GRISEORUBINS, A NEW FAMILY OF ANTIBIOTICS WITH ANTIMICROBIAL AND ANTITUMOR ACTIVITY

I. TAXONOMY OF THE PRODUCING STRAIN, FERMENTATION, ISOLATION AND CHEMICAL CHARACTERIZATION

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A new antibiotic complex has been obtained from the cultures of *Streptomyces* strain No. IMET 20978 isolated from the shrimp *Crangon crangon* L. On the basis of taxonomic studies the producing microorganism is described as *Streptomyces fimicarius* (DUCHÉ) WAKSMAN *et* HENRICI, 1948, type strain IMET 20978. The antibiotic complex, designated as griseorubin, belongs to the polycyclic C-glycosyl antibiotics. It is a red-coloured amorphous material which consists of eight closely related fractions including griseorubins A, B, C, D, E, F, G, and H. The griseorubin complex exhibits antibiotic activity against Gram-positive and -negative bacteria as well as against mycoplasma and protozoa. The griseorubin complex is also effective on leukemia L1210 and ZAJDELA ascites hepatoma.

In the course of our screening program for new antibiotics produced by strains of *Streptomyces* from marine environments, we found that the *Streptomyces* strain No. IMET 20978 isolated from the intestinal tract of the shrimp *Crangon crangon* L. collected in Baltic Sea, produces an antibiotic complex which inhibits *in vitro* the growth of Gram-positive and -negative bacteria as well as mycoplasma and protozoa. In addition to its antimicrobial activity the antibiotic complex shows cytostatic action against experimentally induced tumors in mice and rats. The antibiotic complex was isolated and separated into eight related fractions named griseorubins A, B, C, D, E, F, G, and H. The most potent fraction E was further purified and subdivided into eight components, $E_1 \sim E_8$. On the basis of its physico-chemical properties, the griseorubins represent a new type of polycyclic C-glycosyl microbial metabolites and are related to hedamycin[1], kidamycin[2], neopluramycin[3], and pluramycin[3].

In this report, the taxonomy of the producing strain, the production, the isolation and preliminary investigations of the physico-chemical properties of the griseorubins are presented. The antimicrobial characteristics of the antibiotic complex and the antitumor activity against transplantable animal tumors will be dealt with the following paper.

Taxonomy

The griseorubins-producing strain IMET 20978 was detected by means of the "BIP" test[4] applied as a microbiological screening model *in vitro* in the search for potential carcinostatic agents. Since the strain was found to represent a species of *Streptomyces* the taxonomic characterization was carried out according to the methods used in the International Streptomyces Project (ISP)[5].

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Microscopic Characteristics

The strain produced abundant aerial mycelium with tufts of straight or wavy spore chains and therefore it belongs to the section *Rectus-Flexibilis* (RF). The mature spore chains were generally long with 10~50 or more spores per chain. The spores were oval in shape (1.0~1.6 $\mu \times 0.6$ μ) and smooth as seen by electron microscopy.

Cultural Characteristics

The aerial mycelium was light yellowish-greenish-gray coloured depending on the agar medium (Table 1) and thus it represents the colour-series "yellow" and the colour-type "griseus", respectively[6]. The pink colour occassionally ocurring in aged cultures results from the red reverse mycelium pigment.

On several media the surface of the substrate mycelium was colourless to pale yellow and its reverse side was orange to red, therefore the strain belongs to the colour-group "yellow-brown + red (or orange)". This reverse mycelium pigment and the soluble brownish or brown-red-violet pigment formed in several media were pH-sensitive changing from red-violet to yellow when tested with acid.

No melanin was produced on peptone-iron agar and tyrosine agar (ISP media 6, 7).

Physiological Characteristics

The carbohydrate utilization was determined according to the method of PRIDHAM and GOTTLIEB[7]. The following carbohydrates, as the sole carbon source, supported growth: D-glucose, D-xylose, L-arabinose, rhamnose, D-fructose, D-mannitol, sucrose. There was no growth on raffinose, *m*-inositol and cellulose. Gelatine was liquefied by the strain, starch was hydrolyzed and milk peptonized.

