NEW IRON-CONTAINING ANTIBIOTICS

ISOLATION AND PROPERTIES OF VIRIDOMYCINS A, B, and C

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In 1964, in some actinomycetes new green pigments were discovered [1] which were similar to the microbial pigment ferroverdin studied earlier [2]. Investigations of unfractionated preparations of the new pigments showed the presence of nitrogen and iron in them [3]. Preparations of these pigmentary antibiotics, which have been called viridomycins contain, according to paper chromatography, not less than three components [4-6].

In the present paper we describe methods for separating viridomycins and their properties. The viridomycins are formed by a number of actinomycetes: *Actinomyces atroolivaceus*, *A. herbaricolor*, *A. intermedius*, *A. olivoviridis*, *A. roseoviridis*, *A. streptomycini*, *A. viridans*, *A. viridaris* and *A. viridobrunneus*. For the isolation of the viridomycins we took *Act. viridaris*, strain 1876. The scheme of their isolation is given below.

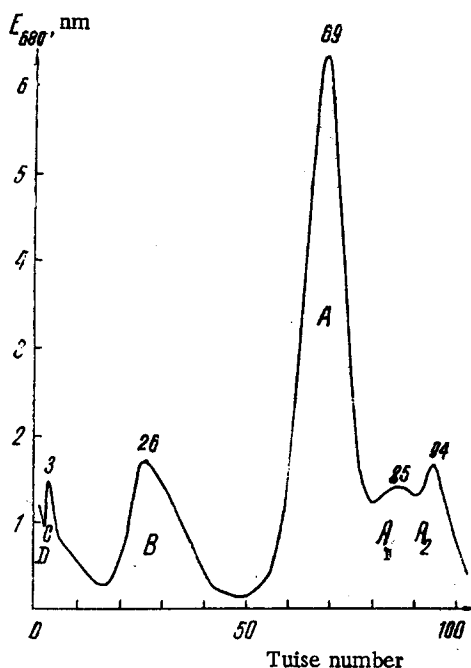

Fig. 1. Curve of the countercurrent distribution of a total preparation of viridomycins in the system of phases: n-butyl acetate, n-butanol, ethanol, water (10:5:2:8).

A mixture of viridomycins can also be obtained from a filtrate of the culture liquid by extraction with n-butanol. The yellow-brown pigments were separated by chromatography on Sephadex LH-20. To separate the viridomycins we used countercurrent distribution in a system of the phases n-butyl acetate, ethanol, and water (10:5:2:8). The curve for the distribution of the total preparation, which is given in Fig. 1, shows the presence in it of a main component (viridomycin A) and of minor components (viridomycins A_1, A_2, B, C, and D). It has been possible to isolate viridomycins A, B, and C in the individual state. A chromatogram showing their homogeneity is given in Fig. 2.

Viridomycins B and C were obtained in the form of amorphous yellow-green powders, and it was possible to crystallize viridomycin A from a mixture of acetone and heptane (5:1): mp above 300°C (decomp). The elementary analysis corresponded to the formula $(C_7H_4NO_2)_2Fe$.

The viridomycins are moderately soluble in the lower alcohols, acetone, and water, readily soluble in pyridine, in glacial acetic acid, and dimethylformamide, and insoluble in petroleum ether, hexane, benzene, carbon tetrachloride, and chloroform. Viridomycins A, B, and C are characterized by very similar electronic absorption spectra with maxima at 680, 430, and 290 nm (Table 1). The IR spectra are given in Fig. 3.

