GUNACIN, A NEW QUINONE ANTIBIOTIC FROM *USTILAGO* SP.

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In a screening program for antibiotics which were antagonized by cysteine, a strain, which was characterized as *Ustilago* sp., was found to produce a new quinone antibiotic, gunacin. The molecular weight $M^+=348.084$ determined by mass spectroscopy, corresponds to a molecular formula of $C_{17}H_{16}O_8$. Further spectroscopic data prove that gunacin is a new antibiotic. The antibiotic possesses a good inhibitory effect against mycoplasmas and Gram-positive bacteria including multi-resistant strains. It also possesses a weak activity against Gram-negative bacteria with the exception of *Proteus vulgaris*, which is more strongly inhibited. The main activity against fungi is found against *Trichophyton mentagrophytes*. Gunacin shows an inhibition of the DNA synthesis *in vivo*, is antagonized by mercapto compounds and possesses an acute toxicity of $LD_{50}=16$ mg/kg i.p. and $LD_{50}=12$ mg/kg i.v. in mice. Against HeLa-cell the antibiotic shows an $ED_{50}=12.11$ μg/ml. Thirty five μg/ml of gunacin induces 1,063 interferon units.

Antibiotics which contain a quinone ring system are synthesized by a wide range of organisms such as higher plants[1,2] and microorganisms[3~11]. These antibiotics interact in the regulation of RNA synthesis[12,13], and also cross-link with DNA molecules[14]. These properties confer an antitumour[15,16] or interferon-inducing activity[17] upon the quinone antibiotics. Cysteine antagonizes the antimicrobial activity of the quinone antibiotics. This fact can be applied in a screening program for antibiotics with a presumed antitumour activity.

The present paper deals with the taxonomy of the producing strain, and the isolation, characterization and the biological activities of gunacin, a new quinone antibiotic.

Taxonomic Studies

The producing strain BC 5317 was isolated from a soil sample from the island Raab in Jugoslavia and maintained as an agar slant culture on a medium containing 4% malt extract and 1.5% agar. The characterization of the strain was carried out according to VON ARX[18] by morphological studies of the growing cultures on malt agar consisting of 2% malt extract and 1.5% agar and on oatmeal agar with 2% oatmeal and 1.5% agar. The strain possesses yeast-like colonies, which are slimy, grey-brown with hyaline septed hyphae, but without a true mycelium. On oatmeal agar clamp connections are sometimes observed. According to these characteristics, the strain is determined to be *Ustilago* sp.

Fermentation

As a production medium for gunacin, CZAPEK-DOX broth with 30 g saccharose, 3 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $Fe_2SO_4 \cdot 7H_2O$ in 1 liter distilled water at pH 7.3, and Goldschmidt Silicon K 1435 as an antifoaming agent was used. Standard fermentations in Giovanola b 20 fermentors were carried out under the following conditions: two agar plates were inoculated with

the producing organism and incubated at 28°C for 8 days. From these plates, a spore suspension was obtained by washing each plate twice with 250 ml of sterile physiological NaCl solution. The fermentor containing 25 liters of the production medium, was inoculated with 1 liter of the spore suspension. The fermentation was conducted at 28°C with an agitation of 400 rpm and an aeration of 5 liters/min. Gunacin in the fermentation broth was analysed every 12 hours by the paper disc-plate method using *Escherichia coli* ATCC 27166 as a test organism having high sensitivity to gunacin. After a 13-day fermentation period, the mycelium was separated by filtration on Hyphlo Super Celite. The yield of the antibiotic was 100 mg/liter fermentation broth.

Isolation

The filtered fermentation broth was adjusted to pH 3.0 and extracted twice with 0.5 volume of chloroform. The combined extracts were concentrated under reduced pressure and a crude powder was obtained. The further purification was a column chromatography on silica gel with a solvent system of chloroform - methanol (100: 0.5). The active fractions were combined and concentrated under reduced pressure to dryness to give a purified orange powder of gunacin.

Physical and Chemical Properties

Gunacin possesses Rf values of 0.3 and 0.46 in a thin-layer chromatography system with silica gel 60 F_{254} (Merck, Darmstadt) and chloroform - methanol (100: 2) as a solvent system. A two dimensional chromatography in the same solvent system confirmed that the two Rf values of gunacin are the result of two isomers which are in equilibrium. The antibiotic was detected as the visible orange spots and by bioautography using *E. coli* ATCC 27166 as test organism.