The strain was inhibited by streptomycin. The NaCl tolerance was $\geqq 4\%$, but $< 7\%$.

The organism showed abundant growth and good sporulation at temperatures between 22 and 30°C, but no growth at 50°C. Analysis of above results indicates that the strain IMET 20978 can be identified as *Streptomyces fimicarius* (DUCHÉ) WAKSMAN and HENRICI 1948[8,9].

A culture of the type strain has been deposited with the culture collection of the Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena, DDR, as IMET 20978.

Table 1. Cultural characteristics of strain IMET 20978.

Medium	Growth	Aerial mycelium	Reverse colour	Soluble pigment
Yeast extract malt agar (ISP medium 2)	abundant, pale yellowish	moderate, yellowish-greenish-gray, later faint pink	yellow-brown to red-brown	faint violet-brown
Oatmeal agar (ISP medium 3)	abundant, pale yellowish	abundant, yellowish-greenish-gray, later faint pink	orange-yellow to red-brown	faint brown
Inorganic salts starch agar (ISP medium 4)	moderate, colourless to ivory	thin, light yellowish	yellow-brown	no distinctive pigment
Glycerol asparagin agar (ISP medium 5)	abundant, pale yellowish	abundant, light greenish-brownish	yellow-brown to orange-brown	no distinctive pigment
CZAPEK saccharose agar	abundant, ivory to yellowish	abundant, yellowish-greenish-gray, later faint pink	orange-brown to violet-brown	faint brown to brown-red-violet

Fermentation

For the best production of the griseorubins a well sporulated agar slant culture of the *Streptomyces* strain No. IMET 20978 was used as a seed culture. Spore suspensions from a 7-day-old culture of the agar slant were used to seed 50 ml of inoculum medium in a 500-ml wide-mouth flask. The inoculum medium consisted of 1.5% glucose, 1.5% soy bean meal, 0.5% NaCl, 0.1% $CaCO_3$, 0.03% KH_2PO_4, and tap water. The pH was adjusted before sterilization to 6.8. The seed flask was incubated at 27~29°C on a rotary shaker (180 r.p.m.) for 2 days. For a second preculture, a 2-liter flask containing 400 ml of the medium mentioned above, was inoculated with 10 ml of the first preculture. The incubation was carried out at 180 r.p.m. and 27~29°C for 2 days. After that a 5% amount of this second preculture was transferred into 150 liters of a production medium in a 300-liter fermentor and incubated at 28°C for 5 days (agitation, 300 r.p.m., aeration, 150 liters/min.). The fermentation medium was composed of 2% glucose, 2% soy bean meal, 0.5% NaCl, 0.3% $CaCO_3$ and tap water. The pH was adjusted before the sterilization to 7.2. Soy bean oil was used as an antifoam agent. The antibiotic level in the culture broth was determined by the "BIP" test method.

Isolation and Purification

The antibiotic complex was isolated from both the culture filtrate and the mycelium by extraction with *n*-butanol and methanol, respectively. A flow diagram for the isolation procedures is given in Fig. 1.

All griseorubins consist of several components as shown by TLC (Fig. 2). For example, griseorubin E can be separated into eight major and minor components by preparative TLC.

