GRISORIXIN, AN IONOPHOROUS ANTIBIOTIC OF THE NIGERICIN GROUP

I. FERMENTATION, ISOLATION, BIOLOGICAL PROPERTIES AND STRUCTURE

PIERRE GACHON* and ALAIN KERGOMARD

Laboratoire de Chimie Organique Biologique, Université de Clermont-Ferrand
B. P. 45, 63170 Aubière, France

T. STARON and C. ESTEVE

Station Centrale de Pathologie Végétale, Centre National de la Recherche Agronomique,
78000 Versailles, France

(Received for publication January 21, 1975)

Grisorixin is an ionophorous antibiotic of the nigericin group isolated from cultures of a strain of *Streptomyces griseus*. It shows activity against Gram-positive bacteria and fungi but is also very toxic. The isolation and purification procedures are reported. Its structure and physico-chemical properties are also described.

In the course of our screening for new antibiotics, several actinomycetes of the genus *Streptomyces* were isolated from soil from the Versailles area. From the mycelium of one of them, a *Streptomyces griseus*, we have isolated an antibiotic related to nigericin which we have named grisorixin,[1] and whose structure was elucidated by X-ray crystallography of its silver and thallium salts.[2,3] It is an open-chain polycyclic polyether with an acidic function and it shows strong antibacterial and antifungal properties as well as high toxicity.

The present paper describes the fermentation, isolation, biological properties, and structure of this antibiotic.

Description of the Producing Strain

The morphology of the producing *Streptomyces* changes according to the nature of the medium on which it is grown. A description and morphologic study of the producing strain will appear in a further paper.

Fermentation

The propagation of this strain of *Streptomyces griseus* was performed on potato agar slant (200 g of filtered potato broth, 20 g of agar, 1 g of corn steep and 20 g of sucrose per liter) at 27°C. Nine day old cultures were used as inoculum.

The strain was at first grown on two different media:

(1) Medium 1: potatoes were boiled in water (200 g of potatoes per liter of water) for 20 minutes. The pulp was filtered off and 20 g per liter of sucrose and 3 g per liter of corn steep were added to the resulting juice.

(2) Medium 2: One liter of this medium contained 30 g of glucose, 20 g of soy bean

* To whom correspondence should be addressed. Present address: Dr. PIERRE GACHON, Institute for Enzyme Research, 1710 University Avenue, Madison, Wisconsin 53706, U.S.A.

meal, and 2 g of corn steep.

Each of these two media was adjusted to pH 7 with NaOH, and 1 % of $CaCO_3$ was added. Erlenmeyer flasks (500 ml) were charged with 100 ml of either medium 1 or medium 2 and autoclave-sterilized at 118°C for 20 minutes. The Erlenmeyer flasks were inoculated with a water suspension of the spores obtained on the potato agar medium. The incubation was carried out on a rotary shaker (100 turns/min) at 27°C for 7 days.

A study aimed at optimizing conditions for the fermentation has been carried out by M. F. LHOMME, M. DUTEURTRE, G. KERGOMARD and A. KERGOMARD (unpublished data).

Isolation

The mycelium was filtered, dried, and extracted with 96 % ethanol (5 ml of ethanol per g of fresh mycelium). The ethanolic extracts were then concentrated, evaporated to dryness, and dissolved in distilled water. The aqueous solution thus obtained was extracted with ether, the ethereal extracts were dried over Na_2SO_4, and the solution was evaporated to dryness. A thick, brown oil was obtained, which was then chromatographed on a silica gel column. Elution was begun with an ethyl acetate-cyclohexane mixture (20:80), with the percentage of ethyl acetate gradually increasing to 50 % at the end of the run. The active fractions were concentrated and purified again on silica gel, using the same elution procedure. Pure antibiotic was thus obtained as a white solid form after the eluant had been evaporated under vacuum.

Grisorixin has an Rf of 0.40 when subjected to tlc on Merck silica gel sheets, using an ethyl acetate-cyclohexane mixture (25:75) for development.

The average yield of antibiotic was 70 mg per liter of medium.

