5-(2′-Oxoheptadecyl)-resorcinol and 5-(2′-Oxononadecyl)-resorcinol, Cytotoxic Metabolites from a Wood-Inhabiting Basidiomycete

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5-(2′-Oxoheptadecyl)-resorcinol and 5-(2′-Oxononadecyl)-resorcinol, Basidiomycete, Cytotoxic Activities

5-(2′-oxoheptadecyl)-resorcinol (**1**) and 5-(2′-oxononadecyl)-resorcinol (**2**) were isolated from fermentations of an imperfect basidiomycete. The structures of the compounds were determined by spectroscopic techniques. Both compounds exhibit cytotoxic effects against the human colon tumor cell lines COLO-320, DLD-1 and HT-29 and the human promyeloid leukemia cell line HL-60, the human leukemia T cell JURKAT, the human hepatocellular carcinoma cell line HEP-G2 as well as the J774 mouse macrophage cell line. The compounds induce morphological and physiological differentiation of HL-60 cells into granulocytes, which subsequently die by apoptosis.
Both compounds show no antibacterial and antifungal activity.

Introduction

Colorectal cancer is one of the major causes of death from cancer in developed countries. Since it is particularly recalcitrant to therapeutic intervention the search for new compounds interfering with its growth and the development of metastases has gained momentum. In our search for new bioactive metabolites with cytostatic or cytotoxic activities against the colon-cancer derived cell line COLO-320 a screening of 500 fungal cultures resulted in the detection and isolation of 5-(2′-oxoheptadecyl)-resorcinol (**1**) and 5-(2′-oxononadecyl)-resorcinol (**2**) (see Fig. 1 for structures) from fermentations of the basidiomycete TA96244. In the following we describe the production, isolation, structure elucidation and the biological activities of both compounds.

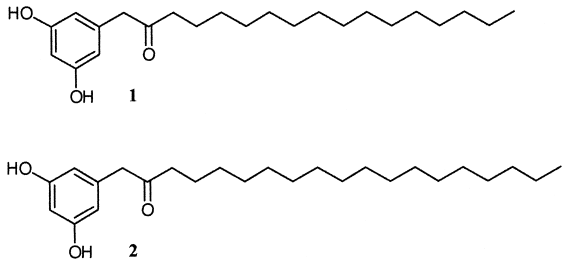

Fig. 1. 5-(2′-oxoheptadecyl)-resorcinol (**1**) and 5-(2′-oxononadecyl)-resorcinol (**2**).

Materials and Methods

General

^{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 $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). FAB mass spectra were recorded with a Jeol SX102 spectrometer. UV and IR spectra were recorded with a Perkin Elmer λ 20 and a Perkin Elmer Spectrum One spectrometer. For analytical HPLC

a Hewlett Packard 1090 series II and for HPLC/MS a Hewlett Packard LC/MSD 1100 series were used.

Producing organism

Mycelial cultures of the basidiomycete TA 96244 were obtained from pieces of a pinkish mycelium covering a piece of rotting wood in Southern France. Neither basidia nor conidia could be found. The mycelial septa bear clamp connections both in the herbarium specimen and in culture. For maintenance and submerged culture YMG medium (malt extract 10 g/l, glucose 4 g/l, yeast extract 4 g/l, pH 5.5) was used. To solid media 2% agar were added. Voucher specimen and mycelial cultures of the fungus are deposited in the herbarium of the LB Biotechnology, University of Kaiserslautern.

Fermentation and isolation

Submerged cultures were carried out in YMG medium in a 20 l fermenter (Braun Biostat U) at 22 °C with aeration (3 l/min) and agitation (120 rpm). A well grown culture (120 h, 250 ml) in the same medium was used as inoculum. During fermentation, aliquots of the culture (100 ml) were taken and the mycelia separated by filtration. The culture fluid was extracted twice with an equal volume of ethyl acetate and the mycelia with 100 ml of methanol-acetone 1:1 (v/v). After evaporation of the solvents in vacuo (40 °C), the residues were dissolved in 1 ml of MeOH and tested for cytotoxicity against COLO-320 cells.

The fermentation was terminated after 290 h, when the glucose was used up. The mycelia were separated by filtration and extracted with 2 l of methanol-acetone 1:1 yielding 8 g of crude extract. The culture fluid containing none of the compounds was discarded. The crude mycelial extract was applied onto a silica gel column (Merck 60, 0.063 ~ 0.2 mm; 30 × 6 cm). Upon elution with cyclohexane-ethyl acetate 1:1, 310 mg of an enriched product were obtained. Further purification by preparative HPLC (Nucleosil 100–7 OH, 7µ, column 250 × 25 mm) with a cyclohexane (CH) *tert*-butyl methyl ether (*t*-BME)gradient (flow 5 ml/min, CH:*t*-BME 95·5, Rt 80 min) yielded a mixture of **1** and **2** (70 mg). Final purification was achieved by preparative HPLC (Nucleogel GPC 50–10, column 250 × 25 mm, flow 5 ml 2-propanol/min) yielding 31 mg of **1** (Rt 35 min) and 6 mg of **2** (Rt 42 min).