General Characterization

All griseorubins have very similar physico-chemical properties. They form amorphous red-violet powders and are weakly basic compounds. The free bases of the griseorubins are readily soluble in lower alcohols, acetone, chloroform, dimethylformamide and pyridine and are insoluble in benzene, petrolether, hexane, ether, and water. The hydrochlorides are soluble in methanol, ethanol, and water. The UV spectrum of the griseorubin complex in methanol is characterized by maxima at 244 nm ($E^{1\%}_{1cm}$ 380), 428 nm ($E^{1\%}_{1cm}$ 58) and a shoulder at 274 nm ($E^{1\%}_{1cm}$ 274) as shown in Fig. 3. In dilute acid the spectrum undergoes only minor changes, whereas in 0.1 N methanolic NaOH the absorption maxima are at 256, 333, and 551 nm. An alkaline solution of the griseorubin complex is thus violet. A bathochromic shift of the visible maxima results by complexing with boroacetic acid. Moreover, the griseorubin complex is easily reduced with Zn in acetic anhydride and with dithionite, respectively. The IR spectrum of the griseorubin complex discloses a broad band at 3400 cm^{-1} due to hydroxyl groups and a strong band at 1640 cm^{-1} corresponding to carbonyl groups. All these characteristics are indicative of a hydroxyquinone moiety. Mild treatment with dilute acid does not lead to any defined degradation products, suggesting that the griseorubins do not contain any simple glycosidic linkages. The absorption pattern in the visible and ultraviolet region indicates a close similarity of the griseorubins to the antibiotics hedamycin[1], indomycins[10], iyomycins[11], kidamycin[2], neopluramycin[3], pluramycin[3], and rubiflavin[12] produced by other *Streptomyces* strains.

The UV data of the griseorubins E_1~E_6 are given in Table 2. As shown in Fig. 4 the IR spectra of the griseorubins E_1~E_6 were quite similar to each other and typical for antibiotics like kidamycin.

Fig. 1. Isolation and purification procedure for the griseorubins.

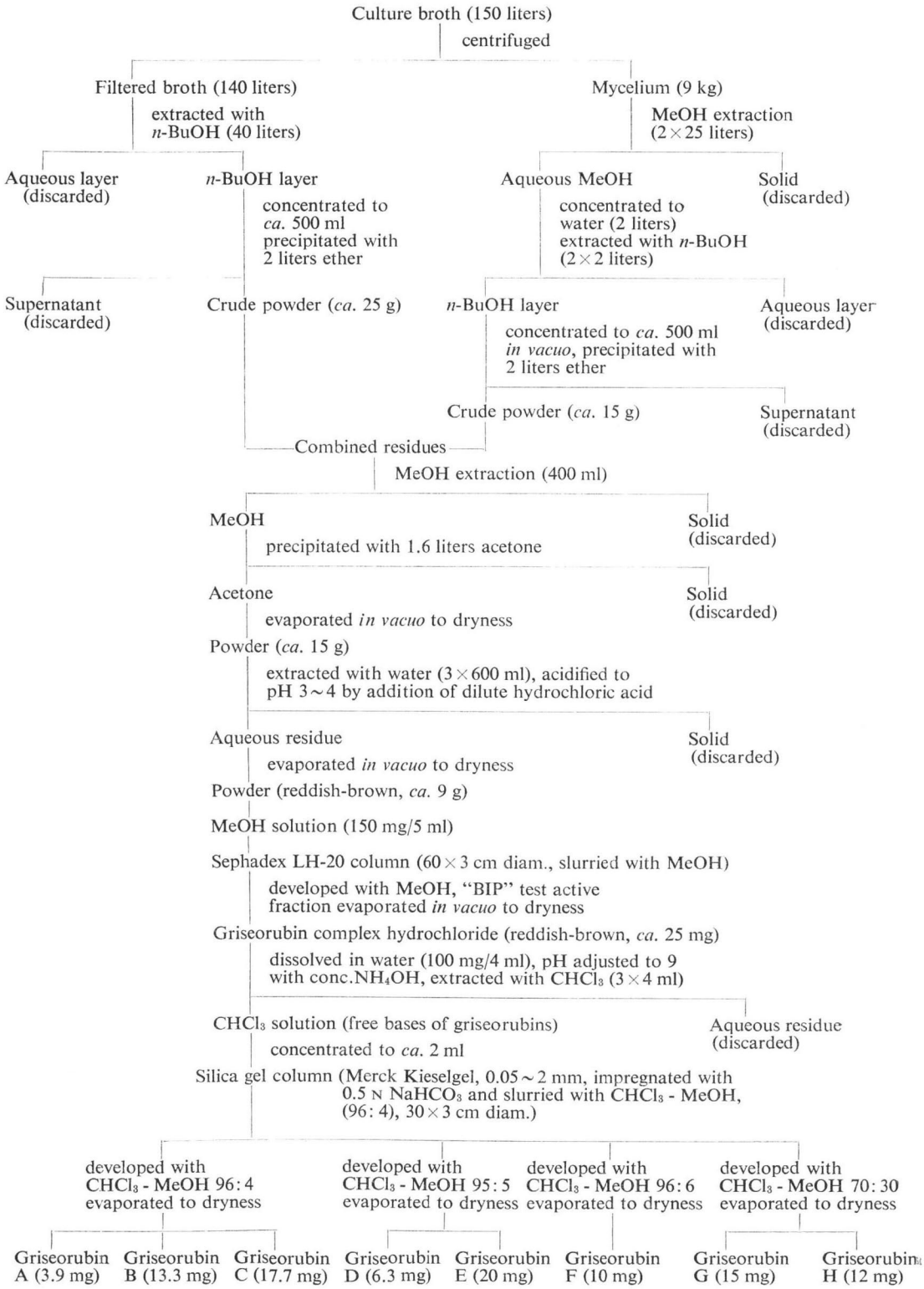
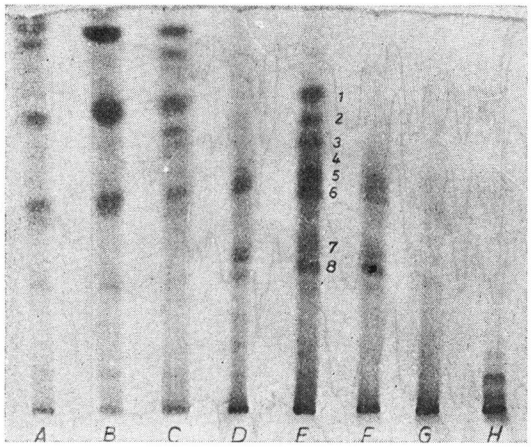


