MYCENON, A NEW METABOLITE FROM A *MYCENA* SPECIES TA 87202 (BASIDIOMYCETES) AS AN INHIBITOR OF ISOCITRATE LYASE

R. HAUTZEL, H. ANKE and W. S. SHELDRICK†

University of Kaiserslautern, Department of Biotechnology,
D-6750 Kaiserslautern, FRG
†University of Bochum, Department of Analytical Chemistry,
D-4630 Bochum, FRG

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Mycenon ($C_{11}H_5Cl_3O_3$), a new inhibitor of isocitrate lyase (EC 4.1.3.1) was isolated from the culture broth of a basidiomycete, *Mycena* sp. Mycenon is a novel chlorinated benzoquinone derivative which is also active against bacteria and fungi. Malate synthase (EC 4.1.3.2) the second key enzyme of the glyoxylate cycle was not affected by mycenon. Isocitrate lyase preparations from plants, bacteria and fungi were sensitive. The following *Ki*-values for mycenon have been determined: *Ricinus communis*, 5.2 μM; *Acinetobacter calcoaceticus*, 11 μM; *Neurospora crassa*, 7.4 μM. The structure of mycenon has been determined by a single crystal X-ray analysis.

In the course of our screening for new inhibitors of the key enzymes of the glyoxylate cycle a new fungal metabolite, mycenon was isolated from the culture broth of *Mycena* sp. TA 87202. Mycenon is an inhibitor of isocitrate lyase (EC 4.1.3.1); malate synthase (EC 4.1.3.2) was not affected by 50 μg/ml. The producing strain TA 87202 was isolated by T. ANKE from a fruiting body collected on Vancouver Island, Canada. The strain is deposited in the collection of the Lehrbereich Biotechnologie, Universität Kaiserslautern, FRG. In this paper the fermentation, isolation, physico-chemical properties, structure determination and biological properties of mycenon are described.

Materials and Methods

General

The mp was determined with a Büchi 510 melting point apparatus and is uncorrected. The UV-spectrum was recorded on a Hitachi spectrometer model 100-60, the IR-spectrum on a Beckman AccuLab 8 spectrometer. TLC was performed on aluminium foils, coated with Silica gel 60 F_{254} Merck, Darmstadt, No. 5554.

Organisms and Preparation of Cell Free Extracts

a) *Acinetobacter calcoaceticus* ((Beijerinck) Baumann, Doudoroff et Stanier) was cultivated in Erlenmeyer flasks (5 liters) containing 2 liters of acetate medium I ($MgSO_4 \cdot 7H_2O$ 0.2 g/liter; K_2HPO_4 10 g/liter; $(NH_4)_2HPO_4$ 1.8 g/liter; biotin 5 mg/liter; thiamine 5 mg/liter; folic acid 5 mg/liter; sodium acetate 50 mM, pH 7.4). The incubation was carried out at 27°C on a rotary shaker (120 rpm). At the end of the logarithmic phase of growth the cells were harvested by centrifugation (4°C, 15 minutes, 10,000 × *g*), washed twice with potassium phosphate buffer (100 mM, pH 7.0) and suspended in 8 ml of the same buffer. The cell free extract was prepared by passage through the X-press (AB Biox, Nacka, Sweden), the supernatant (4°C, 60 minutes, 30,000 × *g*) contained the key enzymes of the glyoxylate cycle.

b) *Neurospora crassa* (Davies) *arg*-5, *ota*, *aga* was cultivated in a 500-ml Erlenmeyer flask containing 200 ml of acetate medium II (VOGEL's medium[1]) with 50 mM sodium acetate; 100 mg/liter putrescine and 50 mg/liter arginine). The incubation was carried out at 27°C on a rotary shaker (120 rpm). The mycelia were harvested in the trophophase, washed twice with potassium phosphate buffer (100 mM, pH 7.0) and disrupted by passage through the X-press. After centrifugation (4°C, 60 minutes, 30,000 × *g*) isocitrate

lyase and malate synthase were present in the supernatant.

c) Cell free extracts from seeds of *Ricinus communis* (Var. Zansibarensis) were prepared as described by COOPER and BEEVERS[2].

