METABOLIC PRODUCTS OF MICROORGANISMS. 207* HALOQUINONE, A NEW ANTIBIOTIC ACTIVE AGAINST HALOBACTERIA

I. ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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(Received for publication June 8, 1981)

Haloquinone, a new antibiotic produced by *Streptomyces venezuelae* ssp. *xanthophaeus* (Lindenbein) strain Tü 2115, was isolated from the mycelium (pH 4.5) by extraction with methanol and chromatography on acid-treated silica gel. The new compound, molecular formula $C_{17}H_{12}O_5$, has been isolated with respect to its activity against halobacteria; it also inhibits Gram-positive and to a smaller extent Gram-negative bacteria. The antibiotic has an effect on DNA synthesis.

In the course of our research for new antibiotics produced by actinomyces, we screened for activity against halobacteria. These, classified as archaebacteria, possess besides other characteristics a special kind of cell wall, containing a glycoprotein very similar to that of eucariotic cells[1~5]. Compared with *Bacillus subtilis* several strains of halobacteria showed a remarkably low sensitivity to most of the 50 known antibiotics tested (see below). These observation led us to search for other antibiotics active against halobacteria. In the following we describe the fermentation, isolation, physicochemical characterization and biological properties of such a new compound, named haloquinone. The determination of the structure is the subject of the following publication[6].

Fermentation and Isolation

Strain Tü 2115 was isolated in 1978 from a soil sample from the Peruvian jungle near Pucallpa and classified as *Streptomyces venezuelae* ssp. *xanthophaeus* (Lindenbein) according to BERGEY's Manual of Determinative Bacteriology (1974). The strain was grown on agar slants containing a yeast extract - malt extract medium (YM, 0.4% yeast extract, 1.0% malt extract, 0.4% glucose, 2.0% agar). Spores from the surface were taken to inoculate a Kolle-flask with a 70 ml YM medium (pH 7.3). After standing for 2 weeks at 27°C the spores were floated off with 100 ml water containing 2 drops of Tween 80. The spore suspension of two Kolle-flasks was filtered through glass wool and used as inoculum for a fermentor (Giovanola Fréres S.A., Monthey Swiss, Model 1976) holding 25 liters of a fermentation medium containing 2% soybean and 2% mannit (pH 7.5). Fermentation conditions were as follows:

* 206. ANKE, H.; T. KEMMER & G. HÖFLE: Deflectins, new antimicrobial azaphilones from *Aspergillus deflectus*. J. Antibiotics 34: 923~928, 1981

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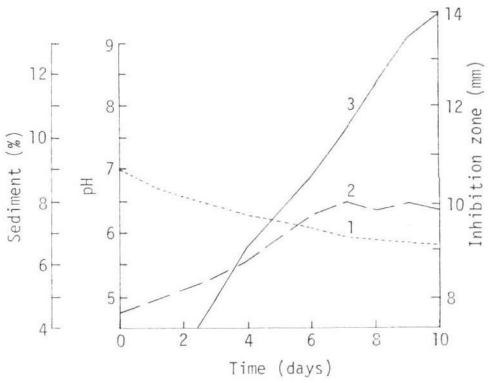
agitation 750 rpm, temperature 27°C, aeration 6 liters/minute. Activity against *Halobacterium cutirubrum* was assayed by agar diffusion tests using 6 mm paper disks. A typical fermentation profile is shown in Fig. 1. At day 10 the production of haloquinone was about 100 μg/ml.

Fig. 1. Time course of haloquinone production by *Streptomyces venezuelae* ssp. *xanthophaeus*.

Mycelial growth as % sediment (1), pH (2), inhibition zone against *Halobacterium cutirubrum* (3).



