Nitidon, a New Bioactive Metabolite from the Basidiomycete *Junghuhnia nitida* (Pers.: Fr.) Ryv.

Alexandra Gehrt[a], Gerhard Erkel[a], Timm Anke[a] and Olov Sterner[b]

[a] Lehrbereich Biotechnologie der Universität Kaiserslautern, D-67663 Kaiserslautern, FRG
[b] Department of Organic Chemistry 2, Lund University, S-221 00 Lund, Sweden

Z. Naturforsch. **53c**, 89–92 (1998); received November 7/December 3, 1997

Basidiomycete, *Junghuhnia nitida*, Nitidon, Antibiotic Activities, Induction of Differentiation of HL-60 Cells

Nitidon, a highly oxidised pyranone derivative produced by the basidiomycete *Junghuhnia nitida*, has been isolated and its biological activities evaluated. The structure was determined by spectroscopic methods. Nitidon exhibits antibiotic and cytotoxic activities and induces morphological and physiological differentiation of tumor cells at nanomolar concentrations.

Introduction

The arrest of uncontrolled growth of tumor cells leading to differentiated stages and eventually apoptosis, offers an attractive concept for the treatment of leukaemias. During our ongoing screening of fungal cultures for new metabolites inducing morphological and physiological differentiation of HL-60 (human promyelocytic leukemia) cells, several new metabolites have been isolated from basidiomycetes. Among them are pinicoloform from *Resinicium pinicola* (Becker *et al.*, 1994), lepistal from *Lepista sordida* (Mazur *et al.*, 1996), puraquinonic acid from *Mycena pura* (Becker *et al.*, 1996), pholiotic acid from *Pholiota destruens* (Becker *et al.*, 1994), nidulal and niduloic acid from *Nidula candida* (Erkel *et al.*, 1996) and pulvinatal from *Nidularia pulvinata* (Becker *et al.*, 1997). In the following we like to describe the isolation and the characterisation of a new antibiotic metabolite inducing morphological and physiological differentiation of HL-60 cells, for which we suggest the name *nitidon*.

Experimental

Junghuhnia nitida strain 95055

Mycelial cultures were derived from spore prints of fruiting bodies showing all characteristics of the genus and species (Breitenbach and Kränzlin, 1986). The strain is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

Reprint requests to Prof. T. Anke.
Fax: 0631/2052999.

Fermentation and isolation of Nitidon

For maintenance on agar slants and submerged cultivation, *J. nitida* was grown in YMG medium composed of (g/l): glucose, 4; malt extract, 10; yeast extract, 4. Fermentations were carried out in a Biolafitte C-6 fermenter containing 20 l of YMG medium with aeration (2 l/min) and agitation (120 rpm) at 22 °C. After 12 days of fermentation the culture broth (18 liters) was extracted with EtOAc (10 liters). Evaporation of the organic phase yielded a crude extract (889 mg) which was applied to a column containing silica gel (Merck 60, 0.063–0.2 μm; column 3x30 cm) with cyclohexane-EtOAc (1:1) as eluant. Further purification of the extract (260 mg) by repeated column chromatography on silicic acid (Sigma, SIL-A-200, 60–200) with cyclohexane-EtOAc (1:1) yielded 124.1 mg of intermediate product. From this intermediate product, 61.7 mg of nitidon were obtained by preparative HPLC (LiChrosorb RP18, column 2.5x25 cm) with H_2O-methanol (1:1) as eluant.

Spectroscopy

^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respec-

0939–5075/98/0100–0089 $ 06.00 © 1998 Verlag der Zeitschrift für Naturforschung. All rights reserved.

