MICROBIAL CONVERSION OF GRISORIXIN, A MONOVALENT CATION IONOPHOROUS ANTIBIOTIC

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The isolation and identification of a bioconversion product of grisorixin from a strain of *Streptomyces rimosus* is reported. The structure of this product was elucidated from physicochemical data, in particular ^{13}C NMR spectra. Its ionophorous and antibiotic properties are markedly different from those of grisorixin.

Grisorixin is a carboxylic ionophorous antibiotic, active against Gram-positive bacteria and fungi, produced by a strain of *Streptomyces griseus* 2142 N 6. The production of this antibiotic in our laboratory has been previously described[1]. Anticoccidial and feed-efficiency-improving properties have been reported for many polyether antibiotics, especially for monensin[2,3]. Four of them (monensin, lasalocid, narasin and salinomycin) are widely employed as poultry and livestock feed additives. The use of ionophores in feeding farm animals is of particular interest[4].

We are studying ionophorous antibiotic metabolism by means of a microbial model method described by ROSAZZA[5]; the objective of this work is to elucidate detoxication mechanisms, and modifications of properties (*i.e.* antibiotic, cation transport, complexation) of these compounds.

The present paper describes the ability of the microorganism, *Streptomyces rimosus*, to modify grisorixin, and the isolation and identification of a bioconversion product.

Materials and Methods

Materials

Grisorixin was obtained in our laboratory[1] from a culture of *Streptomyces griseus* 2142 N 6.

Microorganism

A strain of *Streptomyces rimosus* NRRL 2234 (ATCC 10970) was used.

Culture and Reaction Conditions

Streptomyces rimosus was maintained on Emerson agar slant for 4 days at 28°C. Surface growth from the slant was removed by mixing with sterile water and then inoculated in 100 ml vegetative sterile medium containing 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.5% soluble starch (Difco), 0.25% yeast extract (Bio-Mérieux) and 0.25% $CaCO_3$ in 500-ml Erlenmeyer flask. After incubation at 28°C on a rotatory shaker at 140 rpm for 2 days, 5 ml of this culture were used to inoculate 100 ml of a sterile medium containing 1.5% soybean meal, 2.5% glucose and 0.25% $CaCO_3$ in a 500-ml Erlenmeyer flask. Incubation was continued for 24 hours; grisorixin was then added at 30 mg/100 ml, and the reaction was allowed to proceed under the same conditions for a further 3 to 4 days.

Detection of the Conversion Product

After reaction, the culture was filtered; the filtrate was saturated with ammonium sulfate and extracted with ethyl acetate. The extract was chromatographed on silica gel TLC plates (Merck 60 F-254) with chloroform - methanol (9: 1). The conversion product was detected by spraying with 50% sulfuric

acid and heating to 100°C, and had an Rf value of 0.5 (Rf value of grisorixin: 0.8).

Isolation of the Conversion Product

Ethyl acetate extracts were chromatographed using medium-pressure column chromatography, with Merck Lobar prepacked columns and chloroform - methanol (9: 1). Final purification of the product was achieved by preparative thin-layer chromatography using silica gel LS 254 plates developed with the same solvent mixture as above. Pure conversion product was obtained in 20% yield.

Results and Discussion

Grisorixin (Fig. 1) exhibits antibacterial (Gram-positive) and antifungal activity. It shows acute toxicity[1], complexes monovalent cations[6] and transports potassium in mitochondria[7]; it is water insoluble.

On the contrary, conversion product G_1 (Fig. 1) is devoid of antibiotic activity; it exhibits a very low K^+ efflux (10^{-12} mole/s/cm^2) through a bulk liquid membrane (made with chloroform) compared to that of grisorixin in the same conditions (7×10^{-11} mole/s/cm^2); it is partially water soluble, this property might enable this substance to be eliminated in biological fluids during detoxication.

Microanalysis gave the molecular formula $C_{40}H_{66}O_{13}$. Physico-chemical properties of grisorixin and its conversion product are shown in Table 1.

The IR spectrum of G_1 free acid in KBr (Fig. 2) shows the same absorption frequencies as grisorixin; a wide band between 3400 and 3100 cm^{-1} corresponding to OH functions, intense bands at 1725 and 1705 cm^{-1} (carboxylic C=O) and several maxima between 1120 and 1020 cm^{-1} due to C–O–C bonds.

The mass spectrum of G_1 free acid shows a fragmentation pattern similar to that observed for grisorixin[1,8] for rings A, B, C and D (Fig. 1), the main fragment ions being due to cleavage of C–C bonds between the rings; the corresponding peaks appear at *m/z* 481, 449, 397, 365, 171. Fragmentations are different for E and F rings. The molecular ion peak is at *m/z* 754.

