Chondramides A~D, New Antifungal and Cytostatic Depsipeptides from *Chondromyces crocatus* (Myxobacteria)

Production, Physico-chemical and Biological Properties†

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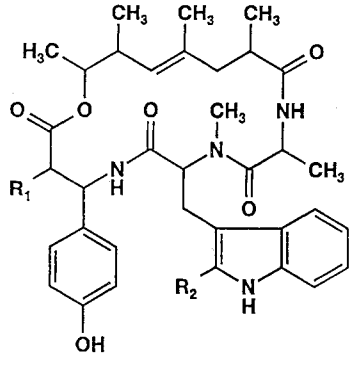
Novel depsipeptides, named chondramides were produced at levels up to 4.3 mg/liter by several myxobacteria of the genus *Chondromyces*. The compounds are structurally closely related to jaspamide/jasplakinolide from marine sponges of the genus *Jaspis*. Initially the chondramides were detected in acetone extracts of the biomass of *Chondromyces crocatus*, strain Cm c2. So far, four structural variants could be characterized, the chondramides A~D. They inhibited the growth of a few yeasts and showed high cytostatic activity against cultivated human and animal cells.

The introduction of the more fastidious and unusual genus *Chondromyces* into our continuous antibiotic screening of myxobacteria resulted in a number of interesting novel activities. Besides the recently described antifungal crocacin[1] we detected in several culture extracts of the species *C. crocatus* a second compound showing weak activity against some yeasts and high cytotoxicity against cultivated mammalian cells. Structure elucidation revealed the activity to be due to a mixture of new depsipeptides, which we named chondramides. In their structures the so far characterized chondramides A~D (Fig. 1) show a remarkable similarity to jaspamide[2], or jasplakinolide[3,4], isolated from the soft bodied marine sponge *Jaspis johnstoni* (order Astrophorida, family Jaspidae). In this article we describe the production, isolation, and some of the physico-chemical and biological properties of chondramides A~D. The structure elucidation will be published elsewhere[5].

Characterization of the Producing Organisms and Culture Conditions

Initially the producing organism was *C. crocatus*, strain Cm c2, isolated at the GBF in 1985 from a soil sample collected on Madeira. Later on the chondramides were found to be produced by all strains of the species *C. crocatus* tested by us, *viz.* Cm c1, Cm c3, Cm c4, Cm c5, Cm c6 and Cm c7. On a larger scale, the chondramides were mostly 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 crocacin[1], they were grown 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 was: 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, 1 ml/liter each of standard vitamin

Fig. 1. The structure of the chondramides A, B, C and D[5]



Chondramide A $R_1 = OCH_3$ $R_2 = H$
Chondramide B $R_1 = OCH_3$ $R_2 = Cl$
Chondramide C $R_1 = H$ $R_2 = H$
Chondramide D $R_1 = H$ $R_2 = Cl$

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and trace element solutions, 50 mM HEPES, pH 7.0. 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 3~5 days. As mentioned previously for strain Cm c3[1], a second bacterium was also observed in cultures of Cm c2 and several other *C. crocatus* strains. The characterization of the companion bacterium has been described[6].

Production

The total yields of the chondramides produced by the different *C. crocatus* strains were determined by HPLC after cultivation of the strains in 100 ml Probion liquid medium in shake cultures for 4 days and extraction of the cell mass with acetone. The production varied from strain to strain from 0.15 mg/liter with Cm c6 to 4.3 mg/liter with Cm c5. The amounts of chondramides produced by Cm c5 on various technical substrates are shown in Table 1.