The structural formula of gunacin is based on spectroscopic data (Table 1).

The high resolution mass spectrum gave a peak for the molecular ion of *m/e* 348.084, which corresponds to a molecular formula of $C_{17}H_{16}O_8$. Acetylation was carried out with acetic anhydride for 6 hours at room temperature followed by chromatography on silica gel with a solvent system of chloroform - methanol (100: 0.5). This yielded a mono- and diacetyl derivative of gunacin, which was demonstrated by the corresponding mass spectra and structural formula. After saponification of the antibiotic with 4.5 N methanolic HCl for 8 hours at reflux temperature, a product was obtained which possessed a molecular ion of 306.075 and a molecular formula of $C_{15}H_{14}O_7$. This implied that one acetyl group was saponificated in the antibiotic. Therefore the data from the mass spectra indicated that two hydroxyl groups and one acetyl group were present in the molecule.

A comparison of the characteristic UV peaks at 228, 267, 311~317 and 430~445 nm with the UV spectra of other naphtoquinone derivatives showed similarity to 3,6,8-trihydroxy-1,4-naphtoquinone or methoxy-1,4-naphtoquinone[19]. This indicated the gunacin structure in the aromatic ring system. Because in the monoacetyl derivative of the antibiotic the UV spectra remained unchanged, the acetylated hydroxyl group was not located in the phenolic ring system. In contrast, the diacetyl derivative provided a UV spectrum which was shifted to a shorter wavelength and which was unchanged in 1% 1 N NaOH in ethanol. This indicated, that the second acetylated hydroxyl group was situated on the phenolic ring system. The UV spectrum of the saponificated antibiotic is identical with that of the untreated gunacin. This signified that the acetyl group is linked to an alcoholic hydroxyl group.

The IR spectrum of gunacin demonstrated an ester or lactone group with a peak at 1730~1740

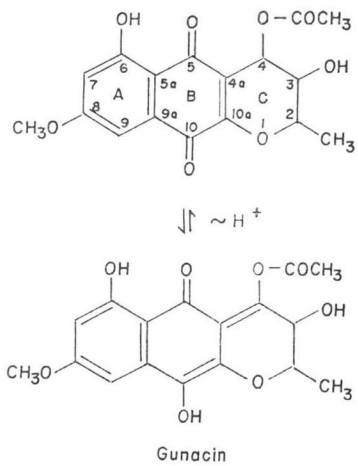
Table 1. Physicochemical properties of gunacin.

Physicochemical parameter	Physicochemical values			
	Gunacin	Saponificated gunacin	Acetylgunacin	Acetylgunacin
Nature	Orange powder	Orange powder	Orange powder	Orange powder
MS-spectra molecular ion (*m/e*)	348.084	306.075	390	432
Molecular formula	$C_{17}H_{16}O_8$	$C_{15}H_{14}O_7$	$C_{19}H_{18}O_9$	$C_{21}H_{20}O_{10}$
UV-spectra				
λ_{max}^{EtOH} nm	228, 267, 311～317 430～445	228, 267, 311～317 430～445	228, 267, 311～317 430～445	262, 295, 320～345
$\lambda_{max}^{1N\ KOH}$ nm (1%)	238, 290 310～320 520～545	238, 290, 310～320 520～545	238, 290, 310～320 520～545	262, 295, 320～345
IR-spectra				
ν cm^{-1} (KBr)	1730～1740, 1635, 1250, 1000	—	—	—
NMR-spectra				
δ ppm	7.17 1Hd J=2.5 Hz 6.65 1Hd J=2.5 Hz 6.2 1Hd J=5.0 Hz +1.2 H 4.4 1H m 4.15 1H m 3.9 3H s 2.1 3H s 1.7 3Hd J=6.0 Hz +1 OH	7.2 6.6 4.9 4.4 4.1 3.9 — 1.7	7.2 6.6 6.35 4.5 5.4 3.9 2.1 2.15 3H 1.7	7.2 6.6 6.35 4.5 5.4 3.9 2.1 2.15 2.4 3H 1.7

cm^{-1}, but no phenolic ester. The peak at 1635 cm^{-1} was explained by an α, β unsaturated carbonyl function. The signal between 1250 and 1000 cm^{-1} was due to an ether or hydroxyl group.