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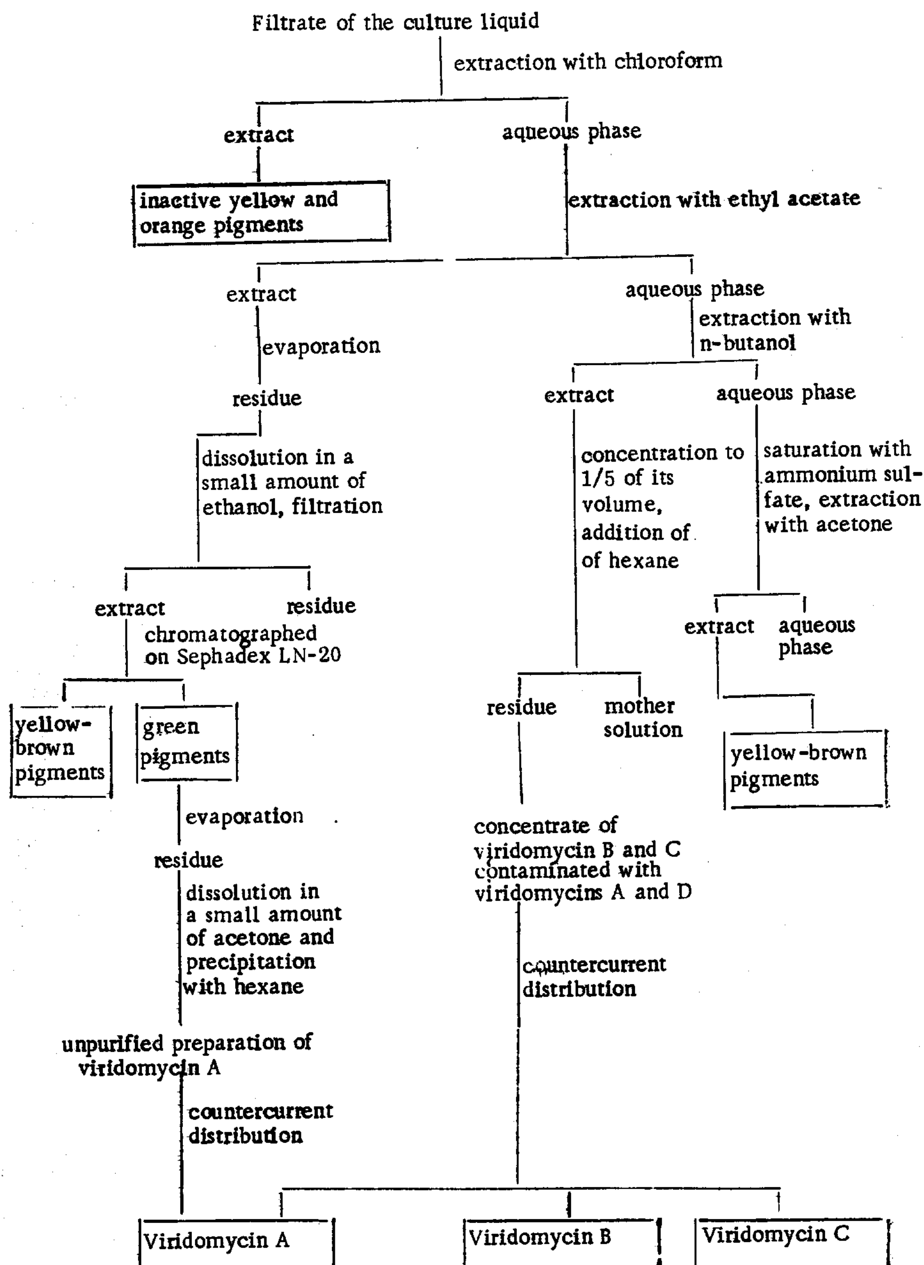
Scheme of the Isolation of Viridomycins A, B, and C



In a concentration of 1-4 γ/ml, the preparations suppress some Gram-negative bacteria (Staph. aureus, Bac. mycoides, Bac. subtilies, Sarcina lutea) and actinomycetes but have no effect on gram-negative bacteria, mycobacteria, fungi, and yeasts. The absorption spectra and elementary compositions show that viridomycins A, B, and C differ from iron-containing antibiotics described previously: the sideromycins [7, 8], gluconimycin [9] and ferramidochloromycetin [10]. On hydrolysis, the latter give amino acids, while there are no amino acids in hydrolyzates of the viridomycins. Furthermore, the viridomycins differ from the sideromycins by their absorption spectra and the greater stability of the bond with the iron; ions of this metal cannot be separated from the viridomycins by such strong complexones as 8-hydroxyquinoline and EDTA or by treatment with hydrogen sulfide or solutions of acids and alkalis at room temperature.

In a number of properties, the viridomycins are similar to the iron-containing green pigment ferroverdin (a derivative of o-nitrosophenol), which possesses no antibacterial action. However, the viridomycins differ from ferroverdin by their antibacterial activity, their composition, and the nature of their spectra in the visible and infrared regions. In par-

Fig. 2. Chromatogram of viridomycins A (2), B (3), and C (4) on paper in water-saturated ethyl acetate: 1) mixture of viridomycins A, B, and C.

