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Cruentaren, a New Antifungal Salicylate-Type Macrolide from *Byssovorax cruenta* (Myxobacteria) with Inhibitory Effect on Mitochondrial ATPase Activity

Fermentation and Biological Properties†

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Abstract The novel macrolide cruentaren A was produced at levels up to 3.2 mg/liter by cultures of the myxobacterium *Byssovorax cruenta*. The new compound strongly inhibited the growth of yeasts and filamentous fungi and showed high cytotoxicity against L929 mouse fibroblast cells. A minor co-metabolite of cruentaren A, named cruentaren B, and identified as a six-membered lactone isomer of cruentaren A, showed only marginal cytotoxicity and no antifungal activity. Cruentaren A inhibited F_0F_1 mitochondrial ATP-hydrolysis in submitochondrial particles of yeasts and beef heart.

Keywords myxobacteria, cruentaren, benzolactone class antifungal, cytotoxic, F_0F_1 ATPase

Introduction

Over the last 25 years myxobacteria have proved to be a consistent source of interesting and novel bioactive secondary metabolites [1, 2]. In continuation of our screening program for new secondary metabolites, strain By c1 (=Ha r1) of the new myxobacterial genus *Byssovorax cruenta* [3], attracted attention because of its pronounced antifungal and high cytotoxic activity. Bioassay-guided fractionation of culture extracts by analytical RP-HPLC showed that the antifungal and cytotoxic activity was due to one and the same major metabolite, which was isolated and structurally characterized as a 12-membered lactone ring with a *N*-acylallylamine side chain. The new compound, named cruentaren A, was accompanied by a small amount of the much less active cruentaren B, which was identified as six-membered lactone isomer of cruentaren A. Fig. 1 shows the structures of the cruentarens, the elucidation of which is reported elsewhere [4, 5]. Chemically, cruentarens A and B, share a common core structure with the growing class of benzolactones, including the salicylihalamides from the marine sponge *Haliclona* sp. [6] and the lobatamides isolated from the tunicate *Aplidium lobatum* [7]. Closest structural similarity exists to the V-ATPase inhibitor apicularen A, produced by several species of the myxobacterial genus *Chondromyces* [8~10]. Here we mainly report on the production, and some of the biological properties of cruentarens A and B.

Materials and Methods

Producing Organism and Culture Conditions

Byssovorax cruenta gen. sp. nov., nom. rev., strain By c1

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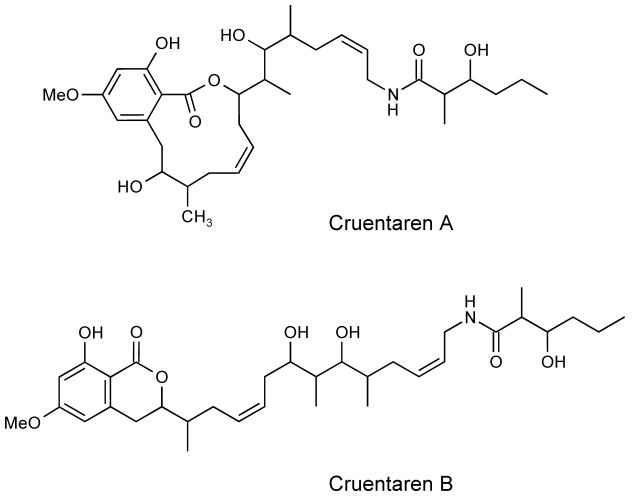

Fig. 1 The structures of cruentarens A and B.

(formerly *Byssophaga cruenta*, Ha r1) was isolated at the GBF in 1997 from a soil sample collected in a sagebrush steppe near Holbrook, Arizona, USA. The organism could be obtained by the standard methods described by Reichenbach and Dworkin [11]. It grew well on living *Escherichia coli*, lyzing the food bacterium. Preferentially, the strain was cultivated on VY/2 agar composed of baker yeast 0.5%, $CaCl_2 \cdot 2H_2O$ 0.1%, vitamin B_{12} 0.5 mg/liter, agar 1.5%, pH 7.2. With pieces of filter paper on the agar or when maltose was added to the medium, reinforced growth was observed. In liquid culture the strain was cultivated in VY/2 medium with the addition of 0.2～0.5% maltose (added after sterilization) and 50 mM HEPES, pH 7.2. Batch cultures of 100 ml or 400 ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a gyratory shaker at 160 rpm for 4～7 days. Strain By c1 grew in small red clumps. Stock cultures were preserved at −80°C or in liquid nitrogen. The strain was deposited under the accession number DSM 14567 at the DMSZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany.

