Disorazol A, an Efficient Inhibitor of Eukaryotic Organisms Isolated from Myxobacteria*

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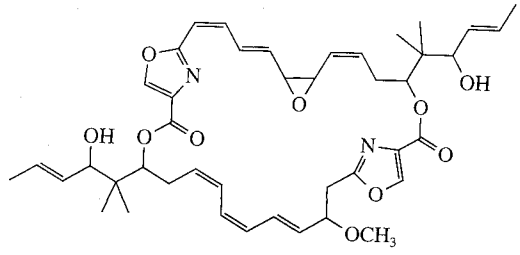
A new antibiotic, disorazol, was isolated from the culture broth of the myxobacterium, *Sorangium cellulosum* strain So ce 12. It is a macrocyclic compound containing two oxazole rings. The antibiotic acted against many fungi and mammalian cell cultures. The latter responded to extremely low doses (MIC 3~30 pg/ml). None of the tested bacteria and yeasts were inhibited.

During fermentation of *Sorangium cellulosum* strain So ce 12 for the isolation of sorangicin[1,2], we discovered in the XAD eluate a new substance by chemical screening. The substance was isolated and its chemical structure was elucidated (Fig. 1). It turned out to be a new compound consisting of a macrocyclic ring with two oxazole rings[3]. The substance showed remarkable biological activity against eukaryotic organisms. We propose the name disorazol.

Occurence and Production

Disorazol A was first detected in the *Sorangium cellulosum* strain mentioned above. During subsequent screening of other strains of this species, it was found that about 13% of the 208 strains tested produced disorazol A, in most cases together with other antibiotics. With the original producer, strain So ce 12, some efforts were made to improve the yield. A mutant with high sorangicin production also showed good yields of disorazol. The medium was: $MgSO_4 \cdot 7H_2O$, 0.15%; $CaCl_2 \cdot 2H_2O$, 0.1%; KNO_3, 0.2%; K_2HPO_4, 0.0125%; fructose, 0.5%; Na-Fe-III-EDTA, 8 mg/l (Merck, Darmstadt); peptone from casein, tryptically digested, 0.1% (Marcor); Tris · HCl, 0.2% (Sigma). The pH was adjusted to 7.4 before autoclaving. As with sorangicin, a high yield was only reached in the presence of the adsorber resin XAD-16 (Rohm and Haas, Darmstadt). In the late log to stationary phase of growth, a fed batch strategy was applied (250 ml Erlenmeyer flasks containing 100 ml of medium, cultivation under shaking, 160 rpm, 30°C). The following additions were made (per day): fructose, 0.3%; glycerol, 0.1%; peptone from casein,

Fig. 1. The chemical structure of disorazol A[3].



Disorazol A

Table 1. Production of disorazol A.

Culture conditions	Average production rate (mg/l.d)	Maximal yield (mg/liter)
Without fed batch		18
With fed batch	21	292
With fed batch + 0.1 mg sodium barbital/liter, added at early log phase ($OD_{623} = 1.4$)	2.7[a]	14
With fed batch + 0.1 mg sodium barbital/liter, added at late log phase ($OD_{623} = 10.9$)	2.3[a]	51

[a] After the addition of barbital.

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0.04%; potassium nitrate or nitric acid, depending on the pH of the culture, 7.5 mM, (in this way keeping the pH between 7.5 and 8.0). For determination of the production rate, all the XAD was removed from the culture, and new adsorber resin was added. After extraction of the XAD with methanol, the content of disorazol A was determined by TLC (HPTLC Silica gel F_{254}, Merck, Darmstadt; solvent system: dichloromethane/methanol = 85 : 15, Rf = 0.72). Quantities were determined by measuring the absorbance of spots at 270 nm with a thin layer scanner (Shimadzu CS-920). Pure disorazol A served as a reference. Table 1 gives the production rates and final yields of disorazol. In the given experiment, the production rate was 21 mg/l.d and the production phase could be maintained for 13 days. In other experiments, synthesis decreased after 5 to 10 days. During our studies on yield improvement it turned out that sodium barbital, which is known to stimulate streptomycin and rifamycin formation[4,5], inhibited disorazol synthesis. It had no effect on sorangicin formation. Table 1 shows that after the addition of

Fig. 2. Electronic absorption spectrum of disorazol A in methanol.

