A Novel Halogenated Compound Possessing Antibiotic and Cytotoxic Activities Isolated from the Fungus *Resinicium pinicola* (J. Erikss.) Erikss. & Hjortst.

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Z. Naturforsch. **49 c,** 772–774 (1994); received June 27, 1994

Dedicated to Professor Hans Zähner on the occasion of his 65th birthday

Fungus, *Resinicium pinicola,* Antibiotic, Cytotoxic, Chlorinated

Pinicoloform, a novel unbranched acyclic compound containing a trichloromethyl group, has been isolated from extracts of the mycelia of the Basidiomycete *Resinicium pinicola.* It was isolated because of its ability to induce morphological and physiological differentiation of mammalian cells, although it also exhibits antibiotic and cytotoxic activities. The structure of pinicoloform was determined by spectroscopic methods.

Introduction

Tumor cells differ from their normal counterparts by their ability to evade the regulatory mechanisms of their environment and to propagate in an uncontrolled fashion. Among the phenotypic abnormalities in acute leukemia is a lack of granulocytes, macrophages and platelets caused by the inability of the neoplastic cells to undergo terminal differentiation. The human HL-60 cell line is an excellent model to study functional and morphological differentiation *in vitro,* because the cells can be induced to differentiate into granulocytes or monocytes/macrophages which can be easily recognized by their ability to reduce the water-soluble nitro-blue tetrazolium chloride to dark-blue cell-associated diformazan deposits. In the course of a screening of fungal extracts for new metabolites inducing morphological and physiological differentiation of HL-60 cells, several new metabolites have been isolated (G. Kocksch *et al.,* 1992). In the following we wish to describe the isolation and characterization of a new compound, which has been named pinicoloform (structure shown in Fig. 1), from fermentations of the Basidiomycete *Resinicium pinicola.*

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Experimental

Resinicium pinicola strain 90230

Fruiting bodies of *Resinicium pinicola* (J. Erikss.) Erikss. & Hjortst. were collected from rotten wood in the U.S.A. (Mount Baker, Wash.). They showed all characteristics of the species as described by J. Eriksson *et al.* (1981). Mycelial cultures were derived from spore prints. The strain is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

Fermentation

For maintenance on agar slants and submerged cultivation, *R. pinicola* 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 (3 l air/min) and agitation (130 rpm) at 22 °C.

Biological assays

The induction of morphological and physiological differentiation of HL-60 cells (ATCC CCL 240, human promyelocytic leukemia) (T. Anke *et al.,* 1993) and antimicrobial activity (H. Anke *et al.,* 1989) were assayed as described previously. For the differentiation assay the cells were grown for

0939–5075/94/1100–0772 $ 06.00

4 days with or without the compounds to be tested. Differentiated cells (monocytes/granulocytes) reduce 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. The cytotoxic activity was assayed with HL-60 cells (grown in RPMI 1640 medium), BHK 21 cells (ATCC CCL 10, grown in G-MEM medium), L1210 cells (ATCC CCL 219, grown in F-12 medium), and HeLa-S3 cells (ATCC CCL 2.2, grown in D-MEM medium). Cytotoxicity was determined as lysis of the cells after 2 days of incubation (K. Leonhardt *et al.,* 1987). The incorporation of [2-^{14}C]thymidine into DNA, [2-^{14}C]uridine into RNA and [1-^{14}C]leucine into proteins was assayed with HL-60 cells (K. Leonhardt *et al.,* 1987). Cells grown for 4 days were harvested by centrifugation (1000×*g*) and resuspended in phosphate-buffered saline containing 0.01% of glucose. After incubation for 30 min with or without pinicoloform 0.1 μCi of the radioactive precursors were added and the cells incubated for 30 min with gentle shaking at 37 °C. The incorporated radioactivity was determined in the 5% trichloroacetic acid insoluble precipitate in a liquid scintillation counter.

Isolation and identification of pinicoloform

After 20 days the culture was harvested, and the mycelia (50 g dry weight/18 l culture fluid) were separated from the culture fluid and extracted with 2 l of EtOAc. The solvent was evaporated, and the crude product was separated by chromatography on silica gel (Merck 60) with cyclohexane:EtOAc (90:10) as eluant. Pure pinicoloform was obtained by preparative HPLC (Merck LiChrosorb Diol, 7 μm) with cyclohexane as eluant. The NMR spectra were recorded with a Bruker ARX500 spectrometer, the UV spectra with a Perkin-Elmer λ16, the IR spectra with a Bruker IFS48, and the mass spectra with a Jeol SX102 spectrometer. Pinicoloform [(2*Z*,5*Z*,7*E*)-1,1,1-trichloro-2-hydroxy-2,5,7-undecatrien-4-one] was obtained as a yellowish oil (6 mg). UV (methanol) λ_{max} (ε): 242 nm (2900) and 357 nm (13,500). IR (KBr): 3415, 2960, 1600, 1555, 1430, 1335, 1150, 995, 955, 835, 800 and 705 cm^{-1}. ^{1}H NMR (500 MHz, recorded in C_6D_6 with the solvent peak at 7.16 ppm as reference, coupling constants in Hz): 14.20, brs, 2-OH; 7.33, dd, J_{6-7} = 11.5, J_{7-8} = 15.1, 7-H; 6.09, dd, J_{5-6} = 11.5, J_{6-7} = 11.5, 6-H; 5.96, s, 3-H; 5.71, dt, J_{7-8} = 15.1, J_{8-9} = 7.1, 8-H; 5.06, d, J_{5-6} = 11.5, 5-H; 1.89, dt, J_{8-9} = J_{9-10} = 7, 9-H_2; 1.23, tq, J_{9-10} = J_{10-11} = 7, 10-H_2; 0.77, t, J_{10-11} = 7, 11-H_3.^{13}C NMR (125 MHz, recorded in C_6D_6 with the solvent peak at 128.0 ppm as reference): 186.5 C-2; 180.3 C-4; 147.0 C-8; 144.3 C-6; 128.9 C-7; 119.0 C-5; 95.7 C-1; 94.8 C-3; 35.3 C-9; 22.1 C-10; 13.7 C-11. MS (EI, 70 eV), *m/z:* 281.9992 (M^+, 16%, $C_{11}H_{13}O_2Cl_3$ requires 281.9981), 247 (6%), 239 (85%), 219 (6%), 205 (10%), 165 (88%), 123 (100%).

