Pulvinatal, a Novel Bioactive Metabolite from the Basidiomycete *Nidularia pulvinata* (Schw.) Fr.

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Basidiomycete, *Nidularia pulvinata*, Differentiation of HL-60 Cells, Pulvinatal

Pulvinatal (**1**) was isolated from fermentations of *Nidularia pulvinata* as an inducer of differentiation of HL-60 promyelocytic leukemia cells. Its structure was elucidated by spectroscopic methods. In addition, *N. pulvinata* was found to produce 2,4,5-trihydroxy-6-methylbenzenecarbaldehyde (**2**) and orsellinic acid (**3**). Pulvinatal exhibits weak antifungal and only marginal cytotoxic activities.

Introduction

Nidularia pulvinata is a small gasteromycete belonging, due to ist appearance, to the "bird's nest fungi". This group, the *Nidulariaceae*, produces the basidiospores in small lentil-shaped peridioles in cup shaped fruiting bodies. The family comprises the genera *Mycocalia*, *Nidularia*, *Nidula*, *Crucibulum*, and *Cyathus* (Brodie, 1975). From these, *Cyathus* species have been found to be the producers of interesting antibiotic compounds, the cyathins (Ayer and Taube, 1972, Ayer and Carstens, 1973) and striatins (Anke *et al.*, 1977, Hecht *et al.*, 1978). More recently, nidulal and niduloic acid have been isolated from *Nidula candida* in our screening for inducers of differentiation of HL-60 cells (Erkel *et al.*, 1996). In the following we wish to describe the production, isolation, structure elucidation and biological activities of pulvinatal, a new metabolite of *Nidularia pulvinata*.

Experimental

General

For TLC aluminium foils coated with silica gel Merck 60 F_{254} were used. Melting points (uncorrected) were determined with a Reichert microscope.

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Spectroscopy

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature, with an inverse 5 mm probe equipped with a shielded gradient coil. COSY, HMQC and HMBC experiments were performed with gradient enhancements using sine shaped gradient pulses, and for the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$=145 Hz and $^2J_{CH}$= 10 Hz. Chemical shifts were given in d units relative to TMS with the solvent signals (7.26 and 77.0 ppm in $CDCl_3$, and 2.50 and 39.5 ppm in CD_3SOCD_3). UV spectra were recorded with a Perkin Elmer l16, the IR spectra with a Bruker IFS48, and the mass spectra with a Jeol SX102 spectrometer. The optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Nidularia pulvinata (Schw.) Fr. strain 8681

Fruiting bodies of *Nidularia pulvinata* were collected in Highlands, U. S. A. The specimen showed all characteristics of the genus and species (H. J. Brodie, 1975). Mycelial cultures were derived from peridioles of a young fruit body. Herbarium specimen and cultures of strain 8681 are deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern.

Fermentation

For maintenance on agar slants and for submerged cultivation the fungus was grown in a medium composed of (g/l): glucose, 4; malt extract, 10; yeast extract, 4. The pH was adjusted to 5.5.

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For fermentations 200 ml of a well grown seed culture were used to inoculate 15 l of the same medium in a Biostat U apparatus (B. Braun, Melsungen) equipped with a MFCS process control system. The fermenter was incubated at 22 °C with aeration (4 l air/min) and agitation (130 rpm). Glucose and maltose were determined by enzymatic assays (Boehringer Mannheim). The oxygen content in the medium, oxygen consumption (Magnos 4G, Hartmann & Braun) and carbon dioxide production (Ultramat, Siemens) in the exhaust were measured on line. The content of pulvinatal in the culture fluid was determined by analytical HPLC (HP1090, Series II, Hewlett-Packard; column: LiChrospher RP18, 5 μm; column 124 x 4 mm, elution with water:methanol 0–100% in 30 min.).

Isolation and identification

After 380 hours of fermentation the culture was harvested, and the culture broth (14 l) extracted with 10 l of EtOAc. Evaporation of the organic phase yielded to 2 g of a crude extract which was separated by chromatography on silica gel (Merck 60) with cyclohexane:EtOAc (1:1) as eluant. Pulvinatal was purified by crystallization in methanol (yield: 50 mg). Pure **2** (yellow oil, yield: 7 mg) and orsellinic acid (**3**) (yellow oil, yield: 3 mg) were obtained by preparative HPLC (Merck Lichroprep CN, 7 μm, column 2.5 x 25 cm; eluant: cyclohexane: *tert*-butyl methyl ether 6:4 for **2** and 1:1 for **3**). Orsellinic acid was identified by comparison of the UV-, IR-, HPLC- and GC-MS-data with an authentical sample (Semar, 1993).

