THE CORALLOPYRONINS, NEW INHIBITORS OF BACTERIAL RNA SYNTHESIS FROM *MYXOBACTERIA**,**

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From the culture broth of the myxobacterium, *Corallococcus* (*Myxococcus*) *coralloides*, three new antibiotics have been isolated: corallopyronin A, B and C. The compounds, which are chemically related to the recently discovered myxopyronins, act mainly on Gram-positive bacteria, with MIC values between 0.1 and 10 μg/ml, and only exceptionally or at much higher concentrations (MIC values; 100 and more μg/ml) on Gram-negatives. They do not inhibit eukaryotic organisms and show no toxicity for mice (sc). The corallopyronins appear to block specifically eubacterial RNA polymerase.

In our screening program for antibiotics from myxobacteria an activity against Gram-positive bacteria was found, isolated, and chemically characterized. It consisted of three components, which were named corallopyronin A, B and C, and proved chemically related to myxopyronin[1,2]. This paper describes the production and some chemical and biological properties of the new antibiotics. The structure elucidation of the corallopyronins has been reported elsewhere[3].

Production of the Antibiotics

The producing organism was *Corallococcus* (=*Myxococcus*=*Chondrococcus*) *coralloides* strain Cc c127 (=*C. coralloides* HI1; Deutsche Sammlung von Mikroorganismen, Göttingen, DSM 2550), isolated in 1980 from a soil sample from Gabès, Tunesia. It was cultivated in modified MD1 liquid medium: peptone from casein, tryptically digested (Merck, Darmstadt) 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.04%, pH 7.2.

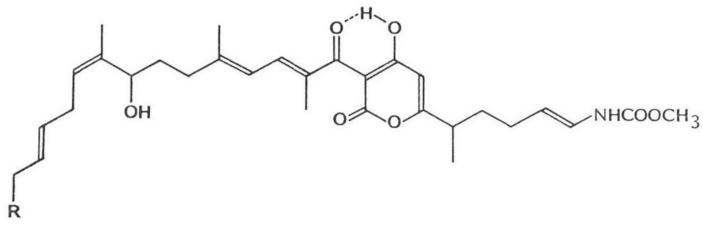
In an effort to optimize antibiotic production, the peptone concentration in the culture medium was varied. As the strain belongs to those myxobacteria that are able to utilize starch[4], this polysaccharide was also added (in the form of soluble starch). Table 1 shows that the variation of the peptone and starch concentrations had a considerable effect on cell mass and antibiotic yield.

Fermentations were then performed with medium No. 4 of Table 1. Bioreactors from Giovanola Frères, Manthey, Switzerland, with total volumes of 70 liters and 320 liters were used. The fermentors were equipped with circulating pump stirrer systems. The 70-liter fermentor was inoculated with 6 liters of a log phase culture grown in a 10-liter flask under forced aeration in modified MD1 liquid medium +0.1% soluble starch. The aeration rate was 0.138 v/v·minute, the stirring rate 550 rpm. The initial pO_2 of about 90~100% saturation decreased to about 20% after 20 hours. It remained then constant or increased slightly during the next 20 hours. The pH was not regulated and rose during the

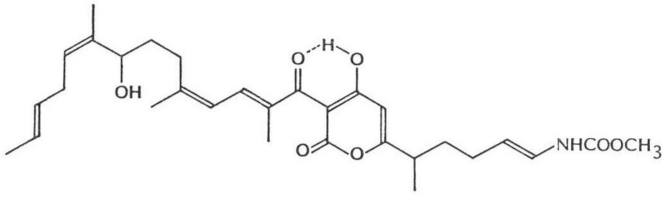
* Article No. 25 on antibiotics from gliding bacteria. Article No. 24: JANSEN, R.; H. IRSCHIK, H. REICHENBACH & G. HÖFLE, Liebigs Ann. Chem., 1985, in press.