The fermentation broth (25-liters) was adjusted to pH 4.5 with 6 N hydrochloric acid and filtered using 2% celite. Haloquinone could only be found in the mycelium, which was thoroughly extracted 7 times with 2.5-liter portions of methanol. After concentration of the extracts *in vacuo* the water containing oily residue was extracted with chloroform. The red colored organic layer was washed with 5% Na_2CO_3 solution. The blue aqueous extracts were combined and reacidified to pH 4.5. The red solution which contained precipitated antibiotic was extracted with chloroform. A crude powder of haloquinone was obtained by evaporating the solvent layer dried with sodium sulfate (anhydrous). The crude material was chromatographed on a column of oxalic acid-treated silica gel (Macherey and Nagel, 0.07 mm) with chloroform. The fraction containing the red colored substance was concentrated *in vacuo* to a small volume of chloroform and added dropwise to *n*-pentane to precipitate the antibiotic. The amorphous powder that was obtained sublimes at 120°C/0.01 mm.

Test-Organisms and Media

Halobacteria were grown on ATCC-medium containing 25% NaCl, 1% $MgSO_4 \cdot 7H_2O$, 0.5% KCl, 0.02% $CaCl_2$, 1% yeast-extract, 0.25% trypton and 1.5% agar (pH 7). A seed culture of halobacteria was incubated in liquid ATCC-medium at 37°C under good aeration in a water-bath. After reaching an $OD_{578}=1.0$, 2.5 ml was added to 100 ml of ATCC-medium and agar plates were made using 17.5 ml portions.

Physicochemical Properties

Haloquinone is an indicator substance, red in acidic or neutral solution, blue in alkali. It is soluble in chloroform (5.3 mg/ml), less soluble in methanol (2.0 mg/ml), acetone (1.3 mg/ml) or in ethanol (0.4 mg/ml) and nearly insoluble in water or pentane. It is also soluble in 2 N NaOH or Na_2CO_3.

On TLC (Merck silica gel plates 60 F_{254}) haloquinone had Rf 0.32 (chloroform) and 0.55 (chloroform - acetone, 9: 1). Haloquinone melts at 226°C. The red solution of haloquinone in acetic anhydride changes to yellow when heated in the presence of sodium acetate and decolorizes if zinc dust is added. The blue solution of haloquinone in 2 N NaOH turns to yellow after addition of zinc dust and becomes blue again by oxidation with air-oxygen. Hydrogen peroxide decolorizes the blue alkali solution within a minute (irreversible reaction). The reactions characterize the antibiotic as a hydroxy quinone. This is also demonstrated by the UV spectra given in Table 1 and Fig. 2. The IR spectrum (Fig. 3) has characteristic absorption bands in the carbonyl region (1691, 1621 cm^{-1}). The molecular ion by MS-EI (*m/z* 296.068) and the elemental analysis fit the molecular formula $C_{17}H_{12}O_5$:

Calcd.: C, 68.92; H, 4.08; 2 *C*-methyl, 10.15 (%); MW 296.28
Found: C, 68.89; H, 4.08; *C*-methyl, 11.25 (%); MW 296.068.

Table 1. UV absorption bands of haloquinone (**1a**) in different solvents.

Solvent	Color	λ_{max} (ε) nm
Chloroform	red	522 (5,900), 398 (4,100), 287 (16,600), 280 sh, 244 (20,600)
Ethanol	red	512 (6,000), 395 (4,300), 284 (20,800), 277 sh, 259 sh, 232 (34,300), 208 (27,100)
0.01 N Ethanol - NaOH	blue	582 (11,100), 463 (4,300), 235 (39,800)
Pyroboracetate in acetic anhydride (heated)	green	568, 439, 422 sh, 317, 287
Conc. H_2SO_4	blue	675, 414, 308, 291, 238, 210

Fig. 2. UV spectrum of haloquinone (**1a**) in ethanol and in 0.01 N ethanolic NaOH.