Biological assays

The induction of morphological and physiological differentiation of HL-60 cells (ATCC CCL 240, human promyelocytic leukemia) was assayed as described previously (Erkel *et al.*, 1996). For the differentiation assay cells were grown for 4 days with or without the compounds to be tested. 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. Apoptosis in HL-60 cells was measured as described by Nagy et *al.* (1995) with slight modifications (Erkel *et al.*, 1996). HeLa S3 cells (ATCC CCL 2.2), BHK-21 cells (ATCC CCL 10) and RBL-1 cells (ATCC CRL 1378) were grown in D-MEM medium, L1210 cells (ATCC CCL 219) were grown in HAM's F12 medium and HL-60 cells and U-937 cells (ATCC CRL 1593) were grown in RPMI 1640 medium supplementd with 10% fetal calf serum and 65 μg/ml benzylpenicillin and 100 μg/ml streptomycin sulfate in a humidified atmosphere containing 5% of CO_2 at 37 °C. Cytotoxicity towards cells growing as monolayers was determined with Giemsa stain as described by Erkel (1990). Metabolic activity of cells growing in suspension culture was measured by the XTT test (Boehringer Mannheim). Macromolecular syntheses in whole HL-60 cells were measured as described previously (Becker *et al.*, 1994). Mutagenicity was tested as described by Ames *et al.* (1975). Mutants of *Salmonella typhimurium*, strain TA98 and strain TA100 were used for the spot test with and without rat liver microsomes. Phytotoxic activities against germinating plant seeds were measured according to Anke *et al.* (1989).

Pulvinatal

Pulvinatal (**1a**) was obtained as yellow crystals, m.p. 208–210 °C. $[\alpha]_D$ 0° (*c* 1.0 in methanol). UV (methanol) λ_{max} (ε): 230 (28,800), 248 (infl., 17,800), 231 (infl., 10.000) and 355 (13,000). IR (KBr): 3420, 1675, 1640, 1575, 1520, 1415, 1330, 1315, 1220, 1080, 1020, 970 and 885 cm^{-1}. ^1H NMR ($CDCl_3/CD_3OD$ 95:5, 500 MHz), δ, mult. *J* (Hz): 10.33, s, 1-H; 6.87, s, 8-H; 6.79, dq, J_{14-15}=15.6, J_{15-16}=7.0, 15-H; 6.17, s, 12-H; 6.07, dq, J_{14-16}=1.7, 14-H; 4.00, s, 3-OCH_3; 3.57, s, 8-OCH_3; 1.92, dd, 16-H_3. ^1H NMR (CD_3SOCD_3, 500 MHz), δ, mult. *J* (Hz): 11.90 and 9.37, brs, 4- and 5-OH; 10.25, s, 1-H; 6.77, s, 12-H; 6.75, s, 8-H; 6.73, dq, J_{14-15}= 15.6, J_{15-16}=7.0, 15-H; 6.35, dq, J_{14-16}=1.7, 14-H; 4.03, s, 3-OCH_3; 3.52, s, 8-OCH_3; 1.93, dd, 16-H_3. ^{13}C NMR ($CDCl_3/CD_3OD$ 95:5, 125 MHz), δ: 190.7 C-1; 165.7 C-11; 165.4 C-9; 159.0 C-13; 151.7 C-3; 147.8 C-5; 139.7 C-4; 137.5 C-15; 121.7 C-14; 121.1 C-2; 117.5 C-7; 108.4 C-6; 101.6 C-10; 101.0 C-12; 96.6 C-8; 61.7 3-OCH_3; 56.7 8-OCH_3; 18.4 C-16. ^{13}C NMR (CD_3SOCD_3, 125 MHz), δ: 190.2 C-1; 165.5 C-11; 164.7 C-9; 159.0 C-13; 152.1 C-3; 148.4 C-5; 140.3 C-4; 137.1 C-15; 122.0 C-14; 120.3 C-2; 117.4 C-7; 108.4 C-6; 101.2 C-10; 101.2 C-12; 96.3 C-8; 61.5 3-OCH_3; 56.2 8-OCH_3; 18.4 C-16. EIMS [*m/z* (% rel. int.)]: 360.0852 (M^+, 65%, $C_{18}H_{16}O_8$ requires 360.0845), 345 (100%), 329 (39%), 277 (10%), 261 (15%), 235 (20%).