TABLE 1. Absorption Spectra of the Viridomycins and Related Substances

Compound	Solvent	Position of the absorption maxima, nm
Viridomycin A	Ethanol	219, 294, 432, 680
	Water	218, 292, 435, 680
	0.1 N HCl in ethanol	293, 430, 680
	0.1 N NaOH in ethanol	296, 430, 680
Viridomycin B	Ethanol	222, 294, 435, 680
	0.1 N HCl in ethanol	294, 430, 680
	0.1 N NaOH in ethanol	296, 430, 680
Viridomycin C	Ethanol	228, 294, 435, 680
	0.1 N HCl in ethanol	294, 435, 680
	0.1 N NaOH in ethanol	294, 438, 680
Deferriviridomycin A	Ethanol	304, 450
	Phosphate buffer, pH 6	305, 450
	Phosphate buffer, pH 3	265, 380
Cobalt analog of viridomycin A	Chloroform	300, 352, 556
	n-butanol	298, 350, 558
Copper analog of viridomycin A	n-butanol	295, 523
Nickel analog of viridomycin A	n-butanol	300, 420 (inf.), 485
Ferroverdin	Ethanol	208, 278, 430, 680
Cobalt analog of ferroverdin	Chloroform	300, 352, 558*

*Literature data [11].

ticular, the IR spectra of the viridomycins lack the band (1730 cm^{-1}) that is characteristic for the ester grouping present in the ferroverdin molecule. Thus, viridomycins A, B, and C are new natural compounds.

When a producing agent of ferroverdin was cultivated on a medium in which iron had been replaced by cobalt, the cobalt analog of ferroverdin was obtained [11]. When producing agents of the viridomycins were grown in media containing cobalt, instead of green pigments — viridomycins — brown-red pigments presumed to be the cobalt analogs of viridomycins were obtained. However, the yields of these analogs were very small, and another, more effective route, to the production of viridomycin analogs has been discovered.

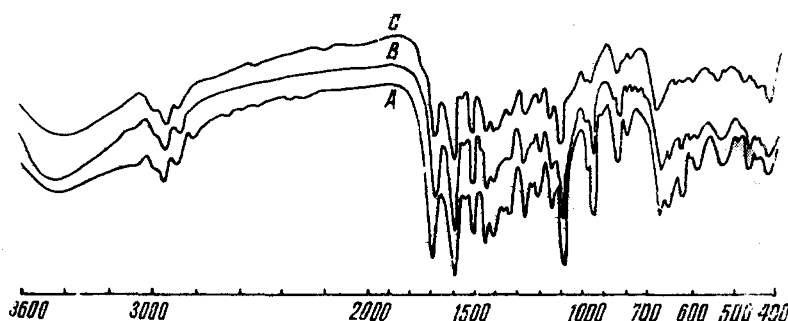


Fig. 3. IR spectra of viridomycins A, B, and C (tables with KBr).

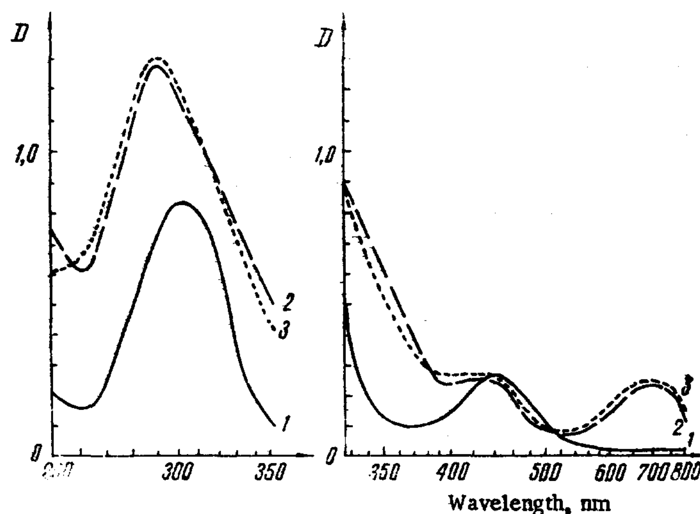


Fig. 4. Electronic absorption spectra of deferriviridomycin A (1) and the product of its reaction with Fe^{++} salts (2); 3) viridomycin A, n-butanol.

An interesting feature of the biosynthesis of the viridomycins is the formation of a precursor when cultivation is carried out on media with a reduced concentration of iron salts. For the maximum formation of the viridomycins themselves, the medium must contain a fairly considerable concentration of iron salts (0.5 g/liter). When the producing agents are media without the addition of iron, a yellow pigment called deferriviridomycin A is formed [12], which, on being treated with iron salts, is converted into viridomycin A.* Deferriviridomycin A is an unstable substance which rapidly decomposes under neutral and alkaline conditions (it loses its capacity for binding metal ions) but is far more stable in acid solutions. Details of its absorption spectrum are given in Table 1 and in Fig. 4. It is characteristic that the absorption maxima at 450 and 305 nm shift on acidification to 380 and 255 nm, respectively. The change in the spectra is reversible: on neutralization the reverse displacement of the maxima takes place.