The production of the chondramides on a larger scale was performed in Poll 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, 1 ml/liter of a standard trace element solution, pH 7.0). To give an example, 5 liter of a culture of strain Cm c5 grown for 4 days in Pol 1 liquid medium with the addition of 50 mM HEPES on a gyratory shaker at 160 rpm were inoculated into 90 liter Poll medium in a 150-liter bioreactor equipped with a flat-blade turbine stirrer (Bioengineering, Wald, Switzerland). The fermentation was run at 30°C, with an aeration rate of 200 liter air per hour and a stirrer speed of 150 rpm. After 4 days, the content of this seed fermenter was inoculated into the production bioreactor (Giovanola Frères, Monthey, Switzerland; periphery modified by GBF) containing 600 liter Poll medium. Because of foam problems, 0.05% silicone antifoam agent (Tegosipon, Goldschmidt AG, Essen) had to be added to both fermentors. The production bioreactor was kept at 30°C, aerated with 80 liter air per minute and agitated with a turbine plate stirrer (50 rpm). Fig. 2 shows the time course of a fermentation in a 690-liter production bioreactor. The pH, which initially drifted slightly into the acid range (pH 6.9), rose during fermentation to 7.6 after 74 hours and was then kept at 7.4 by titration with 30% acetic acid till the end of the fermentation. The pO_2, recorded continuously with a polarographic oxygen electrode, was around 90% saturation at the beginning of the fermentation and fell to about 40% at the end of the fermentation after 93.5 hours. Under these conditions the chondramides, determined by HPLC analysis as described above, accumulated essentially during growth. At the end of the fermentation the concentration of the individual chondramides was 1.5 mg/liter for chondramide B, 0.20 mg/liter for chondramide C, and 0.35 mg/liter for chondramide D. In this case the concentration of chondramide A was below the limit of determination.

Table 1. Effect of various technical substrates on the production of chondramides by *Chondromyces crocatus*, strain Cm c5.

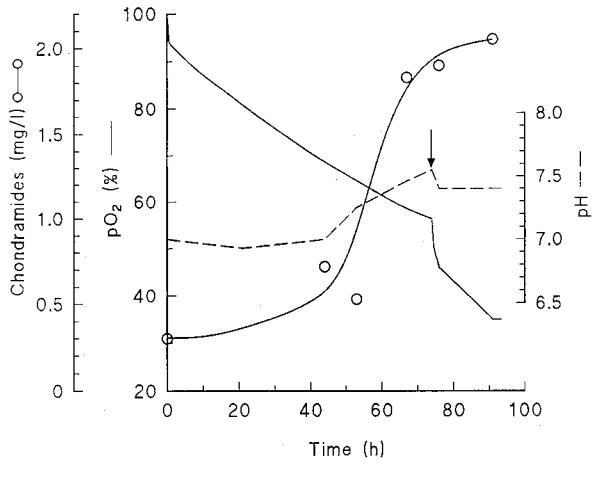
Substrate[a]	Concentration (%)	Chondramides[b] (mg/liter)
Probion[c]	0.4	3.1
Probion	0.9	4.3
Skim milk powder	0.4	1.0
Skim milk powder	0.9	0.8
Soy meal	0.4	2.3
Soy meal	0.9	3.4
Peanut meal	0.4	2.6
Peanut meal	0.9	2.0
Cornsteep powder	0.4	0.0
Cornsteep powder	0.9	0.3
Zein	0.4	1.0
Zein	0.9	1.6
Oat meal	0.4	0.9
Oat meal	0.9	2.3

[a] The basal medium was: soluble starch 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%; $CaCl_2 \cdot 2H_2O$ 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 total concentration of the chondramides was determined in acetone extracts of the cell mass by HPLC analysis (column ET 125,25″ with precolumn 15 mm; Nucleosil 120-5-C_{18}; solvent: gradient water - methanol; flow rate 0.3 ml/minute; detection 222 nm).

[c] Single cell protein (Hoechst Comp.).

Fig. 2. Fermentation of *Chondromyces crocatus*, strain Cm c5 in a 690-liter bioreactor.



Isolation

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. The concentrated extract was further purified by solvent partitions, RP-MPLC, Si-HPLC and RP-HPLC[5]. From a 250-liter fermentation with strain Cm c2, 81 mg of chondramide A, 75 mg of chondramide B, 86 mg of chondramide C, and 14 mg of chondramide D were recovered in this way.