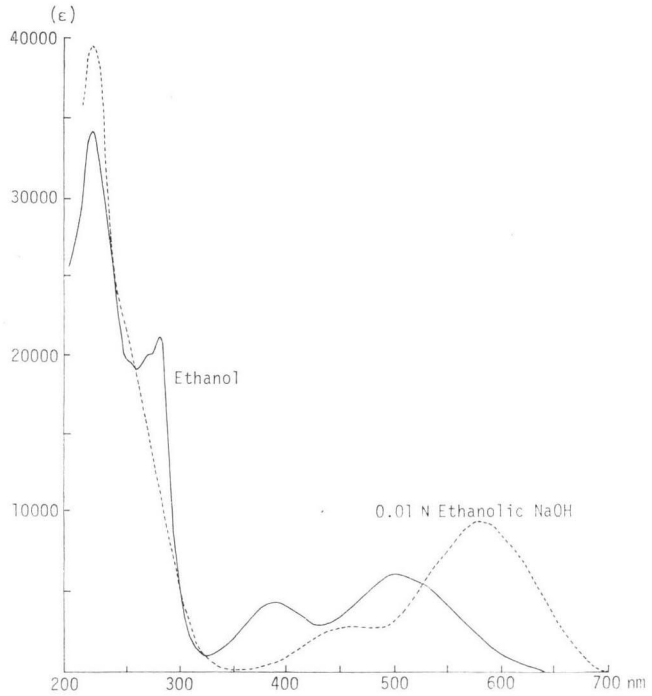
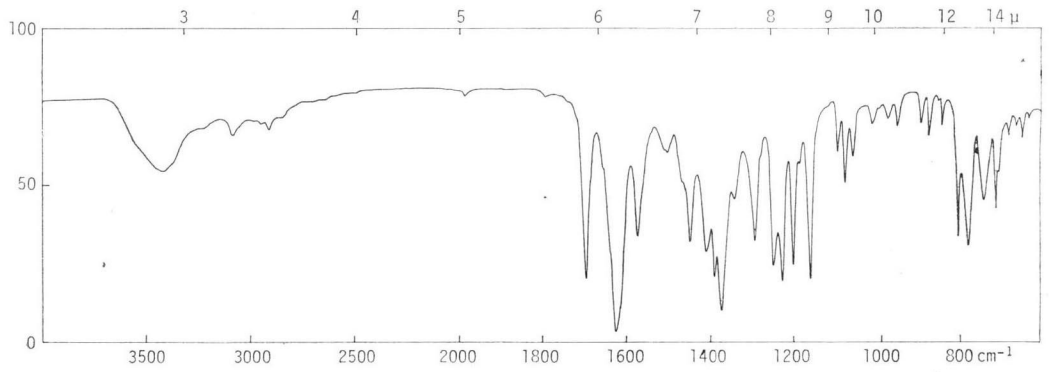
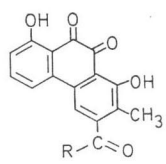


Fig. 3. IR spectrum of haloquinone (**1a**) in KBr.



The structure of haloquinone (**1a**)[6] is very similar to that of the known piloquinone (**1b**)[7].



1a R=CH_3

1b R=$CH_2-CH_2-CH(CH_3)-CH_3$

Biological Properties

The antimicrobial spectrum of haloquinone is shown in Table 2. Haloquinone is active against Gram-positive and less active against Gram-negative bacteria. It mainly acts against halobacteria, arthrobacteria and mycoplasmas. Insects and fungi of 15 genera remain unaffected. In many cases a bacteriostatic effect was observed first, and only after an increase in concentration of 10 to 100 fold, a bactericidal dose was reached. In agar diffusion tests the inhibition zones are quite small (8~20 mm) probably because of the poor diffusion properties of haloquinone.

Biophotometric tests with *Halobacterium cutirubrum* in complex and chemically defined media produced similar growth curves. By using an inoculum of cells in the stationary growth phase (*i.e.* resting cells) a concentration of merely 0.1 μg/ml haloquinone produced irreversible cell damage in chemically defined medium (Fig. 4a). When an inoculum of cells in the logarithmic growth phase was used, a 5-fold increase in the concentration of the antibiotic only resulted in an earlier leveling off of turbidity and a 10-fold increase produced cell damage after a short period of growth (Fig. 4b). Growth of *Halobacterium cutirubrum* on complex medium showed similar results. However, the required minimal inhibition concentrations were 5~10-fold higher (data not shown).

