CROCACIN, A NEW ELECTRON TRANSPORT INHIBITOR FROM *Chondromyces crocatus* (MYXOBACTERIA)

PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES†

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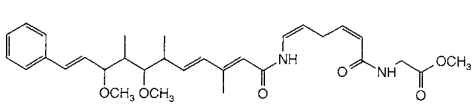
Crocacin was isolated from the biomass of the myxobacterium *Chondromyces crocatus*, strain Cm c3. It inhibited the growth of a few Gram-positive bacteria and a wide spectrum of yeasts and molds. In beef heart submitochondrial particles, crocacin blocked the electron transport within the bc_1-segment (complex III) and caused a red shift in the reduced spectrum of cytochrome *b* with a maximum at 569 nm.

In recent years we succeeded in handling some of the more fastidious and unusual myxobacteria of the genus *Chondromyces*. When we screened crude extracts of *Chondromyces* cultures for new antibiotics, we found that *Chondromyces crocatus* strain Cm c3 produced a potent activity against a wide spectrum of yeasts and fungi. The activity turned out to be due to a new compound named crocacin. In this article we describe the production, isolation, and some of the physico-chemical and biological properties of crocacin. Fig. 1 shows the chemical structure of crocacin, the elucidation of which will be published elsewhere[1].

Microorganism and Culture Conditions

The producing organism was *C. crocatus* strain Cm c3, isolated in 1985 at the GBF from a soil sample collected on Madeira. The organism was initially grown in a standard peptone liquid medium (MD1 l.m.: peptone from casein, tryptically digested, from Merck, Darmstadt 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.0; supplemented with 1 ml/liter of each of a standard vitamin and a trace element solution). Batch cultures of 100 ml or of 400 ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a rotary shaker at 160 rpm for 3~5 days. Cm c3 grew in small lumps. In order to obtain smooth growth and reasonable cell densities, the strain had to be adapted to growth in liquid media by a long series of transfers in shaken cultures. After that, the strain also could be cultivated in media containing technical substrates, *e.g.*, Probion (single cell protein prepared from *Methylomonas clarae*; Hoechst AG, Frankfurt), soy flour, peanut meal or skim milk powder.

Fig. 1. The structure of crocacin[1].



It should be mentioned that during the cultivation of strain Cm c3 in liquid media a second bacterium was observed in the cultures, which

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appeared to stimulate the growth of *C. crocatus*. The vegetative cells of this bacterium are small rods, and first investigations into its identification suggest that it belongs to the genus Flavobacterium[2]. By plating mixed cultures on nutrient agar, the bacterium could be separated from strain Cm c3. When tested for crocacin production, no activity was detected either in the cell mass or in the culture supernatant, so that the producing organism appeared to be *C. crocatus*, strain Cm c3.

Production

In shake cultures with 100 ml of MD1 l.m. in 250-ml Erlenmeyer flasks, the average yield of crocacin was below 1 mg/liter. Among the technical substrates tested for crocacin production, media based on 0.9% of either soy flour, peanut meal or Probion gave the best results (Table 1). Crocacin production on a larger scale was performed in media with technical substrates 0.4%, because in fermentations with high concentrations of technical substrates, strain Cm c3 sometimes lysed after inoculation. To give an example, 5 liters of culture grown for 3~4 days in Pol 1 liquid medium (Probion 0.4%, soluble starch 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, vitamin B_{12} 0.25 mg/liter, and 1 ml/liter of the no-fold concentrated trace element solution SL 6[3], pH 7.0) on a rotary shaker at 160 rpm was inoculated into 65 liters of the same medium in a type b 50 bioreactor (Giovanola Frères, Monthey, Switzerland). Because of foam problems, 0.05% silicone antifoam agent (Tegosipon, Goldschmidt AG, Essen) had to be added. The bioreactor was kept at 30°C and agitated at 200 rpm with a turbine plate stirrer. The aeration rate was 200 liters of air per hour. The pH, which initially drifted slightly into the acid range, increased till the end of the fermentation up to 7.4. The pO_2 in the culture was recorded continously with a polarographic oxygen electrode. At the beginning of the fermentation, oxygen was at about 90% saturation and fell till the end of the fermentation after 90 hours to about 65%.