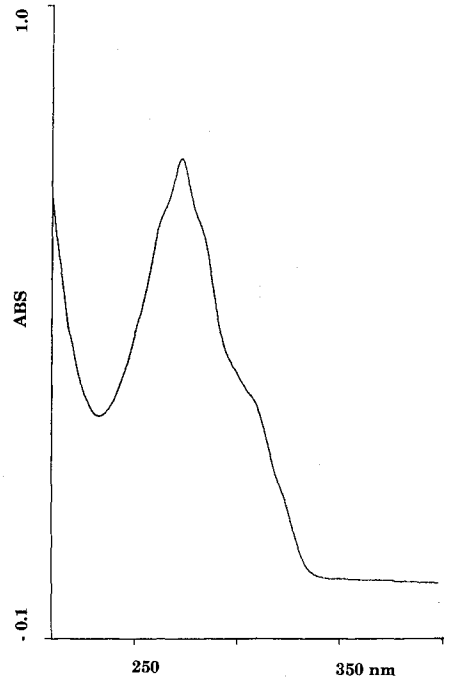


Fig. 3. IR spectrum of disorazol A in KBr.

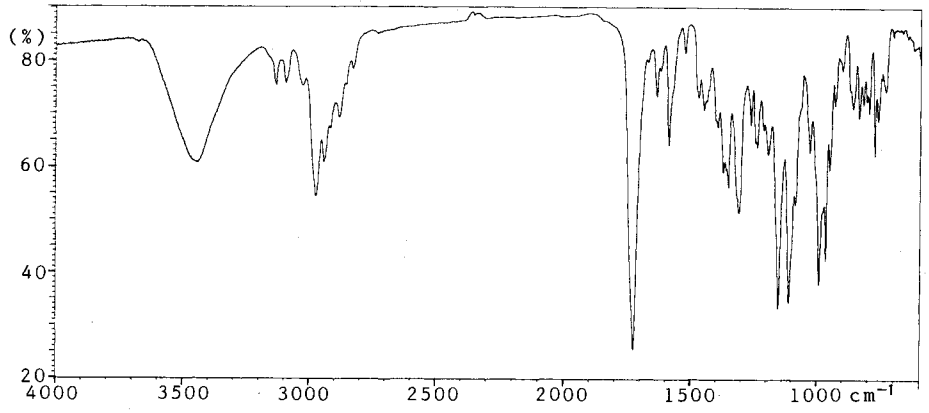


Table 2. The antibiotic spectrum and MIC values of disorazol A.

Test organism	Diameter of inhibition zones (mm) with 2 μg/disc[a]	MIC (μg/ml)	Test organism	Diameter of inhibition zones (mm) with 2 μg/disc[a]	MIC (μg/ml)
Mucor hiemalis	20	0.3	*Saccharomyces cerevisiae*	0	>50
Rhodotorula glutinis	22	0.16	*Candida albicans*	0	>50
Aspergillus niger	23	0.16	*Debaryomyces hansenii*	0	
Pythium debaryanum	20		*Hansenula anomala*	0	
Botrytis cinerea	23		*Bacillus subtilis*	0	
Fusarium oxysporum	27		*Staphylococcus aureus*	0	>50
Rhizopus arrhizus	22		*Micrococcus luteus*	0	
Trichoderma viride	35		*Streptococcus faecalis*	0	
Trichoderma harzianum	30	0.62	*Escherichia coli*	0	
Sclerotina sclerotiorum	22	0.5	*Salmonella typhimurium*	0	
Ustilago maydis	21	0.08			

[a] The antibiotic was applied to paper discs of 6 mm diameter. The media for the test organisms were as described elsewhere[6].

barbital the production rate decreased sharply.

Isolation and Physico-chemical Properties

For production of larger quantities, the organism was cultivated in bioreactors of 60 to 300 liter as described previously[1]. Briefly, the substance was isolated in the following way. The adsorber resin, XAD-16, which was present in the culture from the beginning, was separated from the broth and eluted with methanol. After partition of the extract between ethyl acetate and aquous ammonia, disorazol was detected in the organic phase as a distinct peak by HPLC with diode array detection. Several chemical variants could be separated and elucidated, with disorazol A as the main component. Figures 2 and 3 show the electronic absorption spectrum of disorazol A in methanol and the IR spectrum in KBr.