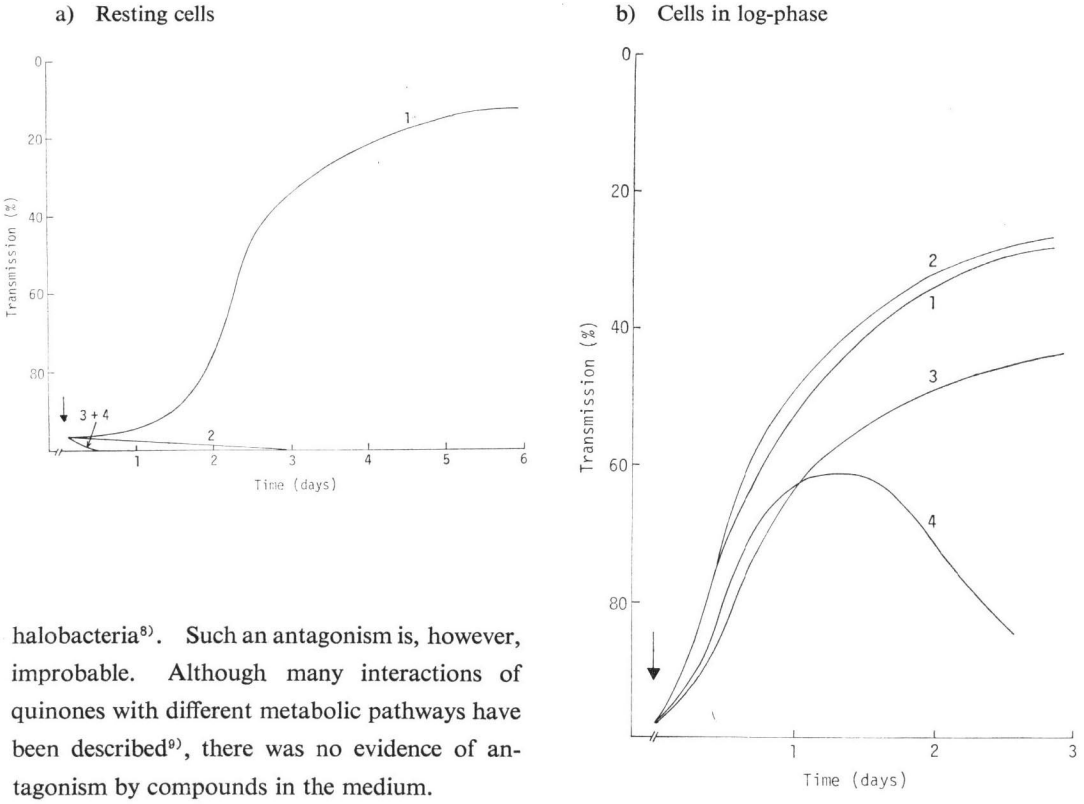
Any possible antagonism with haloquinone by substances in the medium is very difficult to ascertain due to the large number of essential compounds, required for the chemically defined medium of

Table 2. Antimicrobial spectrum of haloquinone in liquid medium.

Test organism	MIC (μg/ml)	MBC* (μg/ml)	Test organism	MIC (μg/ml)	MBC* (μg/ml)
Pseudomonas fluorescens	>100	>100	*Brevibacterium sterolicum*	>100	>100
Escherichia coli K 12	>100	>100	*Micrococcus luteus*	10	>100
Proteus vulgaris	1	50	*Arthrobacter aurescens*	<0.1	5
Proteus mirabilis	<0.1	5	*Arthrobacter citreus*	1	10
Achromobacter geminiani	0.5	100	*Arthrobacter crystallopoites*	0.5	5
Salmonella typhimurium	>100	>100	*Arthrobacter globiformis*	0.1	1
Alcaligenes faecalis	>100	>100	*Arthrobacter oxydans*	0.5	5
Mycoplasma pneumoniae	—	25	*Arthrobacter pascens*	0.1	1
Mycoplasma hominis	—	>100	*Lactobacillus casei*	25	>100
Mycoplasma pulmonis	—	1.56	*Lactobacillus brevis*	>100	>100
Mycoplasma gallisepticum	—	6.25	*Streptococcus faecalis*	5	>100
Mycoplasma laidlawii	—	>100	*Leuconostoc mesenteroides*	10	>100
Ureaplasma	—	>100	*Staphylococcus aureus*	10	>100
Propionibacterium freudenreichii	>100	>100	*Bacillus subtilis*	10	100
Mycobacterium sp.	0.1	1	*Bacillus brevis*	0.1	10
Nocardia brasiliensis	0.5	10	*Clostridium pasteurianum*	>100	>100
Corynebacterium rathayi	<0.1	10	*Halobacterium cutirubrum*	0.5	5
Corynebacterium poinsettiae	0.5	>100	*Halobacterium halobium*	1	10
Brevibacterium linens	10	100	*Halobacterium salinarium*	0.1	1
Brevibacterium flavum	10	>100			