Isolation

At the end of the fermentation, the cells were separated from the culture broth by centrifugation. The cell mass containing the antibiotic was extracted with acetone three times. The combined extracts were concentrated and the remaining oil-water mixture was extracted with dichloromethane. After

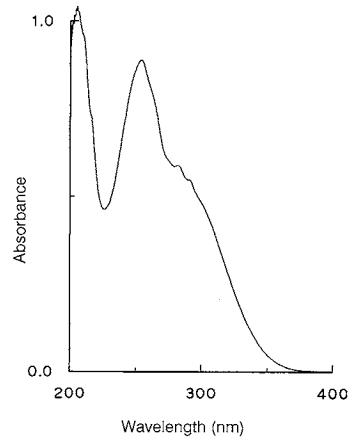
Table 1. Effect of various technical substrates on the production of crocacin.

Substrate[a]	Concentration (%)	Antibiotic titer[b] (mg/liter)
Probion	0.4	4.5
Probion	0.9	4.1
Skim milk powder	0.4	0.3
Skim milk powder	0.9	0.4
Soy flour	0.4	3.4
Soy flour	0.9	5.7
Peanut meal	0.4	3.0
Peanut meal	0.9	5.1
Cornsteep powder	0.4	0.4

[a] The basal medium was: soluble starch 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%; $CaCl_2 \cdot 2H_2O$ 0.05%; HEPES buffer 50 mM (pH 7.2); supplemented with standard vitamin and trace element solutions, 1 ml/liter each. Harvest was at the end of the growth phase after about 4 days.

[b] The concentration of crocacin was determined in acetone extracts of the cell mass by HPLC analysis (column Nucleosil 120-5-C_{18}; solvent: methanol-water 80:20; flow rate 0.3 ml/minute; detection 200~450 nm).

Fig. 2. UV spectrum of crocacin in methanol.



evaporation of the organic solvent, the residue was partitioned between methanol and heptane. Reversed phase chromatography and Si-HPLC of the methanol soluble material, resulted in the isolation of 57 mg of crocacin from a 60 liters fermentation[1].

Physico-chemical Properties

Crocacin is soluble in methanol, acetone, chloroform and ethyl acetate, sparingly soluble in ether, and almost insoluble in hexane. After TLC on silica gel 60 F_{254} (Merck), the Rf value was 0.34 with

Fig. 3. IR spectrum of crocacin in KBr.

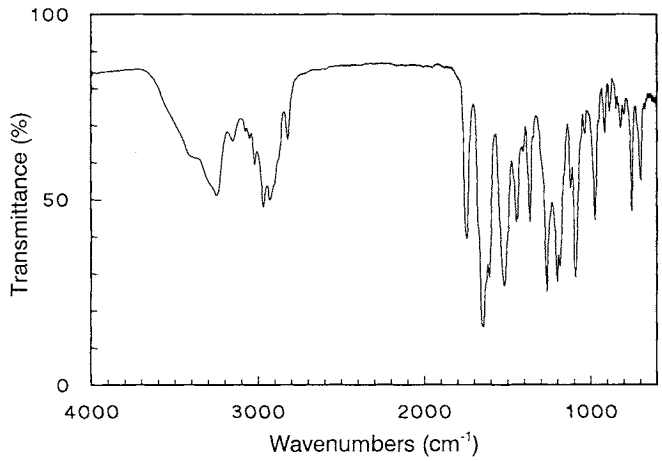


Table 2. Crocacin: biological activity.

Test organism[a]	Diameter of inhibition zone[b] (mm)	Test organism[a]	Diameter of inhibition zone[b] (mm)
Gram-negative bacteria		*Saccharomyces cerevisiae*[e] GBF 36	29
Escherichia coli DSM[c] 423	0	*S. cerevisiae*[f] GBF 36	0
Salmonella typhimurium DSM 5091	0	*Schizosaccharomyces pombe*[g] Tü 501	(24)
Pseudomonas aeruginosa DSM 1117	0	*Torulopsis glabrata* DSM 70398	23
Gram-positive bacteria		Filamentous fungi	
Bacillus subtilis DSM 10	17	*Botrytis cinerea* DSM 877	20
Micrococcus luteus GBF[d] 26	0	*Gibberella fujikuroi* DSM 893	24
Staphylococcus aureus GBF 16	0	*Trichoderma koningii* DSM 63060	23
Brevibacterium ammoniagenes DSM 20306	0	*Mucor hiemalis* DSM 2655	9
		Pythium debaryanum DSM 62948	17
Corynebacterium fascians DSM 20131	14	*Rhizopus arrhizus* DSM 905	(13)
Yeasts		Mammalian cells	MIC [ng/ml]
Candida albicans GBF 129	16	L929, Mouse fibroblasts	0.2
Metschnikowia pulcherrima DSM 70321	22		

