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Two New Biologically Active Cyclopentenones from *Dasyscyphus* sp. A47-98

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Two new biological active cyclopentenones VM 4798-1a (**1a**) and VM 4798-1b (**1b**) were obtained as a 3 : 1 inseparable mixture from fermentations of *Dasyscyphus* sp. A47-98. The mixture of the two isomers showed cytotoxic and weak antibacterial and antifungal properties. VM 4898-1 (2-hydroxy-3,4-dimethyl-2-cyclopenten-1-one, **4**), produced by another *Dasyscyphus* sp. showed no significant biological activity. The structures were elucidated by NMR techniques.

The genus *Dasyscyphus* (*Hyaloscyphaceae*) is characterized by small (usually=1 mm), more or less stalked, cup shaped fruiting bodies always covered with well differentiated hairs. The ubiquitous genus can be found growing on plant debris both in temperate and tropical regions[1,2]. Although quite common, not many secondary metabolites have been described from the genus. Among them is scyphostatin[3,4] a neutral sphingomyelinase inhibitor from *Trichopezizella mollissima* (Lasch) Fuckel (syn. *Dasyscyphus mollissimus* (Lasch) Dennis). In our ongoing search for new compounds derived from fungal cultures we found that a *Dasyscyphus*-strain produced highly cytotoxic compounds. In the following we wish to describe the isolation, structure elucidation and biological characterization of these metabolites.

Materials and Methods

Producing Organism

The strain *Dasyscyphus* sp. A47-98 was found growing on tree bark. The specimen shows all characteristics of the genus. The apothecium is yellow and of 1～2 mm diameter with short hyaline marginal hairs and paraphyses. The spores are fusoid and non septate (30 μm×3 μm). *Dasyscyphus* sp. A48-98 while sharing the other characteristics has septate spores. The specimens were kindly provided by H. ANKE and are deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

Fermentation

Fermentations were carried out in 20 liters of YMG medium composed of (g/liter): Yeast extract 4, malt extract 10 and glucose 4, pH 5.5 in a Biolafitte C6 fermenter at 22°C with aeration (2.5 liters/minute) and agitation (120 rpm). A well-grown culture (250 ml) in the same medium was used as inoculum. During fermentation 100 ml samples were taken. The culture fluid was separated by filtration from the mycelia and then extracted with an equal volume of ethyl acetate. The residue obtained after evaporation of the organic solvent was taken up in 0.5 ml of methanol. 10 μl of the concentrated solutions were assayed for antifungal and antibacterial activity in the agar plate-paper disc diffusion assay using *Nematospora coryli*, *Candida krusei* and *Bacillus brevis* as test organisms.

Isolation of the Compounds

After 8～10 days when the antibiotic activity had reached a maximal value the culture fluid of *Dasyscyphus* sp. A47-98 was separated from the mycelia and passed through a column containing Mitsubishi Diaion HP 21 (30×5.5 cm) adsorber resin. The column was washed with H_2O, then methanol/H_2O (1 : 1), and finally with methanol. The

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methanol eluate was concentrated and the crude product (1.75 g) was applied onto a silica gel column (Merk 60, 6×2.5 cm). Elution with ethyl acetate 100% yielded 430 mg containing **1a** and **1b** (Fig. 1). Final purification was achieved by preparative HPLC on Nucleosil RP18 (7 μm; column 250×21.2 mm; flow 5 ml/minute). Elution with water - methanol 51 : 49 v/v yielded 86.5 mg of **1a** and **1b** in a 3 : 1 mixture. VM 4898-1 (**4**) was isolated from *Dasyscyphus* sp. A48-98, which was grown in 20 liters of YMG medium under the same conditions as described above for *Dasyscyphus* sp. A47-98. The culture fluid was passed through a Mitsubishi Diaion HP 21 column. The column was washed with H_2O, then methanol/H_2O (1 : 1). **4** was eluted with methanol. The methanol eluate was concentrated and the crude product (1.1 g) was applied onto a silica gel column (Merk 60, 6×2.5 cm). Elution with cyclohexane/ethyl acetate 1 : 1 yielded 96 mg of **4** (Fig. 1).