* MBC: Minimal bactericidal concentration

Fig. 4. Effect of haloquinone on the growth of *Halobacterium cutirubrum* in minimal medium.
The arrow marks the application of the antibiotic. 1=control, 2=0.1 μg/ml, 3=0.5 μg/ml, 4=1 μg/ml of the antibiotic.



halobacteria[8]. Such an antagonism is, however, improbable. Although many interactions of quinones with different metabolic pathways have been described[9], there was no evidence of antagonism by compounds in the medium.

Antibiotic Sensitivity of Halobacteria

A main difference of halobacteria compared with eubacteria and even other archaebacteria is the structure of the cell wall, which contains no murein or pseudomurein[10] but instead a complex glycoprotein very similar to that of eucariotic cells. Surprisingly most of the 50 antibiotics tested showed no or only traces of inhibitory effects against halobacteria in agar diffusion tests up to concentrations of 1 mg/ml. Among these were known inhibitors of nucleic acid and protein synthesis, of energy metabolism and as expected of cell wall synthesis: actinomycin D, alanyl-aminoethyl-phosphonic acid, albomycin, antimycin A, cephalosporin C, chloramphenicol, chlorothricin, cladosporin, cycloserine, desferritriacetylfusigen, elaiophyllin, erythromycin, filipin, gentamicin, imacidin, ketomycin, kirromycin, kirrothricin, lankamycin, α-lipomycin, lysolipin I, γ-naphthocyclinon, neomycin, nigericin, nikkomycin, nonactin, nystatin, penicillin G, phosphinothricyl-alanyl-alanine, rubromycin, sepedonin, streptolydigin, streptomycin, streptothricin, tetracycline, tirandamycin B, tylosin, valinomycin, venturicidin X.

A moderate inhibitory effect (zone of inhibition from 12 to 20 mm) was shown by aranciamycin, boromycin, cerulenin, ethericin A and tetracenomycin C. Avilamycin, cinerubin A and rifampicin showed good activity (zone of inhibition from 20 to 30 mm). The only potent inhibitors (inhibition zone up to 48 mm) were bacitracin, granaticin A and mitomycin C. When EDTA was added to the medium, no inhibition or increased inhibition was achieved with the inactive or weak antibiotics (actinomycin D, chloramphenicol, erythromycin, neomycin, streptomycin and tetracycline), but only with those

Fig. 5. Effect of haloquinone on DNA, RNA and protein synthesis of *Halobacterium cutirubrum* cells.
1=control, 2=1 μg/ml haloquinone. The arrow marks the application of the antibiotic.