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

tively) were used as reference. 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 UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. The melting point (uncorrected) was determined with a Reichert microscope, and the optical rotation measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Nitidon (**1**) was obtained as white crystals, m.p. 115–117°. $[\alpha]_D$ – 34° (*c* 1.1 in $CHCl_3$-MeOH 1:1). UV (MeOH), λ_{max} (e): 333 nm (18,500), 262 (3,950) and 248 (4,690). IR (KBr): 3505, 3080, 2925, 2225, 1735, 1615, 1535, 1320, 1220, 1115, 1090, 1070, 890, 850, 810, 620 and 570 cm^{-1}. See Table I for NMR data. EIMS (70 eV), *m/z* (rel. int.): 216.0420 (36%, M^+, $C_{12}H_8O_4$ requires 216.0422), 156.0211 (100%, M^+ – $C_2H_4O_2$, $C_{10}H_4O_2$ requires 156.0214), 128 (19%), 100 (62%), 74 (22%). CIMS (CH_4), *m/z* (rel. int.): 257 (M + C_3H_5+, 5%), 245 (M + C_2H_5+, 12%), 217 (M + H^+, 100%), 201 (23%), 187 (92%), 159 (16%).

Biological assays

The induction of morphological and physiological differentiation of HL-60 cells (ATCC CCL 240, human promyelocytic leukemia) and U-937 cells (ATCC CRL 1593, human histiocytic leukemia) was assayed as described previously (Becker *et al.*, 1997). For the differentiation assay, cells were grown 4 days with or without the test compound. Differentiated cells (monocytes/granulocytes) reduced the water-soluble nitro-blue tetrazolium chloride (NBT) to blue-black cell-associated nitro-blue diformazane deposits. For quantification the percentage of blue-black cells was determined. The cytotoxic activity was assayed with HL-60 cells and U-937 cells (grown in RPMI 1640 medium, Gibco), BHK 21 cells (ATCC CCL 10, grown in D-MEM medium, Gibco), and HeLa S3 cells (ATCC CCL 2.2, grown in D-MEM medium), all supplemented with 10% fetal calf serum. L1210 cells (ATCC CCL 219) were cultivated in Ham's F-12 medium (Gibco) supplemented with 20% horse serum. All media contained 65 μg/ml benzylpenicillin and 100 μg/ml streptomycin sulphate. The incorporation of appropriate precursors into DNA, RNA and proteins in whole HL-60 cells was measured as described previously (Becker *et al.*, 1994). Cells grown for 4 days were harvested by centrifugation (1000 × *g*) and resuspended in phosphate-buffered saline containing 0.01% glucose to a cell density of 4.5 x 10^6 cells/ml. After incubation for 30 min with or without nitidon, 1 ml of the cell suspension was incubated at 37 °C with gentle shaking with 0.1 μCi of the radioactive precursors [2-^{14}C]thymidine (56.5 mCi/mmol), [2-^{14}C]uridine (62.4 mCi/mmol) and [2-^{14}C]leucine (55.2 mCi/mmol). After 30 min the cells were suspended in 5% trichloroacetic, the precipitate was collected on cellulose nitrate filters and the radioactivity measured in a liquid scintillation counter. Mutagenicity was tested according to the method of Ames *et al.* (1975). Histidine auxotrophic mutants of *Salmonella typhimurium*, strain TA98 and TA100 (Ames *et al.*, 1975) were used for the plate incorporation assay with and without rat liver microsomes and revertant colonies counted as described by Venitt *et al.*(1984). The antimicrobial activity was assayed as described previously by Anke *et al.* (1989).

Results and Discussion

Structural elucidation

Nitidon was obtained as white crystals, and high resolution measurements of the apparent molecular ion in the EIMS spectrum indicated that its elemental composition is $C_{12}H_8O_4$. The molecular weight of nitidon was confirmed by CI mass spectrometry, and the compound consequently has an unsaturation index of 9. The ^{13}C NMR data suggest the presence of one carbonyl group and two double bonds, leaving 6 additional unsaturations to be accounted for. An IR band at 2225 cm^{-1} indicates that nitidon may contain an unsymmetrical acetylene moiety, and four signals appearing as singlets between 67 and 84 ppm in the ^{13}C NMR spectrum suggest that nitidon actually may contain 2 carbon-carbon triple bonds. C-12 and 12-H_2 have all characteristics of a -CH_2OH group, and COSY as well as HMBC (summarised in Fig. 1) correlations from 12-H_2 to 11-H/C-11 and C-10, from 11-

Table I. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of Nitidon in $CDCl_3$-CD_3OD 20:1. The chemical shifts are given in ppm relative to the $CHCl_3/CDCl_3$ signals (7.26 and 77.0 ppm, respectively), and the coupling constants (*J*) are given in Hz.