Fermentation and Isolation

Batch fermentations of *B. cruenta* strain By c1 were performed in VY/2 liquid medium with the addition of 0.3% maltose, 10～50 mM HEPES buffer and 1.0% (v/v) of the adsorber resin Amberlite XAD-16 (Rohm and Haas, Darmstadt, Germany). As an example, 2.0 liters of well-grown shake cultures were inoculated into 8 liters of medium (50 mM HEPES) in a 15-liter bioreactor (Giovanola Frères, Monthey, Switzerland). The fermentation was carried out at 30°C, with an aeration rate of 0.5 liter air per minute and an agitation at 45～55 rpm with a flat-blade turbine stirrer. After 7 days, the content of this seed bioreactor was inoculated into a 150-liter bioreactor equipped with a flat-blade turbine stirrer (Bioengineering, Wald, Switzerland), containing 80 liters of medium (with 10 mM HEPES) and 1.0% (v/v) of the adsorber resin Amberlite XAD-16. The production bioreactor was kept at 30°C, aerated with 3 liters air per minute, and agitated at 100 rpm. The pH value rose till the end of the fermentation after 7 days from about 7.2 to 7.4 and the pO_2, recorded with a polarographic oxygen electrode decreased from 95% to 85%.

At the end of the fermentation the cell mass together with the Amberlite XAD-16 adsorber resin was collected by centrifugation and extracted with acetone. Subsequently, the combined and concentrated crude extract was further purified by partition between water and ethyl acetate, followed by chromatography on silica gel Si 60 and preparative HPLC (RP18, Kronlab ODS AQ 120 16 μm) as described in detail elsewhere [4].

Analysis of Secondary Metabolites

The spectrum and quantitative analysis of secondary metabolites produced by *B. cruenta* was determined in aliquots of concentrated acetone extracts by diode-array-detected analytical HPLC using a Hewlett Packard HP-1100 instrument. Chromatographic conditions were as follows: column 125×2 mm, 5 μm, Nucleosil C18, solvent: acetonitrile/water isocratic 55 : 45, flow 0.3 ml/minute, diode array detection.

Biological Spectrum

Standard strains for testing the biological activity were obtained from the DSMZ and the stock collection of our laboratory at the GBF.

The antimicrobial spectrum of cruentarens A and B was determined by an agar-plate diffusion assay using paper discs as described previously [12]. The minimal inhibition concentrations were determined with the serial two-fold dilution method. As an inoculum 1×10^6 cells were used. The antibiotics were dissolved in methanol, giving MeOH concentrations in the cultures of not more than 3%. Cytotoxicity against L929 mouse cells (connective tissue, ATCC CCL 1) was assayed as reported [13].

Preparations of Mitochondria, NADH-Oxidation and ATPase Assays

Yeast mitochondria were isolated as described previously [14]. In some cases the yeast cells mixed with glass beads were only homogenized in a mini-shaker (Ika). Beef heart mitochondria were isolated by differential centrifugation as reported earlier [10]. Submitochondrial particles (SMP)

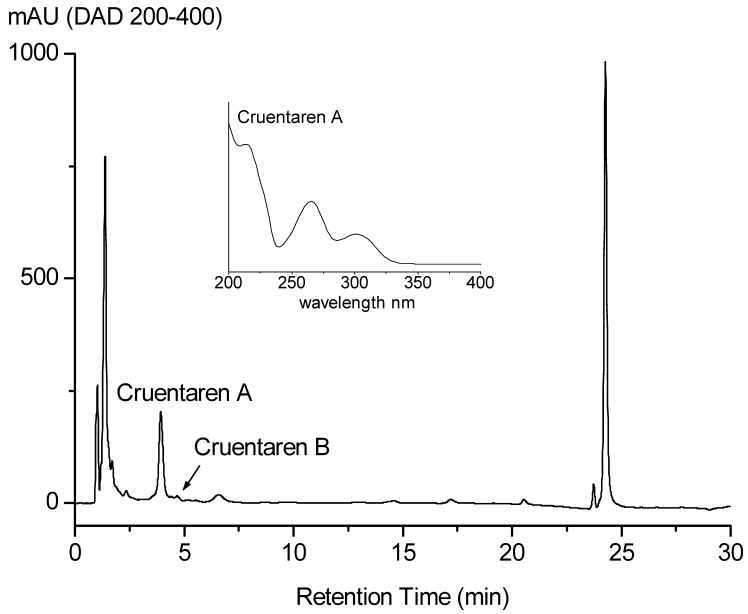

Fig. 2 HPLC profile of a crude extract from a fermentation of *Byssovorax cruenta* Ha r1, and overlaid the UV spectrum of cruentaren A.