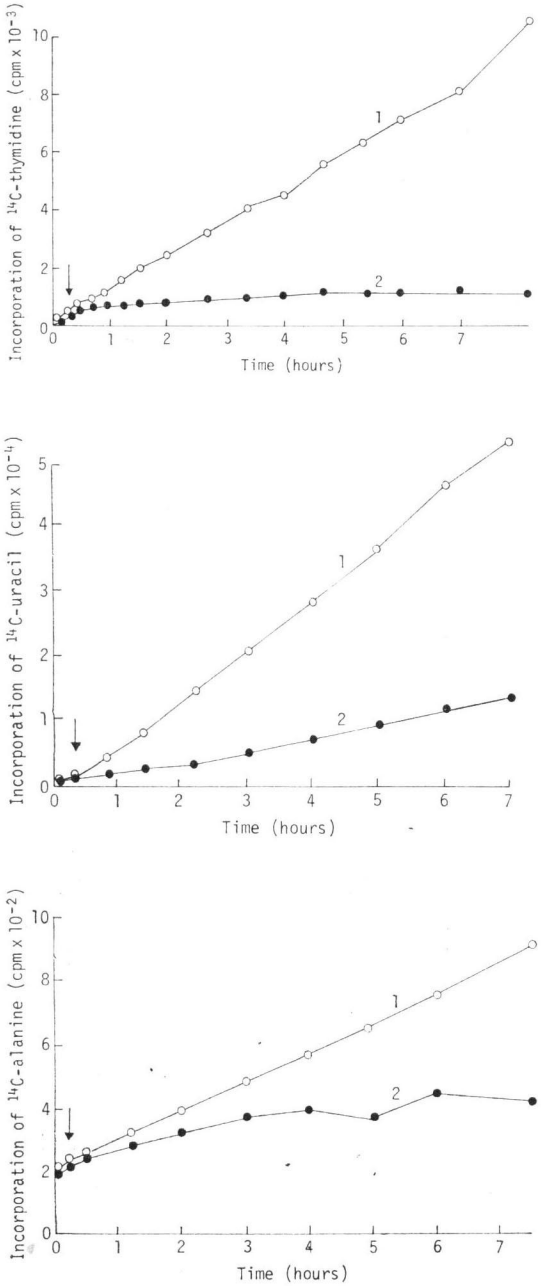
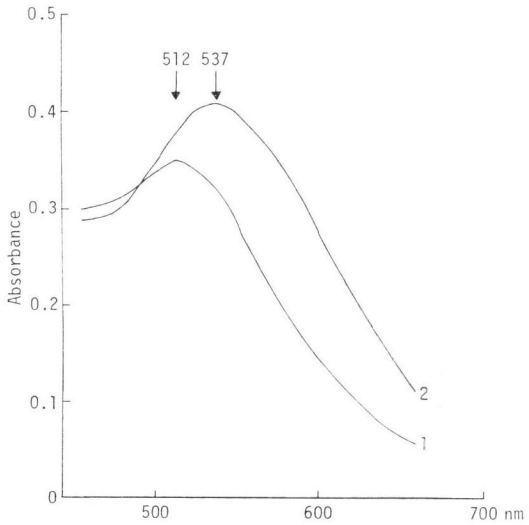


Fig. 6. Shift of the absorption maximum of haloquinone after incubation with calf thymus DNA.
1=haloquinone in buffer, 2=haloquinone in buffer with DNA.



that were already strongly active before. This indicates that a different permeation barrier of halobacterial compared with other bacterial outer membranes is perhaps less important.

The strong inhibitory effect of bacitracin must be explained by its target site—the polyisoprenoid carrier. This molecule allows the transport of cell wall precursors across the cell membrane in halobacteria as in all other bacteria[4,10,11]. The other two potent inhibitors belong to the chemical class of quinone antibiotics and interfere with nucleic acid metabolism[9].

Mode of Action

Fig. 5 shows the effect of haloquinone on the DNA, RNA and protein synthesis in *Halobacterium cutirubrum* cells. At a concentration of 1 μg haloquinone per ml, incorporation of ^{14}C-thymidine is reduced immediately to 10~12% of the control, incorporation of ^{14}C-uracil to 25% whereas incorporation of ^{14}C-alanine is only affected after 45~60 minutes and reduced to 45% of the control. This means that DNA or perhaps RNA seem to be the first target of the antibiotic action.

To test a direct interaction of haloquinone with DNA, changes in the absorption maximum in the

visible light region were measured[12]. When incubated with calf thymus DNA in buffer solution (pH 7.8) the absorption maximum of haloquinone at 512 nm was shifted about 25 nm to 537 nm (Fig. 6). This red shift is a strong indication that the DNA is the primary target of haloquinone and suggests that an interaction (or even a covalent bonding) with the double helix strands occurs.

An interaction between an antibiotic and DNA suggests the possibility of antitumor activity. However, at present, haloquinone has not shown any antitumor properties, possibly because of its relatively high toxicity.

A further discussion of the biological properties of haloquinone is presented in reference 13. The structurally related piloquinone (**1b**) is only weakly active against halobacteria.

Acknowledgement

For a kind gift of piloquinone we thank Prof. Dr. E. LEDERER, Laboratoire de Biochemie, CNRS, Gif/Yvette, France. We thank the Deutsche Forschungsgemeinschaft for generous financial support.

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