Diacetylpulvinatal

Diacetylpulvinatal (**1b**) was obtained as yellowish oil. $[\alpha]_D$ 0° (*c* 0.5 in methanol). IR (KBr): 1780, 1725, 1690, 1575, 1540, 1465, 1360, 1315, 1190, and 1020 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz), δ, mult.

J (Hz): 10.46, s, 1-H; 6.80, dq, J_{14-15}=15.6, J_{15-16}= 7.0, 15-H; 6.78, s, 8-H; 6.04, dq, J_{14-16}=1.7, 14-H; 6.02, s, 12-H; 3.97, s, 3-OCH_3; 3.62, s, 8-OCH_3; 2.36 and 2.33, 4- and 5-Ac; 1.94, dd, 16-H_3. ^{13}C NMR ($CDCl_3$, 125 MHz), δ: 189.5 C-1; 167.2 and 167.0 4- and 5-Ac; 164.2 C-11; 163.7 C-9; 159.8 C-13; 156.5 C-3; 144.3 C-5; 138.7 C-4; 136.6 C-15; 128.9 C-2; 122.8 C-7; 122.6 C-14; 117.9 C-6; 99.9 C-10; 99.2 C-12; 96.3 C-8; 63.6 3-OCH_3; 56.9 8-OCH_3; 20.9 and 20.3 4- and 5-Ac; 18.5 C-16. EIMS [*m/z* (% rel. int.)]: 444.1044 (M^+, 27%, $C_{22}H_{20}O_{10}$ requires 444.1056), 402 (28%), 360 (39%), 345 (100%), 329 (22%).

2,4,5-trihydroxy-6-methyl-benzenecarbaldehyde (**2**)

(**2**) was obtained as yellowish oil. ^{1}H NMR ($CDCl_3$/CD_3OD 95:5, 500 MHz), d, mult. *J* (Hz): 9.95, s, 1-H; 6.17, s, 4-H; 2.38, s, 8-H_3. ^{13}C NMR ($CDCl_3$/CD_3OD 95:5, 125 MHz), d: 191.1 C-1; 159.4 C-3; 154.2 C-5; 136.2 C-6; 125.9 C-7; 111.8 C-2; 99.6 C-4; 9.7 C-8. EIMS [*m/z* (% rel. int.)]: 168.0435 (M^+, 100%, $C_8H_8O_4$ requires 168.0422), 139 (7%), 122 (9%), 69 (12%).

Results and Discussion

A typical fermentation of *Nidularia pulvinata* is shown in Fig. 1. The production of pulvinatal starts after 200 hours. The highest content of the compound is reached after 380 hours when the glucose is used up and the pH rises. Pulvinatal, 2,4,5-trihydroxy-6-methyl-benzenecarbaldehyde (**2**) and orsellinic acid were isolated as described in the experimental section.

Structural eludication

HRMS experiments with pulvinatal (**1a**) showed that its elemental composition is $C_{18}H_{16}O_8$, while acetylation led to the diacetyl derivative with the elemental composition $C_{22}H_{20}O_{10}$. The unsaturation index of pulvinatal (**1a**) is therefore 11, and as the NMR data suggest the presence of 8 double bonds pulvinatal (**1a**) should be tricyclic. From the NMR data it is evident that it contains a 1-propenyl moeity, a methyl acetal, an aldehyde group, a methoxy group, and two phenolic hydroxy groups (which may be acetylated, OH protons visible in the ^{1}H NMR spectrum recorded in CD_3SOCD_3), the remaining proton being olefinic. The structure of pulvinatal (**1a**) was essentially determined from the NMR data of the diacetate **1b**. Starting from the propenyl chain, in which the double bond is *trans* according to the coupling constant (J_{14-15}= 15.6 Hz), there is a HMBC correlation to C-13, from both 14-H and 15-H, and to C-12, from 14-H. 12-H gives a NOESY correlation to 14-H, and HMBC correlations to C-6, C-10 and C-11 in addition to C-13 and C-14. The chemical shifts for C-12 and C-13 suggest that C-13 is oxygenated. The

