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Cyrmenins, New β-Methoxyacrylate Inhibitors of the Electron Transport

Production, Isolation, Physico-chemical and Biological Properties†

FLORENZ SASSE*, THOMAS LEIBOLD††, BRIGITTE KUNZE,
GERHARD HÖFLE†† and HANS REICHENBACH

GBF, German Research Centre for Biotechnology, Department of Natural Product Biology
and †† Department of Natural Product Chemistry,
Mascheroder Weg 1, D-38124 Braunschweig, Germany

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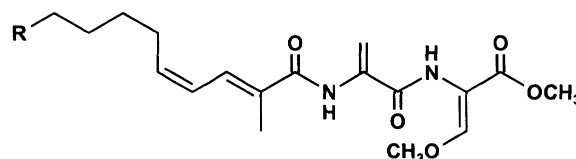
New antibiotic compounds, named cyrmenins, were isolated from the culture broth of strains of the myxobacteria *Cystobacter armeniaca* and *Archangium gephyra*. The compounds belong to the group of β-methoxyacrylate (MOA) inhibitors and are the first naturally occuring nitrogen-linked MOAs. The cyrmenins show nearly the same antifungal activity as strobilurin A, but are less toxic in a growth inhibition assay with L929 mouse cells. Cyrmenins inhibit NADH oxidation by submitochondrial particles from beef heart. Investigations by difference spectroscopy showed that cyrmenin B_1 blocks the electron transport within the cytochrome bc_1-segment (complex III) of the respiratory chain.

During our screening for antibiotics from myxobacteria, we found antifungal activities in the culture broth of a strain of *Cystobacter armeniaca* Cb a24. The activities were due to new compounds, which we named cyrmenins. Later on we also found these compounds in a strain of *Archangium gephyra*, Ar 9944. In this paper we describe the production, isolation, and the physico-chemical and biological properties of the cyrmenins. Fig. 1 shows the chemical structures of the isolated compounds, the elucidation of which will be published elsewhere[1]. The cyrmenins contain a β-methoxyacrylate (MOA) system and are related to strobilurin[2]. Strobilurin served as lead compound to develop agricultural fungicides such as kresoxim-methyl and azoxystrobin[3].

Microorganisms and Culture Conditions

The producing organisms were isolated at the GBF as follows: *Cystobacter armeniaca* strain Cb a24 in 1999 from a sample of saw dust and other organic material with some soil from Mallorca, Spain, *Archangium gephyra* strain Ar 9944 in 1996 from a sample of rotting wood from a rain forest near Sao Paulo, Brazil. Both strains were normally grown on a modified VY/2 agar[4] (bakers' yeast 0.5%, $CaCl_2 \cdot 2H_2O$ 0.1%, HEPES 1%, glucose 0.2%, vitamin B_{12} 0.1 mg/liter, agar 1.8%, pH 7.2) and on M7 liquid medium[5]. Batch cultures of 100 or 500 ml in 250-ml or

Fig. 1. The chemical structures of the cyrmenins.



Cyrmenin A $R = -CH_2-CH(CH_3)_2$
Cyrmenin B_1 $R = -CH_2-CH_2-CH_3$
Cyrmenin B_2 $R = -CH(CH_3)_2$

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* Corresponding author: fsa@gbf.de

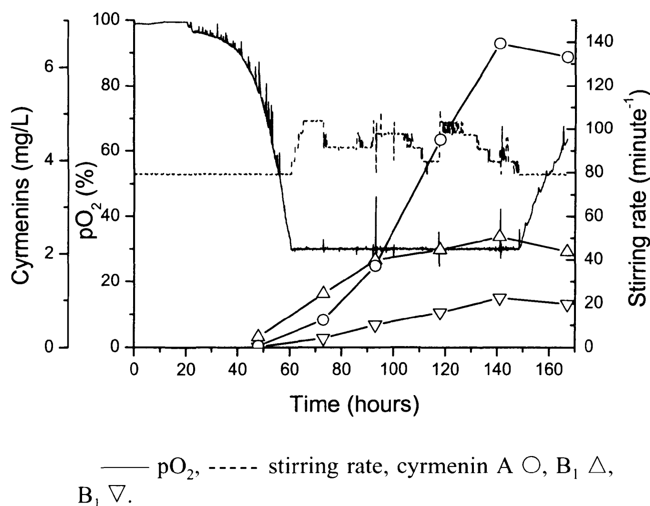
1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a gyratory shaker at 160 rpm for 3~5 days.

