New Caloporoside Derivatives and their Inhibition of Fungal Spore Germination

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In our ongoing screening culture fluid extracts of *Gloeoporus* (*Caloporus*) *dichrous* strain 83065 inhibited the germination of *Magnaporthe grisea* and *Fusarium graminearum* spores. While isolating the active metabolites two new caloporosides, caloporoside G and caloporoside H, in addition to the known caloporoside derivatives F-16438G, caloporoside A, and 2-hydroxy-6-(16-hydroxyheptadecyl)benzoic acid were obtained.

Key words: Caloporoside, Spore Germination Inhibition, *Gloeoporus dichrous*

Introduction

The basidiomycete *Gloeoporus* (*Caloporus*) *dichrous* is known to produce several salicylic acid derivatives with different biological activities. In the following, two new caloporoside derivatives, caloporosides G (**1**) and H (**2**), as well as already known compounds, the derivatives F-16438G (**3**), caloporoside A (**4**), and 2-hydroxy-6-(16-hydroxyheptadecyl)benzoic acid (**5**) were identified. The first example of the series, caloporoside A (**4**), had been found in a search for new inhibitors of pro- and eukaryotic phospholipase C (Weber *et al.*, 1994). The derivatives deacetylcaloporoside and 2-hydroxy-6-[16-(β-D-mannopyranosyloxy)-heptadecyl]benzoic acid were isolated from the imperfect fungus HA 137-89 because of their affinity to the *t*-butylbicyclophosphorothionate binding site of the $GABA_A$ receptor (Shan *et al.*, 1994). Recently, inhibition of cyclin-dependent kinases (Eder *et al.*, 2002) and inhibition of the binding of hyaluronic acid to its receptor CD44 (Harada *et al.*, 2006) by caloporoside derivatives have been described.

Material and Methods

Producing organism

Gloeoporus dichrous (Fr.) Bres. syn. *Caloporus dichrous* (Fr.) Ryv. strain 83065 is a producer of caloporoside A (**4**) (Weber *et al.*, 1994). The mycelial culture is deposited in the collection of the Institute of Biotechnology and Drug Research (IBWF e. V.), Kaiserslautern, Germany. For maintenance, the fungus was grown on YMG medium (4 g/l yeast extract, 4 g/l malt extract, 4 g/l glucose). The pH value was adjusted to 5.5 before autoclaving. Solid medium contained 2% of agar.

Fermentation and isolation

Caloporus dichrous strain 83065 was grown in PD medium (4 g/l dried mashed potatoes, 20 g/l glucose, the pH value was adjusted to 5.5 before autoclaving) in a 20-l fermenter (Biolafitte) at 22–24 °C with agitation (130 rpm) and aeration (3 l/min). This culture was inoculated with 1 l of a well-grown starter culture in the same medium. The fermentation was stopped after 7 d when the production of caloporosides had reached a maximum as judged daily by analytical HPLC. The mycelia were separated from the culture fluid by filtration and lyophilized (yield: 53.3 g). A first extraction was performed with ethyl acetate followed

by a second one with isopropanol. The isopropanol extract was dried *in vacuo* yielding 700 mg of a crude extract with caloporoside derivatives as the main components. Preparative HPLC (Merck Lichrosorb RP 18, 7 μm, 250 × 25 mm) with a MeCN/0.1% HCOOH gradient (60% to 90% MeCN in 20 min, to 100% MeCN in 5 min and finally 5 min isocratic with 100% MeCN) and a flow of 20 ml/min yielded compounds **1** (6.7 mg; RT = 20.5 min), **5** (3.2 mg; RT = 28 min) and an intermediate fraction containing compounds **3** and **4** (229.5 mg; RT = 12–14 min). The intermediate fraction (75 mg) was again subjected to preparative HPLC under isocratic conditions (72% MeCN) yielding 10 mg of compound **4** (RT = 13 min) and 6.7 mg of **3** (RT = 17 min). Compound **2** is an artefact first isolated after preparative HPLC with 0.1% H_3PO_4 instead of 0.1% HCOOH. To examine the formation of this artefact, H_3PO_4 (4%) was added to 20 mg of **3** dissolved in 0.5 ml MeOH and heated to 80 °C for 1 h. Preparative HPLC (Phenomenex Luna C18, 10 μm, 250 × 10 mm, MeCN/HCOOH gradient, 50% to 80% MeCN in 15 min) yielded 0.2 mg (6%) of compound **2** (RT = 13.7 min) and 20% of the starting material **3** along with side products.