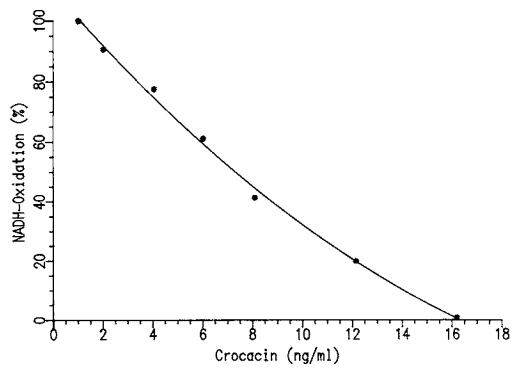
[a] Bacteria were tested on nutrient agar, fungi on malt extract peptone agar.
[b] Determined by the agar diffusion test with 20 μg crocacin per 6-mm paper disc. Figures in parentheses indicate an incomplete inhibition.
[c] Deutsche Sammlung von Microorganismen.
[d] Strain of the GBF, Dept. of Microbial Secondary Metabolites.
[e] The organism was cultivated in a medium containing 2% glycerol.
[f] The organism was cultivated in a medium containing 1% glucose.
[g] Collection university Tübingen.

toluene-methanol (85:15) as the solvent, and 0.67 with dichlormethane-methanol (9:1). Crocacin can be visualized as brown spots by spraying with vanillin/sulfuric acid reagent and heating to 120°C. Low and high-resolution EI mass spectroscopy furnished the molecular ion *m/z* (%) 538 which corresponded to the elemental composition $C_{31}H_{42}N_2O_6$. The specific optical rotation $[\alpha]_D$ of crocacin was +109.6 (*c* 1.0, MeOH). The UV spectrum of crocacin in methanol was recorded with a Hitachi U 3200 spectrophotometer (Fig. 2.). The IR spectrum of crocacin in KBr was measured with a Nicolet 20 DXB FT-IR spectrometer (Fig. 3). Detailed data will be published with the structure elucidation[1].

Biological Activity

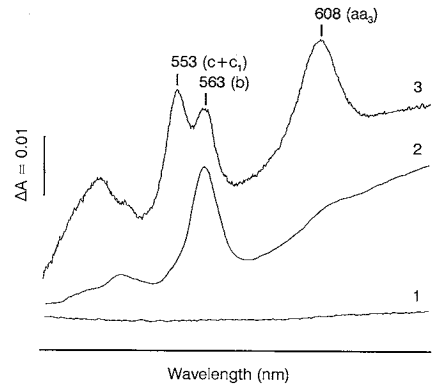
The antibiotic activity of crocacin was determined by the agar diffusion test using paper discs. As Table 2 shows, crocacin moderately inhibited the growth of a few Gram-positive bacteria and was a potent inhibitor of the growth of several yeasts and fungi. The MIC for *Ustilago maydis* was 0.625 μg/ml and for *Saccharomyces cerevisiae* when grown in glucose free medium 0.01 μg/ml. Crocacin proved to be also highly inhibitory to animal cell cultures. It was noticed that *S. cerevisiae* became less sensitive to crocacin when grown in the presence of glucose. Since *S. cerevisiae* is able to metabolize sugars by fermentation, the antagonistic effect of glucose suggested that crocacin might interfere with respirative energy metabolism. For a more detailed study on the mechanism of action of crocacin, beef heart submitochondrial particles (SMP) were chosen as the experimental system. The isolation and characterization of SMP as well as the execution of the experiments has been described previously[4]. In all assays, SMP were suspended in 75 mM sodium phosphate buffer pH 7.4, plus 1 mM each of EDTA and $MgCl_2$. Crocacin was dissolved in methanol, and the methanol concentration in all tests did not exceed 2%. The effect of crocacin on NADH oxidation in SMP was recorded with a 551S UV/VIS spectrophotometer (Perkin-Elmer, Uberlingen, FRG). Fig. 4 shows, that crocacin indeed blocked NADH oxidation in SMP. At a concentration of 5.9 nM (7.5 ng/ml), an inhibition of 50% was achieved. The site of inhibition within the electron transport chain was investigated

Fig. 4. The effect of crocacin on NADH oxidation in beef heart submitochondrial particles (SMP).