Fig. 2. Thin-layer chromatogram of the griseorubins.



Solvent: $CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1), lower phase

Sheet: Polygram Sil G/UV_{254} (Macherey & Nagel, FRG) impregnated with 0.5 N $NaHCO_3$.

Fig. 3. UV spectrum of the griseorubin complex in methanol.

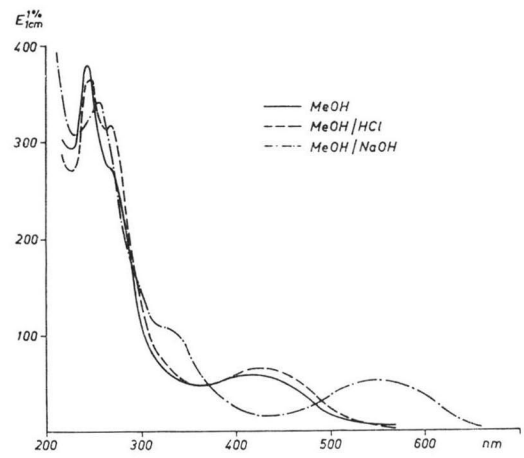


Table 2. UV data of griseorubins $E_1 \sim E_6$ in methanol.

	λ_{max} (nm)	$E^{1\%}_{1cm}$		λ_{max} (nm)	$E^{1\%}_{1cm}$
Griseorubin E_1	243	556	Griseorubin E_4	243	665
	274	366		273	416
	428	106		432	222
Griseorubin E_2	243	582	Griseorubin E_5	246	487
	271	345		263	457
	422	100		430	55
Griseorubin E_3	244	476	Griseorubin E_6	245	490
	270	334		263	385
	429	90		434	49

Griseorubin E_1, the main component, melts with decomposition at 220°C. The elemental analysis is as follows: C 67.61, H 6.29, N 3.62, O (diff.) 22.48%. The minimal molecular formula, calculated for two atoms of N in the molecule is $C_{44}H_{50}N_2O_{11}$ with a molecular weight of 782. Due to the thermal lability of the antibiotic it has not been possible to determine the exact mass of the molecular ion by electron impact, electron attachment, and field desorption mass spectroscopy. The molecular weight of griseorubin E_1 determined by vapour pressure osmometry in acetone was 728.