Production

Both strains were used for production on a larger scale. They were grown on M7 liquid medium without HEPES. For example, three 1-liter cultures in 2-liter Erlenmeyer flasks grown for three days on a gyratory shaker were inoculated into a bioreactor with 300 liters of M7 medium without HEPES to which 1% (v/v) of the adsorber resin, Amberlite XAD-16 (Rohm & Haas, Frankfurt) was added. The fermentor was kept at 30°C and agitated at 150 rpm. In order to reduce foam formation, 10 g behenyl alcohol was added before heat sterilization, and a mixture of dodecanol, molten at 50°C, and 20% methanol was pumped into the fermentor at a rate of 14 ml/day during the whole fermentation process[6]. The pump rate was increased when needed. The aeration rate was 0.1 volume air per culture volume and minute. The pH was kept between 7.2 and 7.6 using 5 M KOH and 20% acetic acid. At harvest, the adsorber resin was separated from the culture broth by passing the culture through a process filter of 210 μm mesh size.

Fig. 2 shows a fermentation of Ar 9944 in a 15-liter draft tube bioreactor (Giovanola Frères SA, Monthey, Switzerland). As the strain grew in lumps, the oxygen consumption was monitored as a growth parameter. The initial stirring rate was 80 rpm and was raised when the pO_2 level dropped under 30%. In the beginning of the fermentation cyrmenin B_1 was the main product, but after 4 days the production of cyrmenin A surpassed that of B_1 and became the main product. In the given example, the final yields were 6 mg/liter cyrmenin A, 2 mg/liter cyrmenin B_1, and 0.9 mg/liter cyrmenin B_2.

Fig. 2. Fermentation of *Archangium gephyra* Ar 9944 in a 15-liter bioreactor with a flat-blade turbine stirrer (10 liters culture volume, aeration rate 1 liter/minute, temperature 30°C).



—— pO_2, ----- stirring rate, cyrmenin A ○, B_1 △, B_1 ▽.

Isolation and Quantitative Determination

The cyrmenins were eluted from the resin with methanol. The organic solvent was evaporated, and the remaining aqueous phase extracted with ethyl acetate. The organic phase was separated and evaporated again. The resulting residue was dissolved in methanol and washed with heptane. Concentration of the polar phase yielded a raw product, which was separated by silica gel chromatography (LiChroprep Si 100, sovents: CH_2Cl_2 and CH_2Cl_2 - diethyl ether, 95 : 5). The HPLC fractions containing the cyrmenins were combined and evaporated to dryness.

Sephadex LH20 chromatography (solvent: CH_2Cl_2 - MeOH, 4 : 1) and final RP-MPLC separation (HD Sil; solvent: acetonitril - H_2O, 6 : 4, +0.05 M ammonium acetate; detection at 277 nm) yielded the cyrmenins as colorless oils after concentration of the main fractions. All partition and chromatography steps were done under controlled pH (pH 6.5) in order to prevent decomposition of cyrmenins under basic conditions.

The cyrmenins were quantitatively determined by HPLC (column 125×2 mm, nucleosil 100-5 C18 ec, Macherey-Nagel, Oensingen, Switzerland; solvent: MeOH - H_2O, 63.5 : 36.5, +5 mM ammonium acetate; flow rate: 0.4 ml/minute; detection at 270 nm). The retention times were as follows: cyrmenin A-6.3 minutes, cyrmenin B_1-4.7 minutes, and cyrmenin B_2-4.3 minutes.

Physico-chemical Properties

The cyrmenins were soluble in methanol, acetone and ethyl acetate. TLC on silica gel 60 F-254 (Merck) with CH_2Cl_2 - diethyl ether (85 : 15) as solvent gave an Rf value of 0.41 for all cyrmenins. For detection the plates were sprayed with vanillin/sulphuric acid reagent and heating to 120°C, which resulted in light gray spots. High resolution EI mass spectroscopy with a Finnigan MAT 95 gave the following molecular masses and calculated elemental composition: 424.2821 ($[M+NH^4]^+$, calculated 424.2811) for cyrmenin A ($C_{22}H_{34}N_2O_5$); 410.2701 ($[M+NH^4]^+$, calculated 410.2655) for cyrmenin B_1 ($C_{21}H_{32}N_2O_5$); and 410.2678 ($[M+NH^4]^+$, calculated 410.2655) for cyrmenin B_2 ($C_{21}H_{32}N_2O_5$). The UV spectrum of cyrmenin B_1 in

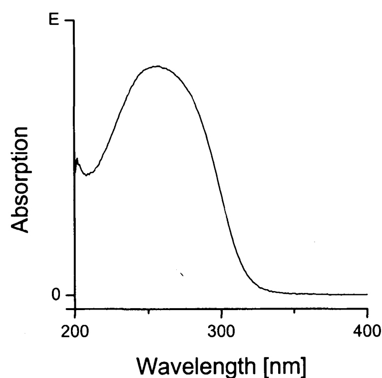


Fig. 3. The UV spectrum of cyrmenin B_1 in methanol.