Results and Discussion

Pinicoloform induces the differentiation of 20–30% of the HL-60 cells to monocytes/granulocytes, as measured by NBT reduction, at concentrations between 0.5–1 μg/ml (1.8–3.5 μM). Cytotoxic activities were observed starting from 2.5 μg/ml (8.8 μM) towards HL-60 cells, BHK 21 cells and L1210 cells. HeLa-S3 cells were lysed at 3 μg/ml (10 μM). In HL-60 cells the incorporation of [2-^{14}C]thymidine into DNA was inhibited by 15% at 10 μg/ml (35 μM) while no effect on the incorporation of [2-^{14}C]uridine into RNA and [1-^{14}C]leucine into proteins was observed. The cytotoxicity of pinicoloform is therefore not primarily caused by the inhibition of macromolecular syntheses. The compound exhibits unselective antimicrobial activities towards bacteria and fungi. In a serial dilution assay the following minimal inhibitory concentrations (μM) were observed: *Acinetobacter calcoaceticus* (66), *Corynebacterium insidiosum* (16), *Mycobacterium phlei* (16); *Paecilomyces variotii* (6.5), *Saccharomyces cerevisiae* strain is 1 (6.5).

The structure determination of pinicoloform is based on mass spectrometry and NMR spectroscopy data. The isotope pattern of M^+ obtained by electron impact ionization, as well as the exact

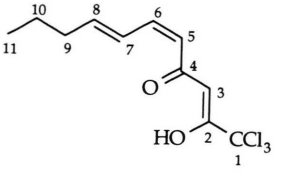

Fig. 1. The structure of pinicoloform.

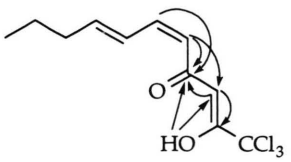

Fig. 2. Significant $^1H-^{13}C$ long-range correlations observed for pinicoloform.

mass recorded by high resolution measurements, showed that the compound contains three chlorine atoms. Major fragments are observed at *m/z* 239 (with the trichloro isotope pattern retained), *m/z* 165 and *m/z* 123 (no chlorine is left in the two latter). High resolution measurements indicated that 239 is formed after the loss of $-CH_2CH_2CH_3$, 165 after the loss of $-CCl_3$, and 123 after the loss of $C_3H_2OCl_3$. The presence of a propyl group was evident from the NMR data, and $^1H-^1H$ correlation spectroscopy showed that it is connected to a $-CH=CH-CH=CH-$ chain. $^1H-^{13}C$ long-range correlation experiments (data summarized in Fig. 2) confirmed this and also suggested that the chain continues with a keto-enol moiety. The ^1H NMR shift for the hydrogen-bonded hydroxyl proton is 14.2 ppm and the ^{13}C NMR shifts for C-2 and C-3 are 186.5 and 94.8 ppm, respectively. These are typical NMR values for a β-diketo functionality present in the enol form, as in pinicoloform. The trichloromethyl group must consequently be attached to C-2, although we could not observe any $^1H-^{13}C$ long-range correlations to C-1. The observed fragmentation in the mass spectrum of pinicoloform discussed above is caused by a α-cleavage of the keto function (between C-3 and C-4), by which the fragment $-CH=C(OH)-CCl_3$ is lost. The signal for C-1 in the normal ^{13}C NMR spectrum of pinicoloform is very weak, probably due to the different relaxation properties of the trichloromethyl carbon. However, by increasing the inter-pulse delay time (from 0.5 to 2 s), or by decreasing the pulse angle (to 10°), the signal for C-1 became relatively stronger. The ^{13}C NMR shift for the trichloromethyl carbon of pinicoloform (95.7 ppm) can be compared with that of hexachloropropene in the same solvent (92.6 ppm). The dysidenins (isolated from the sponge *Dysidea herbacea*) contain a trichloromethyl group attached to a saturated carbon, and for these chemical shifts close to 105 ppm have been reported (R. Kazlauskas *et al.,* 1977). For compound KS-504e (isolated from the fungus *Mollisia ventosa*) containing a trichloromethyl group attached to the β-carbon of an α,β-unsaturated aldehyde the chemical shift for the chlorinated carbon is less than 90 ppm (S. Nakanishi *et al.,* 1989). The stereochemistry of the carbon–carbon double bonds was suggested by the magnitude of the $^1H-^1H$ coupling constants, and confirmed by the NOESY correlations observed between 3-H and 5-H, between 5-H and 6-H, and between 7-H and 9-H_2. In addition to pinicoloform, small amounts of what appears to be the 5*E*-isomer was obtained from the extracts, although it was not possible to isolate this compound. Its ^1H NMR data are very similar to those of pinicoloform, except for J_{5-6} which is 15.2 Hz.

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