The NMR spectra confirmed the methoxy group at the phenolic ring with a signal at 3.9 ppm and the acetyl group at 2.1 ppm. The signal 7.2 ppm was due to the proton in 9-position; the signal 6.6 ppm represented the proton in 7-position of the phenolic ring. The cyclization of the lactone ring was explained by the lack of a olefinic proton signal at the aromatic ring system in 10a-position and also by the lack of a CH_2 signal between 2.5～3.0 ppm. The signal at 6.2 ppm was due to a proton between an ester oxygen and an olefinic or aromatic double bond. The 4.4 ppm multiplet was caused by the position of a proton at an aryl

Fig. 1. Structural formula of gunacin.



Gunacin

ether and an aliphatic carbon atom in the neighbourhood of a methyl group indicated the proton in 2-position. The 4.1 multiplet was coupled to the CH groups in 2- and 4-position. The signal at 1.7 ppm corresponded to the methyl group substituted in 2-position.

The broadened proton signals in ring C indicated the isomeric structure and explained the keto-enol tautomerism. From these spectroscopic data the following structure was suggested (Fig. 1).

Although the spectroscopic data were compared with those of known quinone antibiotics such as aquamycin, bostrycin, deoxyfrenolicin, frenolicin, granaticin, griseusin, julimycin, kalafungin, kinamycin, nanaomycin, SS-228 Y and tetrangomycin, no agreement could be determined between gunacin and the known quinone antibiotics.

Biological Properties

In order to determine the minimal inhibitory concentration (MIC) of gunacin, a wide range of microorganisms, including multi-resistent strains were investigated in the agar diffusion test. The test organisms were obtained from ATCC. The *Escherichia coli* strains DB 10, 222, 57b were kind gifts of Dr. NAOMI DATTA of the Bacteriology Department, Royal Postgraduate Medical School, London. Other strains were derived from clinical isolates. The agar medium for the bacteria consisted of 10 g peptone, 8 g meat extract, 3 g NaCl, 2 g Na_2HPO_4 and 15 g agar in 1,000 ml distilled water (pH was adjusted to 7.2); except for the staphylococci strains the agar medium contained additionally 10 g of glucose. For the fungi a SABOURAUD-agar was used consisting of 10 g peptone, 40 g glucose, 1 g NaCl, 1 g Na_2HPO_4 and 15 g agar. *Mycoplasma* was tested on agar plates containing 35 g PPLO-agar, 5 g Vitambact (Vitam GmbH, Hameln), 10 g yeast extract, 200 ml beef serum per liter, pH 7.6.

As can be seen from Table 2 the antibiotic possesses a strong inhibitory effect against Gram-positive bacteria and mycoplasma, and a relatively good effect against *Proteus vulgaris* and *Trichophyton mentagrophytes* (Table 2).

Table 2. Antimicrobial activity of gunacin determined by the minimal inhibitory concentration (MIC) in the agar dilution test.
APC=ampicillin, CER=cephaloridin, CP=chloramphenicol, MCI-PC=cloxacillin, EM=erythromycin, FA=fusidic acid, MTC=methicillin, NB=novobiocin, PC-G=penicillin G, TC=tetracyclin, TYC=tyrothricin

Test organisms	Resistance	MIC (μg/ml)
Staphylococcus aureus SG 511		0.025
Staphylococcus aureus E88 R1000	PC-G, APC, MCI-PC, MTC, TC, CER	0.080
Staphylococcus aureus 80R 580	PC-G, APC, MCI-PC, MTC, TC, EM	0.095
Streptococcus Aronson		0.070
Streptococcus pyogenes ATCC 8668		0.020
Escherichia coli ATCC 9637		8.0
Escherichia coli DB 10		1.25
Escherichia coli 222	CP, APC, NB, PC-G, FA, TYC	9.5
Escherichia coli R 57 B	CP, TC, FA, APC, NB, TYC	18.0
Proteus vulgaris		1.25
Serratia marcescens ATCC 13 880		9.5
Klebsiella pneumoniae ATCC 10 031		50.0
Pseudomonas aeruginosa Hbg.		50.0
Candida albicans ATCC 10 231		10.0
Trichophyton mentagrophytes ATCC 9129		1.0
Aspergillus niger		2.0
Mycoplasma pneumoniae ATCC 15 531		0.25

Table 3. Influence of beef serum on the MIC against *Streptococcus pyogenes* ATCC 8668 in the agar dilution test.