Biological Properties

Disorazol A was active against many filamentous fungi belonging to different taxonomic groups, but not against yeasts (Table 2). The MIC values for the sensitive organisms ranged from 0.1 to 1 μg/ml. The antibiotic was inactive against bacteria. On the other hand, it was extremely toxic for cultivated animal cells (Table 3). Disorazol was fungicidal, as shown with *Rhodotorula glutinis*. First, *Rhodotorula glutinis* was plated to get colonies from single cells. Then, 100 colonies were tested

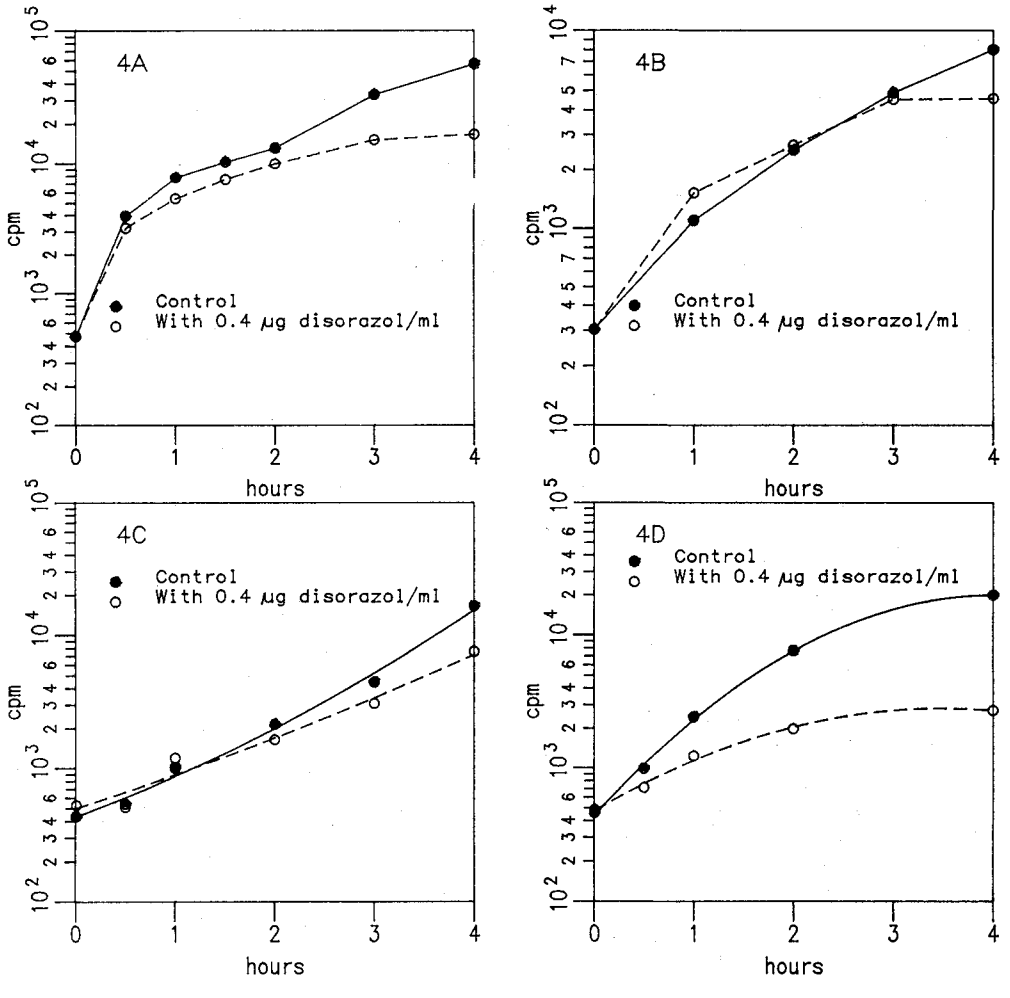
Table 3. MIC values of disorazol A for mammalian cells.

Cell line	MIC (ng/ml)
Mouse fibroblasts L 929	3×10^{-3}
HeLa cells ATCC CCL 2	30×10^{-3}

Fig. 4. Effect of disorazol A on macromolecule syntheses in *Rhodotorula glutinis*.

[4A] On protein synthesis; [1-^{14}C]methionine (specific activity 55 Ci/mol).
[4B] On cell-wall synthesis; *N*-acetyl-D-[1-^{14}C]glucosamine (specific activity 56 Ci/mol).
[4C] On DNA synthesis; [2-^{14}C]thymine (specific activity 55 Ci/mol).
[4D] On RNA synthesis; [2-^{14}C]uracil (specific activity 52 Ci/mol).

