	
6	25	50
Ivermectin	0.1	0.5

Table 2. Antimicrobial effects of compounds **1**~**6** in the agar diffusion assay towards filamentous fungi (100 μg/paper disk, diameter: 6 mm).

Organism	Diameter of inhibition zone (mm)					
	1	**2**	**3**	**4**	**5**	**6**
Mucor miehei	12	11	8	13	9	11
Penicillium notatum	14	—*	—	12	—	—
Paecilomyces variotii	15	11	—	13	—	12

* No inhibition zone.

Table 3. Antimicrobial effects of compounds **1**~**6** in the serial dilution assay towards yeasts (YMG medium) and bacteria (nutrient broth).

Organism	MIC (μg/ml)					
	1	**2**	**3**	**4**	**5**	**6**
Bacteria:						
Acinetobacter calcoaceticus	25	100	>100	25	>100	50
Bacillus brevis	2	25	100	25	100	5
Bacillus subtilis	10	25	100	25	100	10
Micrococcus luteus	10	50	>100	25	>100	25
Yeasts:						
Candida albicans	50	100	nt*	50	nt	nt
Nematospora coryli	5	50	100	50	>100	10
Rhodotorula glutinis	25	100	>100	100	>100	25
Saccharomyces cerevisiae	10	50	>100	25	>100	50

* nt: not tested.

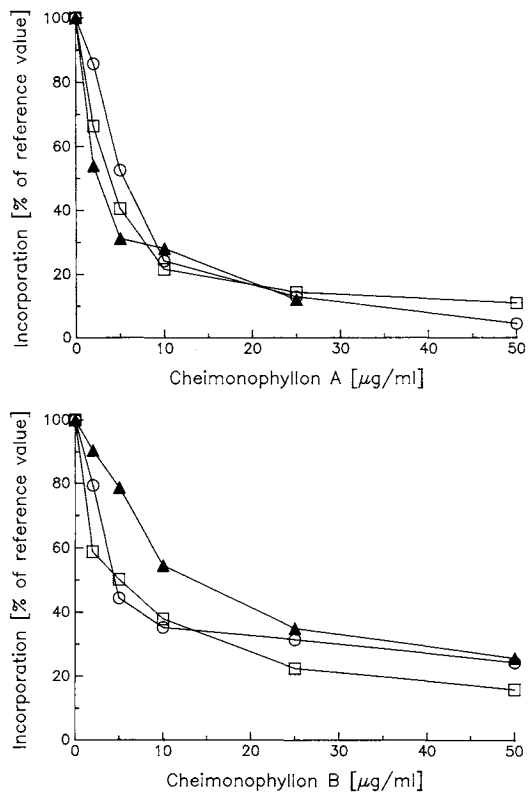
Table 4. Cytotoxic effects of compounds **1**~**6** against mammalian cell lines. IC_{90}: Concentration causing more than 90% lysis after 24 hours.

Cell line	IC_{90} (μg/ml)					
	1	**2**	**3**	**4**	**5**	**6**
L 1210	10	25	100	10	100	5
HL 60	2	25	50	2	100	5
BHK 21	25	50	100	25	>100	10

nematicidal, cytotoxic, and antimicrobial activities, whereas **3** and **5** showed weaker effects. The LD_{50} for *C. elegans* was 10 μg/ml for **1** and **4** and 25 μg/ml for **2** and **6**. (Table 1). Growth of filamentous fungi was weakly inhibited by **1**~**6** (Table 2). *Nematospora coryli* and *Bacillus brevis* were the most sensitive microbes in the serial dilution assay (Table 3). The cytotoxic activities, listed in Table 4, show that HL 60 cells were more sensitive than other mammalian cell lines. Therefore this cell line was chosen to determine the effect of **1** and **2** on macromolecular syntheses. In comparison to protein biosynthesis, the syntheses of nucleic acids were slightly more sensitive to cheimonophyllon B (**2**). Cheimonophyllon A (**1**) affected the incorporation of all three precursors to the same extent as shown in Fig. 4. Upon incubation with L-cysteine, cheimonophyllon A (**1**), cheimonophyllon B (**2**), cheimonophyllon D (**4**), and cheimonophyllal (**6**) lost their biological activities after 30 minutes and ninhydrin-positive adducts were detectable on TLC plates, suggesting that the activities of these compounds are due to the reactive α,β-unsaturated carbonyl moieties[9]. Compounds **3** and **5** did not form cysteine adducts which is in accordance with their lower activities.

Fig. 4. Effects of cheimonophyllons A (**1**) and B (**2**) on the incorporation of labeled precursors into macromolecules in HL 60 cells.

(○) thymidine (reference value: 2,105 cpm), (□) uridine (reference value: 12,950 cpm), (▲) leucine (reference value: 23,400 cpm).



Cheimonophyllon A (**1**) showed weak mutagenic activity in the Ames test at 10 μg/plate. In strains with base pair mutations the number of revertants was 3 to 10 times higher as compared to the background indicating DNA-alkylating properties of **1**. At 25 μg/plate **1** inhibited the growth of all *Salmonella* strains. The mutagenic activities of compounds **2**~**6** remain to be investigated.

None of the compounds exhibited phytotoxic activities towards *Setaria italica* or *Lepidium sativum*. They were not hemolytic towards bovine erythrocytes at 150 μg/ml. **1** and **2** inhibited the collagen-induced aggregation of bovine thrombocytes[10] at 33~66 μg/ml. **3**, **5**, and **6** were inactive. **4** has not yet been tested.

So far only one bioactive bisabolane type sesquiterpene has been isolated from cultures of a basidiomycete, namely bisabolol from *Merulius tremellosus*[11]. Bisabolane sesquiterpenoids, including bisabolol are known from a considerable number of higher plants[12]. *Aspergillus sydowi* and another *Aspergillus* species have been found to produce compounds of this type[13,14]. However, in these compounds the ring system is aromatic.

Acknowledgments

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