VOL. 51, NO. 12, DEC. 1998 THE JOURNAL OF ANTIBIOTICS pp. 1075 — 1080

Apicularens A and B, New Cytostatic Macrolides from *Chondromyces* Species (Myxobacteria): Production, Physico-chemical and Biological Properties†

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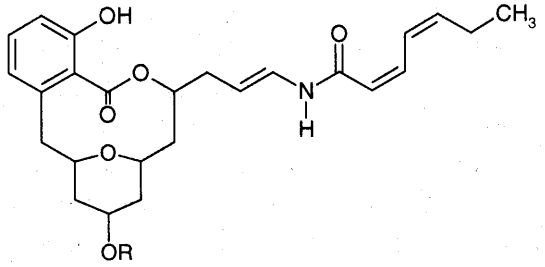
(Received for publication September 4, 1998)

A novel macrolide, apicularen A, was produced by several species of the genus *Chondromyces*. Initially it was discovered by bioassay-guided RP-HPLC-fractionation of culture extracts of *Chondromyces robustus*, strain Cm a13. Apicularen A showed no antimicrobial activity, but was highly cytotoxic for cultivated human and animal cells, with IC_{50} values ranging between 0.1 and 3 ng/ml. A cometabolite of apicularen A, the *N*-acetylglucosamine glycoside apicularen B, was distinctly less cytotoxic with IC_{50} values between 0.2 and 1.2 μg/ml, and showed weak activity against a few Gram-positive bacteria. Apicularen A is chemically closely related to the salicylihalamides A and B from the marine sponge *Haliclona* sp.

During our screening for new, biologically active metabolites from myxobacteria, we discovered within the more fastidious and unusual genus *Chondromyces* a number of interesting novel activities. While strains of the species *C. crocatus* produce the antifungal crocacin[1] and the cytotoxic chondramides[2~4], culture extracts of further *Chondromyces* species, *viz. C. apiculatus*, *C. robustus*, *C. pediculatus* and *C. lanuginosus* also showed high cytotoxicity against cultivated mammalian cells without containing one of the known compounds of *C. crocatus*. Bioassay-guided fractionation of culture extracts in the analytical RP-HPLC, in particular of strain Cm a13, revealed that the high cytotoxicity was due to a common main metabolite, which was isolated and subsequently characterized as a novel macrolide with a highly unsaturated amide side chain. It was named apicularen A (Fig. 1). The crude extracts contained in addition a much less cytotoxic, more polar cometabolite with an identical UV spectrum. That compound was identified as the *N*-acetyl-glucosamine glycoside, of apicularen A, apicularen B (Fig. 1). Apicularen A shows a remarkable structural similarity to the salicylihalamides A and B, novel cytotoxic macrolides isolated from the marine sponge *Haliclona* sp.[5] .There is also a structural relationship to the lobatamides A and B[6], novel cytotoxic macrolides, isolated from the tunicate *Aplidium lobatum*. In this article we describe the production, isolation, and physico-chemical and biological properties of apicularens A and B. Details of the structure elucidation will be published elsewhere[7].

Fig. 1. The structures of apicularens A and B[7].



Apicularen A R=H
Apicularen B R=*N*-acetyl-β-D-glucosamine

† Article No. 82 on antibiotics from gliding bacteria. Article No. 81: HERRMANN, M.; B. BÖHLENDORF, H. IRSHIK, H. REICHENBACH & G. HÖFLE: Angew. Chem. Int. Ed. 37: 1253~1255, 1988.

Table 1. Effect of various technical substrates on the production of the apicularens A and B by *Chondromyces robustus*, strain Cm r8.

Substrate[a]	Concentration (%)	Apicularen A[b] (mg/liter)	Apicularen B[b] (mg/liter)
Probion[c]	0.4	1.6	2.9
Probion	0.9	2.4	1.4
Skim milk powder	0.4	1.3	1.2
Skim milk powder	0.9	1.3	1.5
Soy flour	0.4	1.5	1.8
Soy flour	0.9	1.4	2.1
Peanut meal	0.4	0.7	0.5
Peanut meal	0.9	0.5	0
Cornsteep powder	0.4	0.6	0.2
Cornsteep powder	0.9	0.6	0.1
Zein	0.4	1.8	0.9
Zein	0.9	1.6	0.4
Oat meal	0.4	1.1	0.8
Oat meal	0.9	1.2	1.4

[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); containing 1 % of the adsorber resin XAD-16 (Rohm & Haas) and supplemented with standard vitamin and trace element solutions, 1 ml/liter each. Harvest was at the end of the growth phase after 6~7 days.