** Patent Application P 3,305,974.8, Deutsches Patentamt München, Feb. 18, 1983.

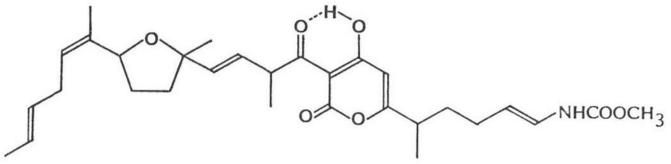
Fig. 1. The chemical structures of corallopyronins A, A', B and C[3].



Corallopyronin A R = H
Corallopyronin B R = CH_3



Corallopyronin A'



Corallopyronin C

Table 1. *Corallococcus coralloides* Cc c127: Influence of peptone and starch concentration in the medium on cell mass and antibiotic yield.

Medium No.	Peptone concentration (%)	Starch concentration (%)	Cell mass* (OD_{623})	Antibiotic concentration** (μg/ml)
1	0.3	—	0.95	2
2	0.3	0.2	1.55	5
3	0.6	—	1.95	3.7
4	0.6	0.2	3.0	10

* Measured as optical density at 623 nm, Eppendorf filter photometer, 1 cm lightpath.
** Determined by HPLC.

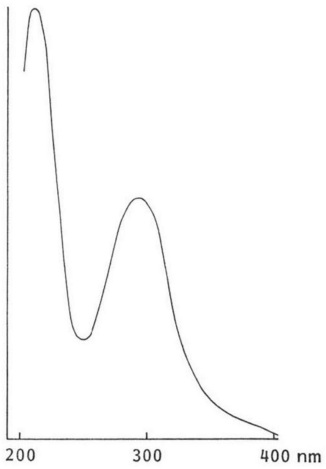
fermentation from 7.2 to 8. After 40 hours the fermentation was stopped by removing the cells by centrifugation. The cell yields were about 6 g/liter wet weight, the antibiotic concentration in the supernatant 4~5 mg/liter. The 320-liter fermentor was inoculated with 7 liters of log phase culture, the aeration rate was 0.05 v/v·minute. The stirring rate of 300 rpm. was increased to 500 rpm. after 24 hours, because by then the pO_2 had fallen to 10%. Harvest was after 52 hours.

Isolation of the Corallopyronins

The isolation procedure for the corallopyronins was worked out by following the biological activity against *Staphylococcus aureus*, either by the agar diffusion test with paper discs, or on TLC plates

(bioautograms). As an example we describe the processing of a 250-liter batch of fermentation broth. The broth was extracted with ethyl acetate using a three step counter-current extraction unit. Vacuum evaporation of the solvent resulted in 34 g of an oily residue, containing 1.2 g of corallopyronins A and B. The oil was partitioned between methanol and heptane (600 ml each). After two additional washings with heptane (250 ml each) the methanol phase was evaporated and yielded 8.3 g of viscous oil. A second distribution between chloroform and water (500 ml each) reduced the weight of the crude antibiotic to 6.3 g. This material was dissolved in 30 ml of dichloromethane and applied to a column containing 240 g of Florisil (Floridin Company, USA, obtained *via* Merck, Darmstadt). The column was eluted stepwise with dichloromethane (fraction 1) and dichloromethane containing 1% (fraction 2) or 5% (fraction 3) acetic acid, respectively (2.5 liters each). Prior to concentration *in vacuo*, the acidic fractions were shaken with sodium bicarbonate solution and dried over sodium sulfate. The first fraction consisted of 320 mg, the second of 750 mg and the third fraction of 1.85 g of oily material; the latter contained the antibiotic activity.

Fig. 2. Electronic absorption spectrum of corallopyronin A in ethanol.



Florisil fraction 3 was further fractionated by preparative HPLC in three runs on silica gel (prepacked Lobar LiChroprep Si 60 column type C, Merck, Darmstadt) with dichloromethane - heptane - methanolic buffer, 55: 43: 2, as the mobile phase (methanolic buffer: methanol - formic acid - triethylamine, 5: 10: 37). The main peak (UV absorption at 280 nm) contained a mixture of corallopyronins A and B (660 mg). Finally, the components A and B were separated by reversed phase HPLC (prepacked Lobar LiChroprep RP-8 column type C, Merck) with methanol - aqueous buffer, 65: 35, as the eluant (aque-

Fig. 3. IR spectrum of corallopyronin A in chloroform.