were obtained by ultrasonic treatment of the mitochondria. NADH oxidation in SMP was tested in a UV2 Unicam UV/VIS spectrophotometer as described previously [15].

ATPase assays were performed in a final volume of 1000 μl and at a pH of 8.0 with 2.7~3.4 μg protein, 50 mM Tris, 50 mM KCl and 2.5 mM $MgCl_2$. After 5 minutes of preincubation with or without inhibitors, 5 mM ATP was added, and after an additional incubation time of 15 minutes the reaction was stopped by the addition of 0.4 ml of 20% TCA. Inorganic phospate produced in the assays was measured as blue coloured phosphomolybdate complex at 740 nm according to the method of Fiske and Subarrow [16] using ascorbic acid as reducing agent.

Results and Discussion

Strain By c1 (=Ha r1) shows a conspicious red pigmentation untypical for myxobacteria. The vegetative cells are typical of the suborder Sorangineae, and like *Sorangium*, the organism is able to degrade cellulose. Yet it also grew well on living *Escherichia coli*, lyzing the food bacterium, which is in contrast to *Sorangium*, that can neither grow on living *E. coli*, nor lyze it. The special characteristics of this strain made it necessary to assign it to a new genus of the order Myxococcales. It has been named *Byssovorax cruenta* [3].

After the organism had been adapted to growth in liquid media by a long series of transfers in shaken cultures, the production of cruentarens on a larger scale could be performed as described in the experimental section. In a 90-liter fermentation cruentaren A accumulated at the end of the fermentation at 7 days up to about 3.2 mg/liter as determined by HPLC. Isolation and purification yielded about 270 mg of pure cruentaren A and 7 mg of cruentaren B, respectively. Figure 2 shows an analytical HPLC/DAD profile of a fermenter crude extract of Ha r1 after 7 days of cultivation.

Biological Properties

The determination of the antimicrobial activity showed that cruentaren A strongly inhibited the growth of several yeasts and filamentous fungi. Additionally, it was slightly inhibitory against a few Gram-positive bacteria but completely inactive against a series of Gram-negative bacteria (Table 1). The MIC for *Saccharomyces cerevisiae* grown in glucose free medium was 0.4 μg/ml. Cruentaren A also proved to be highly cytotoxic. For cultivated L929 mouse fibroblasts the IC_{50} was 1.2 ng/ml. Cruentaren B showed no antimicrobial activity up to 20 μg/disc and with an IC_{50} of 1.0 μg/ml, it was only marginal cytotoxic for L929 cells. Because of higher activity of cruentaren A against *S. cerevisiae* when grown on glucose free medium we suspected that the new compound acted on a target associated with mitochondria. Our experiments to examine the mitochondrial electron transport chain (complex I, complex bc_1 and complex IV), showed that, up to a concentration of 8 μg/ml, cruentaren A did not influence

NADH oxidation in submitochondrial particles of yeasts and beef heart, respectively (data not shown). In a further attempt to uncover the mechanism of action of cruentaren A, we subsequently tested the inhibitory efficacy of cruentaren A on the F-ATPase activity in both submitochondrial particles of *S. cerevisiae* and beef heart. As shown in Fig. 3, cruentaren A indeed significantly inhibited mitochondrial ATPase hydrolysis. At concentrations of 0.1 μM and 1.0 μM, respectively, the ATPase activity in both systems was reduced to values around 10∼20%. While the structural related V-ATPase inhibitor apicularen A reduced this activity only slightly, the inhibitory values of cruentaren A were in the same concentration range as those measured for the specific F-ATPase inhibitor oligomycin [17].