Analytical Methods

For analytical HPLC a Hewlett Packard 1100 series instrument was used and for preparative HPLC a Gilson Model 302 (Fa. Abimed, Langenfeld) with variable wavelength UV-Detector (Fa. Knauer, Bad Homburg). UV and IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. 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 optimised for $^{1}J_{CH}=145$ Hz and $^{n}J_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra (HREI) were recorded with a Jeol SX102 spectrometer, and with a HP Series 1100 LCD-MSD HPLC-MS set-up (Hewlett-Packard, Waldbronn, Germany).

Compounds **1a** and **1b** were obtained as a 3 : 1 mixture, as a colourless oil. UV λ_{max}^{MeOH} 276 nm (log ε 4,2). IR (KBr): 3436, 2956, 1730, 1619, 1581, 1438, 1216, 1126, 950, 880, 824, 805, 742 and 697 cm^{-1}. HREIMS $[M]^+$ m/z 263.9944 (required for $C_{10}H_{10}O_4{}^{35}Cl_2$, 263.9956) and 229.0265 (required for $C_{10}H_{10}O_4{}^{35}Cl$, 229.0268). Major isomer (**1a**): ^{1}H NMR at 500 MHz in $CDCl_3$ (δ, mult., J in Hz) 6.68, q, J=6.8, 7-H; 6.65, s, 2-H; 4.68, s, 5-H; 4.43, br s, 4-OH; 3.83, s, 10-H_3; 1.99, d, J=6.8, 8-H_3. ^{13}C NMR at 125 MHz in $CDCl_3$ (δ) 192.7 C-1, 170.9 C-9, 162.2 C-3, 136.3 C-7, 130.7 C-2, 127.1 C-6, 83.7 C-4, 67.7 C-5, 54.4 C-10, 16.1 C-8. Minor isomer (**1b**): ^{1}H NMR at 500 MHz in $CDCl_3$ (δ, mult., J in Hz) 6.66, q, J=6.8, 7-H; 6.61, s, 2-H; 4.59, s, 5-H; 4.43, brs, 4-OH; 3.87, s, 10-H_3; 2.00, d, J=6.8, 8-H_3. ^{13}C NMR at 125 MHz in $CDCl_3$ (δ) 195.4 C-1, 172.2 C-9, 163.9 C-3, 135.8 C-7, 129.5 C-2, 127.7 C-6, 78.2 C-4, 64.1 C-5, 54.2 C-10, 16.1 C-8.

Biological Assays

Biological and cytotoxic activities were assayed as described previously[5,6]. Hela S3 cells (ATCC CCL 2.2 human cervix carcinoma) were grown in DMEM-medium, L1210 (ATCC CCL 219 mouse lymphocytic leukemia), HL-60 (ATCC CCL 240 human promyelocytic leukemia), Jurkat (ATCC TIB 152 human acute T cell leukemia) in RPMI with 10% of fetal calf serum. All media contained 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Viable cells were counted after 24 hours.

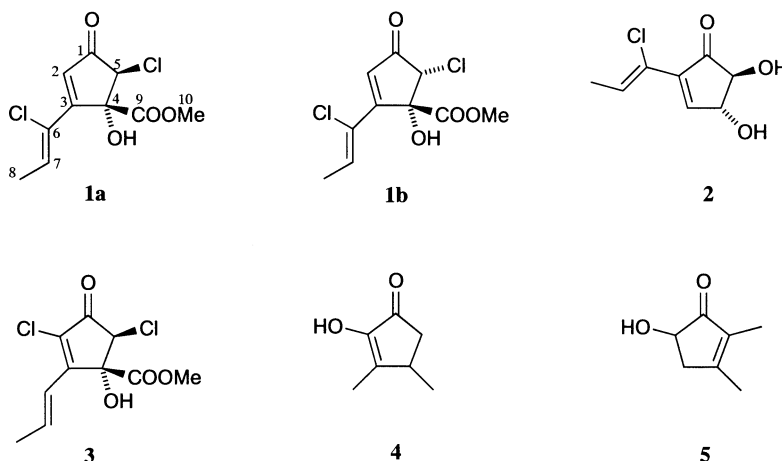
The incorporation of [2-^{14}C]-uridine (1.96 GBq/mMol), [2-^{14}C]-thymidine (2 GBq/mMol) and [1-^{14}C]-leucine (2 GBq/mMol) into RNA, DNA and proteins was assayed in Jurkat cells as described previously[7].