Deferriviridomycin A forms stable complexes with cobalt, nickel, and copper ions, and the spectra of these compounds are given in Fig. 5.

Of the analogs of viridomycin A, the cobalt complex has been studied in most detail because, in particular, this complex is the only compound of the viridomycin A series that gives good mass spectra. The isolation of the cobalt analog of viridomycin A was based on

*In the case of ferroverdin no analogous precursor has been discovered [13]. Our attempts to obtain the presumable precursor of ferroverdin by cultivating the corresponding producing agents on media with low concentrations of iron also proved unsuccessful.

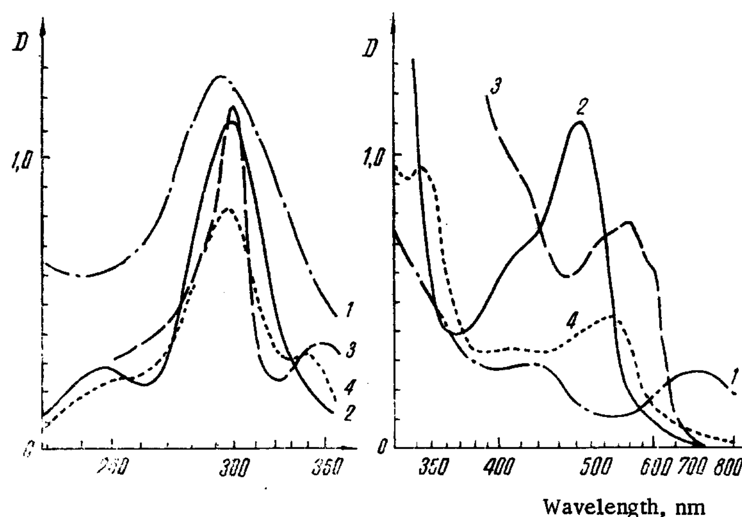


Fig. 5. Electronic absorption spectra: 1) viridomycin A; 2) nickel analog of viridomycin A; 3) cobalt analog of viridomycin A; 4) copper analog of viridomycin A (spectra 1, 2, and 4 were measured in n-butanol, and spectrum 3 in chloroform).

the fact that this analog, unlike its precursor, readily dissolves in chloroform. A filtrate of the culture liquid was extracted first with n-butanol to eliminate traces of viridomycin and then exhaustively with chloroform to eliminate all lipophilic impurities. Then a cobalt salt was added to the culture liquid and the cobalt analog formed was extracted with chloroform. The absorption spectrum of the cobalt analog of viridomycin A is very similar to that of the cobalt analog of ferroverdin (see Table 1), but there is a bathochromic shift of one of the absorption maxima, as in the spectra of the iron complexes of ferroverdin and viridomycin A. The IR spectrum of the cobalt analog of viridomycin A is similar to the spectrum of viridomycin A and differs from that of the cobalt analog of ferroverdin.

The strength of the complexes depends on the nature of the metals. Iron is bound most strongly. The cobalt complex is not decomposed by EDTA and acids but on treatment with alkalis it is possible to isolate deferriviridomycin A from the complex. The complexes with copper and nickel ions are the least stable: they are decomposed by EDTA and by solutions of acids and bases. Furthermore, iron ions are capable of displacing copper and nickel from their complexes. No complexes are formed with calcium, magnesium zinc, cadmium, aluminum, or manganese ions.

EXPERIMENTAL METHOD

Isolation of Viridomycins A, B, and C

Actinomyces viridaris (strain 1876) was cultivated in a 100-liter fermenter in Sr-1 medium of the following composition (g/liter): glucose 20, ferric citrate 0.5, K_2HPO_4 0.5, KNO_3 1, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5, calcium carbonate 1, and water from the mains. The seed material was prepared in the following way: a 24-h culture of the producing agent on an agarized medium of the same composition was seeded into the liquid medium. After shaking for 24 hours on shaking machines at 28°C, the grown mycelium was transferred into fermenters. After growth for 4-5 days, the mycelium was separated off by centrifuging. The green aqueous phase was extracted with chloroform (1/4 volume) and the chloroform extract was separated off and discarded, since it contained inactive yellow and brown pigments. Then the liquid was extracted five times with ethyl acetate. All the extracts were combined and evaporated to an oily dark green residue.