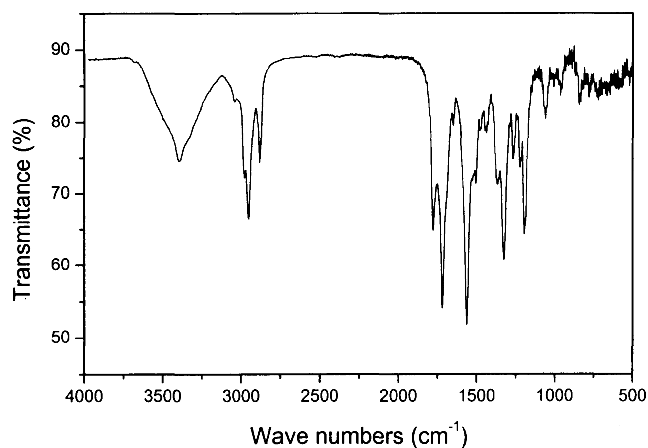


Fig. 4. IR spectrum of cyrmenin B_1 in KBr.

Table 1. Antimicrobial activity of cyrmenins in comparison with strobilurin A.

Test organisms[a]	Diameter of inhibition zone[b] (mm)			
	Cyrmenins			Strobilurin
	A	B_1	B_2	A
Gram-negative bacteria				
Escherichia coli DSM498	0	0	0	-
Pseudomonas aeruginosa DSM 1117	0	0	0	-
Gram-positive bacteria				
Bacillus subtilis DSM 10	0	0	0	-
Staphylococcus aureus GBF 16	0	0	0	-
Yeasts				
Candida albicans GBF 129	18	19	19	37
Hansenula anomala DSM 70263	15	16	14	21[7]
Metschnikowia pulcherrima DSM 70321	14	14	13	24[7]
Saccharomyces cerevisiae GBF 36	25	28	28	41
Filamentous fungi				
Aspergillus niger DSM 823	17	18	19	23
Botrytis cinerea DSM 877	23	21	23	24[7]
Pythium debaryanum DSM 62946	29	23	23	28[7]
Trichoderma koningii DSM 3121	30	18	17	15
Ustilago zeae DSM 3121	22	16	17	18

[a] The organisms were grown on agar (1.5 %) media (bacteria: peptone 1%, meat extract 0.1%, yeast extract 0.1%, pH 7.0; fungi: glycerol 2 %, casein peptone 1%, yeast extract 1%, sodium phosphate 50 mM, pH 6.3). The strains were from the collection at the GBF or from the German Collection of Microorganism and Cell Cultures (DSMZ).

[b] Determined by agar diffusion assay using paper discs of 6 mm diameter with 2 µg of the compound in 20 µl methanol.

Table 2. Cytotoxicity of cyrmenins and their inhibition of NADH oxidation in beef heart submitochondrial particles in comparison with strobilurin A.

Compound	Cytotoxicity[a] IC$_{50}$ (ng/ml)	NADH oxidation[b] IC$_{50}$ (ng/ml)
Cyrmenin A	2000	27
Cyrmenin B$_1$	1200	41
Cyrmenin B$_2$	2000	159
Strobilurin A	180[7]	80

[a] Cytotoxicity was measured by a growth inhibition assay with the mouse fibroblast cell line L929 (ATCC CCL1).

[b] NADH oxidation was measured with beef heart SMP suspended in air-saturated buffer at a concentration of 90 µg protein/ml. NADH was added to a final concentration of 0.16 mmol/liter.

ethanol is shown in Fig. 3. λ_{max} (lg ε)=258 nm (4.215). The IR spectrum of cyrmenin B$_1$ in KBr (Fig. 4) was measured with a Nicolet 20 DXD FT-IR spectrometer.

Biological Activity

Because of their structural relationship, the toxicity and antimicrobial activity of the cyrmenins were compared to those of strobilurin A. The cyrmenins had no effect on bacteria, but high antifungal activity (Table 1) that were comparable to the activity of strobilurin A. The toxicity of the compounds was tested by a growth inhibition assay with L929 mouse fibroblast cells as described[7]. Shortly, cells were seeded into each well of a 96-well microtiter plate containing serially diluted concentrations of the test compounds, and the metabolic activity of the grown cells was measured by an MTT assay after 5 days. The IC$_{50}$ values derived from the resulting inhibition curves showed that the cyrmenins were clearly less toxic than strobilurin A (Table 2).