For the measurement of the mitochondrias membrane potential ($\Delta\Psi$) Jurkat cells were seeded in 24 well tissue plate (2×10^5/ml) and treated with different concentrations of VM 4798-1a,b for 24 hours. The cells were then loaded with 40 nM fluorochrome 3,3′-dihexyloxacarbocyanide iodide (DiOC6); (Molecular Probes, Leiden, The Netherlands) for 30 minutes, after which the dye is accumulated in mitochondria that possess an intact membrane potential[8]. 10.000 cells were measured on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuestPro software.

Results and Discussion

Fermentations of *Dasyscyphus* spp. A47-98 and A48-98 were harvested when the antibiotic activity against *Nematospora coryli* had reached its maximum. **1a**, **b** and **4** were isolated as described in the experimental section. The mixture of VM 4798-1a and VM 4798-1b (3 : 1) could in our hands not be separated in a preparative scale, although in the analytical system separation could be achieved. HPLC-UV and HPLC-MS data indicated that the two components are very similar and have identical molecular weights, and this was confirmed by the spectroscopic

Fig. 1. Structures of cyclopentenones VM 4798-1a (**1a**), VM 4798-1b (**1b**), CPDHC (**2**), (+)-cryptosporiopsin (**3**), 2-hydroxy-3,4-dimethyl-2-cyclopenten-1-one (VM 4898-1 (**4**)), 5-hydroxy-2,3-dimethyl-2-cyclopenten-1-one (**5**).

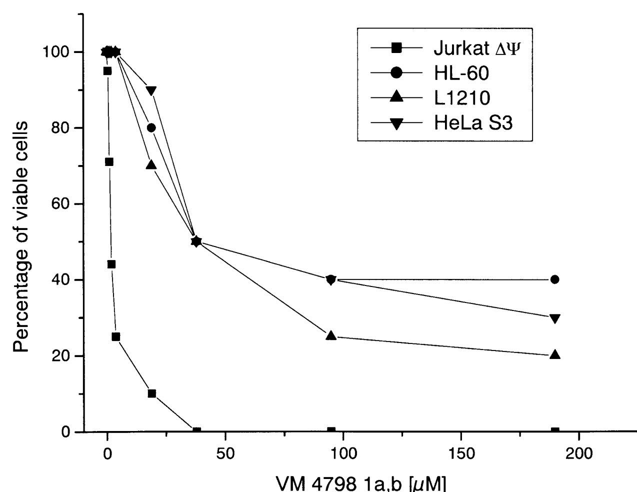


experiments accounted for below. 1D NMR experiments revealed a set of 10 strong and 10 weak signals with similar shifts in the ^{13}C NMR spectrum, and the corresponding two sets of six signals (accounting for 10 protons) in the ^{1}H NMR spectrum. High resolution MS experiments gave a molecular ion with an isotope pattern characteristic for a dichloro compound, and loss of the mass 35 gave a fragment that obviously had only one chlorine. The HR data confirmed the composition of both the molecular ion (as $C_{10}H_{10}O_4{}^{35}Cl_2$) and the fragment, and as the NMR data indicate the presence of two carbonyl groups and two carbon–carbon double bonds the two isomers consequently contain one ring. The COSY correlation between 7-H and 8-H_3 as well as HMBC correlations from 8-H_3 to C-6 and C-7 and from 7-H to C-3 and C-6 demonstrate that the two carbon–carbon double bonds are conjugated. HMBC correlations from 2-H to C-1, C-4, C-5 and C-6, together with HMBC correlations from 4-OH to C-3, C-4, C-5 and C-9 show that C-3 is connected to C-4 which is a quaternary carbon. The HMBC correlations from the methoxy protons (10-H_3) to C-9 (strongly) and C-4 (weakly) show that the carboxylic ester is connected to C-4, and HMBC correlations from 5-H to C-1 and C-4 positions C-5 in a five-membered ring between C-1 and C-4. The two chlorines must therefore be positioned on C-5 and C-6. The relative stereostructures of the two isomers (Fig. 1) were determined by investigating the data from a NOESY experiment. 7-H in both isomers correlated to both 10-H_3 and 4-OH, which only should be expected if the C-6/C-7 double bond is *Z*. A difference between the two isomers was observed in the NOESY correlations from 5-H, to 4-OH on the major isomer and to 10-H_3 in the minor. This shows that the 4-OH and 5-Cl are *trans* in the major and *cis* in the minor isomer.