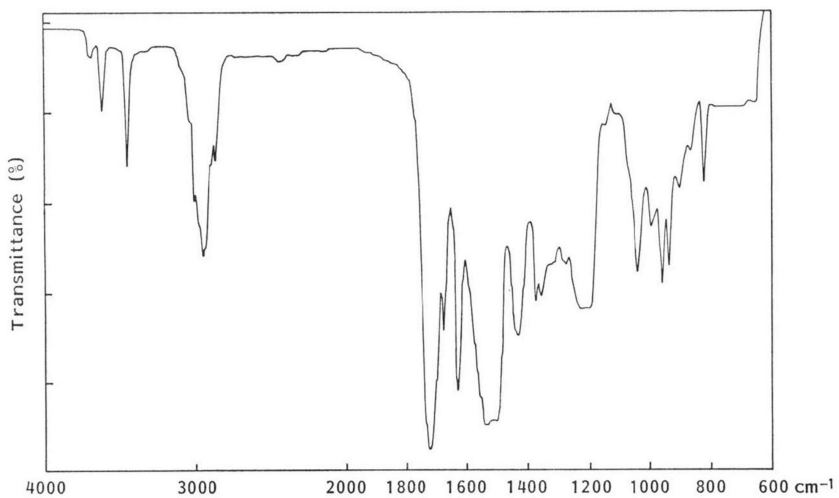


Table 2. The antibiotic spectrum of the different corallopyronins.

Test organisms	Corallopyronin*						
	A		A′		B		C
	0.5	5	0.5	5	0.5	5	5
Arthrobacter simplex	8	17	0	10	0	8	8
Micrococcus lysodeikticus	10	30	10	22	15	26	20
M. luteus	0	14	0	9	0	13	6.5
Nocardia corallina	0	11	0	9	0	10	0
N. flava	0	14	0	8	0	10	0
Corynebacterium mediolanum	7	17	0	12	0	13	9
Bacillus subtilis	0	12	0	9	0	8	0
B. polymyxa	15	26	10	20	14	23	16
B. megaterium	17	27	10	21	10	20	16
Mycobacterium phlei		0		0		0	0
Staphylococcus aureus	15	25	10	20	14	20	16
Brevibacterium ammoniagenes	0	10		0		0	0
Pseudomonas aeruginosa		0		0		0	0
P. acidovorans	0	7	0	7		0	0
Salmonella typhimurium		0		0		0	0
Proteus morganii		0		0		0	0
Klebsiella sp.		0		0		0	0
Rhizobium meliloti	16	28	10	21	14	25	17
Serratia marcescens		0		0		0	0
Aerobacter aeruginosa		0		0		0	0
Escherichia coli		0		0		0	0
E. coli tol C**	8	17	0	9			7
Schizosaccharomyces pombe		0		0		0	0
Saccharomyces cerevisiae		0		0		0	0
Nadsonia fulvescens		0		0		0	0
Debaryomyces hansenii		0		0		0	0
Pichia membranaefaciens		0		0		0	0
Hansenula anomala		0		0		0	0
Mucor hiemalis		0		0		0	0

* The antibiotics were applied on paper discs (0.5 and 5 μg/disc) of 6 mm diameter. The figures give the diameters of the inhibition zones.

** Mutant strain with increased permeability.

Table 3. Minimal inhibitory concentrations (MIC) of the different corallopyronins*.

Test organism	Corallopyronin (μg/ml)			
	A	A′	B	C
Micrococcus luteus	0.78	6.25	6.25	12.5
Bacillus subtilis	6.25	12.5	12.5	25
B. megaterium	0.39	1.56	3.1	6.25
Corynebacterium mediolanum	0.78	3.1		6.25
Staphylococcus aureus	0.097	0.78	0.39	0.78
Pseudomonas aeruginosa	125		>125	
Rhizobium meliloti	0.39	1.56		3.1
Escherichia coli	100	>100	>125	>100
Corallococcus coralloides Cc c127	50	50		>100

* The MIC values were determined by the serial dilution assay as described before[4].