Physico-chemical Properties

The chondramides A~D were obtained as colorless glasses. They were 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) with toluene-methanol (85:15) as the solvent, the chondramides A, B, C and D showed Rf values of 0.19, 0.25, 0.20 and 0.26, respectively, and a common Rf of 0.46 with dichloromethane-methanol (9:1). The chondramides A and C could be visualized as violet spots by spraying with vanillin/sulfuric acid reagent and heating to 120°C, whereas the chondramides B and D gave light-blue spots. The basic structure of the chondramides was elucidated spectroscopically. High-resolution EI mass spectroscopy furnished the elemental composition $C_{36}H_{46}N_4O_7$ for chondramide A, $C_{36}H_{45}ClN_4O_7$ for chondramide B, $C_{35}H_{44}N_4O_6$ for chondramide C and $C_{35}H_{43}ClN_4O_6$ for chondramide D. Detailed data will be published with the structure elucidation[5]. The optical rotation of the chondramides was $[\alpha]_D$ +2.1 (*c* 2.0, MeOH) (A), $[\alpha]_D$ +20.8 (*c* 2.0, MeOH) (B), $[\alpha]_D$ +40.2 (*c* 2.4, MeOH) (C), and $[\alpha]_D$ +49.6 (*c* 1.3, MeOH) (D). The UV spectrum of chondramide A in methanol was recorded with a Hitachi U 3200 spectrophotometer (Fig. 3). It had maxima at λ_{max} (log ε) 222 nm (4.56), 279 nm (3.79), 290 nm (3.67). The spectra of chondramides B, C and D showed the same maxima. The IR spectrum of chondramide A in KBr, measured with a Nicolet 20 DXB FT-IR spectrometer, is given in Fig. 4.

Biological Activity

The antimicrobial activity of the chondramides A~D was determined by agar diffusion tests and is presented in Table 2. The compounds were completely inactive against Gram-positive and Gram-negative bacteria, inhibited the growth of several yeasts, but not that of filamentous fungi. The MIC for *Candida albicans* determined by a serial dilution assay was 25 μg/ml for chondramides B and D, and 50 μg/ml for chondramides A and C. The chondramides proved to be highly effective in mammalian cell cultures (Table 3). Concentration-dependent growth inhibition of different cell lines was determined 5 days after the addition of the various chondramides by an MTT assay, which measures the

Fig. 3. UV spectrum of chondramide A in methanol.

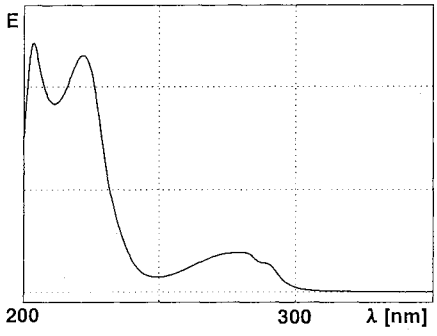
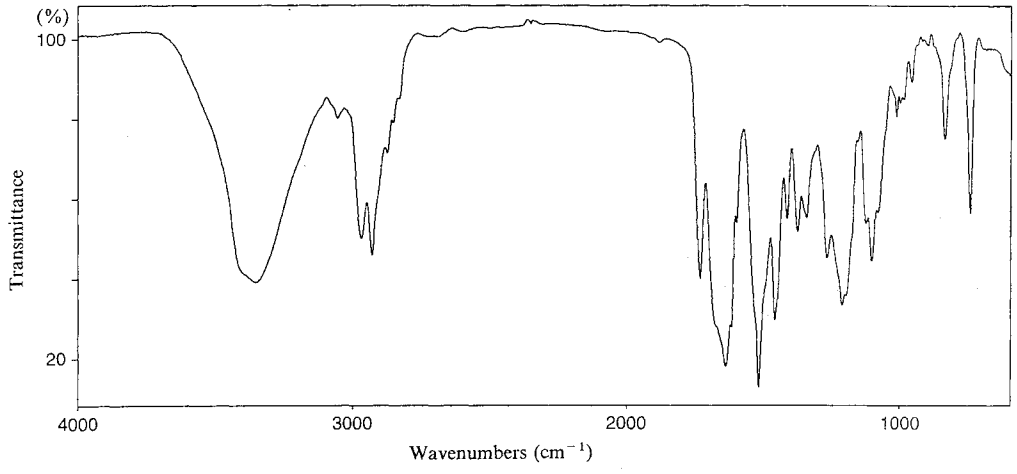