5-(2′-oxoheptadecyl)-resorcinol (**1**) was obtained as colourless crystals, mp. 88–90 °C (EtOAc). UV (MeOH), λ_{max} (ε): 282 nm (1629). IR (KBr): 3425, 2920, 1700, 1630, 1605, 1490, 1465, 1380, 1350, 1290, 1155, 1005 and 840 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$), δ, mult., *J* (Hz): 6.17, 1H, t, *J* = 2.0, 2-H; 6.14, 2H, d, *J* = 2.0, 4-H and 6-H; 3.47, 2H, s, 1′-H_2; 2.41, 2H, t, *J* = 7.5, 3′-H_2; 1.47, 2H, m, 4′-H_2; 1.20, 24H, m, 5′-H_2–16′-H_2; 0.83, 3H, t, *J* = 7.0, 17′-H_3. ^{13}C NMR (125 MHz, $CDCl_3$), δ: 210.4 C-2′, 157.9 C-1 and C-3, 136.2 C-5, 108.0 C-4 and C-6, 101.3 C-2, 50.1 C-1′, 41.8 C-3′, 31.8 C-15′, 29.6 C-6′, C-8′, C-12′ and C-14′, 29.5 C-10′, 29.5 C-11′, 29.3 C-9′, 29.3 C-13′, 29.2 C-7′, 29.0 C-5′, 23.6 C-4′, 22.5 C-16′, 13.9 C-17′. FABHRMS: (363.2908, M+H^+, $C_{23}H_{39}O_3$ requires 363.2899).

5-(2′-oxononadecyl)-resorcinol (**2**) was obtained as colourless crystals, mp. 90–92 °C (EtOAc). UV (MeOH), λmax (ε): 282 nm (1564). IR (KBr): 3425, 2920, 1700, 1630, 1605, 1490, 1465, 1380, 1350, 1290, 1155, 1005 and 840 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$), δ, mult., *J* (Hz): 6.19, 1H, t, *J* = 2.0, 2-H; 6.16, 2H, d, *J* = 2.0, 4-H and 6-H; 3.49, 2H, s, 1′-H_2; 2.42, 2H, t, *J* = 7.5, 3′-H_2; 1.48, 2H, m, 4′-H_2; 1.22, 28H, m, 5′-H_2–18′-H_2; 0.85, 3H, t, *J* = 6.9, 19′-H_3. ^{13}C NMR (125 MHz, $CDCl_3$), δ: 210.4 C-2′, 157.8 C-1 and C-3, 136.3 C-5, 108.2 C-4 and C-6, 101.4 C-2, 50.1 C-1′, 41.9 C-3′, 31.9 C-17′, 29.6 C-6′, C-8′, C-10′, C-11′, C-12′, C-14′ and C-16′, 29.5 C-13′, 29.4 C-9′, 29.3 C-15′, 29.3 C-7′, 29.0 C-5′, 23.7 C-4′, 22.6 C-18′, 14.0 C-19′. FABHRMS: (391.3219, M+H^+, $C_{25}H_{43}O_3$ requires 391.3212).

Biological assays

The antimicrobial spectra were measured as described previously (Anke *et al.*, 1989).

COLO-320 cells (DSMZ ACC 144, human colon adenocarcinoma), Jurkat cells (ATCC TIB 152, human acute T cell leukemia), J774A.1 cells (DSMZ ACC 170, mouse monocytes-macrophages), JURKAT cells (DSMZ ACC 282, human T cell leukemia) and HL-60 cells (ATCC CCL 240, human promyelocytic leukemia) were grown in RPMI 1640 medium supplemented with 10% fetal

calf serum (FCS), 65 μg/ml penicillin G and 100 μl/ml streptomycin sulfate in a humidified atmosphere containing 5% of CO_2 at 37 °C. DLD-1 cells (ATCC CCL 221, human colon adenocarcinoma) HT-29 cells (DSMZ ACC 299, human colon adenocarcinoma), HEP-G2 cells (DSMZ ACC 180, human hepatocellular carcinoma) and HeLa S3 (ATCC CCL 2.2, human cervix carcinoma) were maintained in DMEM medium (+ 10% FCS, 65 μg/ml penicillin G and 100 μl/ml streptomycin sulfate) in a humidified atmosphere containing 5% of CO_2 at 37 °C.