Measured as incorporation of a precursor (from Amersham, Braunschweig) into perchloric acid-insoluble material. The precipitated cells were collected on glass fiber filters (Whatman GF/A). Radio-activity was determined in a Beckman liquid scintillator LS 1801.



for growth on agar containing 2 × MIC of disorazol. All of them were sensitive to the antibiotic. One of these colonies was used for further experiments. The cells were suspended in MYC medium (Phytone peptone, Becton-Dickinson, 1%; glucose 1%) and incubated at 30°C on a rotary shaker with or without the antibiotic. The number of viable cells was determined by serial dilution and plating on MYC agar. In the presence of 2 times the MIC of disorazol, the number of viable cells had declined after two hours to 38% of the initial cell number. This reduction continued to about 0.2% after 24 hours. Of the remaining cells, 18% were resistant to disorazol. With 10 times the MIC, the killing effect of disorazol was only slightly greater.

The effect of disorazol on the synthesis of various cellular macromolecules is shown in Figs. 4A to 4D. During the first 3 to 4 hours, incorporation of methionine, thymine and *N*-acetyl-glucosamine into perchloric acid (PCA) insoluble material was only little influenced. The incorporation of uracil was inhibited immediately after the addition of the antibiotic. However, even with 10 times the MIC (data not shown), the inhibition of RNA synthesis was incomplete during the first two hours after the addition of the antibiotic. Table 4 shows the response of one of the three eukaryotic RNA polymerases, polymerase II (from wheat germ) to disorazol. The data indicate that this enzyme was not the target for disorazol.

The mutation rate for resistance against disorazol A was measured as followed: A sensitive culture of *Rhodotorula glutinis* with 2.5×10^7 cells/ml was incubated in MYC medium at 30°C. At different times, 0.1 ml of the culture was plated on nutrient agar containing 2 × MIC of disorazol and, after dilution, on agar without the antibiotic. The mutation rate was calculated as follows:

$$\frac{R_n - r_n}{TCN_n - TCN_m}$$

with: R = number of resistant colonies at time $t = n$

r = number of resistant colonies at $t = n$, calculated from the number of resistant cells at $t = m$ ($n > m$) under the assumption that the generation times of the resistant mutants are the same as that of the sensitive strain. This was confirmed with 10 resistant strains. The cell number at $t = m$ was multiplied with the quotient of

$$\frac{TCN_n}{TCN_m}$$

TCN = total cell number.

Table 4. Effect of disorazol A on wheat germ RNA polymerase II[a].

Inhibitor	Activity of the polymerase (cpm)	(%)
Control	17,370	100
Disorazol A, 8 μg/ml	18,898	109
Disorazol A, 128 μg/ml	17,664	102
α-Amanitin, 2.5 μg/ml	0	0

[a] From BIOZYM, Hameln, Germany. The tests were done according to the application sheet.

Table 5. Occurence of resistant mutants of *Rhodotorula glutinis*, and calculation of the mutation rate.

	A	B	C
Time (hour)	Cells/ml ($\times 10^7$)	Resistant cells/ml	Calculated number of resistant cells/ml
0	2.5	9	
2.5	4.6	24	16.56
6	14.35	86	51.66

	D	E	F
Time (hour)	B − C	$TCN_n - TCN_0$[a] ($\times 10^7$)	D/E ($\times 10^{-7}$)
0			
2.5	7.44	2.1	3.5
6	34.34	11.85	2.9

[a] Total cell number at time n minus total cell number at time 0.

The values of the different cell numbers are given in Table 5.

The average mutation rate was 3.2×10^{-7}.

The resistant colonies were transfered to nutrient agar without the antibiotic. After growth they were transfered again to plates containing 2 × MIC of disorazol and to plates with 20 × MIC. All cells formed colonies on both plates.

Discussion

Disorazol is a novel macrocyclic compound with two oxazol rings. It was first detected as a by-product of sorangicin fermentations and was found to be active against many fungi belonging to the Ascomycetes, Basidiomycetes, Zygomycetes, Oomycetes and Deuteromycetes (Table 2). Further, there was an extremely high toxicity for mammalian cells (Table 3). This toxicity exceeded that for fungi by 5 to 6 orders of magnitude and is among the highest toxicities ever recorded for a natural product. It appears that normal and transformed cells do not differ in this respect.

Measurements of macromolecular syntheses in *Rhodotorula glutinis* showed that RNA synthesis was inhibited first and to the greatest extent, but the inhibition was not complete, even with high concentrations of disorazol. The antibiotic appears not to influence DNA dependent RNA polymerase II. This suggests that disorazol interferes perhaps specifically with RNA polymerase I or III. The spontaneous mutation rate for disorazol resistance is rather high (Table 5). All of the tested mutants selected against $2 \times$ MIC were also resistant to $20 \times$ MIC. This indicates that resistance occured in a one-step mutation.

Acknowledgement

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