# The Ripostatins, Novel Inhibitors of Eubacterial RNA Polymerase Isolated from Myxobacteria†

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A new antibiotic, ripostatin, was isolated from the culture supernatant of the myxobacterium, *Sorangium cellulosum* strain So ce377. It is a macrocyclic lactone carbonic acid containing an unsubstituted phenyl ring in a side chain. The antibiotic acts especially on *Staphylococcus aureus*, but seems not to penetrate most bacteria.

The MIC values are in the range of 1 μg/ml. Ripostatin is an inhibitor of eubacterial RNA polymerase. It interferes with the initiation of RNA synthesis.

In our screening program we found in the culture supernatant of *Sorangium cellulosum*, strain So ce377 an activity against certain Gram-positive bacteria. The antibiotic complex contained two compounds. They were named ripostatin A and B.

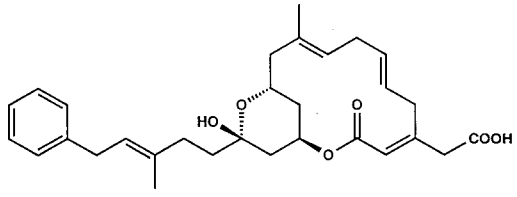
In this paper we describe the production and some chemical and biological properties. The structure elucidation is described elsewhere[1].

## Production of Ripostatin

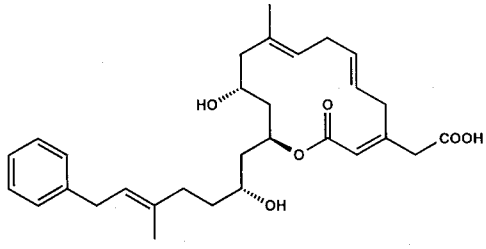
The producing organism was isolated by us in 1989 from a soil sample from Kenya.

In preliminary experiments in shake flasks, it was found that antibiotic synthesis occured during the growth phase (Table 1). The following medium was used: Soluble starch, 0.3%; $MgSO_4 \cdot 7H_2O$, 0.15%; $CaCl_2 \cdot 2H_2O$, 0.1%; $K_2HPO_4$ 0.0125%; Glucose·$H_2O$, 0.3%; Na-Fe-(III)-EDTA, 8 mg/liter (all from Merck, Darmstadt); yeast extract, 0.17% (Difco); HEPES, 0.1% (Serva); soya meal, 0.05%; pH 7.5. In order to obtain larger quantities, the antibiotic was produced in a fermentor containing 300 liters of medium and equipped with a circulating pump stirrer system (Giovanola Frères, Monthey, Switzerland). The medium used was as mentioned above, but soluble starch was replaced by potato starch, HEPES buffer was omitted, and 0.3% fructose and 0.01% pyruvic acid were added, because these substances had a positive effect on the production of ripostatin. Further, the adsorber resin XAD-16 (1%, v/v; Rohm and Haas) and silicone antifoam (0.1 ml/liter; Tegosipon, Goldschmidt AG) were added. The fermentation was started with 30 liters from a seed fermentor. The temperature was 30°C, the aeration rate 0.3 $m^3$ air/hour, the stirring rate was 300 rpm. After 65 hours the $pO_2$ had dropped from 100% to 30% saturation. It slowly decreased further to 15% until the end of fermentation 144 hours after inoculation. At this time the glucose was consumed, and 30 mg ripostatin/liter had been produced. All antibiotic was bound to the

Fig. 1. The chemical structure of ripostatins A and B.




Ripostatin A




Ripostatin B

† Article No. 68 on antibiotics from gliding bacteria. Article No. 67: IRSCHIK, H.; R. JANSEN, K. GERTH. G. HÖFLE and H. REICHENBACH: Sorangiolid A, a new antibiotic isolated from the myxobacterium *Sorangium cellulosum* So ce12. J. Antibiotics 48: 886~887, 1995.

adsorber resin. In other, small-scale fermentations potato starch was replaced with good results by wheat or rye flour, which were much cheaper than purified starch.