Cytotoxicity was measured in microtiter plates with ~ $1 \times 10_5$ cells/ml. Cells were incubated with or without the test compounds. After 24, 48 and 72 hours the cells were examined under the microscope and the percentage of lysed cells counted. In addition, the effect on the growth of monolayer cell lines was measured with Giemsa stain as described by Weber *et al.*, 1990. Cell viability of cell growing in suspension was measured by the XTT test (Boehringer, Mannheim/Roche) as described in the product information.

The influence of 5-(2′-oxoheptadecyl)-resorcinol on the synthesis of macromolecules in COLO-320 cells was analyzed as described previously. (Weidler *et al.*, 2000).

Nematicidal activities (Mayer *et al.*, 1996) and phytotoxic activities against germinating plant seeds were measured according to Anke *et al.*, 1989.

The induction of morphological and physiological differentiation of HL-60 cells was assayed as described previously (Erkel *et al.*, 1996). For the differentiation assay cells were grown for 4 days with or without the test compounds. Differentiated cells reduced the water-soluble nitro-blue tetrazolium chloride (NBT) to blue-black cell-associated nitro-blue diformazan deposits. For quantification the percentage of blue-black cells was determined.

Results and Discussion

Fermentation and isolation

The production of 5-(2′-oxoheptadecyl)-resorcinol (**1**) and 5-(2′-oxononadecyl)-resorcinol (**2**) by strain TA 96244 in 20 l batches started 200 h after inoculation. The highest cytotoxic activity of the crude extracts from the mycelia against COLO-320 was reached when the glucose was used up after approximately 11 days. The extraction of the mycelia and isolation of 5-(2′-oxoheptadecyl)-resorcinol (**1**) and 5-(2′-oxononadecyl)-resorcinol (**2**) is described in the experimental section.

Structural elucidation

The two compounds are obviously closely related, as the NMR data appear to be almost identical. However, high resolution MS experiments suggest that **2** contains two additional methylene groups compared to **1**, and this is confirmed if the integrals of the signals in the ^{1}H NMR spectra are closely compared. The UV absorption suggest that the compounds contain a benzene ring, and the 1D NMR data are consistent with 5-substituted resorcinols. HMBC correlations from 1′-H_2, a singlet in the ^{1}H NMR spectrum, to C-5, C-4/C-6, C-2′ and C-3′ shows that C-1′ is benzylic and that the resorcinol is substituted in position 5, and the position of the keto function. COSY as well as HMBC correlations between 4/5-H and 2-H, and between 2-H and C-1/C-3 as well as C-4/C-6 confirm the presence of the resorcinol system, and the signals for the remaining protons and carbons are all typical for an unbranched alkyl chain. Both compounds have been suggested to be produced by rice (Suzuki *et al.*, 1998), and **1** has also been reported from rye grains (Kozubek and Tyman, 1995). However, this is the first report where the metabolites are isolated and properly characterised spectroscopically, and it is also the first report of their presence in fungi.

Biological properties

Cytotoxic activities of 5-(2′-oxoheptadecyl)-resorcinol (**1**) were observed against the colon cancer cell lines COLO-320, DLD-1, HT-29 and HL-60 cells at 2.5–5 μg/ml. Hep-G2, HeLa S3, Jurkat and J774.1a cells were lysed at 7.5–10 μg/ml. 5-(2′-oxononadecyl)-resorcinol (**2**) exhibits somewhat less cytotoxic activity (see Table I).

Cellular DNA-, RNA-, and protein syntheses were measured in COLO-320 cells by determining the incorporation of [^{14}C]-thymidine, [^{14}C]-uridine and [^{14}C]-leucine in TCA-insoluble fractions. At a concentration of 7.5 μg/ml 5-(2′-oxoheptadecyl)-resorcinol a selective 50% inhibitory effect on the

Table I. Cytotoxic activities of 5-(2′-oxoheptadecyl)-resorcinol (**1**) and 5-(2′-oxononadecyl)-resorcinol (**2**).

Cell line	IC_{50} [μg/ml; μM] Compound **1**	**2**
COLO-320	2.5; 6.9	15; 38.3
DLD-1	5; 13.8	15; 38.3
HT-29	5; 13.8	15; 38.3
HL-60	5; 13.8	20; 51.1
HeLa S3	7.5; 20.6	20; 51.1
Jurkat	7.5; 20.6	15; 38.3
HepG2	7.5; 20.6	15; 38.3
J774.1a	10; 27.6	25; 63.9

biosynthesis of proteins- could be observed after 24 h of incubation (Fig. 2).

As measured by NBT reduction **1** and **2** induced 20 ~ 25% of HL-60 cells to differentiate at concentrations of 5 μg/ml and 12.5 μg/ml respectively (Table II). No antifungal (*Penicillium notatum*, *Mucor miehei*, *Paecilomyces variotii*, *Nematospora coryli*), antibacterial (*Bacillus brevis*, *B. subtilis*, *Enterobacter dissolvens*, *Micrococcus luteus*), phytotoxic (*Lepidium sativum*, *Setaria italica*) and nematicidal (*Meloidogyne incognita*, *Caenorhabditis elegans*) activities were observed for **1** and **2** at concentrations of 100 μg per ml or filter disc.