The single broad band ^1H decoupled ^{13}C spectrum of the potassium salt of G_1 in CD_3OD gave 39 resonances (Fig. 3a). To determine signal multiplicities, a new method based on *J*-modulation of a spin echo[9] was used; "even signals" (CH_3, CH) were separated from "odd signals" (CH_2, quaternary C) (Fig. 3b). At the same time, an "even signal" which was hidden in the solvent peak in the broad band spectrum was separated from the "odd" solvent signal (Fig. 3b). Thus, a total number of 40 resonances were detected. The spectrum in C_2D_5OD and an off-resonance spectrum of G_1 (unpublished results)

Fig. 1. Grisorixin and bioconversion product G_1.
The stereochemistry of grisorixin is indicated in reference 1.

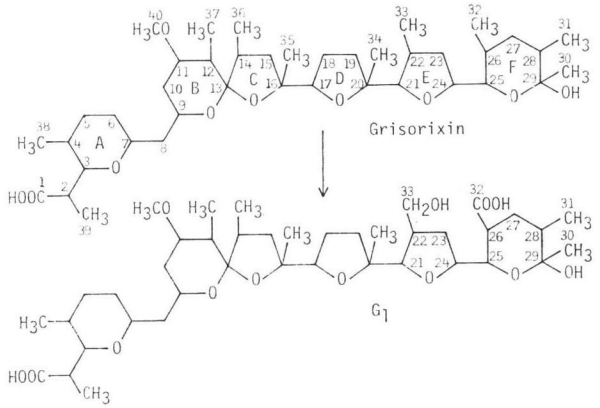

Table 1. Physico-chemical properties of grisorixin and G_1 free acids.

	Grisorixin	G_1
Formula	$C_{40}H_{68}O_{10}$	$C_{40}H_{66}O_{13}$
M. W.	708	754
Melting point	80~85°C	120~123°C
Optical rotation $[\alpha]_D^{20}$	+16° (*c* 0.04, Me_2CO)	+12.3° (*c* 0.025, Me_2CO)

supported these results.

The chemical shifts of the ^{13}C NMR spectra are shown in Table 2. Grisorixin potassium salt chemical shift assignments in CD_3OD were made by comparison with the known values of nigericin sodium salt[10].

^{13}C Spectrum of the potassim salt of G_1 showed 2 COOH signals (1 in grisorixin spectrum)

Fig. 2. IR spectrum of G_1 (free acid in KBr).

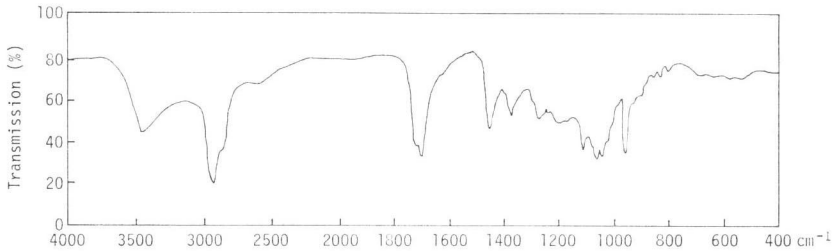

Fig. 3. ^{13}C NMR spectra of G_1 potassium salt (CD_3OD, 100 MHz).
(a) Broad band ^1H decoupled ^{13}C spectrum.
(b) *J*-Modulated spin echo spectrum.