Enzyme Assays

The isocitrate lyase activity was measured according to the method of DIXON and KORNBERG[3]. The reaction mixture contained in a final volume of 1 ml: 1.25 mM *threo*-Ds (+) isocitrate; 3.75 mM $MgCl_2$; 4.1 mM phenylhydrazine hydrochloride; sodium phosphate buffer (60 mM, pH 6.8) and various amounts of the cell free extracts (*R. communis* 80~100 μg protein; *A. calcoaceticus* 25 μg protein; *N. crassa* 50 μg protein). Protein was determined by the method of BRADFORD[4]. The assay was initiated by addition of the enzyme and the formation of glyoxylate phenylhydrazone was followed spectrophotometrically at 334 nm. The malate synthase activity was tested as described by HOCK and BEEVERS[5].

Test for Mutagenicity

Mutagenicity was tested according to the method of AMES and co-workers[1]. Mutants of *Salmonella typhimurium* strain TA 98 and strain TA 100 were used for the spot test, with and without rat liver microsomes.

Test for Cytotoxicity and Antimicrobial Activity

The antimicrobial spectrum and the cytotoxicity against cells of the ascitic form of Ehrlich carcinoma (H. PROBST, University of Tübingen), HeLa-S3 (ATCC CCL 2.2) and L1210 cells (ATCC CCL 219) were measured as described previously[6~8].

Fermentation of *Mycena* sp.

For maintenance and submerged cultivation *Mycena* sp. TA 87202 was grown in YMG-medium (yeast extract 0.4%, glucose 0.4%, malt extract 1%, pH 5.5). Fermentation was carried out in a 150-liter tank (Deutsche Metrohm, Stuttgart) containing 100 liters of YMG-medium with stirring (100 rpm) and aeration (10 liters air/minute). The production of the active compound was calculated by the $E^{1\%}_{1\,cm}$ value at 273 nm.

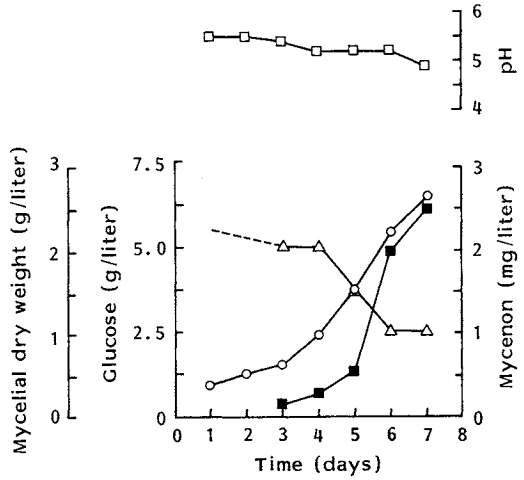
Results and Discussion

Production of Mycenon

During fermentation in the YMG-medium (Fig. 1) the production of mycenon by *Mycena* sp. TA 87202 started 3 days after inoculation. At this time, in the trophophase, there was no limitation of free glucose in the medium. The highest concentration of mycenon was reached after 7 days, a longer fermentation time resulted in a decrease of the inhibitor.

Fig. 1. Fermentation of *Mycena* sp. (TA 87202).

□ pH, ○ dry weight, △ glucose, ■ concentration of mycenon.



Isolation of Mycenon

The culture broth (90 liters) was filtered to remove the mycelium. The active compound from the culture filtrate was adsorbed on Diaion HP-21 (Mitsubishi Chem. Ind.) and the inactive filtrate discarded. The resin was eluted with methanol (5 liters). After evaporation of the solvent, the aqueous solution (3 liters) was extracted twice with ethyl acetate (3 liters). Further purification was achieved by ex-

traction into cyclohexane (3×2 liters) from the acidified (pH 2.5) ethyl acetate fraction. The extract (200 mg) was subjected to silica gel column chromatography (Merck 60, 12×2.5 cm) and the mycenon containing fraction (94 mg) was eluted with 20% methanol in CH_2Cl_2. Pure mycenon (12 mg) was obtained in form of red crystals by crystallization from hexane-acetone.