## Isolation and Some Physico-chemical Data

The XAD was separated from the culture, and the antibiotic was isolated as described elsewhere[1]. Briefly, the XAD was eluted with methanol. Ripostatin was isolated from this extract by preparative HPLC on silica gel RP 8. Fig. 1 shows the chemical structures of the ripostatins. Figs. 2 and 3 give the electronic absorption spectrum of ripostatin A in methanol and the IR spectrum in KBr.

## Biological Properties

The antibiotic spectrum is shown in Table 2. Ripostatin A and B were active only against a few bacteria. The two components had nearly the same inhibitory effects.

Table 1. Production of ripostatin A in Erlenmeyer flasks.

| Time (days) | $OD_{623}$ | pH | Glucose (%) | Ripostatin A ($\mu$g/ml)[a] |
|---|---|---|---|---|
| 0 (Start) | 0.21 | 7.5 | | |
| 3 | 0.7 | 7.1 | 0.25 | 0 |
| 4 | 1.4 | 6.9 | 0.15 | 2.1 |
| 5 | 2.6 | 6.9 | 0.15 | 5.8 |
| 7 | 4.5 | 6.5 | 0.1 | 12 |
| 8 | 4.8 | 6.9 | 0.05 | 25.4 |
| 9 | 4.1[b] | 7.0 | 0 | 28.1 |
| 10 | 3.6[b] | 7.0 | 0 | 23.2 |

[a] At maximal yield, the content of ripostatin B was about 1/10 of that of component A.
[b] Cells began to lump.
100 ml of medium in 250-ml flasks, cultivated on a rotary shaker at 160 rpm, 30°C. Medium as described in the text.

Yeasts and fungi were not influenced by ripostatin A. But component B showed minor activity against several yeasts and fungi. The MICs of ripostatin A and B against cultivated mouse fibroblasts were identical and rather high. The effect of ripostatin A on viability of *E. coli* tol C is shown in Fig. 4. The bacteria were cultivated in nutrient broth in the presence of ripostatin A. At different times, the cell suspension was diluted and plated on nutrient agar. After 24 hours incubation colonies were counted. As can be seen, the cell number decreased only slightly during the experiment.

The effect of ripostatin A on various macromolecular syntheses in *Staphylococcus aureus* is shown in Fig. 5. The experiments were done as recently described[2]. Ripostatin was added at the same time when the reaction was started with labeled precursors. DNA synthesis (Fig. 5A) was barely influenced during the 45 minutes of the experiment. Protein synthesis (Fig. 5B) was affected 15 minutes after the addition of the antibiotic, and RNA synthesis (Fig. 5C) was completely inhibited 15 minutes after application of ripostatin.

To confirm that RNA synthesis was the primary target, the effect of ripostatin on isolated RNA polymerase from *E. coli* (EC 2.7.7.6, Boehringer Mannheim) was studied (Figs. 6 and 7). The antibiotic blocked the enzyme with an $IC_{50}$ of 0.1 $\mu$g/ml. Complete inhibition was achieved at 50 $\mu$g/ml. Fig. 7 shows the kinetics of uridine-phosphate incorporation into RNA. The mixture was divided 3 minutes after the reaction had been started. To one half, 45 $\mu$g ripostatin A/ml was added, to the other one an equivalent volume of methanol. As can be