Biological Properties and Toxicity of the Antibiotic

The antimicrobial spectrum of grisorixin has been determined by the liquid dilution method. Bacteria and yeast sensitivity was tested in glucose peptone water. Fungicidal activity was determined on a culture medium that allowed satisfactory growth of all the tested micro-organisms (STARON's medium[4]).

Fungi pathogenic to man or animals were incubated at 37°C, while vegetable parasites were incubated at 25°C. The results obtained after 8 days are given in Table 1. The minimum inhibitory doses of antibiotic are given in μg/ml of culture medium.

Determination of the toxicity of grisorixin in mice revealed an LD_{50} of 15 mg/kg when the antibiotic was given subcutaneously.

In HeLa cells, grisorixin showed a cytotoxic effect, with cell destruction when given at 10 μg/ml and an inhibitory effect on the growth and multiplication of these cells at a dose of 1 μg/ml.

All the tests made *in vitro* clearly show the antibacterial and antifungal properties of grisorixin. Unhappily, trying to treat streptococcal and staphylococcal infections *in vivo* in mice always failed. The great toxicity of this molecule indeed restricts its use at curative doses.

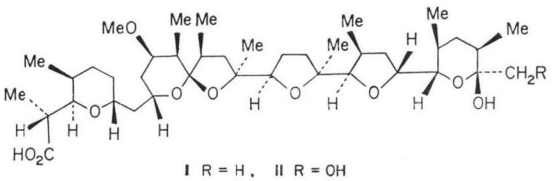
Structure and Physical Properties

Grisorixin is a polycyclic polyether bearing an acidic function, with molecular formula

Table 1. Antimicrobial spectrum of grisorixin.

Test organism	M.I.C. in μg/ml	Test organism	M.I.C. in μg/ml
Escherichia coli CIP 54127	>200	*Aspergillus ochraceus* CLA 1714	50
Proteus vulgaris CIPA 232	>200	*Endothia parasitica* CLA 516	1.5
Salmonella cholerae CIP 5857	>200	*Cercospora beticola* CLA 32	2
Pseudomonas aeruginosa CIPA 22	>200	*Rhizoctonia solani* CLA 1718	12
Bacillus subtilis CIP 5262	0.1	*Phoma betae* CLA 162	20
Staphylococcus aureus CIP 53156	0.4	*Sclerotinia sclerotiorum* CLA 183	0.7
Streptococcus pyogenes CIP 561	0.5	*Pythium ultinum* CLA 1620	50
Mycobacterium chelonii CLA 1952	2	*Phytophthora infestans* CLA 169	100
Streptomyces antibioticus CLA 3430	2	*Fusarium alii* CLA 61	50
Saccharomyces cerevisiae CLA 15	1	*Monilia laxa* CLA 1312	0.7
Candida albicans CLA 31	20	*Phomopsis mali* CLA 1613	0.3
Geotrichum candidum CLA 33	50	*Botrytis cinerea* CLA 23	0.3
Cryptococcus neoformans CLA 314	25	*Verticillium albo-atrum* CLA 211	0.3
Trichophyton mentagrophytes CLA 1313	25	*Epichloe typhina* CLA 519	6
Microsporum canis CLA 133	50	*Helminthosporium festucae* CLA 76	20
Blastomyces dermatidis CLA 24	12	*Dactylium dendroides* CLA 44	50
Sporotrichum schenckii CLA 1818	50	*Trichothecium roseum* CLA 1810	0.7
Madurella mycetoni CLA 1313	12	*Colletotrichum lindemuthianum* CLA 311	1.5
Penicillium roqueforti CLA 1617	1	*Ascochyta pisi* CLA 116	1.5

$C_{40}H_{68}O_{10}$, whose structure (**I**) closely resembles that of nigericin (**II**). Chronologically, it is the fourth representative to have been described in a new family of antibiotics composed of monensin,[5] nigericin,[6,7] X-537A,[8] X-206,[9] dianemycin,[10] A204A,[11] salinomycin,[12] and septamycin.[13]



I R = H, II R = OH

Grisorixin is a white solid, very soluble in organic solvents but insoluble in water, which is easily isolated as an amorphous powder, m.p. 80~85°C (microscope). It can be crystallized as a monohydrate from water-ethanol mixtures, yielding white crystals with m.p. 110~113°C (microscope). Its pK, determined at 25°C in methanol, is 10.02.