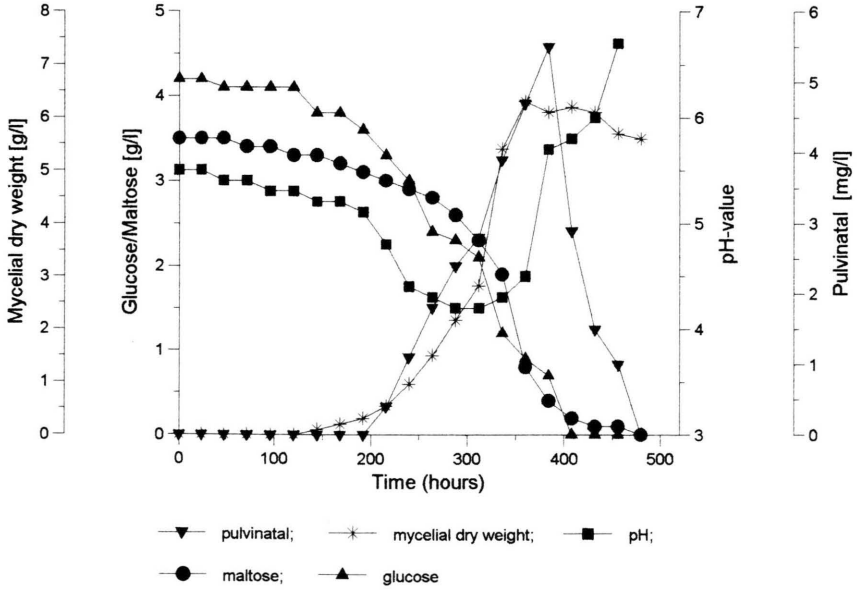
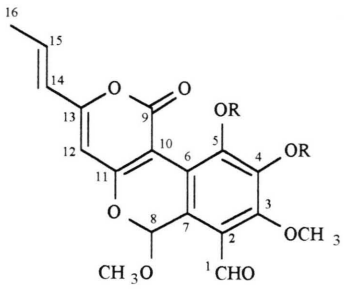
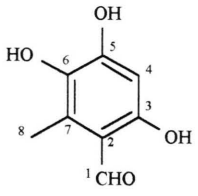


Fig. 1. Fermentation of *Nidularia pulvinata* 8681 in 15 l scale and production of pulvinatal.



1



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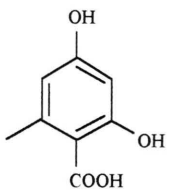

3

1a: R = H; **1b:** R = Ac.

Fig. 2. Structures of pulvinatal (**1a**), diacetylpulvinatal (**1b**), 2,4,5-trihydroxy-6-methyl-benzenecarbaldehyde (**2**) and orsellinic acid (**3**).

methyl acetal can be assigned because of the chemical shifts of C-8 and 8-H, and the correlations observed between 8-H and 8-O**C**H_3 as well as between 8-O**C**H_3 and C-8. In addition, 8-O**C**H_3 gives a weak NOESY correlation to 12-H. 8-H also gives HMBC correlations to C-2, C-6, C-7 and C-11, all sp^2 carbons of which C-11 due to its chemical shift must be bound to the second C-8 oxygen. H-8 is close to the aldehyde proton (1-H), as they give a correlation in the NOESY spectrum, and 1-H also gives a NOESY correlation to the remaining methoyx group (3-OCH_3) suggesting that the aldehydes function is positioned between the two methoxy groups. 1-H gives HMBC correlations to C-2 and C-7, although not to C-3. Of the remaining three carbons, C-4, C-5 and C-9, the former two are hydroxylated aromatic carbons while the latter is a carbonyl group. The chemical shifts for C-4 and C-5 are upshifted a few ppm by the acetylation, and a weak HMBC correlation can be observed between the acetyl methyl groups and the two carbons. No other correlations can be seen to C-4 and C-5, and none at all indicate the position of the C-9 carbonyl group. However, by positioning C-9 as a lactone function between C-10 and C-13, and C-4 as well as C-5 in the aromatic ring, the resulting structure fits all spectroscopic data. The existence of a hydrogen bond between the 5-OH and the lactone carbonyl oxygen in pulvinatal (**1a**) is indicated by the observed signal for an exchangable proton at 11.90 ppm in the ^1H NMR spectrum in $(CD_3)_2SO$, and by the shift of the lactone carbonyl IR band (1640 cm-1 for pulvinatal (**1a**) and 1780 cm-1 for the diacetate **1b**). In addition, the structure of pulvinatal is reasonable from a biogenetic point of view, being generated by the condensation of two tetraketides (C-1 to C-8, and C-9 to C-16).