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

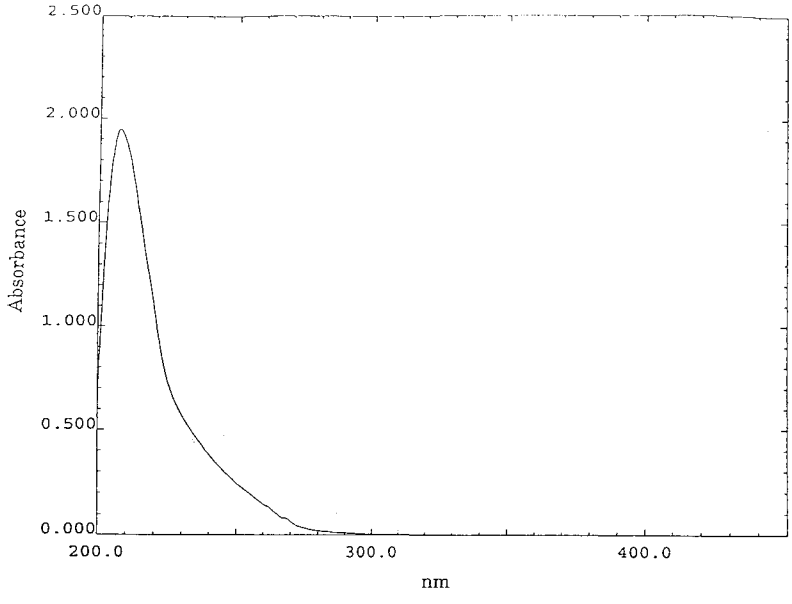


Fig. 3. IR spectrum of ripostatin A in KBr.

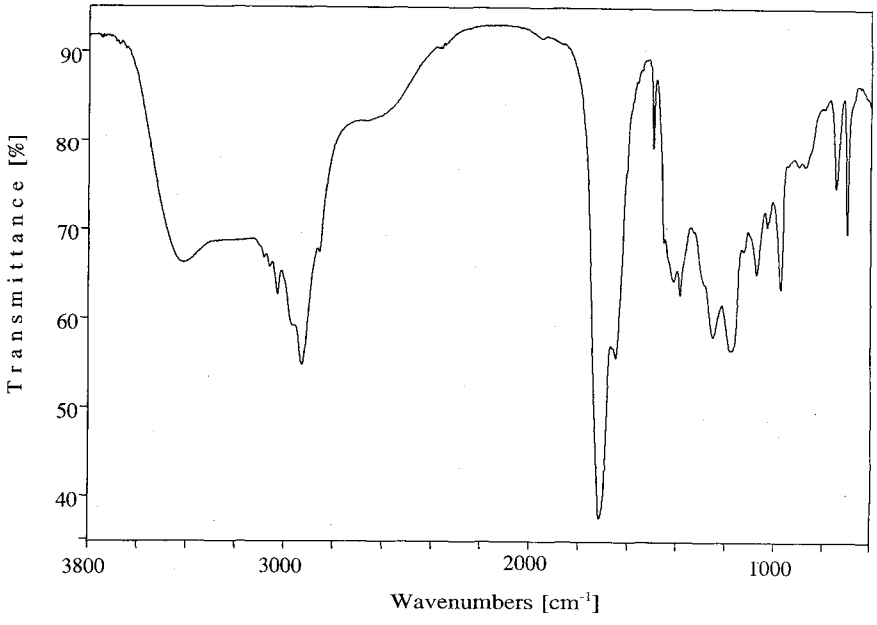


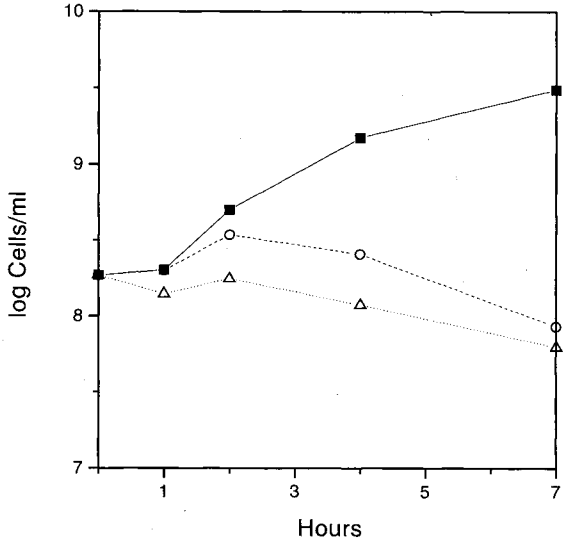

Table 2. Antibiotic spectrum of ripostatins A and B.