Fig. 4. Effect of corallopyronin A on DNA synthesis in *Staphylococcus aureus*, measured as incorporation of [*U*-^{14}C]thymidine into perchloric acid insoluble material.

At the time indicated by the arrow the antibiotic (1 μg/ml) was added to the culture (○).

●: Control culture without antibiotic.

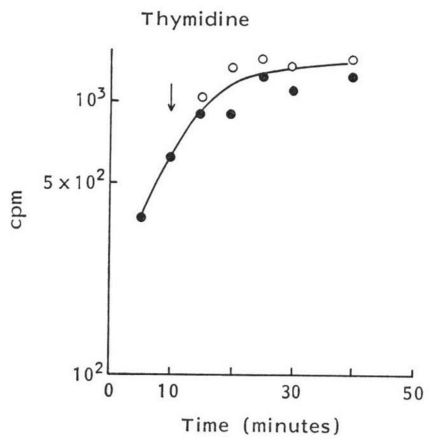


Fig. 5. Effect of corallopyronin A (1 μg/ml) on protein synthesis in *S. aureus*, measured as incorporation of [*U*-^{14}C]isoleucine into perchloric acid insoluble material.

Symbols as in Fig. 4.

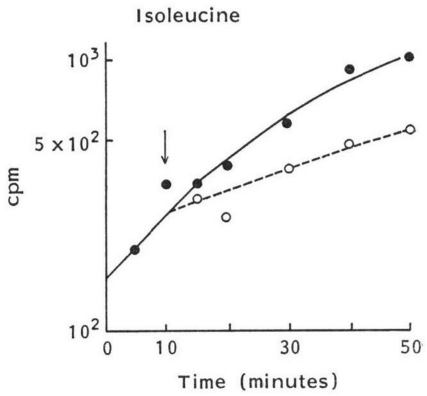


Fig. 6. Effect of corallopyronin A (1 μg/ml) on RNA synthesis in *S. aureus*, measured as incorporation of [2-^{14}C]uracil into perchloric acid insoluble material.

Symbols as in Fig. 4.

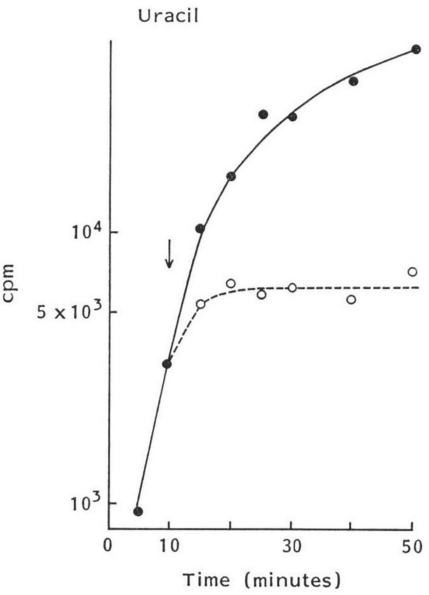
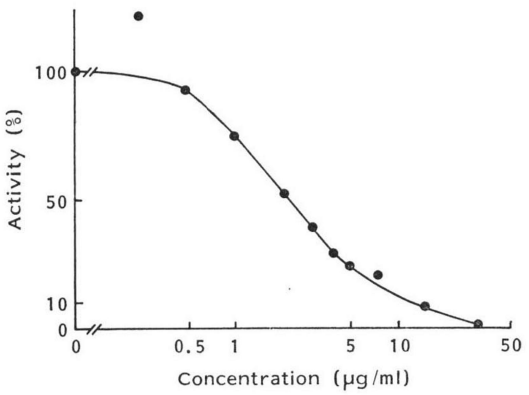


Fig. 7. Inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by corallopyronin A, dose response curve.

The incorporation of [4-^{14}C]UTP into TCA insoluble material was measured.



ous buffer: 0.5% formic acid in water, neutralized with triethylamine) yielding 460 mg of corallopyronin A and 40 mg of corallopyronin B. After each chromatographic step the buffer was removed by partition between dichloromethane and water.