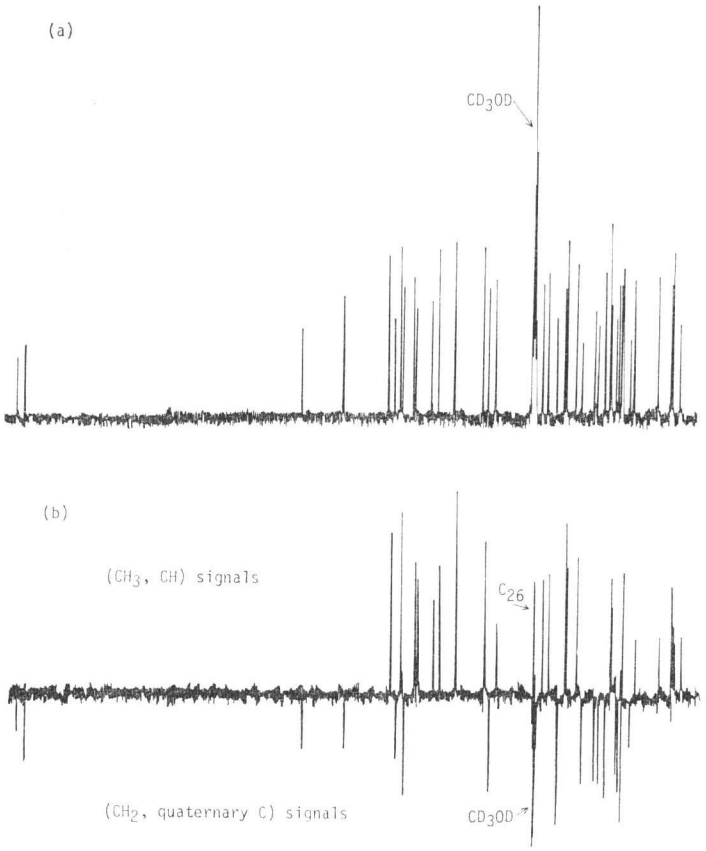

Table 2. ^{13}C NMR spectral data of grisorixin and G_1 potassium salts.*

C-No.	Functional group	Grisorixin (δ in ppm)	G_1 (δ in ppm)	$\Delta\delta$**	C-No.	Functional group	Grisorixin (δ in ppm)	G_1 (δ in ppm)	$\Delta\delta$**
1	-COOH	183.8	183.8		21	-CH(O)	87.4	86.7	−0.7
2	$-CH(CH_3)$	46.7	46.7		22	-CH(R)°	36.6	45.2	+8.6
3	-CH(O)	75.3	75.3		23	$-CH_2$	33.2	27.9	−5.3
4	$-CH(CH_3)$	29.3	29.2		24	-CH(O)	77.9	79.3	
5	$-CH_2$	27.4	27.3		25	-CH(O)	78.7	73.7	−5.0
6	$-CH_2$	24.6	24.6		26	-CH(R')°	33.6	49.5	+15.9
7	-CH(O)	69.6	69.6		27	$-CH_2$	37.9	33.5	−4.4
8	$-CH_2$	36.9	36.7		28	$-CH(CH_3)$	41.5	41.1	−0.4
9	-CH(O)	62.0	61.9		29	-O-C-OH	98.4	98.4	
10	$-CH_2$	32.6	32.5		30	$-CH_3$	26.3	26.4	
11	$-CH(OCH_3)$	80.2	80.1		31	$-CH_3$	17.8$^\theta$	17.3$^\theta$	
12	$-CH(CH_3)$	38.0	38.0		32	$-CH_3$	17.3	—	+164.3
13	-O-C-O	109.3	109.4		32	-COOH	—	181.6	
14	$-CH(CH_3)$	40.7	40.6		33	$-CH_3$	15.8	—	+ 44.9
15	$-CH_2$	42.9	42.9		33	$-CH_2OH$	—	60.7	
16	$-C-O(CH_3)$	83.2$^+$	83.0$^+$		34	$-CH_3$	23.0	23.3	
17	-CH(O)	83.2	83.6		35	$-CH_3$	29.4	29.4	
18	$-CH_2$	26.6	26.7		36	$-CH_3$	13.5	13.6	
19	$-CH_2$	30.8	30.8		37	$-CH_3$	13.5	13.5	
20	$-C-O(CH_3)$	85.6$^+$	85.0$^+$		38	$-CH_3$	11.7	11.6	
					39	$-CH_3$	14.1$^\theta$	14.0$^\theta$	
					40	$-OCH_3$	58.6	58.9	

° $R=R'=CH_3$ in grisorixin.
$R=CH_2OH$ and $R'=COOH$ in G_1.

* ^{13}C NMR spectra were taken on a Brüker WM-400 spectrometer operating at 100.62 MHz, in CD_3OD solution. Chemical shifts (δ) are in ppm downfield from internal TMS.

** $\Delta\delta=\delta(G_1)-\delta$ (grisorixin).

$^{+,\theta}$ Assignments may be interchanged.

Table 3. ^{13}C Substituent effects*.

	H⟶OH			CH_3⟶COOH			
	Literature[11]	Experimental	C-No.	Literature[12]	Literature[13]	Experimental	C-No.
Cα	+35 to +52	+44.9	33	+12	+14	+15.9	26
Cβ	+5 to +12	+8.6	22	−3	−5	−5	25
						−4.4	27
Cγ	0 to −6	−0.7	21	−1	0	−0.4	28
		−5.3	23				

* Positive values in ppm represent shifts to lower field.

and 14 C-O carbons (13 in grisorixin spectrum), the extra C-O was a CH_2 signal in the CH_2OH region (60.7 ppm). In addition the 2 methyl groups linked to C_{22} and C_{26} in grisorixin had disappeared. Substituent effects between G_1 and grisorixin potassium salts compared to literature values (Table 3) are consistent with the following conclusion: the methyl group linked to C_{22} has been oxidized to CH_2OH and the methyl group linked to C_{26} to COOH.

Comparison of ^1H NMR spectra of potassium salts of grisorixin and G_1 confirmed G_1 structure: the grisorixin salt spectrum exhibited 10 methyl group signals (7 doublets and 3 singlets) and 8 signals

due to protons adjacent to C–O, while G_1 salt spectrum exhibited 8 methyl group signals (5 doublets and 3 singlets) and 10 signals due to protons adjacent to C–O (2 extra signals corresponding to CH_2OH). The total assignment was obtained on a Brüker WM-400 giving ^1H resonances at 400.13 MHz (to be published).

Bioconversion of grisorixin gives a product devoid of antibiotic properties and which exhibits reduced ionophorous transport properties. It should be noted that this bioconversion product is slightly water soluble. All these modifications correspond to a detoxication process. Given the above findings, the present study is to be extended to other ionophores used as poultry and livestock feed additives.

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