[b] The concentration of apicularen A and B was determined from XAD- and cell extracts, successively extracted with methanol and acetone, by HPLC analysis as described in Fig. 2. Each value gives the average of two experiments.

[c] Single cell protein (Hoechst Comp.)

Producing Organisms and Culture Conditions

Initially the producing organism was *C. robustus*, strain Cm a13, isolated at the GBF in 1980 from a soil sample collected in 1978 on Isola Mujeres, Yucatan, Mexico. Later the apicularens A and B were also found in many strains of the species *C. apiculatus*, *C. robustus*, *C. pediculatus*, *C. lanuginosus*. For production of the apicularens on a larger scale we used *C. robustus* strain Cm r8, which was isolated at the GBF in 1995 from a soil sample from Lucknow, India.

After the organisms had been adapted to growth in liquid media as described for the production of crocacin[1], they were cultivated in media containing 0.3~0.9% 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%, 1 ml/liter each of standard vitamin 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 4~7 days. All strains grew in small lumps.

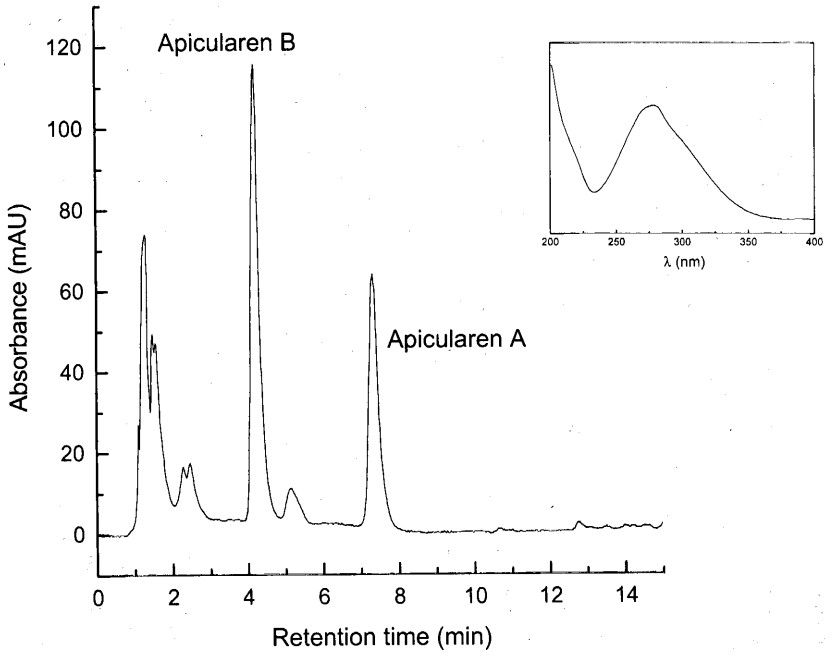
Production

The effect of various technical substrates on the production of apicularens A and B by *C. robustus*, strain Cm r8, was investigated in shake cultures containing 100 ml of liquid medium supplemented with 1% of the neutral adsorber resin Amberlite XAD-16 (Rohm & Haas) and is shown in Table 1. The total yields of the apicularens were determined by HPLC after cultivating the strain for 6~7 days and extracting the cell mass together with the adsorber resin first with methanol and then with acetone. Fig. 2 shows a typical HPLC-profile of a concentrated culture extract from strain Cm r8, cultivated with 0.4% Probion as the technical substrate.

The production of the apicularens A and B on a larger scale was performed in Probion 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} 0.5 mg/liter, 1 ml/liter of a standard trace element solution, pH 7.0) for 7~10 days at 30°C in the presence of 1% of the neutral adsorber resin Amberlite XAD-16 (Rohm & Haas). To give an example, 5 liters of a culture of strain Cm r8 grown for 6 days in Probion liquid medium with the addition of 50 mM HEPES on a gyratory shaker at 160 rpm were