| Test strain | Diameter of inhibition zone with 10 μg/disc[a] | | MIC (μg/ml) | |
|---|---|---|---|---|
| | A | B | A | B |
| *Arthrobacter simplex* | 0 | 0 | | |
| *Nocardia corallina* | 10 | 0 | | |
| *Corynebacterium mediolanum* | 0 | 0 | | |
| *Mycobacterium lacticola* | 0 | 0 | | |
| *Bacillus megaterium* | 8 | 0 | | |
| *B. subtilis* | 0 | 8 | | |
| *Micrococcus luteus* | 0 | 8 | | |
| *Staphylococcus aureus* GBF | 27 | 27 | 1.25 | 1 |
| *S. aureus* DSM 799 | 25 | 25 | | |
| *S. aureus* DSM 20232 | 30 | 30 | 0.63 | 1.25 |
| *Escherichia coli* | 0 | 0 | | |
| *E. coli* tol C[b] | 20 | 21 | 2.5 | 1.25 |
| *Salmonella typhimurium* | 0 | 0 | | |
| *Serratia marcescens* | 0 | 0 | | |
| *Pseudomonas acidovorans* | 0 | 0 | | |
| *Candida albicans* | 0 | 0 | | |
| *Saccharomyces cerevisiae* | 0 | 0 | | |
| *Rhodotorula glutinis* | 0 | 0 | | |
| *Debaryomyces hansenii* | 0 | 10 | >80 | 80 |
| *Pichia membranaefaciens* | 0 | 8 | >80 | >40 |
| *Nadsonia fulvescens* | 0 | 10 | >80 | 20 |
| *Mucor hiemalis* | 0 | 10 | | |
| *Aspergillus niger* | 0 | 10 | | |
| *Trichoderma harzianum* | 0 | 0 | | |
| Mouse fibroblasts L 929 | 74 | 74 | | |

[a] Diameter 6 mm. Test conditions as described[2].
[b] Mutant with altered outer membrane.

Fig. 4. Effect of ripostatin A on viability of *Escherichia coli* tol C.

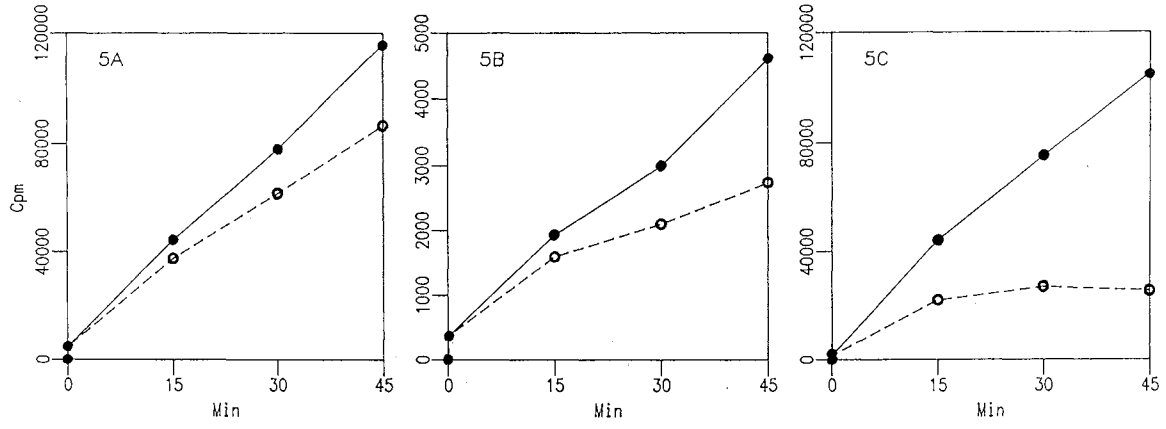
(■) Control, (○) with 2 × MIC, (△) with 10 × MIC.




seen the rate of RNA synthesis was only slightly reduced under these conditions. Table 3 shows the effect of ripostatin A on wheat germ RNA polymerase II. This enzyme is insensitive to the antibiotic up to at least 20 μg/ml. Table 4 gives the number of spontaneous mutants of *Staphylococcus aureus* resistant to ripostatin A and cross-resistance data for ripostatin and two other inhibitors of eubacterial RNA polymerase, *viz.*, rifampicin and sorangicin. *Staphylococcus aureus* was cloned and 100 clones were examined for sensitivity against