The bioautograms of some crude extracts showed a third, more lipophilic biological activity. This substance eluted from the Florisil column in fraction 2 and was purified by reversed phase HPLC on a prepacked Lobar LiChroprep RP-8 column with methanol - aqueous buffer, 84: 16, as the eluant, followed by HPLC on silica gel (LiChrosorb Si 100, 10 μm, Merck, Darmstadt; column 25 cm × 16 mm)

Fig. 8. Kinetics of RNA synthesis by *E. coli* RNA polymerase.

After 6 minutes (arrow) the mixture was divided and corallopyronin A (20 μg/ml) added to one half (○). ●: Control without antibiotic. Enzyme activity was measured as in Fig. 7.

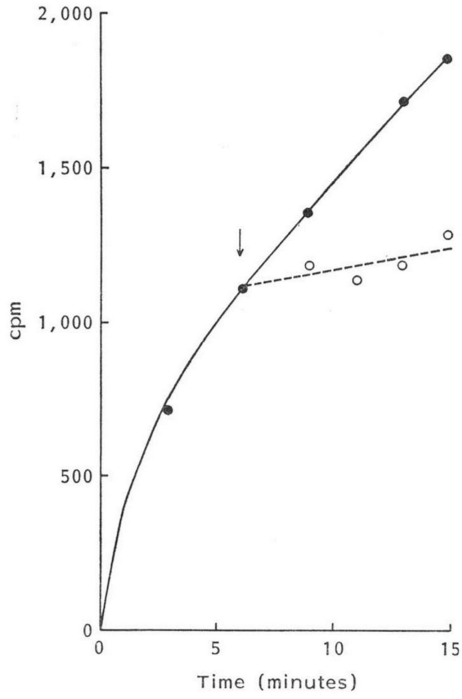


Table 4. The activity of wheat germ RNA-polymerase II in presence of corallopyronin A or α-amanitin*.

Corallopyronin A (μg/ml)	α-Amanitin (μg/ml)	Activity (%)
0		100
10		83
40		93
	1	0

* For experimental details, see text.

with 1% acetic acid in heptane - dichloromethane - ethanol, 92.5: 5: 2.5, as the eluant. This compound turned out to be another structural type of corallopyronin and was named corallopyronin C. All corallopyronins were obtained as colorless, highly viscous oils. Corallopyronin A', an isomer of corallopyronin A, was obtained from A by acid catalysis.

Physico-chemical Properties

The UV spectrum of corallopyronin A (Fig. 2) was recorded with a Zeiss DMR 21 spectrophotometer in ethanol as the solvent. The IR spectrum (Fig. 3) was measured in chloroform with a Perkin-Elmer 297 IR spectrophotometer. Corallopyronins A and C had the molecular formula $C_{30}H_{41}NO_7$, that of corallopyronin B was $C_{31}H_{43}NO_7$[3].

Biological Properties

As Tables 2 and 3 show the antibiotics act mainly against Gram-positive bacteria and, with few exceptions, only at much higher concentrations if at all against Gram-negative ones. Yeasts and molds are not affected. At concentrations of up to 100 mg/kg (sc) corallopyronin A showed no acute toxicity for mice.

Experiments were done with corallopyronin A to get information about its mechanism of action. Incorporation of labeled precursors into macromolecules was studied with *Staphylococcus aureus* as described recently[1], with the exception that leucine was replaced by isoleucine in the experiments on protein synthesis. The antibiotic was added in a concentration of 1 μg/ml.

The incorporation of thymidine was not affected by corallopyronin (Fig. 4), but protein and RNA synthesis were both inhibited (Figs. 5 and 6). While protein synthesis continued at a reduced rate for a considerable time after addition of the antibiotic, the incorporation of uracil stopped completely after 10 minutes.

To show that RNA polymerase is the primary target of corallopyronin, inhibition studies were performed with *Escherichia coli* RNA polymerase (E.C. 2.7.7.6; from Boehringer, Mannheim). The tests were done as described earlier[1]. As Fig. 7 shows, the enzyme was inhibited by 2 μg of coral-

lopyronin per ml by 50%. With 30 μg/ml, the inhibition was complete. Fig. 8 shows the kinetics of uridine incorporation into RNA by the enzyme. The reaction mixture was divided after 6 minutes. To one half corallopyronin A, dissolved in methanol, was added (20 μg/ml), to the other half an identical amount of methanol as a control. As can be seen, the synthesis of RNA is interrupted immediately after the addition of the antibiotic.