Pos.	dH; mult.; *J*	dC; mult.
1	–	160.7; s
2	6.33; dd; 0.9, 9.4	118.3; d
3	7.23; dd; 6.7, 9.4	142.6; d
4	6.46; dd; 0.9, 6.7	112.7; d
5	–	143.2; s
6	–	68.6; s
7	–	78.9; s
8	–	66.8; s
9	–	83.6; s
10	3.49; d; 2.1	42.2; d
11	3.29; ddd; 2.1, 2.6, 3.4	60.5; d
12a	3.77; dd; 2.6, 13.1	59.2; t
12b	3.62; dd; 3.4, 13.1	

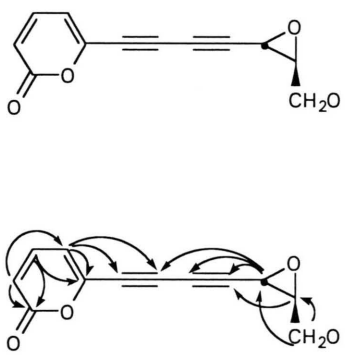

Fig. 1. Structure of Nitidon (top). Pertinent correlations observed in the HMBC spectrum (bottom).

H to 10-H/C-10 and 12-H_2/C-12, and from 10-H to 11-H/C-11 and C-12 establish that the three carbons are connected. However, the ^{1}H-^{1}H coupling constants between 11-H and 12-H_2 are small (2.6 and 3.4 Hz), as is that between 10-H and 11-H (2.1 Hz), but reasonable if C-10 and C-11 are part of an epoxide ring. This is also in agreement with the observed chemical shifts of C-10 and C-11. 11-H in addition gives a HMBC correlation to C-9, while 10-H correlates strongly to both C-8 and C-9 and weakly to C-7. The three olefinic protons give COSY and HMBC correlations that are typical of a pyranon substituted in position 6, and the HMBC correlations from 4-H to C-5, C-6 and weakly to C-7 establish the structure of nitidon. The conjugation of the diacetylenic chain with the pyranone is in agreement with the observed UV spectrum, with a strong absorbance at 333 nm. The relative stereochemistry of the epoxide ring could be established by comparison of the 10-H/11-H coupling constant with similar compounds, and by the NOESY correlation observed between 10-H and 12-H_2. Obviously nitidon is a highly reactive compound, and attempts to prepare derivatives (e.g. by acetylation with acetic anhydride in pyridine at room temperature) resulted in unseparable mixtures.

Biological properties

Many polyacetylenes have been described from basidiomycetes (for review see Jones *et al.*, 1973). Structures more closely related to nitidon have been described by Jones *et al.* (1963) and Dagne *et al.* (1994).

At a concentration of 100 ng/ml (0.46 μM) nitidon induces the differentation of 25–30% of the HL-60 cells into granulocyte-monocyte-like cells and a differentation of 20% of the U-937 cells into the monocyte-like cells as measured by NBT reduction (Tables II and III). Cytotoxic activities (lysis of cells) of nitidon were observed at 250 ng/ml for both cell lines. L1210 cells, HeLa S3 cells and BHK-21 cells were lysed starting from 500 ng/ml. Nitidon exhibits antibacterial and antifungal activities (minimal inhibitory concentrations in μg/ml): *Streptomyces* sp. ATCC 23836 (10), *Bacillus subti-*

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

Compound	Concentration [μM]	Differentiated cells [%]	Cell number [cells/ml]
–	–	4–8	5×10^5
DMSO	1.5% (v/v)	70–80	1.3×10^5
Nitidon	0.46	25–30	1.2×10^5

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

Compound	Concentration [μM]	Differentiated cells [%]	Cell number [cells/ml]
–	–	1–2	5×10^5
Retinoic acid	6	30–40	1.4×10^5
Nitidon	0.46	20	1.3×10^5

lis (10), *Rhodotorula glutinis* (25), *Saccharomyces cerevisiae* S288c (25), *Penicillium notatum* (5), *Fusarium oxysporum* (25), *Mucor miehei* (5), and *Nematospora coryli* (5). In the test for mutagenicity, nitidon did not induce revertants of *Salmonella typhimurium* TA98 and TA100 at concentrations up to 5 μg/plate (plate incorporation assay with and without addition of rat liver microsomes). In phosphate buffered saline an inhibitory effect of nitidon on the incorporation of [^{14}C]-thymidine into DNA and [^{14}C]-leucine into protein of HL-60 cells was observed (IC_{50} = 16.1 μM and 11.5 μM; Table IV).