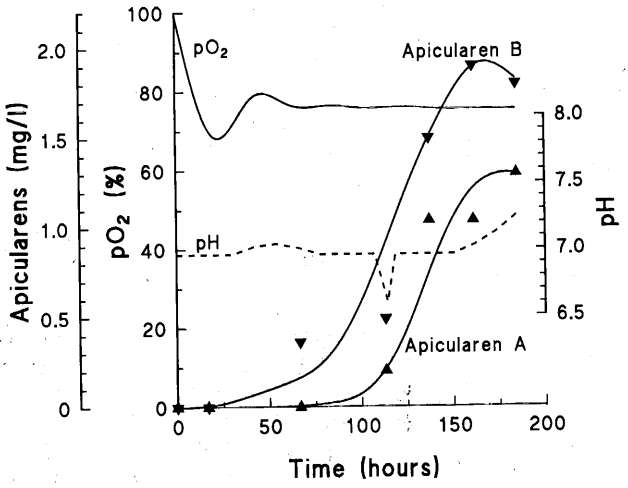
Fig. 2. HPLC-profile of a culture extract of *Chondromyces robustus*, strain Cm r8, and as insert the UV-spectrum of apicularen A in acetonitrile.

Chromatographic conditions: Column 125 × 2 mm, Nucleosil 120-5-C_{18}; solvent: water (A) - methanol (B) gradient, 50% B - 4 minutes to 65% B at 10 minutes; flow rate 0.3 ml/minute; detection 200 ~ 400 nm.



inoculated into 90 liters of the production medium in a 150-liter bioreactor (Bioengineering, Wald, Switzerland). Because of foam problems, 0.1% of an antifoam emulsion (Dow Corning, USA) had to be added. The bioreactor was agitated at 120 rpm with a flat-blade turbine stirrer. The aeration rate was 4.6 liter of air per minute. Fig. 3 shows the time course of the fermentation in a 90-liter production bioreactor. The pH was kept within 150 hours around 7.0 by titration with 1 N KOH (between 107.5 and 118.5 hours the pH-regulation failed) and increased then till the end of the fermentation at 184.75 hours up to 7.25. The pO_2 was recorded continuously with a polarographic oxygen electrode. It was around 95% saturation at the beginning of the fermentation and fell within 20 hours to about 70%; it remained between 80 ~ 70% till the end of the fermentation. Under these conditions the apicularens A and B determined by HPLC analysis as described above reached 1.3 mg/liter (A) and 1.8 mg/liter (B), respectively.

Fig. 3. Time course of a fermentation of *Chondromyces robustus*, strain Cm r8, in a 150-liter bioreactor with 90 liters of Probion liquid medium.



Isolation

At the end of the fermentation the adsorber resin together with most of the cells (little clumps) was separated from the culture by passing the content of the fermentor through a process filter of 210 μm pore size (PTFE, Fa. Seitz). The cell mass was collected from the filtrate by centrifugation. Both the cell mass and the adsorber resin contained the activity and were extracted together with acetone. Firstly apicularen B, was isolated from the concentrated crude extract by partition

between dichloromethane and water, and by RP-MPLC. Apicularen A was isolated from the concentrated extract by partition between methanol and heptane, followed by silica gel chromatography and crystallization from methanol[7]. From a 90-liter fermentation with strain Cm r8, thus 210 mg apicularen A and 42 mg apicularen B were obtained.

Physico-chemical Properties

Apicularen A, obtained as colorless crystals, was soluble in methanol, acetone, chloroform, and ethyl acetate. The amorphous, more polar apicularen B was very well soluble in water. After TLC on silica gel 60 F_{254} (Merck) with dichloromethane/methanol (9:1) as the solvent, the apicularens A and B showed Rf values of 0.39 and 0.1 respectively. They could be visualized as brown-pink spots after spraying with vanillin/sulfuric acid reagent and heating to 120°C. The structures of the apicularens A and B were elucidated spectroscopically. High-resolution EI mass spectroscopy furnished the elemental composition $C_{25}H_{31}NO_6$ for apicularen A and $C_{33}H_{44}N_2O_{11}$ for apicularen B. Detailed data will be published with the structure elucidation[7].The optical rotation of the apicularens was $[\alpha]_D$ −36.1 (*c* 1.0,