From 40 liters of centrifugate 1.5 g of preparation was obtained. The raw material contained mainly viridomycin A. The aqueous phase was extrated with n-butanol. The butanolic extracts were combined and evaporated. The weight of the raw material was 8.5 g. This preparation contained a mixture of viridomycins A, B, and C. The oily residues from the ethyl acetate and butanol extracts were each separately extracted repeatedly with ethanol. The extracts were concentrated in vacuum to small volume, and were deposited on a column of

Sephadex LH-20, which was eluted with ethanol. The main fraction with an intense green color was evaporated, the residue was dissolved in acetone, the solution was filtered and concentrated, and the product was precipitated with hexane.

The precipitates were filtered off and dried in the air. The powder from the ethyl acetate extract contained mainly viridomycin A and the powder from the butanol extract, a mixture of viridomycin A (the main component) and small amounts of viridomycins B and C and other components. These mixtures were separated by countercurrent distribution.

Countercurrent distribution was performed in a Quickfit and Quartz Ltd. apparatus, series A-6, with 100 cells each having a capacity of 26.5 ml for the upper and lower phases. Butyl acetate—butanol—ethanol—water (10:5:2:8) and (7:3:2:8) systems were used. Good separation was observed with a number of transfers ranging from 90 to 130. The solutions of the viridomycins separated by countercurrent distribution were evaporated and the resulting green mass was reprecipitated from acetone with hexane.

Crystallization. Viridomycin A was crystallized from a mixture of acetone and hexane (5:1).

Isolation of Deferriviridomycin A

Deferriviridomycin A is formed when *Act. viridaris* (strain 1876) is grown on the medium Sr-1 without the addition of iron. The actinomycete was grown on a circular shaking machine (200 rpm) in 250-ml flasks each containing 50 ml of medium at 28°C, the time of growth being 36 h. The yellow culture liquid was filtered from the mycelium and the filtrate was extracted with butanol three times (1/5 of the volume of the culture liquid) to eliminate viridomycins and with chloroform (1/3 volume) to eliminate lipoid substances. The culture liquid purified in this way which contained the deferriviridomycin A was used to obtain the analogs of viridomycin A.

Preparation of the Cobalt, Copper, and Nickel Analogs of Viridomycin A

The Cobalt analog was obtained from the culture liquid treated as described above by the action of a solution of $CoCl_3$. A 0.1% aqueous solution of $CoCl_3$ was added with stirring to a solution of deferriviridomycin A in a proportion of 50 mg of cobalt salt to 1 liter of culture liquid. The cherry-red-violet complex was extracted several times with chloroform. The combined extracts were washed with water and evaporated to dryness. This gave a lustrous dark-violet powder; one liter of the culture liquid yielded 8-10 mg of product.

The Copper Analog. An aqueous solution of copper sulfate was added to a solution of deferriviridomycin A (100 mg of $CuSO_4 \cdot 5H_2O$ to one liter of culture liquid). The copper complex was extracted three times with butanol. The extract was washed twice with water and was evaporated to dryness. The red-brown oily residue was dissolved in methanol, and the solution was filtered and was evaporated to small volume. The concentrate was separated by gel filtration through Sephadex LH-20 into three fractions colored, respectively, yellow, pink-violet (main component), and green. The main fraction was evaporated to dryness. This gave a red-brown powder of the copper analog of viridomycin A. Yield 10 mg from one liter of culture liquid.

Nickel Analog. A 5% solution of $NiSO_4 \cdot 7H_2O$ was added to an aqueous solution of deferriviridomycin A in a proportion of 55 mg of salt per liter of culture liquid. The bright-red solution of the complex that was formed was extracted three times with butanol. When chromatographed on a "Silufol" plate in the acetone—ethyl acetate (1:4) system, the substance separated into three fractions: the chromatogram had three orange-colored spots with R_f 0, 0.1, and 0.4. The main component (R_f 0.1) was isolated by preparative chromatography under the same conditions. Yield 20 mg per liter of culture liquid.

Study of Spectral and Chromatographic Properties. The UV and IR spectra were taken on Karl Zeizz (GDR) Specord and UR-10 spectrophotometers.

Viridomycins A, B, and C were chromatographed on paper by the circular method in the water-saturated ethyl-acetate system. Before the chromatograms were treated to reveal the spots, they were kept in a desiccator saturated with water vapor and ethyl acetate for 12 h.

SUMMARY

1. New iron-containing antibiotics, viridomycins A, B, and C, have been isolated from the actinomycete Actinomyces viridaris.

2. Deferriviridomycin A — the precursor of viridomycin A — possesses the capacity for binding not only iron ions but also cobalt, nickel, and copper ions. The copper, nickel, and cobalt analogs of viridomycin A have been obtained.

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