Spectroscopical characterization

Optical rotations were measured with a Krüss P8000 polarimeter at 589 nm. UV and IR spectra were measured with a Perkin-Elmer Lambda-16 spectrophotometer and a Bruker IFS48 FTIR spectrometer, respectively. NMR spectra were recorded with a Bruker Avance II spectrometer (400 MHz), the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.16 ppm; CD_3OD: δ_H = 3.31 ppm, δ_C = 49.00 ppm; Gottlieb *et al.*, 1997). APCI mass spectra were measured with a Hewlett Packard MSD1100 instrument. ESI-HR mass spectra were recorded on a MicroMass/Waters ESI Q-TOF mass spectrometer equipped with a LockSpray interface using NaI/CsI or trialkylamines as external reference.

Physicochemical properties

Caloporoside G (**1**): Colourless oil. – $[\alpha]_D^{26}$ –27.0° (*c* 0.40, $CHCl_3$). – UV (MeCN): λ_{max} (log ε) = 233 (3.60), 301 nm (3.37). – IR (KBr): ν = 3427, 2925, 1747, 1658, 1454, 1248, 1077 cm^{-1}. – ^1H and ^{13}C NMR data: see Table I. – APCIMS neg.: *m/z* (%) = 637.3 $[M–H]^-$ (100). – HRESIMS: *m/z* = 661.3538; $[C_{34}H_{54}O_{11}+Na]^+$ requires *m/z* = 661.3564.

Table I. ^1H (400 MHz) and ^{13}C NMR data (101 MHz, $CDCl_3$) of caloporoside G (**1**). Coupling constants are given in Hz. Multiplicities were determined indirectly by HSQC.

Position	δ_H	δ_C
1	–	174.1; s
2	–	110.9; s
3	–	163.7; s
4	6.84; dd; 8.3, 1.1	115.8; d
5	7.33; dd; 8.3, 7.6	135.1; d
6	6.75; dd; 7.6, 1.1	122.7; d
7	–	147.6; s
8	2.94; m	36.7; t
9	1.58; m	32.3; t
10–21	1.43–1.23; m	29.9–29.4; t
22	1.57; m	36.9; t
	1.41; m	
23	3.81; pseudo sext; 6.1	75.2; d
24	1.12; d; 6.1	19.6; q
1'	4.75; d; 1.0	96.6; d
2'	5.42; dd; 3.3, 1.0	70.0; d
3'	4.93; dd; 9.9, 3.3	74.0; d
4'	4.03; t; 9.9	65.8; d
5'	3.43; dt; 9.9, 3.8	75.7; d
6'	3.99; d; 3.8	62.3; t
2'-OAc	–	170.5; s
	2.15; s	20.8; q
3'-OAc	–	170.8; s
	2.07; s	20.8; q

Caloporoside H (**2**): Colourless oil. – $[\alpha]_D^{26}$ –19.2° (*c* 0.33, MeOH). – UV (MeOH): λ_{max} (log ε) = 238 (sh, 3.46), 302 nm(3.29). – IR (KBr): ν = 3433, 2924, 2853, 1739, 1376, 1241, 1070 cm^{-1}. – ^1H and ^{13}C NMR data: see Table II. – APCIMS neg.: *m/z* (%) = 915.3 $[M–H]^-$ (100). – HRESIMS: *m/z* = 967.4396; $[C_{44}H_{61}D_7O_{20}–D+2Na]^+$ requires *m/z* = 967.4392 (all hydroxy groups were deuterated by the use of CD_3OD as the NMR solvent).

Biological assays

Antimicrobial activities against bacteria and fungi were determined using the serial dilution assay (minimal inhibitory concentration, MIC) as described previously (Anke *et al.*, 1989). The spore germination was tested with *Magnaporthe grisea* as described previously (Kettering *et al.*, 2005) and was adapted for the spore germination assay with *Fusarium graminearum*. Cytotoxicity was assayed as described previously (Schoettler *et al.*, 2006).

Table II. ^{1}H (400 MHz) and ^{13}C NMR data (101 MHz, CD_3OD) of caloporoside H (**2**). Coupling constants are given in Hz. Multiplicities were determined indirectly by HSQC.