Fig. 4. IR spectrum of chondramide A in KBr.



reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to its insoluble formazan[7]. Fig. 5 shows the dose response curves of the chondramides A~D with the continuously growing KB-3-1 cell line (DSM ACC 158), which was established from a primary cervical carcinoma. The course of the inhibition was similar for the different chondramides, the IC_{50} values ranged from 2 to 7 ng/ml. Fig. 6 shows the growth kinetics of K-562 (ATCC CCL 243) leukemia cells in the presence and absence of chondramide A. The cells were cultivated

Table 2. Antimicrobial spectrum of the chondramides A~D.

Test organisms[a]	Diameter of inhibition zone (mm)[b]			
	A	B	C	D
Bacteria:				
Bacillus subtils DSM 10[c]	0	0	0	0
Brevibacterium ammoniagenes DSM 20306	0	0	0	0
Escherichia coli DSM 498	0	0	0	0
Pseudomonas aeruginosa DSM 1117	0	0	0	0
Yeasts:				
Candida albicans GBF[d]	13	14	9	12
Candida albicans DSM70014	12	12	12	13
Candida albicans DSM 1577	12	13	9	12
Hansenula anomala DSM 70263	12	13	7	12
Lipomyces lipofer GBF	11	14	9	12
Schizosaccharomyces pombe Tü 501[e]	0	16	0	0
Torulopsis glabrata DSM 70398	9	14	9	11
Filamentous fungi:				
Botrytis cinerea DSM 877	0	0	0	0
Gibberella fujikuroi DSM 893	0	0	0	0
Mucor hiemalis DSM 2655	0	0	0	0
Rhizopus oryzae DSM 905	0	0	0	0

[a] Bacteria were tested on nutrient agar, fungi on malt extract peptone agar.
[b] Determined by the agar diffusion test with 20 μg chondramides on a 6 mm paper disc.
[c] Deutsche Sammlung von Mikroorganismen.
[d] Strain of the GBF, Abteilung Naturstoffbiologie.
[e] Collection University of Tübingen.

Fig. 5. Concentration-dependent growth inhibition of the human cell line KB-3-1 by the chondramides A~D.

● Chondramide A, ▲ chondramide B, ▼ chondramide C and ◆ chondramide D.

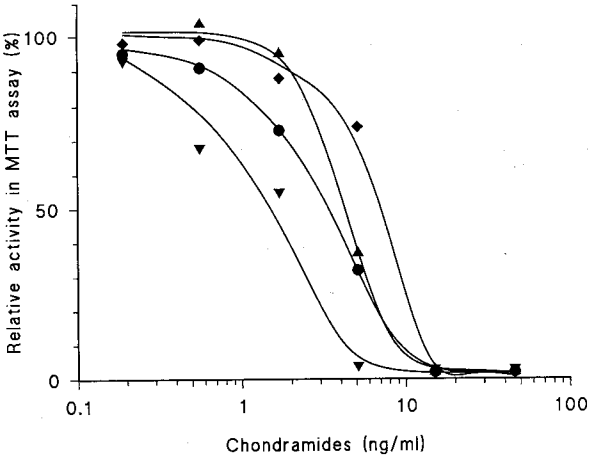


Fig. 6. Growth inhibition of K-562 leukemia cells by chondramide A.

○ Without chondramide A, ● with chondramide A (200 ng/ml), culture medium replaced by fresh medium without inhibitor after 1 day (▲), after 2 days (▼), as indicated by arrows.

