Ajudazols, New Inhibitors of the Mitochondrial Electron Transport from Chondromyces crocatus

Production, Antimicrobial Activity and Mechanism of Action†

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In the course of our screening of myxobacteria for new biologically active compounds, crude extracts of the species Chondromyces crocatus were noticed for their high antifungal and cytotoxic activity. Subsequently these activities could be ascribed to several structural diverse groups of secondary metabolites, which are simultaneously produced by C. crocatus. Crocacin A, the main representative of the at first isolated group, is a complex N-acyl dipeptide, which effectively inhibits the growth of yeasts and fungi, caused by blocking the electron flow within the cytochrome bc1 segment (complex III) of the eukaryotic respiratory chain1,2). Another group, the chondramides A–D3,4) are new cyclo-depsipeptides structurally related to the sponge metabolite jasplamide/jasplakinolide5–7). They show only weak activity against yeasts, but are highly cytostatic for different cultured mammalian cell lines by interfering with the actin cytoskeleton8). Advanced analysis of crude extracts of C. crocatus led to the discovery of the new β-amino styrenes, the chondrochlorens9), and the ajudazols A (1) and B (2) (Fig. 1). Structure elucidation revealed the ajudazols to be unique isochromanone derivatives with an extended side chain containing an oxazole, a Z,Z-diene, and a 3-methoxybutenoic acid amide as characteristic structural features10). Here we report on the production of the ajudazols, their antimicrobial activity, and on experiments on their mechanism of action showing that the ajudazols inhibit the electron transport in beef heart submitochondrial particles (SMP) at the site of complex I, i.e. NADH:ubiquinone-oxidoreductase.

Like crocacins1) and chondramides3), the ajudazols are regularly detected in crude extracts of C. crocatus strains, viz., of strain Cm c1 to Cm c13. On large scale they were produced with strain Cm c5, which was isolated at the GBF in 1988 from a soil sample collected in Brazil.

After the organisms had been adapted to growth in liquid media as described for the production of crocacin1), they were cultured in media containing 0.4–0.9% of a technical substrate, e.g., Probion (single cell protein prepared from...
Methylomonas clarae; Hoechst A.G.), soy flour, peanut meal, or skim milk powder. The basal composition of these media is given in Table 1. Batch cultures of 100ml or 400ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30ºC on a gyratory shaker at 160rpm for 3-5 days.

The total amount of the main component ajudazol A produced by the different C. crocatus strains was determined by HPLC after cultivation in 100ml Probion liquid medium in shake cultures for 4 days and extraction of the cell mass with acetone. In a test series with strains Cm c1 to Cm c7 the production ranged from 0.93mg/liter for strain Cm c2 to 9.92mg/liter for strain Cm c7 (each cultivated with 0.9% Probion). The influence of various technical substrates on the yields of ajudazol A with the producing strain Cm c5 is given in Table 1.

For the isolation of ajudazols on large scale, usually fermentations of strain Cm c5 were used, which were simultaneously run for the production of the other secondary metabolites, e.g., chondramides3). These were performed in PolI liquid medium (Probion 0.4%, soluble starch 0.3%, MgSO₄·7H₂O 0.1%; CaCl₂·2H₂O 0.05%; HEPES buffer 50 mM (pH 7.2); supplemented with standard vitamin- and trace element solutions, 1ml/liter each. Harvest was at the end of the growth phase after about 4 days. The concentration of ajudazol A was determined in acetone extracts of the cell mass by HPLC analysis (column ET 125,25* with precolumn 15 mm; Nucleosil 120-5-C₁₈; solvent gradient water-methanol: 4 min 60% MeOH, 6 min rising to 70%, 5 min to 90%, and 2 min to 100% MeOH; flow rate 0.3 ml/minute; detection 236 nm).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (%)</th>
<th>Ajudazol A (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probion</td>
<td>0.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Probion</td>
<td>0.9</td>
<td>7.6</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Soy meal</td>
<td>0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Soy meal</td>
<td>0.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>0.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>0.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Corn steep powder</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Corn steep powder</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Zein</td>
<td>0.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Zein</td>
<td>0.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Oat meal</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Oat meal</td>
<td>0.9</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*The basal medium was: soluble starch 0.3 %, MgSO₄·7H₂O 0.1 %; CaCl₂·2H₂O 0.05 %; HEPES buffer 50 mM (pH 7.2); supplemented with standard vitamin- and trace element solutions, 1ml/liter each. Harvest was at the end of the growth phase after about 4 days.