The biological activity of nitidon is at least in part due to its high chemical reactivity. Addition of cysteine yielded adducts which were almost devoid of differentiation inducing activity.

Acknowledgements

The financial support of the Deutsche Forschungsgemeinschaft and the Swedish Science Research Council is gratefully acknowledged.

Table IV. Effect of nitidon on DNA, RNA and protein syntheses in HL-60 cells. 4.5×10^6 were incubated with or without nitidon as described in the experimental section.

Compound	Concentration [μM]	Incorporation [pmol] [^{14}C]thymidine	[^{14}C]uridine	[^{14}C]leucine
Control	0	124	281	220
Nitidon	0.46	109.5	261	213.4
	4.6	96.7	227.6	180.4
	23	21.3	185.5	0.22

Ames B. N., McCann J. and Yamasaki E. (1975), Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian mutagenicity test. Mut. Res. **31**, 347–364.

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

Anke T., Erkel G., Koksch G. und Kuschel A. (1993), Inhibitoren der HIV-1-Reversen Transkriptase und Induktoren der Differenzierung menschlicher Zelllinien aus Pilzen, in: Wege zu neuen Produkten und Verfahren der Biotechnologie. DECHEMA Monographien, Band 129. VCH, Frankfurt, pp. 15–25.

Becker, U., T. Anke and O. Sterner (1994) A novel halogenated compound posessing antibiotic and cytotoxic activities isolated from the fungus *Resinicium pinicola* (J. Erikss.) Erikss. & Hjortst. Z. Naturforsch. **49c**, 772–774.

Becker, U., G. Erkel, T. Anke and O. Sterner, Puraquinonic acid, a novel inducer of differentiation of human HL-60 promyelocytic leukemia cells from *Mycena pura* (Pers. ex Fr.). Z. Naturforsch., in print.

Becker, U., T. Anke and O. Sterner (1994), A novel illudalane isolated from the fungus *Pholiota destruens* (Brond.)Quel. Nat. Prod. Lett. **5**, 171–174.

Becker, U., G. Erkel, G. Kocksch, T. Anke and O. Sterner, Pulvinatal, a novel bioactive metabolite from the basidiomycete *Nidularia pulvinata* (Schw.) Fr. Z. Naturforsch. in press.

Breitenbach J. and Kränzlin F. (1986) Pilze der Schweiz, Vol. **2**. Verlag Mykologia, Luzern

Collins S., Gallo R. C. and Gallagher R. E. (1977), Continous growth and differentiation of human myeloid leukemia cells in suspension culture. Nature **270**, 347–349.

Dagne, E., Asmellash, S. and Abate, D. (1994), An antifungal polyacetylenic compound from the culture filtrate of the fungus *Trametes pubescens*, J. Natl. Prod., **Vol.57**, No.3, 390–392.

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

Jones, E. R. H., Stephenson, J. S., Turner, W. B. and Whiting, M. C. (1963), Synthesis of (a) a C_9 triacetylenic epoxy-alcohol, a *Coprinus quadrifidus* metabolite and (b) a C_9 triacetylenic 1,2-diol. The structure of Biformyne I. J. Chem. Soc., 2048–2055.

Koksch G., Anke T. and Hansske F. (1992), Suche nach neuen Induktoren der morphologischen Differenzierung der humanen Zellinie HL-60 aus Pilzen. DECHEMA Biotechnology Conferences, **Vol.5A**. Verlag Chemie, Weinheim, 319–322.

Mazur, X., U. Becker, T. Anke and O. Sterner (1996), Lepistal and lepistol, two novel bioactive diterpenes from *Lepista sordida* (Fr.)Sing. Phytochemistry **43**, 405–407 (1996).

Venitt, S., Crofton-Sleight, C. and Forster, R. (1984), Mutagenicity testing, a Practical Approach. IRL Press, Oxford, New York, 45–98.