Physico-chemical Properties of Mycenon

Mycenon ($C_{11}H_5Cl_3O_3$) is soluble in MeOH, EtOH and acetone, it is insoluble in water. Mycenon exist as red crystals, mp 118 °C; Rf 0.52 (CH_2Cl_2-MeOH, 8:2), 0.8 (BuOH-EtOH-H_2O, 4:4:1), 0.86 (toluene-ethyl formate-formic acid, 10:5:3), 0.71 (BuOAc-BuOH-EtOH-H_2O, 4:4:1:1); UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 228 (430), 273 (442), 305~315 (sh, 270), 358 (264), 467 (41); IR (KBr) cm^{-1} 3620, 3560, 2210, 1680, 1570, 1550, 1365, 1310, 1265, 1220, 1135, 1025, 980, 885, 815, 780, 755.

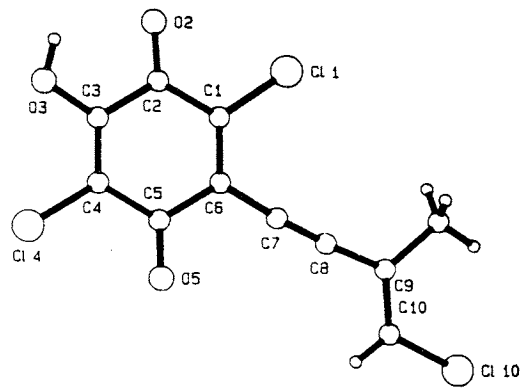
X-Ray Structural Analysis

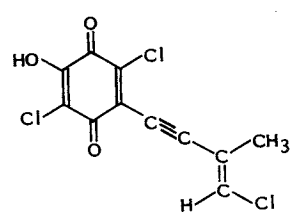
Mycenon, $C_{11}H_5Cl_3O_3$, crystallizes in the orthorhombic space group $P2_12_12_1$ with $a=9.010$ (2), $b=23.845$ (3), $c=5.481$ (2) Å, $z=4$, $D_{calc}=1.70\,g\cdot cm^{-3}$. Unit cell constants were obtained from a least-squares fit to the settings of 25 reflections recorded on an Enraf-Nonius CAD4 diffractometer. Intensities were collected on the diffractometer in the w-mode with graphite-monochromated radiation (Cu$K\alpha$, $2\theta_{max}=140°$, data collection for $+h$, $+k$, $+l$). Empirical absorption corrections [μ(Cu$K\alpha$)=$71.7\,cm^{-1}$] were applied to the reflections intensities. After data reduction 1,231 independent reflections (from 1,332 measured) with $F_o^2 \gg 2\sigma(F_o^2)$ were used for the structure determination. After solution by direct methods, the structure was refined by full-matrix least-squares. Anisotropic temperature factors were introduced for all nonhydrogen atoms. The positions of the five protons were obtained from a difference Fourier synthesis and included in the final refinement cycles. The terminal reliability indices were $R=0.047$ and $R_w=[\Sigma_w(F_o-F_c)^2/\Sigma_w F_o^2]^{1/2}=0.050$ with weights given by the expression $w=(\sigma^2(F_o)+p^2F_o^2)^{-1}$, p=0.007. Calculations were performed with SHELX-76 and with local programs.

Molecular Structure

The molecular structure of mycenon is depicted in Fig. 2. The six membered ring is effectively planar with a maximum deviation of 0.033 Å at C6. Significant deviations from linearity are observed for the angles at the *sp*-hybridized carbon atoms C7 and C8: C6-C7-C8 is 173.2 (7), C7-C8-C9 175.6 (7)°. The C9-C10 double bond displays the *E*-configuration.

Fig. 2. Molecular structure of mycenon.





Biological Properties

Without preincubation of isocitrate lyase from *R. communis* with mycenon, the Lineweaver-Burk plot (Fig. 3) for various inhibitor concentrations showed the kinetic of a competitive inhibition. The inhibition of isocitrate lyase from *A. calcoaceticus* and *N. crassa* by mycenon is also of a competitive type. The *Km*-and the *Ki*-values are listed in Table

1. When the enzyme was preincubated with mycenon for 3 minutes the inhibition was more pronounced and no longer of a competitive type. This prompted us to investigate the influence of SH-reagents. In the presence of cysteine (0.5 mM) mycenon was completely inactivated.