Table 1 Cruentaren A: Antimicrobial spectrum

Test organisms[a]	Diameter of inhibition zone[b] (mm)
Escherichia coli DSM 423	0
Salmonella typhimurium DSM 5091	0
Pseudomonas aeruginosa DSM 1117	0
Bacillus subtilis DSM 10	16
Micrococcus luteus GBF 26	24
Staphylococcus aureus GBF 16	tr
Brevibacterium ammoniagenes DSM 20306	0
Corynebacterium fascians DSM 20131	0
Candida albicans GBF 129	(24)
Metschnikowia pulcherrima DSM 70321	22
Saccharomyces cerevisiae[c] GBF 36	(28)
Saccharomyces cerevisiae[d] GBF 36	33
Rhodotorula glutinis	23
Botrytis cinerea DSM 877	26
Mucor hiemalis DSM 2655	28
Rhizopus arrhizus DSM 905	25

[a] The organisms were tested on standard complex media.
[b] Values represent the means of at least three independent determinations. Figures in parentheses indicate incomplete inhibition.
[c] The organism was cultivated in a medium containing 1% glucose.
[d] The organism was cultivated in a medium containing 2% glycerol.

Altogether the cruentarens are the first novel basic structures isolated from the new myxobacterial genus *Byssovorax cruenta* [3]. Chemically, cruentarens A and B belong to a growing number of structurally related macrocyclic salicylate natural compounds, *e.g.*, salicylihalamide A [6], lobatamide A [7] and apicularen A [8∼10]. While the salicylihalamides and the lobatamides were isolated from an unidentified species of the marine sponge *Haliclona* sp. and from different tunicate species of the genus *Aplidium*, respectively, apicularen A is produced by several species of the myxobacterial genus *Chondromyces*, *e.g.*, *C. apiculatus* or *C. robustus*. Thus the cruentarens represent further myxobacterial compounds which resemble natural products isolated from marine inhabitants. This raises again the question whether marine animals are really the producers of such compounds, or if they are only hosts of bacterial producers. Other examples of myxobacterial compounds that are structurally related to

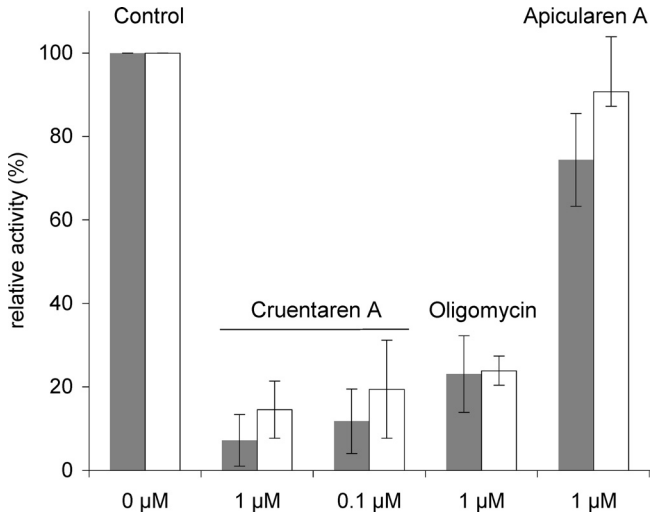

Fig. 3 Inhibition of F-ATPase activity in submitochondrial particles from *Saccharomyces cerevisiae* and beef heart.

Values represent the means of at least four independent experiments. The specific ATPase activity without inhibitor was 15.7±6.9 μmol mg^{-1} min^{-1} in *S. cerevisiae* (grey columns) and 7.19±2.3 μmol mg^{-1} min^{-1} in submitochindrial particles of beef heart (white columns). The control without inhibitor was set as 100%.

compounds isolated from sponges are the chondramides [18], which resemble jaspamide/jasplakinolide from the sponge *Jaspis johnstoni* [19], and saframycin [20] which is chemically related to renieramycin from the sponge *Reniera* sp. [21].

First investigations to unravel the mechanism of action of cruentaren A showed that it inhibited ATP hydrolysis in submitochondrial particles of both the yeast *S. cerevisiae* and beef heart and thus seems to interfere with the proton-pumping mitochondrial F_0F_1-ATPase, which is located in the inner mitochondrial membrane. This is in contrast to the structurally related apicularen A, which had no specific effect on F-type ATPases, but was shown to be a new and powerful inhibitor of the V-ATPase [10].

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