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

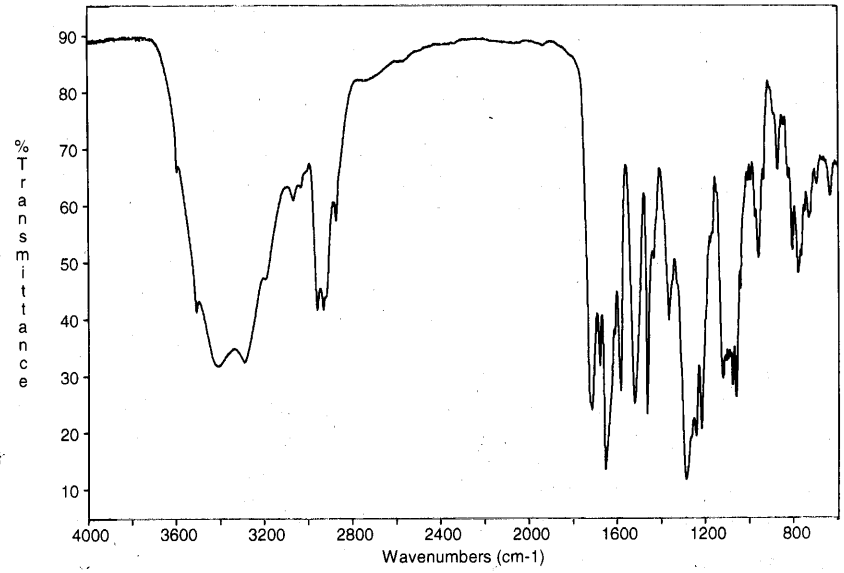


Table 2. Cytostatic effects of the apicularens A and B on human cancer cell lines.

Cell line	Origin	IC_{50} (ng/ml)	
		A	B
KB-3-1 (DSM ACC 158)[a]	Cervix carcinoma	1	1,000
KB-V1 (DSM ACC 149)[a]	Cervix carcinoma	0.4	1,200
K-562 (ATCC CCL 243)[b]	Chronic myelogenous leukemia	2	—
HL-60 (DSM ACC 3)[b]	Acute myeloid leukemia	3	—
U-937 (DSM ACC 5)[b]	Histocytic carcinoma	1.5	100
A-498 (DSM ACC 55)[c]	Kidney carcinoma	0.3	—
A-549 (DSM ACC 107)[a]	Lung carcinoma	0.1	200
PC-3 (ATCC CRL-1435)[d]	Prostata carcinoma	0.5	300
SK-OV-3 (ATCC HTB-77)[e]	Ovarian carcinoma	1.5	—

[a] In Dulbecco's modified Eagle (DME) medium (high glucose; GIBCO).
[b] In RPMI 1640 medium (GIBCO)
[c] In Minimum essential medium (EAGLE) with non-essential amino acids.
[d] In Ham's F12K medium (GIBCO)
[e] In McCoy's 5A medium (GIBCO)

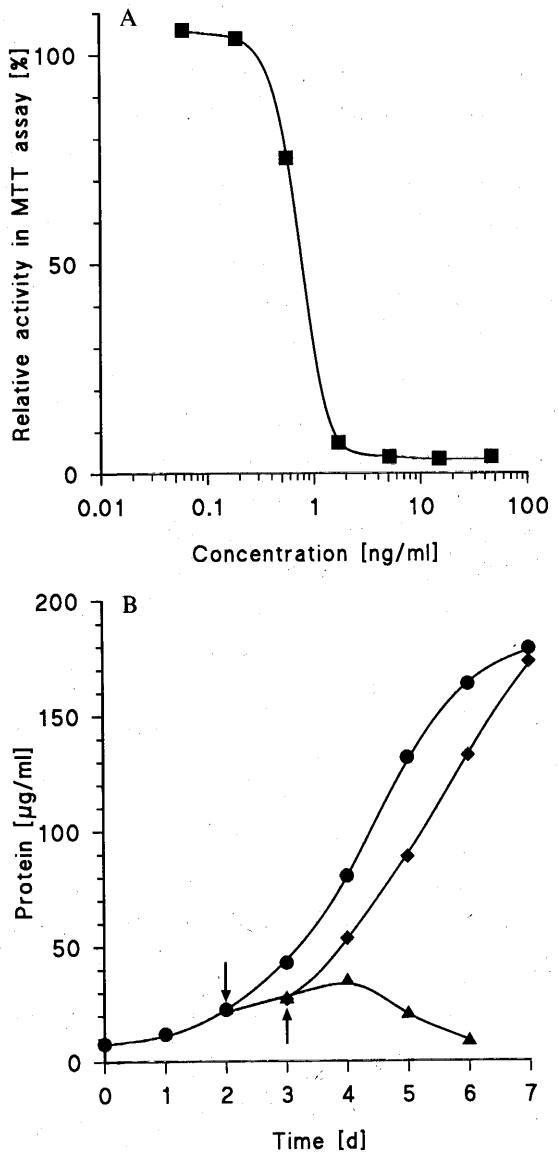
acetonitrile) (A) and $[\alpha]_D$ −5.5 (*c* 0.3, methanol) (B). The UV spectrum of apicularen A in acetonitrile was recorded with a Shimadzu UV-VIS spectrophotometer UV-2102 PC (Fig. 2). It had a maximum at λ_{max} (lg ε) 278 nm (4.43). The spectrum of apicularen B showed the same maximum. The IR spectrum of apicularen A in KBr, measured with a Nicolet 20 DXB FT-IR spectrometer, is given in Fig. 4.