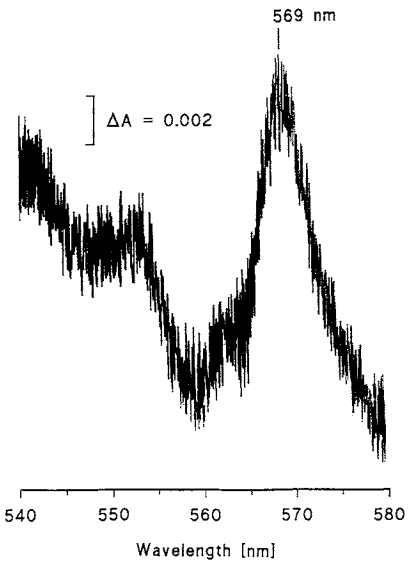
The SMP were suspended in air saturated buffer at a concentration of 70 μg protein/ml. NADH was added to a final concentration of 0.16 mM. The rate of NADH oxidation in the control without crocacin was 1.6 nmol/minute × mg protein. Each value gives the average of 2 to 3 experiments.

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



The SMP were suspended in air saturated buffer at a concentration of 3.2 mg protein/ml. Trace 1: Baseline. Trace 2: Difference spectrum (reduced minus oxidized) of SMP reduced with NADH (final concentration 2 mM) in the presence of 25 μg/ml crocacin. Trace 3: Difference spectrum of SMP reduced with NADH (final concentration 2 mM) without inhibitor.

Fig. 6. Spectral shift induced by crocacin in dithionite reduced beef heart submitochondrial particles.



A suspension of SMP (15 mg protein/ml) was reduced with dithionite and filled into sample and reference cuvettes with a 1 cm light path. After adjustment of the baseline, crocacin (final concentration 20 μg) was added to the sample cuvette, and the volume was corrected in the reference cuvette. The difference spectrum was recorded with a bandwidth of 1.5 nm.

by difference spectroscopy using a DW 2A UV/VIS double-beam spectrophotometer (American Instruments, Silver Springs, MD, USA)[4]. Upon reduction with physiological substrates such as 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 showed the characteristic absorption maxima for the different cytochromes. In the presence of crocacin, only cytochrome *b* of complex III (α band at 563 nm) became reduced, whereas the cytochromes aa_3 (α band at 608 nm) and $c+c_1$ (α band at 553 nm) remained in the oxidized state. This indicated that crocacin inhibited the electron flow within the cytochrome bc_1 segment of the respiratory chain. The binding of certain complex III inhibitors to cytochrome *b*, *e.g.*, antimycin, myxothiazol or stigmatellin causes a spectral shift in the α band of reduced cytochrome *b*[4,5]. When added to a suspension of dithionite-reduced SMP, crocacin also caused a red shift in the reduced spectrum of cytochrome *b* with a maximum at 569 nm (Fig. 6).

Discussion

Crocacin is the first antibiotic which could be isolated from a strain of the genus *Chondromyces*. After its discovery in strain Cm c3, it was detected in all other tested *C. crocatus* strains, *viz.* Cm c1, Cm c2, Cm c4, Cm c5, Cm c7. In the course of our studies on secondary metabolites, the myxobacteria turned out to be an extremely rich source of novel electron transport inhibitors. With crocacin we isolated and characterized the eleventh new compound of this type. While the myxalamids[6], aurachin A and B[7], phenoxan[8] and thiangazole[9] block electron transport specifically in complex I (NADH: ubiquinone oxidoreductase), crocacin interferes at the bc_1 segment (complex III), like myxothiazol[4], stigmatellin[5] and the aurachins C and D[7]. Like the other mentioned complex III inhibitors, crocacin induced in dithionite-reduced SMP a red shift in the spectrum of cytochrome *b*, which points to an interaction with cytochrome *b*. The maximum of that red shift signal at 569 nm might indicate a binding site of crocacin in the vicinity of cytochrome b_{566}. Further experiments will use this spectral shift to analyse the binding behavior of crocacin and to determine interferences with other inhibitors of complex III.

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