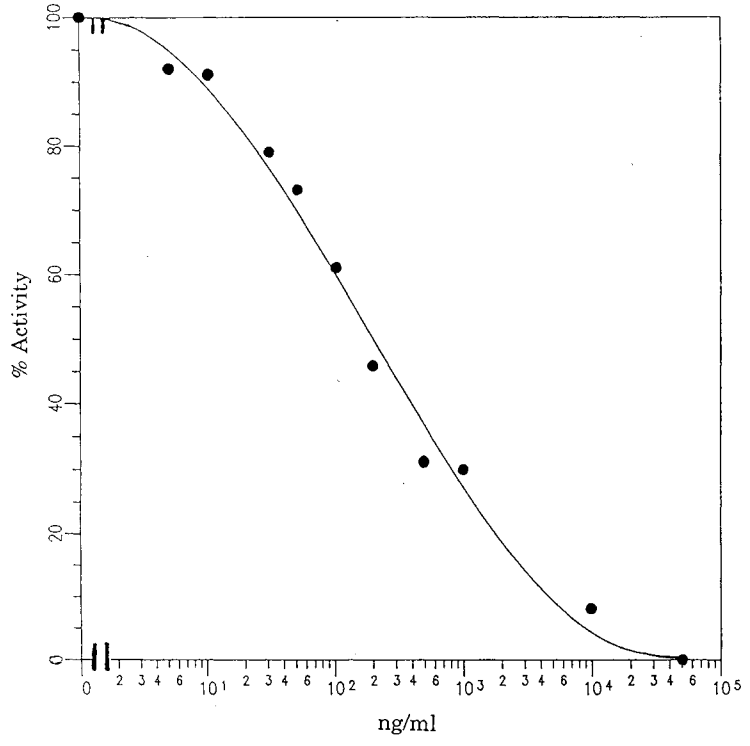
Fig. 5. Effect of ripostatin A on the syntheses of macromolecules by *Staphylococcus aureus*, measured as incorporation of precursors into perchloric acid insoluble material.

A: DNA synthesis with [methyl-$^{14}$C]thymidine (specific activity 56 Ci/mol, Amersham), B: Protein synthesis with [U-$^{14}$C]alanine (specific activity 165 Ci/mol), C: RNA synthesis with [2-$^{14}$C] uracil (specific activity 52 Ci/mol).




The precipitated cells were collected on glass fiber filters, Whatman GF/B. Radioactivity was measured in a Beckman liquid scintillation system LS 1801. Closed circles: control; open circles: with 8 $\mu$g ripostatin A/ml.

Fig. 6. Inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by ripostatin A, dose response curve.

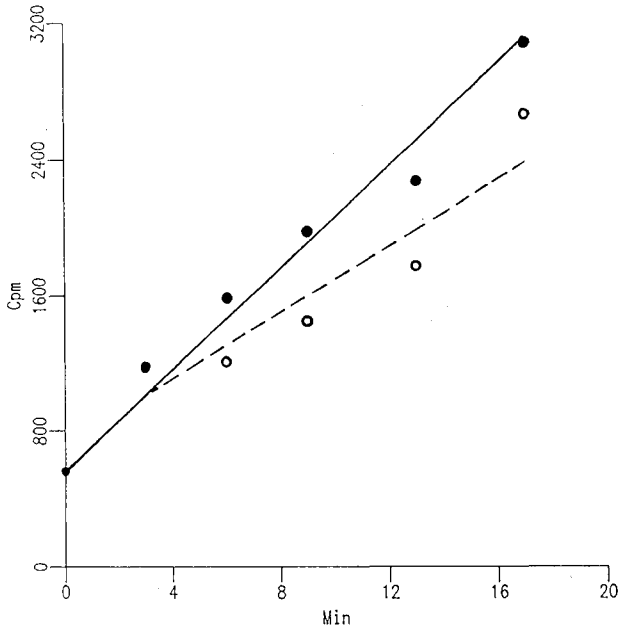



The incorporation of [5,6-$^3$H]UTP (specific activity 44 Ci/mmol) into perchloric acid insoluble material was measured.