The IR spectrum, in KBr (Fig. 1), shows OH functions (wide band between 3400 and 3100 cm^{-1}) and a carboxyl C=O (intense band at 1725 cm^{-1}). Several maxima are also seen between 1120 and 1020 cm^{-1} due to C-O-C bonds.

The NMR spectrum of the antibiotic (CCl_4, 100 MHz) (Fig. 2) shows a singlet at 3.33 ppm due to the methoxyl group. A broad signal centered at 6.65 ppm is also present, corresponding to the protons of the carboxyl and hemiketal functions, and which disappears when D_2O is added. In addition to the CH_3 and CH_2 signals, another broad peak is observed between 3.5 and 4.5 ppm due to the protons on carbons bearing oxygen in the THF and THP rings.

Lastly, the mass spectrum of grisorixin (Fig. 3) shows a fragmentation pattern very similar to that observed for nigericin.[14] The main fragment ions are due to cleavage of the C-C bonds between the rings (Fig. 4). The molecular ion peak is very weak (0.6 % of the base peak) and the peak at *m/e* 690 is due to the loss of a water molecule from the parent ion. The base peak of the spectrum is at *m/e* 365, and corresponds to cleavage of the C-C bond between rings C and D after the loss of a methanol molecule by the *m/e* 397 ion.

In the nigericin mass spectrum, the ion resulting from cleavage between rings E and F

Fig. 1. IR spectrum of grisorixin (KBr).

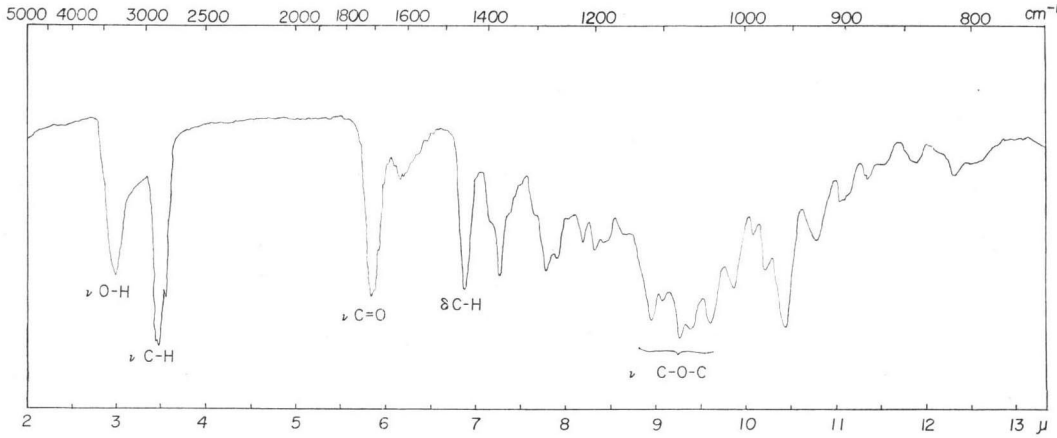


Fig. 2. NMR spectrum of grisorixin (CCl_4, 100 MHz).