Position	δ_H	δ_C
1	–	174.4; s
2	–	115.7; s
3	–	162.2; s
4	6.73; dd; 8.2, 1.2	115.7; d
5	7.24; dd; 8.2, 7.6	134.0; d
6	6.72; dd; 7.6, 1.2	122.9; d
7	–	146.7; s
8	2.88; m	36.7; t
9	1.57; m	33.3; t
10–21	1.37–1.27; m	30.9–30.6; t
22	1.63; m	37.0; t
	1.54; m	
23	4.97; m	73.6; d
24	1.23; d; 6.3	20.2; q
1'	–	171.9; s
2'	4.79; d; 9.7	73.9; d
3'	4.27; dd; 9.7, 1.0	69.9; d
4'	3.57; dd; 9.5, 1.0	69.5; d
5'	4.09; ddd; 9.5, 5.9, 2.2	75.1; d
6'	4.70; dd; 11.8, 2.2	63.8; t
	4.17; dd; 11.8, 5.9	
1"	4.86; d; 0.7	98.9; d
2"	5.31; dd; 3.5, 0.7	73.2; d
3"	3.68; dd; 9.5, 3.5	73.4; d
4"	3.45; t; 9.5	69.3; d
5"	3.30; m	78.1; d
6"	3.91; dd; 11.6, 2.5	63.1; t
	3.61; dd; 11.6, 7.4	
1"'	–	168.2; s
2"'	3.51; s	42.0; t
3"'	–	169.2; s
4"'	3.75; s	53.0; q
2'-OAc	–	171.6; s
	2.10; s	20.4; q
2"-OAc	–	172.4; s
	2.13; s	21.1; q

The cell lines Jurkat (ATCC TIB 152), Colo-320 (DSMZ ACC 144), and L-1210 (DSMZ ACC 123) were grown in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany). HeLa-S3 (DSMZ ACC 161) cells were grown in DMEM medium (Invitrogen). All media were supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 65 μg/ml of penicillin G, and 100 μg/ml of streptomycine sulfate. The viability was measured photometrically with XTT (suspension cell lines) or with Giemsa staining (monolayer cells).

Results and Discussion

Structure elucidation

High-resolution MS suggested an elemental composition of $C_{34}H_{54}O_{11}$ for caloporoside G (**1**). NMR spectroscopy revealed a salicylic acid fragment substituted with a long alkyl chain in position 6. The alkyl chain is terminated by a methyl group which is represented by a doublet resonance in the ^{1}H NMR spectrum; therefore, the penultimate carbon atom should be a methine. Its chemical shift suggested an ether functionality and the HMBC spectrum revealed a substituted pyranose unit attached to the position at its anomeric centre. The pyranose protons showed typical coupling constants and NOE correlations for mannose, and NOE contacts between H-1 to H-3 as well as H-5 proved the β-configuration of the mannoside. Positions 2 and 3 of the mannose were acetylated, and the length of the alkyl chain was deduced from the MS data to be identical to that found in the caloporoside aglycone **5**. The optical rotation of **1** is in agreement with that of the closely related 2-hydroxy-6-[16-(β-D-mannopyranosyloxy)heptadecyl]benzoic acid reported by Shan *et al.* (1994), suggesting D-configuration of the mannose unit.

Caloporoside H (**2**) had an elemental composition of $C_{44}H_{68}O_{20}$ (HRMS). The same aglycone unit as in **1** was found to be acylated by mannonic acid as could be determined by two-dimensional NMR spectroscopy. A β-mannopyranoside was formed with the hydroxy group at position 5 of the mannonic acid, while both the mannonic acid and the mannopyranose were acetylated in position 2. Position 6 of the mannonic acid was esterified with methyl malonate. Thus, compound **2** is the methyl ester of F-16438G (**3**). NMR data and optical rotation were in agreement with those of compound **3** (Hirota-Takahata *et al.*, 2006), and **2** could be prepared from a sample of **3** by esterification with methanol. Again, β-configuration of the mannopyranoside was proven by characteristic NOE contacts between three axial hydrogen atoms in positions 1, 3, and 5.

In both compounds, C-23 was proposed to have *R*-configuration as reported for the isolated aglycone and the other caloporosides (Fig. 1) (Shan *et al.*, 1994).