The ^1H NMR spectrum ($CDCl_3$, 100 MHz) of griseorubin E_1 indicates the presence of six methyl groups, three of them as singlets at 0.86, 2.00 and 2.98 ppm and three as doublets at 1.42 (J=6 Hz), 1.48 (J=6 Hz) and 2.04 (J=5 Hz) ppm. The spectrum further contains two singlets at 2.25 and 2.45 ppm corresponding to two dimethyl amino groups, three aromatic protons as singlets at 6.38, 7.72 and 7.90 ppm as well as a vinyl proton as a multiplet at 7.45 ppm. In the region of $3 \sim 5$ ppm the spectrum shows some multiplets which are very difficult to determine. At about 14 ppm a broad signal

Fig. 4. IR spectra of the griseorubins $E_1 \sim E_6$ (KBr pellets).

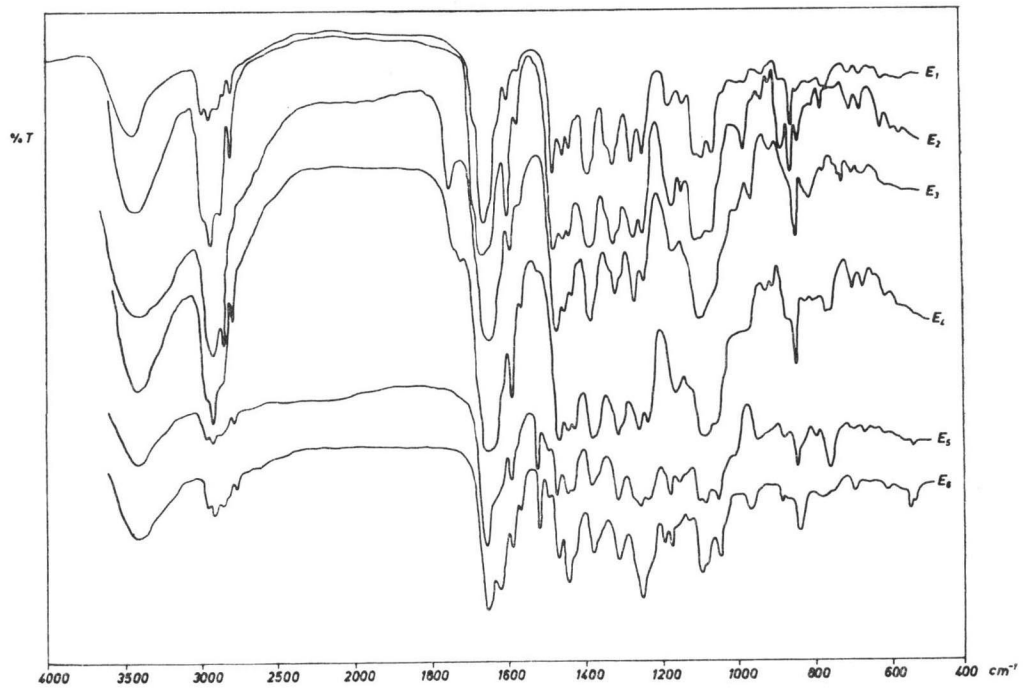


Table 3. UV maxima of hedamycin, kidamycin and griseorubin E_1 in methanol.

	UV maxima (nm)		
Hedamycin	244	264 S*	434
Kidamycin	243	270 S	434
Griseorubin E_1	243	274 S	428

* S=Shoulder

Table 4. Rf values of kidamycin and griseorubin E_1 on TLC.

	I	II	III	IV
Kidamycin	0.73	0.90	0.83	0.60
Griseorubin E_1	0.62	0.89	0.80	0.65

System I: $CHCl_3$ - MeOH - 17% NH_4OH (2:1:1), lower phase, Polygram SIL G/UV_{254} sheets (Macherey & Nagel, FRG)
II: $CHCl_3$ - MeOH - 17% NH_4OH (2:1:1), lower phase, SIL G/UV_{254} sheets impregnated with 0.5 N $NaHCO_3$
III: EtOH - 17% NH_4OH (4:1) SIL G/UV_{254} sheets impregnated with 0.5 N $NaHCO_3$
IV: *n*-BuOH - MeOH - H_2O (4:1:2) Polygram Alox N/UV_{254} sheets (Macherey & Nagel, FRG)

can be seen corresponding to one proton which readily exchanges with D_2O belonging certainly to one H-bonded hydroxyl group.