Many long-chain alkylresorcinols have been found in higher plants including gramineous cereals, brown algae and bacteria. (Suzuki *et al.*, 1998). These resorcinolic lipids, due to their amphiphilic structure, are described to be active biomembrane-altering agents with antioxidant properties (Kozubek and Tyman, 1995). Also antifungal activity (Suzuki *et al.*, 1996) and inhibition of glycerol-3-phosphate dehydrogenase (Tsuge *et al.*, 1992) from alkylresorcinols was reported.

Acknowledgements

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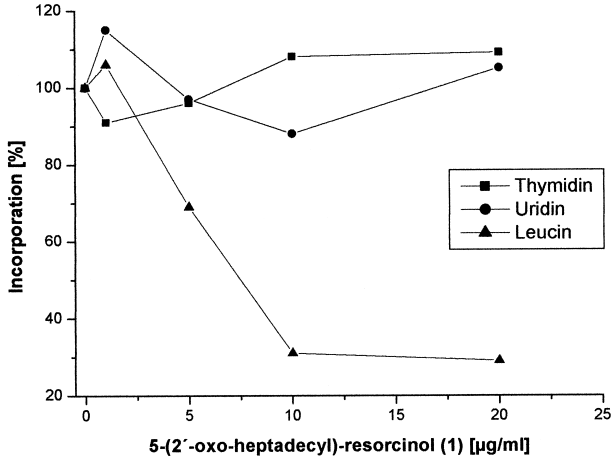

Fig. 2. Incorporation of radiolabelled precursors into macromolecules of COLO-320 cells. The influence of 5-(2′-oxoheptadecyl)-resorcinol on cellular DNA-, RNA-, and protein syntheses was measured in COLO-320 cells by determining the incorporation of [^{14}C]-thymidine, [^{14}C]-uridine and [^{14}C]-leucine in TCA-insoluble fractions (Weidler *et al.*, 2000).

Compound	Concentration [μg/ml; μM]	Differentiated cells (%)	Metabolic activity (%)
Negative control	–	6 ~ 8	185
(1)	5; 13.8	20 ~ 25	130
(2)	12.5; 31.9	20 ~ 25	113
DMSO	1% (v/v)	70 ~ 80	100

Table II. Differentiation of HL-60 cells after 96 hours of incubation. Metabolic activity was measured by the XTT-based colorimetric assay (Roche Diagnostics).

Anke H., Bergendorf O. and Sterner O. (1989), Assays of the biological activities of guaiane sesquiterpenes isolated from fruit bodies of edible *Lactarius* species. Food Chem. Toxicol. **27**, 393–397.

Erkel G., Becker U. and Anke T. (1996), Nidulal, a novel inducer of differentiation of human promyelocytic leukemia cells from *Nidula candida*. J. Antibiotics **49**, 1189–1195.

Kozubek A. and Tyman J. H. P. (1995), Cereal grain resorcinolic lipids: mono and dienoic homologues are present in rye grains. Chem. Phys. Lip. **78**, 29–35.

Mayer A., Kilian M., Hoster B., Sterner O. and Anke H. (1999), *In vitro* and *in vivo* nematicidal activities of the cyclic dodecapeptide omphalotin A. Pest. Sci. **55**, 27–30.

Suzuki Y., Esumi Y., Hyakutake H., Kono Y. and Sakurai A. (1996), Isolation of 5-(8-Z-heptadecenyl)-resorcinol from etiolated rice seedlings as an antifungal agent. Phytochemistry. **41**, 1485–1489.

Suzuki Y., Esumi Y., Saito T., Kishimoto Y., Morita T., Koshino H., Uzawa J., Kono Y. and Yamaguchi I. (1998), Identification of 5-n-(2′-oxo)alkylresorcinols from etiolated rice seedlings. Phytochemistry **47**, 1247–1252,

Tsuge N., Mizokami M., Imai S., Shimazu A. and Seto H. (1992), Adipostatins A and B, new inhibitors of glycerol-3-phosphate dehydrogenase. J. Antibiotics **45**, 886–891.

Weidler M., Rether J., Anke T. and Erkel G. (2000), Inhibition of interleukin-6 signaling by galiellalactone. FEBS Lett. **484**, 1–6.

Weber W., Anke T., Stefan B. and Steglich W. (1990), Antibiotics from basidiomycetes. XXXII. Strobilurin E: A new cytostatic and antifungal *E*-β-methoxyacrylate antibiotic from *Crepidotus fulvotomentosus*. J. Antibiotics **43**, 207–212.