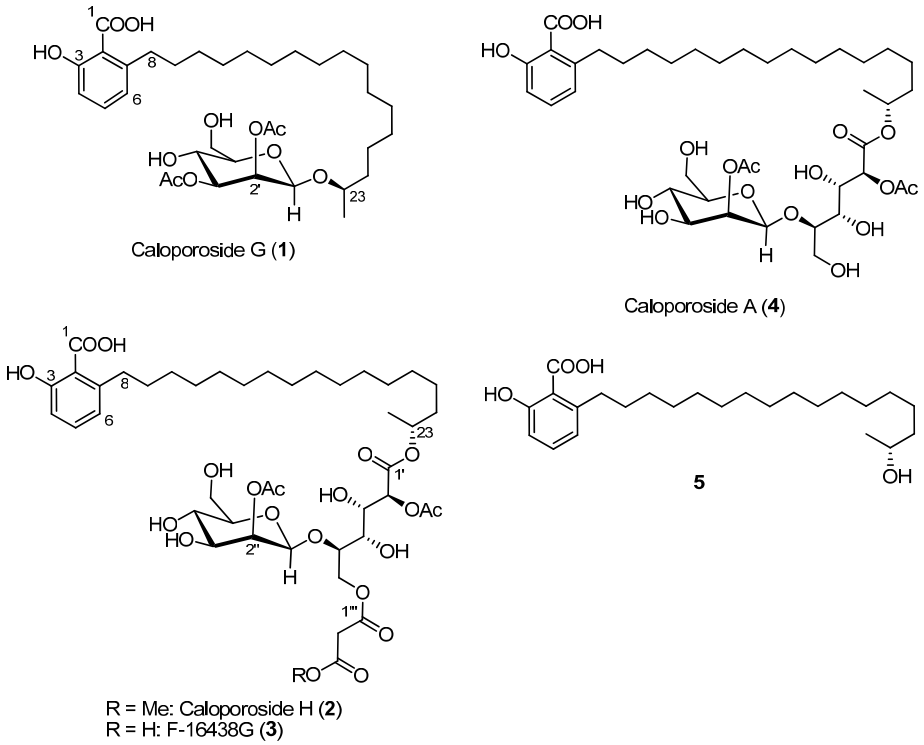

Fig. 1. Chemical structures of caloporosides G (**1**) and H (**2**) and related compounds.

Biological properties

The minimal inhibitory concentrations are listed in Table III. The two mating types of the zygomycete *Absidia glauca* were inhibited by 25 μg/ml of **3** and 10 μg/ml of **4**. *Arthrobacter citreus* was the most sensitive organism inhibited by all isolated caloporoside derivatives with MIC values from 5 to 25 μg/ml. Addition of 5 μg/ml of **5** inhibited the growth of *Corynebacterium insidiosum* and *Micrococcus luteus*. No growth inhibition by all compounds isolated here was observed up to 50 μg/ml for the following organisms: *Ascochyta pisi*, *Aspergillus ochraceus*, *Fusarium fujikuroi*, *Fusarium oxysporum*, *Mucor miehei*, *Penicillium islandicum*, *Paecilomyces variotii*, *Penicillium notatum*, *Zygorhynchus moelleri*, *Nematospora coryli*, *Mycobacterium phlei*, *Enterobacter dissolvens*, *Escherichia coli*, and *Pseudomonas fluorescens*. The spore germination of *Magnaporthe grisea* 70–15 and *Fusarium graminearum* was inhibited by **2**–**4**. Up to 50 μg/ml no inhibition was observed for **1** and **5**. The results are listed in Table IV. Moderate cytotoxic activities were observed

Table III. Minimal inhibitory concentrations of the isolated compounds **1**–**5**.

Microorganism	MIC [μg/ml]				
	1	**2**	**3**	**4**	**5**
Absidia glauca (+)	> 50	> 50	25	10	> 50
Absidia glauca (–)	> 50	> 50	25	10	> 50
Athrobacter citreus	10	25	25	25	5
Bacillus brevis	> 50	> 50	> 50	> 50	50
Bacillus subtilis	> 50	> 50	> 50	> 50	25
Corynebacterium insidiosum	> 50	> 50	> 50	> 50	5
Micrococcus luteus	50	> 50	> 50	> 50	5

Table IV. Inhibition of spore germination by caloporoside H (**2**), F-16438G (**3**), and caloporoside A (**4**).

Microorganism	Inhibition of spore germination [μg/ml]		
	2	**3**	**4**
M. grisea	10[a]	5	5
F. graminearum	25	10	5

[a] About 80% of the spores bore short germ tubes.

only with **1** and **5** showing IC_{50} values between 20 and 50 μg/ml (Colo-320, Jurkat, and L-1210 cells). No cytotoxic activity was observed against HeLa-S3 cells up to 50 μg/ml. The antimicrobial activities of the structurally related caloporoside derivatives correspond to former studies (Weber *et al.*, 1994).

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