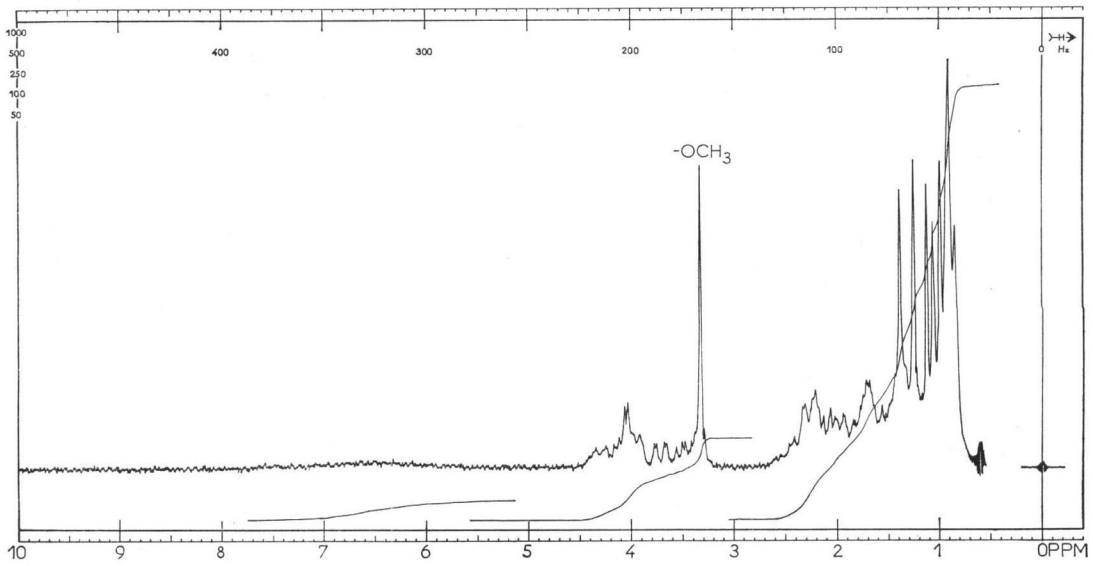


Fig. 3. Mass spectrum of grisorixin.

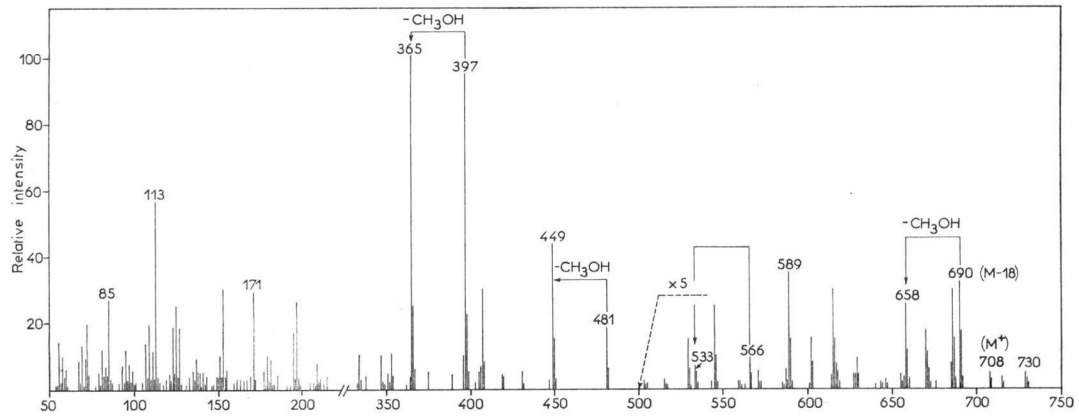
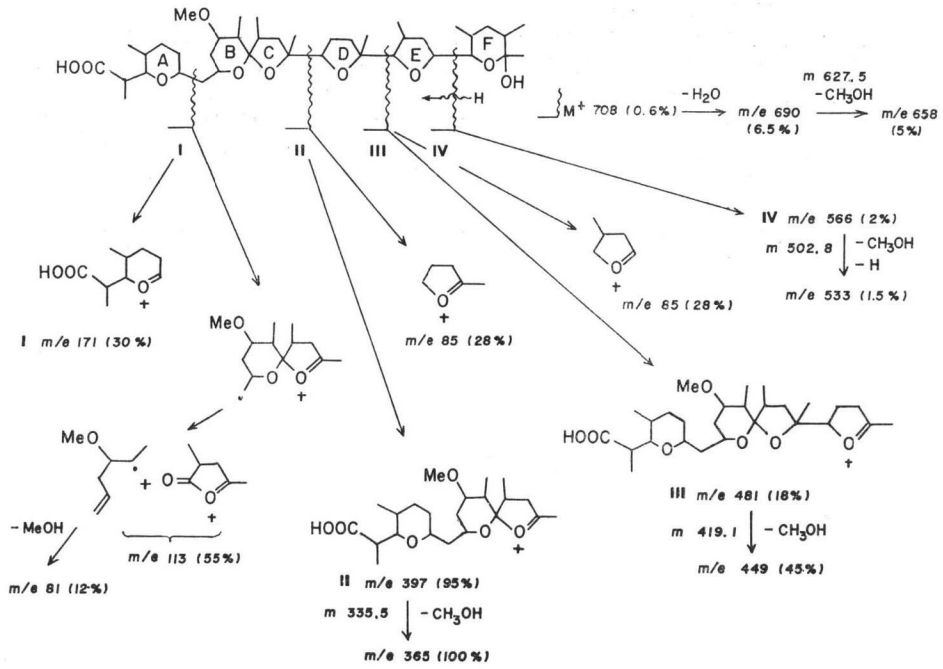