The chromatographic behaviour of griseorubin E_1 on thin-layer sheets in comparison with kidamycin is described in Table 4.*

Comparison of Griseorubin E_1 with other Antibiotics

Griseorubin E_1 was compared with other antibiotics of this class as far as the structures or parts of them are known, such as kidamycin[2], hedamycin[1], pluramycin[3], neopluramycin[3], and the indomycins[10]. Griseorubin E_1 shows similar colour reactions to those of kidamycin. The UV spectra of both antibiotics are similar to each other. In order to ascertain the presence of a quinoid group, re-

* Kidamycin was kindly supplied by Dr. N. KANDA, Research Institute, Daiichi Seiyaku Co., Ltd., Tokyo.

Fig. 5. UV spectra of the reduction products in methanol.

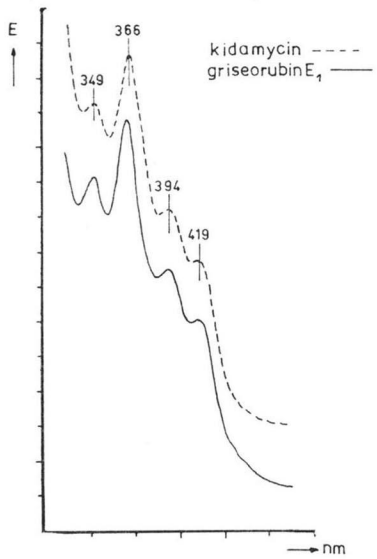
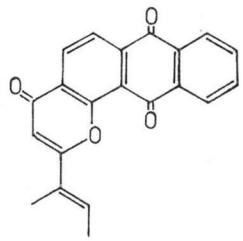


Fig. 6. Chromophore of griseorubin E_1.



ductive acetylation of griseorubin E_1 in comparison with kidamycin with zinc and acetic anhydride was undertaken. As shown in Fig. 5 both reduction products have the same characteristic UV absorption maxima. This suggests that both antibiotics contain the same main chromophore, perhaps even with the same olefinic side chain at the pyrone ring. The IR data of the reduction compounds support this view. Both reduction products give rise to a strong carbonyl absorption at 1640 cm^{-1} indicating the presence of an additional conjugated carbonyl function which belongs to the pyrone ring. The effect of different side chains on this chromophore was investigated in connection with the structural studies of the indomycins[10].

Indomycinone derivatives with different side chains have similar UV spectra. The main differences can be observed in the region of 270 nm, this band is shifted according to the number of double bonds in the side chain conjugated to the pyrone ring. Comparison of the UV-maxima of kidamycin, hedamycin, and griseorubin E_1 (Table 3) shows that griseorubin E_1 probably contains very similar olefinic substitution pattern to kidamycin but is different from hedamycin which also possesses the same chromophore as kidamycin but with a saturated substituent at the pyrone ring (Fig. 6).

Comparison of the ^1H and ^{13}C NMR spectra of hedamycin and kidamycin with those of griseorubin E_1 (unpublished data) shows that griseorubin E_1 most probably contains very similar, if not the same C-glycosyl amino sugar rings as hedamycin and kidamycin. Details will be reported in a separate paper.

However, griseorubin E_1 can be differentiated from kidamycin by its TLC behaviour (see Table 4) and by its biological activities (see the following paper).

Neopluramycin and pluramycin can be distinguished from griseorubin E_1 by their IR spectra which show bands at 1745 cm^{-1} characteristic for non-conjugated carbonyl groups. It was therefore concluded that the antibiotic griseorubin E_1 was a new antibiotic different from related antibiotics which structures are known at present. Further investigations on the chemical structure are in progress.

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