Beef serum (%)	MIC (μg/ml)
—	0.020
5	0.030
10	0.050
20	0.210

Table 4. Antagonism of gunacin (G) with mercapto compounds (M) determined by the MIC against *Streptococcus pyogenes* ATCC 8668 in the agar dilution test in a molar combination of G : M/ 1 : 50.

Mercapto compound	MIC (μg/ml)
—	0.020
2-Mercaptothiazoline	0.025
Penicillamine	0.060
Thiomalic acid	0.310
Cysteine	0.320

The influence of serum on the biological activity of gunacin was determined by the MIC in the agar dilution test in the presence of 5, 10 and 20% beef serum (Table 3).

These data demonstrate that there is a serum concentration dependent inactivation of the antibiotic.

To determine whether the inactivation of the quinone antibiotic by cysteine is confined to the amino acid in particular, or to compounds with a mercapto group in general, effects of several mercapto compounds on the antimicrobial activity of gunacin were examined.

The MIC of gunacin in the presence of the compounds was tested in molar concentrations in a combination antibiotic - antagonist 1 : 50 (Table 4). The results of the experiment demonstrate that the antagonism by cysteine can be generally achieved by mercapto compounds and is not due to a specific interaction with the cysteine metabolism. Because of the known inhibitory effect of the quinone antibiotics on the nucleic acid synthesis the relative inhibition of [^{14}C] thymidine and [^{14}C] uracil incorporation into the corresponding nucleic acids was measured along with the inhibition of the protein synthesis by antagonism of incorporation of [^{14}C] isoleucine. All radiochemicals were obtained from New England Nuclear.

Staphylococcus aureus SG 511 was used as a test organism.

The bacterial culture grown at 37°C in a DAVIS's medium containing 0.5% peptone was transferred to fresh medium during the logarithmic phase to give a cell count of 10^6 cells/ml. To each 5 ml of this suspension was added [^{14}C] thymidine and [^{14}C] uracil in a final concentration of 1 μg/ml or [^{14}C] isoleucine with 2 μg/ml, and gunacin with a final concentration of 0.2 μg/ml. The tubes were incubated at 37°C. Every 15 minutes a sample of 100 μl was taken and the reaction was stopped by adding 2 ml of cold 7% trichloracetic acid. The samples were filtered over Sartorius membrane filters No. 11306, dried with infrared light, and their activity measured (Fig. 2). Fig. 2 demonstrates that gunacin (0.2 μg/ml) has a significant inhibitory effect on the DNA synthesis, whereas protein synthesis is not inhibited, and the RNA synthesis is slightly affected.

For the determination of the cytotoxic effect, a HeLa-cell culture ATCC CCL 2 was adjusted in a minimal essential medium MEM (Flow Laboratories) to 2×10^5 cells/ml. Samples of 100 μl were pipetted into microtiter plates and the activity of gunacin was tested in the final concentrations of 0.035, 0.175, 0.35, 1.75, 3.5, 17.5, 35 and 350 ng/ml with each 6 references. The cultures were incubated for 72 hours at 37°C. The growth inhibitory effect was determined by the cell density at each concentration in relation to the control without antibiotic. The ED_{50}-value of 12.22 ng/ml was calculated according to REED[20].

Fig. 2. Inhibition of the incorporation of [^{14}C] thymidine, [^{14}C] uracil and [^{14}C] isoleucine into the DNA, RNA and protein respectively of *Staphylococcus aureus* SG 511 by 0.2 μg/ml gunacin.

In the control the 100% values of the labeled compounds correspond to 68.443 dpm for [^{14}C] uracil, 10.775 dpm for [^{14}C] thymidine and 39.204 dpm for [^{14}C] isoleucine.