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Table 1. Effect of various technical substrates on the production of ajudazol A by Chondromyces crocatus, strain Cm c5.
about 40% at the end after 93.5 hours. Ajudazol A, determined by HPLC analysis as described in Table 1, accumulated under these conditions up to 3.8 mg/liter. The yield of ajudazol B in this fermentation was about a tenth. At the end of the fermentation the cells were separated from the culture broth by centrifugation. The cell mass containing the activity was extracted with acetone and the concentrated extract was further purified by solvent partitions and consecutive chromatographic separations on RP-18 silica gel and Sephadex LH 20. The ajudazols were obtained as colourless amorphous solids soluble in methanol, acetone, chloroform and ethyl acetate. Detailed data of the isolation and physico-chemical properties have been published with the structure elucidation\(^\text{10}\).

The antimicrobial activity of the ajudazols was determined by the agar diffusion assay using paper discs of 6 mm diameter. With 40 μg ajudazols/disc in 20 μl methanol ajudazol B incompletely inhibited growth of the following fungi (data in parentheses indicate diameter of inhibition zone in mm): Botrytis cinerea (10), Trichoderma koningii (21), Gibberella fujikuroi (17) and Ustilago maydis (13). It was also weakly active against few Gram-positive bacteria. The MIC determined by a serial dilution assay for Micrococcus luteus was 12.5 μg/ml. Ajudazol A showed only minor activity against a few fungi and Gram-positive bacteria.

During studies to investigate the mechanism of action we also tested the influence of the ajudazols on the mitochondrial respiratory energy metabolism of beef heart submitochondrial particles (SMP). The isolation and characterization of SMP as well as details of the experiments has been described previously\(^\text{11}\). NADH oxidation in SMP, was determined in a UV2 Unicam UV/VIS spectrophotometer and was inhibited by 50% at a concentration of 13.0 ng/ml (22 nm) ajudazol A and 10.9 ng/ml (18.39 nm) ajudazol B, 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. 3, the difference spectrum of NADH-reduced minus air-oxidized SMP without inhibitor showed the characteristic absorption maxima of the different cytochromes. However, in presence of ajudazol A and also ajudazol B (not represented) all cytochromes remained in the oxidized state. This indicated that the site of inhibition of ajudazols is on the substrate side of cytochrome b. This can be reduced either by NADH via complex I (NADH: ubiquinone oxidoreductase) or by succinate. To decide whether the ajudazols interfere with complex I, with complex II, or with both, we tested the effect of the compounds on the reduction kinetics of cytochrome b using either NADH or succinate as the substrate. The time course

![Fig. 2. Time course of a fermentation of Chondromyces crocatus, strain Cm c5, in a 900-liter bioreactor with 690-liter culture volume.](image1)

![Fig. 3. The effect of ajudazol A on the reduction of cytochromes by NADH.](image2)

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, ——— and in the presence of 12 μg/ml ajudazol A. ——— Baseline.
of cytochrome $b$ reduction was measured by dual wavelength spectroscopy at the wavelength pair 663 minus 577 nm. As can be seen in Fig. 4, ajudazol A inhibited the reduction of cytochrome $b$ only when NADH was the electron donor. Ajudazol B showed equal effects in this experiment.

The investigations on the mechanism of action of the ajudazols suggest, that the new compounds block the electron flow in SMP specifically at the site of complex I, i.e., NADH: ubiquinone-oxidoreductase, similarly as e.g. phenoxan$^{11}$, thiangazole$^{12}$, myxalamids$^{13}$ and phenalamids$^{14}$, further biologically active compounds found in the myxobacterial screening mentioned above.

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References

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