In the serial dilution assay **1a** and **1b** inhibit the growth of fungi and bacteria at 50~100 μg/disk (Table 1). As shown in Fig. 2, **1a, b** caused 50% lysis of HeLa S3-, HL 60- and L1210-cells at concentrations of 10 μg/ml (37.9 μM). The cytotoxic activity on Jurkat cells is quite significant with 50% lysis at 1.4 μM. The measurement of the mitochondrias membrane potential ($\Delta\Psi$) resulted in a 50% reduction of viable cells at concentrations of 1.5 μM of **1a, b** (Fig. 2).

The 3 : 1 mixture of VM 4798-1a and VM 4798-1b completely inhibited the incorporation of the appropriate radioactive precursors into DNA, RNA and proteins in Jurkat cells at concentrations of 1.9 μM. This is thought to be a consequence of the breakdown of the mitochondrias membrane potential. CPDHC (**2**) a compound structurally related to VM 4798-1a, b has been reported to inhibit the interleukin-6-induced secreted alkaline phosphatase expression in HepG2 cells with IC_{50} values of 4.0~5.3 μM. No effects were observed on cellular DNA, RNA and protein synthesis in HepG2 cells up to concentrations of 30 μM[9]. For cryptosporiopsin[10] (**3**), an isomer of **1a** and **1b**, phytotoxic properties (*Lepidium sativum* and *Medicago sativa*)[11] have been reported. The antifungal activities of **1a, b** are shown in Table 1. They are comparable to those

Fig. 2. Cytotoxic effects of VM 4798-1a,b against HL-60, L1210 and HeLa S3 cells.



In Jurkat cells the mitochondrias membrane potential ($\Delta \Psi$) was measured.

described for **3**[12].

VM 4898-1 (**4**) is a known cyclopentenone isolated from a new fungal source. It has been described as a natural constituent of coffee and as being derived by chemical synthesis[13]. The substance has a characteristic flavor and shows only moderate biological activity. The antimicrobial spectrum is shown on the Table 1. The most sensitive organisms were *Aspergillus ochraceus*, *Paecilomyces variotii* and *Mucor miehei*. No antibacterial and cytotoxic activities were observed above concentrations of 100 μg/ml.

5-Hydroxy-2,3-dimethyl-2-cyclopenten-1-one (**5**), another cyclopentenone, was detected in crude extracts of *Dasyscyphus* sp. 4898 and identified by HPLC-MS. It is an isomer of VM 4898-1 with no reported biological activities[9,14].

Table 1. Minimal inhibitory concentration (MIC) of VM 4798-1 (**1**) and VM 4898-1 (**4**) in the serial dilution assay.

Organism	**Compound, MIC [μg/ml]**	
	1	**4**
Bacteria		
Arthrobacter citreus	100	--
Bacillus brevis	50	--
Bacillus subtilis	50	--
Micrococcus luteus	25	--
Mycobacterium phlei	25	--
Escherichia coli	--	--
Salmonella typhimurium	100	--
Yeasts		
Candida glabrata	50	--
Candida krusei	50	--
Candida parapsilosis	25	--
Nadsonia fulvescens	100	100
Nematospora coryli	50	100
Rhodotorula glutinis	25	--
Saccharomyces cerevisiae is1	100	--
S. cerevisiae αS288c	10	--
Filamentous fungi		
Absidia glauca (+)	50	--
Absidia glauca (-)	50	--
Alternaria porri	50	100
Ascochyta pisi	--	--
Aspergillus ochraceus	10	10
Cladosporium cladosporioides	100	--
Fusarium fujikuroi	100	--
Fusarium oxysporum	50	--
Mucor miehei	100	50
Paecilomyces variotii	50	50
Penicillium islandicum	50	--
Penicillium notatum	50	--
Zygorhynchus moelleri	50	--

-- no effects up to 100 μg/ml

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