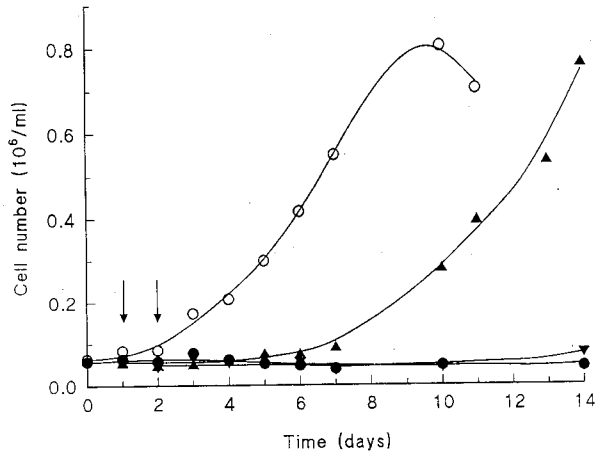


Table 3. Cytostatic effects of the chondramides A~D on mammalian cell lines.

Cell line	Origin	IC_{50} of chondramides (ng/ml)			
		A	B	C	D
KB-3-1 (DSM ACC 158)[a]	Human, cervical carcinoma	3	4	2	7
KB-V1 (DSM ACC 149)[a]	Human, cervical carcinoma	60	20	60	30
K-562 (ATCC CCL 243)[b]	Human, chronic myelogenous leukemia	6	5	2	3
HL-60 (DSM ACC 3)[a]	Human, acute myeloid leukemia	10	3	2	2
L929 (ATCC CCL 1)[a]	Mouse, connective tissue	30	20	20	30
Pt K2 (ATCC CCL 56)[c]	Potorous tridactylis kidney, marsupial	35	10	15	5

[a] DULBECCO's modified EAGLE (DME) medium (high glucose; GIBCO).
[b] RPMI 1640 medium (GIBCO).
[c] Minimum essential medium (EAGLE) with non-essential amino acids.

in RPMI 1640 Medium (GIBCO)+10% newborn calf serum at 37°C and 10% CO_2. The inoculum was 50,000 cells/ml. As a parameter of growth the cell number was determined at different times with a hemocytometer. At a concentration of 200 ng/ml of chondramide A the leukemia cells stopped growth immediately and the effect was hardly reversible. When the culture medium was replaced by fresh medium without chondramide A after 1 day, the K-562 cells started to propagate again with a long lag of 5 days; upon a medium replacement after 2 days, no growth was observed anymore. The growth kinetics of K-562 cells in the presence of the other chondramides was comparable.

Discussion

After crocacin, the chondramides are the second group of biologically active substances isolated from strains of the myxobacterium, *C. crocatus*. We detected the chondramides in varying amounts in seven *C. crocatus* strains tested, which suggests that the chondramides, like crocacin, are widely distributed in this species. Furthermore, the strains of this species belong to those myxobacteria, which synthesize two or more chemically unrelated secondary metabolites with different mechanisms of action[8]. The antimicrobial and cytostatic activity of the chondramides is similar to that of the structurally related japamides/jasplakinolides[2~4]. It is most interesting, that the chondramides also show activity against the KB-V1 cell line, which is resistant against the cytostatic drug, vinblastin. First investigations on the mechanism of action suggest that, like jasplakinolide[9], the chondramides interfere with actin polymerisation. So far no other myxobacterial species has been found to produce chondramides. Thus it is even more remarkable that chemically closely related but otherwise unique compounds, *viz.*, the chondramides and jaspamide[2,3], occur in phylogenetically so distant organisms as a myxobacterium and a marine sponge (*Jaspis*). If one would assume that the sponge harvests jaspamide with planktonic bacterial feed, still the problem remains that no halotolerant myxobacteria are known. While the most of our *C. crocatus* strains only grow in the presence of a (taxonomically identical[6]) companion bacterium, and thus have to be fermented as mixed cultures, there also are strains which grow as pure cultures, *e.g.* Cm c5. As this strain produces the same pattern of secondary metabolites as the symbiotic cultures, biosynthesis of all those substances must be performed exclusively by the myxobacterium.

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