Biological Activity

The antimicrobial activity of the apicularens A and B was determined by agar diffusion tests. Wheras apicularen A was completely inactive against a series of Gram-positive and Gram-negative bacteria as well as against several yeasts and filamentous fungi, apicularen B showed weak activity against a few Gram-positive bacteria. The MIC for *Micrococcus luteus* determined by a serial dilution assay was 12.5 μg/ml, and that for *Corynebacterium fascians* 25 μg/ml. On the other hand, apicularen A proved highly effective in mammalian cell cultures, whereas apicularen B was distinctly less cytotoxic (Table 2). Growth inhibition of various cell lines was determined 5 days after the addition of the compounds by an MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to its insoluble formazan[8]. Fig. 5 A shows the dose response curve of apicularen A with the continuously growing KB-3-1 cell line (DSM ACC 158), established from a primary cervix carcinoma. The IC_{50} value was 1 ng/ml, at which concentration the cells began to round up and loose contact to the substrates. Fig. 5 B shows the growth kinetics of KB-3-1 cells in the presence and absence of apicularen A. The cells were cultivated in Dulbecco's modified Eagle (DME) medium (high glucose; GIBCO) +10% newborn calf serum at 37°C in an atmosphere with 10% CO_2. The inoculum was 50,000 cells/ml. As a parameter of growth, the protein concentration of harvested and washed cells was determined at different times using Bradford reagens (Bio-Rad). At a concentration of 10 ng/ml of apicularen A the KB-3-1 cells stopped growth immediately. When the culture medium was replaced by fresh medium without apicularen A after 1 day, the cells started to propagate again without recognizable delay.

Fig. 5. Effects of apicularen A on the human cancer cell line KB-3-1.

A. Concentration-dependent growth inhibition. Growth was measured by the MTT test as explained in the text. B. Kinetics of the growth inhibition.



● Without apicularen A; ▲ with 10 ng/ml of apicularen A, added after 2 days (first arrow), ◆ culture medium replaced by fresh medium without inhibitor after 1 day as indicated by second arrow. Growth was determined as protein increase (see text for details).

Discussion

After crocacin, which inhibits the mitochondrial respiratory chain at complex III[1], and the chondramides acting on the actin cytoskeleton[2,4], the apicularens A and B are the third basic structures isolated from strains of the genus *Chondromyces*. While crocacin A and the chondramides are so far only found in strains of *C. crocatus*, we detected the apicularens in varying amounts in most of the other *Chondromyces* species, but never in *C. crocatus*. First investigations into the mechanism of

action showed that apicularen A causes several abnormal effects in sensitive cell lines, *e.g.*, the formation of mitotic spindles with multiple spindle poles and of thick stress fiber bundles in the actin cytoskeleton. After the myxobacterial saframycin[9], which is chemically related to renieramycin from the sponge *Reniera* sp.[10], and the chondramides, which resemble jaspamide from the sponge *Jaspis johnstoni*[11,12], the apicularens, which are structurally related to the salicylihalamides from *Haliclona* sp.[5], represent the third group of myxobacterial compounds related to substances isolated from marine sponges. It is remarkable that chemically closely related but otherwise unique compounds occur in phylogenetically so distant organisms as myxobacteria and marine sponges. As marine myxobacteria have been isolated, one could speculate that the sponges harvest those compounds from their planktonic bacterial feed or that they harbor bacterial symbionts synthesizing those substances. In a 60 cell line human tumor screen at the NCI, the salicylihalamides showed a striking pattern of differential cytotoxicity, without any significant correlation to the profiles shown by other known antitumor compounds[5]. Since similar and highly active apicularen A derives from a microbial source, it is much easilier to produce and perhaps opens an attractive possibility for the development of a new class of antitumor compounds.

Acknowledgments

The authors wish to thank Mrs. H. PETRAT, N. MALKOMES, B. VOßWINKEL and M. WEILHARTER for excellent technical assistance, and the colleagues of the fermentation service of the GBF, H. SCHÜLER, R. KRÜTZFELDT, B. EBERT and R. STERLINSKI for their help with large scale fermentations.

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