To see whether a typical eukaryotic RNA polymerase would be sensitive to corallopyronin A, wheat germ RNA polymerase II (Miles Laboratories, Elkhart, Indiana) was tested as described before[1]. Table 4 shows that the enzyme was not inhibited by up to 40 μg of antibiotic per ml.

Discussion

Elucidation of the chemical structure of the corallopyronins[3] (Fig. 1) showed that they are new antibiotics, and that they are closely related to the myxopyronins recently isolated from another myxobacterium[1,2]. Their antibiotic spectrum is also similar to that of the myxopyronins: Both substances act mainly against Gram-positive bacteria (Tables 2 and 3). There could be great differences in sensitivity even within one genus. Eukaryotic cells were completely resistant.

It seems that the side chain at carbon 3 of the pyrone ring is important for the activity of these antibiotics, because the MIC values change when this side chain is altered. Even slight changes may have a considerable effect, as becomes evident *e.g.* when the activities of corallopyronin A and its isomeric compound A′ (Fig. 1) are compared (Tables 2 and 3).

The molecular target of corallopyronin A is the same as that of myxopyronin: bacterial RNA polymerase. But there are remarkable differences in the efficiencies of the two inhibitors. With myxopyronin the inhibition of RNA synthesis was never complete, not even at very high concentrations, neither in growing cells of *Staphylococcus aureus* nor *in vitro* with isolated RNA polymerase from *Escherichia coli*. Corallopyronin A, in contrast, totally inhibited RNA synthesis in *S. aureus* cells at a concentration of 1 μg/ml, and isolated RNA polymerase from *E. coli* at 30 μg/ml (Fig. 7). This difference in efficacy reflects again the importance of the side chain at C-3, because there lies the only difference between corallopyronin and myxopyronin. Corallopyronin inhibits RNA synthesis even after chain elongation has started (Fig. 8), comparable to the mode of action of streptolydigin and pyran copolymer, but different from that of rifampicin[5,6,7]. As with myxopyronin, wheat germ polymerase II was not sensitive to corallopyronin (Table 3). It remains to be investigated where exactly the new inhibitors interfere with RNA biosynthesis.

Acknowledgments

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References

1) IRSCHIK, H.; K. GERTH, G. HÖFLE, W. KOHL & H. REICHENBACH: The myxopyronins, new inhibitors of bacterial RNA synthesis from *Myxococcus fulvus* (Myxobacterales). J. Antibiotics 36: 1651~1658, 1983
2) KOHL, W.; H. IRSCHIK, H. REICHENBACH & G. HÖFLE: Myxopyronin A und B – zwei neue Antibiotika aus *Myxococcus fulvus* Stamm Mx f50. Liebigs Ann. Chem. 1983: 1656~1667, 1983
3) JANSEN, R.; H. IRSCHIK, H. REICHENBACH & G. HÖFLE: Corallopyronin A, B und C – drei neue Antibiotika aus *Corallococcus coralloides* Cc c127 (Myxobacterales). Liebigs Ann. Chem., 1985, in press
4) KUNZE, B.; T. KEMMER, G. HÖFLE & H. REICHENBACH: Stigmatellin, a new antibiotic from *Stigmatella aurantiaca* (Myxobacterales). I. Production, physico-chemical and biological properties. J. Antibiotics 37: 454~461, 1984
5) SCHLEIF, R.: Isolation and characterization of a streptolydigin resistant RNA polymerase. Nature 223:

1068~1069, 1969
6) O'CONNOR, D. L. & S. A. KUMAR: Inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by pyran copolymer. Biochem. Intl. 6: 489~496, 1983
7) CASSANI, G.; R. R. BURGESS, H. M. GOODMAN & L. GOLD: Inhibition of RNA polymerase by streptolydigin. Nature 230: 197~200, 1971