Mycenon showed antimicrobial activity against a variety of bacteria and fungi, especially against *Bacillus brevis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Nematospora coryli* and *Saccharomyces cerevisiae* isl. The MIC values in the serial dilution assay are listed in Table 2.

The cytotoxic activity of mycenon was tested with the ascitic form of Ehrlich carcinoma (ECA), HeLa-S3 and L1210 cells. At 25 μg/ml of mycenon lysis of ECA cells was observed. Lysis of L1210 cells was obtained at 10 μg/ml of mycenon. The growth of HeLa-S3 cells was inhibited by 25 μg mycenon/ml. In the test for mutagenicity according to AMES *et al.*[1] no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed with 100 μg mycenon/disc (spot test with and without addition of rat liver microsomes). With bovine erythrocytes no hemolytic activity was detected at 50 μg/ml.

Conclusions

Mycenon, a novel compound from the basidiomycete *Mycena* sp. TA 87202 is the first quinone metabolite isolated from cultures of the genus *Mycena*. Known biologically active compounds from species of this genus are strobilurin A[9], strobilurin B[9], oudemansin A[10], drosophilin A methyl ether[11] and amino acid derivatives[12]. Other natural products with structural similarities to mycenon are culpin, frustulosin, frustulosinol and siccayne. These compounds are hydroquinone derivatives with an

Fig. 3. Lineweaver-Burk plot of isocitrate lyase from *Ricinus communis* inhibited by mycenon as a function of substrate and inhibitor concentration.

○ Control, ▽ 2.5 μg/ml, □ 5 μg/ml, △ 10 μg/ml.

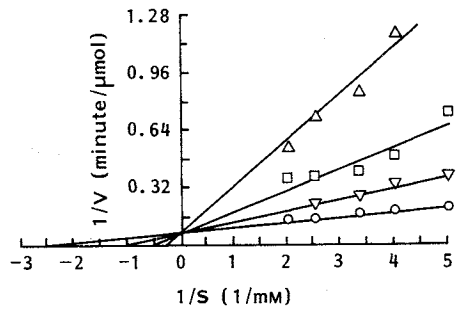


Table 1. Inhibition of isocitrate lyase from various sources by mycenon (the reaction was started with the enzyme).

Organism	*Km* (mM)	*Ki* (μM)
	Isocitrate	Mycenon
Ricinus communis	0.408	5.2
Acinetobacter calcoaceticus	0.295	11
Neurospora crassa	1.11	7.4

Table 2. Antibacterial and antifungal activity of mycenon in the serial dilution assay.

Organism	MIC (μg/ml)	Organism	MIC (μg/ml)
Acinetobacter calcoaceticus	20	*Mucor miehei*	≫50
Bacillus brevis	5	*Nematospora coryli*	5
B. subtilis	20	*Paecilomyces variotii*	≫50
Escherichia coli K-12	≫50	*Penicillium notatum*	50
Pseudomonas fluorescens	≫50	*Rhodotorula glutinis*	50
Micrococcus luteus	10	*Saccharomyces cerevisiae* isl[a]	10
Staphylococcus aureus	5	*S. cerevisiae* 288c	50
Candida albicans	≫50		

[a] Strain obtained from Prof. F. LACROUTE, Straßburg.

Inoculum 10^6 cells/ml.

Fungi were cultivated in YMG-medium at 27°C. Bacteria were cultivated in nutrient broth (Difco) at 37°C (*P. fluorescens* was cultivated at 27°C).

isopentenyne side chain but without chlorine substitutions. They exhibit similar antimicrobial activities as mycenon. Culpin was isolated from cultures of the ascomycete *Preussia* sp. (ATCC 20923)[13]. Frustulosin and frustulosinol were obtained from cultures of the basidiomycete *Stereum frustulosum*[14]. Siccayne was isolated from cultures of the marine fungus *Halocyphina villosa*[15] and from *Helminthosporium siccans*[16].

Acknowledgments

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