Fig. 4. Mass spectral fragmentation of grisorixin.



appears at *m/e* 565. In the case of grisorixin, the corresponding ion has *m/e* 566, implying a rearrangement with hydrogen transfer. It should be noted that the mass spectrum was taken on a sample of grisorixin contaminated with its sodium salt, which accounts for the presence of a peak at *m/e* 730.

Acknowledgment

One of us (P. G.) thanks l'Institut National de la Santé et de la Recherche Médicale for support of this work.

References

1) Gachon, P.; A. Kergomard, H. Veschambre, C. Esteve, & T. Staron: Grisorixin, a new antibiotic related to nigericin. Chem. Comm. 1970: 1421~1422, 1970
2) Alleaume, M. & D. Hickel: The crystal structure of grisorixin silver salt. Chem. Comm. 1970: 1422~1423, 1970
3) Alleaume, M. & D. Hickel: The crystal structure of thallium grisorixin monohydrate. Chem. Comm. 1972: 175~176, 1972
4) Staron, T. & C. Allard: Propriétés antifongiques des 2-(4'-thiazolyle)benzimidazole ou thiabendazoles. Phytiatrie, Phytopharmacie 13: 163~168, 1964
5) Agtarap, A.; J. W. Chamberlin, M. Pinkerton & L. Steinrauf: The structure of monensic acid, a new biologically active compound. J. Am. Chem. Soc. 89: 5737~5739, 1967
6) Steinrauf, L. K.; M. Pinkerton & J. W. Chamberlin: The structure of nigericin. Biochem. Biophys. Res. Comm. 33: 29~31, 1968
7) Kubota, T.; S. Matsutani, M. Shiro & H. Koyama: The structure of polyetherin A. Chem. Comm. 1968: 1541~1543, 1968
8) Westley, J. W.; R. H. Evans, Jr., T. Williams & A. Stempel: Structure of antibiotic X-537A. Chem. Comm. 1970: 71~72, 1970
9) Blount, J. F. & J. W. Westley: X-ray crystal and molecular structure of antibiotic X-206. Chem. Comm. 1971: 927~928, 1971

10) CZERWINSKI, E. W. & L. K. STEINRAUF: Structure of the antibiotic dianemycin. Biochem. Biophys. Res. Comm. 45: 1284~1287, 1971
11) JONES, N. D.; M. O. CHANEY, J. W. CHAMBERLIN, R. L. HAMILL & SUE CHEN: Structure of A204A, a new polyether antibiotic. J. Am. Chem. Soc. 95: 3399~3400, 1973
12) KINASHI, H.; N. OTAKE, H. YONEHARA, S. SATO & Y. SAITO: The structure of salinomycin, a new member of the polyether antibiotics. Tetrahedron Letters 1973-49: 4955~4958, 1973
13) PETCHER, T. J. & H. P. WEBER: X-ray crystal structure and absolute configuration of *p*-bromophenacyl septamycin monohydrate, a polyether antibiotic. Chem. Comm. 1974: 697~698, 1974
14) KUBOTA, T. & S. MATSUTANI: Studies on the antibiotic nigericin (polyetherin A). J. Chem. Soc. (C) 1970: 695~703, 1970