The structure of compound **2**, a new tetraketide, was determined from its NMR and MS data. The elemental composition is $C_8H_8O_4$, it has an unsaturation index of 5 and consequently one ring. It contains one methyl group and one aldehyde group, and a strong NOESY correlation between the two suggests that they are positioned beside each other on the ring. The fifth proton is olefinic, while the remaining three are exchangable. The methyl group gives HMBC correlations to C-2, C-6 and C-7, and the strong HMBC correlations between 4-H and C-2 and C6 show that 4-H and the methyl group are on opposite sides of an aromatic ring. The remaining three hydrogens and oxygens must then be hydroxyl groups on C-3, C-5 and C-6.

Biological properties

Pulvinatal induces the differentiation of 20–25% of HL-60 cells at a concentration of 51 µM as measured by NBT reduction (Table I). Addition of 1.5% of DMSO (positive control) resulted in 70–80% of differentiated cells. In the negative control without additions 4–8% of HL-60 cells

Table I. Differentiation of HL-60 cells after 96 hours of incubation.

Substance	Concentration [µg/ml]	Differentiated cells (%)	Cell number (cells/ml)	Metabolic activity (%)
–	–	4–8	5×10^5	243
DMSO	1.5% (v/v)	70–80	1.3×10^5	100
Pulvinatal	51	20–25	1.1×10^5	110
2	178	15–20	1.6×10^5	120

Metabolic activity was measured by XTT reduction.

differentiated spontaneously. At the same concentration of pulvinatal only 5–10% of the U-937 cells differentiated. Retinoic acid, the positive control, induced 30–40% of cells to differentiate (Table II). **2** induces a differentiation of 15–20% of HL-60 cells at a concentration of 178 μM. Cytotoxic activities towards HL-60 cells and U-937 cells were observed starting from 130 μM of pulvinatal. RBL-1 cells, L-1210 cells HeLa S3 cells and BHK-21 cells were lysed starting from 255 μM. Cytotoxic activities of **2** were observed at concentrations starting from 600 μM.

The mutagenic activity of pulvinatal and **2** and their influence on macromolecular syntheses were tested. In the test for mutagenicity according to Ames **1**, **2** and **3** did not induce revertants of *Salmonella typhimurium* TA 98 and TA 100 at concentrations of up to 100 μg/plate (pour plate assay with and without addition of rat liver microsomes). The inhibitory effect of pulvinatal and **2** on DNA, RNA and protein syntheses in HL-60 cells was low (20% at 100 μg/ml).

In the plate diffusion assay pulvinatal shows antifungal activity against the yeasts *Nadsonia fulvescens*, *Nematospora coryli*, and *Saccharomyces cerevisiae* at concentrations starting from 20 μl/disc. In the serial diffusion assay only *N. coryli* was inhibited (MIC 10–50 μg/ml).

Acknowledgments

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Table II. Differentiation of U-937 cells after 96 hours of incubation.

Substance	Concentration [μg/ml]	Differentiated cells (%)	Cell number (cells/ml)	Metabolic activity (%)
–	–	1–2	5.1×10^5	158
Retinoic acid	6	30–40	1.3×10^5	100
Pulvinatal	51	5–10	1.1×10^5	94
(**2**)	178	1–2	2.6×10^5	132

Metabolic activity was measured by XTT reduction.

TableIII. Antifungal spectrum of pulvinatal measured by agar diffusion assay.
(paper disk: ∅ 6 mm). – no inhibition.

Test organism	Diameter of inhibition zone [mm] μg pulvinatal/disk		
	20	50	100
Alternaria porri	–	–	–
Ascochyta pisi	–	–	–
Aspergillus ochraceus	–	–	–
Botrytis cinerea	–	–	–
Cladosporium cladosporoides	–	–	–
Curvularia lunata	8	10	12
Fusarium oxysporum	–	–	–
Mucor miehei	–	–	–
Nadsonia fulvescens	13	16	19
Nematospora corylii	10	14	22
Paecilomyces variotii	–	–	8
Penicillium islandicum	–	–	–
Penicillium notatum	–	–	–
Phoma clematidina	–	–	–
Phytophtora infestans	–	–	–
Rhodotorula glutinis	–	7	11
Saccharomyces cerevisiae is 1	10	12	15
Ustilago nuda	–	–	–
Venturia cerasi	–	–	–
Verticillium spec.	–	–	–

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