25 × MIC of rifampicin and sorangicin and 10 × MIC of ripostatin. All of them were sensitive to the three antibiotics. Then 60 clones were selected and cultivated in liquid medium to about $10^9$ cells/ml. The cultures of clones 1 to 20 were plated on agar medium with 25 × MIC of sorangicin, of clones 21 to 40 on plates with 25 × MIC of rifampicin, and of clones 41 to 60 on plates with 10 × MIC of ripostatin. Resistant colonies were counted, and the spontaneous mutation frequency was calculated. Then one clone resistant to either one of the three antibiotics, respectively, was used for determination of cross-resistance. While there was a high cross-resistance between rifampicin and sorangicin, no cross-resistance was observed at all between ripostatin and rifampicin or

Fig. 7. Kinetics of RNA synthesis by *Escherichia coli* RNA polymerase in the presence and absence of ripostatin A with calf thymus DNA as the template.

● Control, ○ with 45 μg ripostatin A/ml.




The antibiotic was added 3 minutes after the polymerization reaction had been started.

Table 3. Effect of ripostatin A on wheat germ RNA-polymerase II[a].

| Antibiotic | UMP[b] incorporated (cpm) | Activity (%) |
|---|---|---|
| Control | 2187 | 100 |
| Ripostatin A, 2.5 μg/ml | 2629 | 117 |
| Ripostatin A, 20 μg/ml | 2123 | 97 |
| α-Amanitin, 2.5 μg/ml | 182 | 8 |

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

[b] From [5,6-$^3$H]UTP (specific activity 44 Ci/mmol, Amersham).

Table 4. Isolation of spontaneous mutants of *Staphylococcus aureus* resistant to rifampicin, sorangicin (25 × MIC each) and ripostatin (10 × MIC).

| Resistance to | Cross resistance to | | |
|---|---|---|---|
| | Rifampicin No. of (%) clones[a] | Sorangicin No. of (%) clones[a] | Ripostatin No. of (%) clones[a] |
| Rifampicin | | 16/19 (84) | 0/19 (0) |
| Sorangicin | 17/17 (100) | | 0/17 (0) |
| Ripostatin | 0/17 ( 0) | 0/17 ( 0) | |

[a] Resistant clones/clones tested.

Mutation frequency: rifampicin-resistant $7.0 \times 10^{-8}$, sorangicin-resistant $6.6 \times 10^{-8}$, ripostatin-resistant $2.9 \times 10^{-8}$.

## Discussion

The ripostatins are macrolides of a novel type. The inhibitory spectrum comprised only bacteria and was rather narrow. The reason for the resistance of most bacteria to ripostatin seems to be a penetration barrier and not the destruction of ripostatin. While yeasts and fungi were insensitive against component A, ripostatin B showed minor activity against some yeasts and fungi (Table 2). No differences between the components were seen in cell cultures, which were rather insensitive to the antibiotic, anyway.

The primary target of ripostatin was eubacterial RNA polymerase. Kinetic studies suggest that chain initiation rather than chain elongation of RNA synthesis was inhibited (Fig. 7). This is the same mechanism as is known for rifampicin[3] and sorangicin[2], but it is different from that of another myxobacterial RNA polymerase inhibitor, corallopyronin[4].

Recently a high cross-resistance between rifampicin and sorangicin has been shown with *E. coli*[5]. But ripostatin-resistant mutants of *Staphylococcus aureus* were not resistant to rifampicin or sorangicin, and *vice versa*. So the binding site of ripostatin seems to be well separated from those of rifampicin and sorangicin within the RNA polymerase.

After myxopyronin[6], the chemically related corallopyronins[4,7], and sorangicin[2], ripostatin is the fourth novel antibiotic isolated by us from myxobacteria, which acts specifically on eubacterial RNA polymerase. This is a rare mechanism of action, and its frequent occurence among the relatively few antibacterial substances of myxobacterial origin characterized so far is remarkable.

Acknowledgement

We thank Dr. F. SASSE for determining the toxicity in cell cultures. Mrs. I. KRISTEN and Mrs. A. THIES provided careful technical assistance.

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