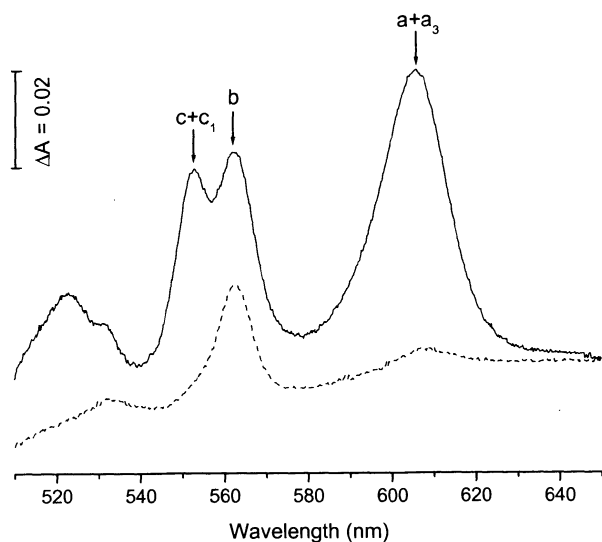
β-Methoxyacrylates are well known inhibitors of the mitochondrial respiratory energy metabolism. Therefore we tested the influence of the cyrmenins on NADH oxidation in beef heart submitochondrial particles (SMP) using a UV2 Unicam UV/VIS spectrophotometer. The isolation and characterization of SMP as well as the execution of the experiment has been described previously[8]. As expected, the cyrmenins inhibited the NADH oxidation by SMP. The concentrations needed for a 50% inhibition are shown in Table 2. The most active compound was cyrmenin A with an IC$_{50}$ of 27 ng/ml (66 nmol/liter). The IC$_{50}$ for strobilurin A in the same test system was 80 ng/ml, or 290 nmol/liter, respectively.

The site of inhibition within the electron transport chain was investigated by difference spectroscopy using a DW 2000 UV/VIS SLM double beam spectrophotometer (SLM Instruments, Inc., Il., U.S.A.). Upon reduction with physiological substrates, *e.g.* NADH, fully oxidized cytochromes in front of the block become reduced, while those behind it remain oxidized. As can be seen in Fig. 5, the difference spectrum of NADH-reduced minus air-oxidized SMP without inhibitor showed the characteristic absorption maxima of the different cytochromes. In the presence of cyrmenin B$_1$, only cytochrome *b* of complex III (α band at 563 nm) became reduced, whereas the cytochromes aa_3 (α band at 608 nm) and cc_1 (α band at 553 nm) remained in the oxidized state. This indicated that cyrmenin B$_1$ inhibited the electron flow within the cytochrome bc_1 segment of the respiratory chain.

Discussion

Inhibition of the respiratory chain is a relatively rare mechanism of action among bacterial compounds, but it is much favored by myxobacteria. Thus far we isolated approximately 20 new substances from myxobacteria which inhibit the electron transport in the respiratory chain.

Fig. 5. The effect of cyrmenin B_1 on the reduction of cytochromes by NADH.



Beef heart submitochondrial particles (SMP) were suspended in air-saturated buffer at a concentration of 3.2 mg protein/ml. —— Difference spectrum (reduced minus oxidized) of SMP reduced with NADH (final concentration 2 mM) without inhibitor. ----- Difference spectrum of SMP reduced with NADH (final concentration 2 mM) in the presence of 20 μg/ml cyrmenin B_1.

The cyrmenins are a new group that contain a β-methoxyacrylate pharmacophore. There are two types of β-methoxyacrylates known, such compounds that are substituted in the α-position, like oudemansin[9] and strobulurin[2], and such that are β-substituted like the myxobacterial compounds myxothiazol[10] and melithiazol[7]. The cyrmenins are the first α-substituted β-methoxyacrylates found in myxobacteria. All these MOA inhibitors have nearly the same antifungal potential, but differ greatly by their toxicity to mammalian cells. The cyrmenins are the least toxic group: Cyrmenin A and myxothiazol A, which shows the highest toxicity, differ by a factor of 4000. Though the chemical structures resemble strobilurin, the cyrmenins are an independent invention of nature. While strobilurin has a polyketide structure, the cyrmenins are apparently non-ribosomal peptides and, from a biological point of view, are analogous compounds. Cyrmenins and strobilurin are an example of a convergent evolution on a molecular level.

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