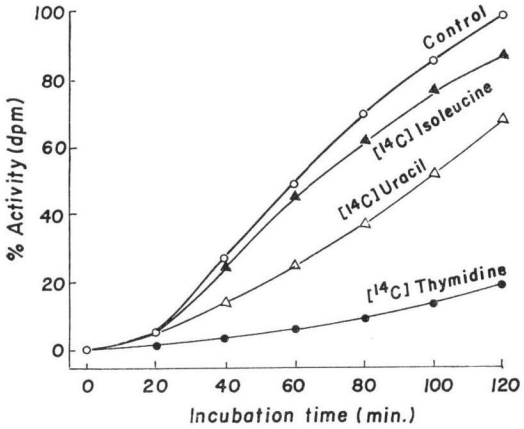
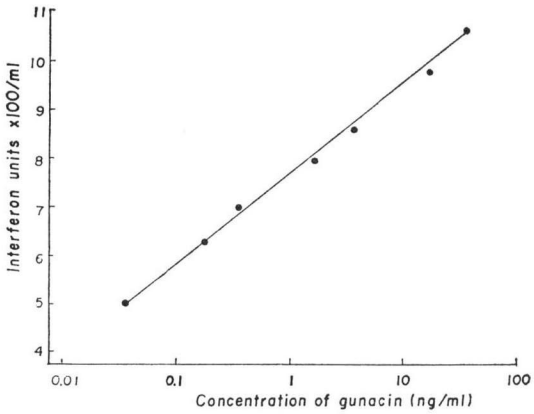


Fig. 3. Concentration dependent interferon induction in fibroblast cells by gunacin.



The interferon-inducing effect of gunacin was tested with fibroblast cells. After a growth period of 6 days at 37°C in MEM, with 10% foetal calf serum the medium was replaced by MEM and the cells were incubated at 37°C for 24 hours. Poly I: C was supplied in a final concentration of 50 μg/ml for 1 hour. The cultures were washed three times with MEM and gunacin was administered in the concentration range of 0.035 ng/ml ~ 35 ng/ml for 24 hours. The interferon units were determined by a dye uptake test according to FINTER[21]. It could be demonstrated that gunacin stimulates a concentration dependent interferon induction (see Fig. 3).

If the toxicity of gunacin in mice is examined, 40 mg/kg i.p. and i.v. are fatal within 24 hours; 8 mg/kg i.v. produces hard tonic and clonic, i.p. light tonic cramps; 2 mg/kg is tolerated without any symptoms. The acute toxicity was determined as $LD_{50} = 16$ mg/kg i.p. and $LD_{50} = 12$ mg/kg i.v.

Experimental

The mass spectra were taken with a Varian-Mat instrument, model CH-5, high resolution mass spectra with an AEI-instrument, model MS-903, equipped with a data system using a PDP-8 computer. IR spectra were recorded on a grating spectrometer, Perkin-Elmer model 237-B, ultraviolet spectra on a Perkin-Elmer model 137-UV.

^{1}H-NMR spectra were taken with a FOURIER-transform spectrometer, Bruker model WP-80 DS (80 MHz spectra) and a Bruker HX-90, equipped with Nicolet 1083 computer and multipulsunit for decoupling experiments in the 90 MHz spectra.

Solvents for ultraviolet spectra: Ethanol and 1% of 1 N KOH in ethanol.

Solvents for NMR spectra: $CDCl_3$ and CD_3OD containing TMS as an internal reference.

Discussion

Gunacin is one of the few quinone antibiotics, produced by fungi. The widespread occurence of the quinone structure and their mode of action by inhibition of the nucleic acid synthesis, suggest a role of these antibiotics in reducing the metabolism of streptomyces and fungi at the level of the nucleic acid synthesis before the sporulation process. This may be the reason why gunacin is produced at the very end of the growth phase of *Ustilago* sp. In general including the higher plants they may have an impor-

tance in the regulation of nucleic acid biosynthesis. The structures of the quinone antibiotics indicate that they are composed of acetate units[22], which are synthesized in the fatty acid metabolism. The structure of gunacin suggests a similar biosynthetic pathway. The relatively strong inhibitory effect of these structures against Gram-positive bacteria in contrast to the Gram-negative strains may be explained by the lipophilic character of the quinone antibiotics. This property does not allow them to penetrate the outer membrane of Gram-negative strains in order to reach their intracellular target[23]. This is demonstrated by the relatively strong inhibitory effect against *E. coli* DB 10, which possesses a reduced penetration barrier for antibiotics[24].

The antagonism by the mercapto compounds other than cysteine demonstrates that gunacin does not specifically inhibit the metabolism of the amino acid but is inactivated by an interaction of the mercapto group with the quinoic carbonyl groups of the antibiotic. This may also be the reason for the serum inactivation by which gunacin may be bound to the marcapto groups of the proteins.

The cytotoxic effect of gunacin can be explained by its inhibitory effect on the DNA synthesis. The fact that actinomycin, a known inhibitor of the DNA synthesis, induces interferon[25], lends support to the theory that the inducing effect of gunacin on interferon is caused by its interaction with DNA.

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