NEW ANTIBIOTICS 4181-A AND B FROM *STREPTOMYCES GRISEUS*; TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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The new antibiotics 4181-A and B were isolated from the fermentation broth of *Streptomyces griseus*, a soil isolate. Their molecular formulae were determined as $C_{29}H_{21}NO_9$ and $C_{28}H_{19}NO_9$, respectively. The UV, IR and NMR spectra suggest that they possess a quinone moiety in their structures. They were found to have antibacterial, antifungal and antitumor activity.

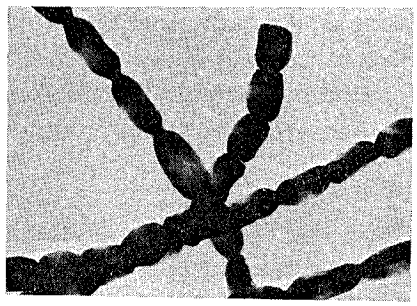
In the course of our screening for new antibiotics from actinomycetes, antimicrobial and antitumor antibiotics, 4181-A and B, were isolated by extraction with ethyl acetate from both culture broth and mycelia of *Streptomyces griseus*, newly isolated from a soil sample. The present paper deals with the taxonomy of the producing strain 4181, the fermentation, isolation and physico-chemical and biological properties of the antibiotics.

Taxonomy of the Producing Strain 4181

The strain 4181 was isolated from a soil sample collected at Emei Mountain in Sichuan Province, China. Taxonomic properties were determined according to the method of the International Streptomyces Project (ISP)[1] using the media recommended by WAKSMAN[2].

Microscopic observation showed the aerial mycelium to be generally straight to flexious and mature spore chain had 8 to 40 or more spores per chain. The spores were cylindrical in shape with smooth surface, as shown in Plate 1. No scleotia, sporangia or flagellated spores were observed.

Plate 1. Electron micrograph of the spores of strain 4181.



The cultural characteristics of strain 4181 on various media are shown in Table 1. Aerial mycelium was often poorly developed and mass

Table 1. Cultural characteristics of strain 4181.

Medium	Growth	Aerial mycelium	Reverse side	Soluble pigment
Sucrose - nitrate agar	Good	Light gray	Dark olive	None
Glucose - asparagine agar	Moderate	Whitish	Light yellow	None
Nutrient agar	Moderate	None	Light yellow	None
BENNET's agar	Moderate	None	Pale yellowish brown	None
Yeast extract - malt extract agar (ISP medium No. 2)	Moderate	Grayish white	Pale yellowish brown	None
Oatmeal agar (ISP medium No. 3)	Good	Grayish	Dull yellow	None
Inorganic salts - starch agar (ISP medium No. 4)	Poor	None	Colorless	None
Glycerol - asparagine agar (ISP medium No. 5)	Moderate	Light yellowish gray ~ light gray	Dark olive	None
Tyrosine agar (ISP medium No. 7)	Good	Light gray	Olive black	None

Color names were assigned according to Guide to Color Standard (Nippon Shikisai Co., Ltd., Tokyo, 1981).

color was almost light gray on some media tested. The vegetative mycelium developed well without fragmentation on most of the media and was usually pale yellowish brown to dark olive or olive black (Table 1). Melanoid pigment was not produced on peptone - yeast extract - iron agar or tyrosine agar.

Table 2. Physiological properties of strain 4181.

Optimum temperature for growth	27~30°C
Melanin production	−
Starch hydrolysis	+
Milk coagulation	+
Milk peptonization	+
Gelatin liquefaction (27°C)	+ (weakly)
Nitrate reduction	−
H_2S production	−

+: Positive, −: negative.

The physiological properties of strain 4181 are shown in Table 2. The utilization of carbon sources by this strain was examined according to the methods of PRIDHAM and GOTTLIEB[3]. D-Glucose, L-arabinose, D-xylose, inositol, D-mannitol, D-fructose, rhamnose, sucrose, raffinose, D-mannose, maltose, D-sorbitol, inuline and sodium citrate were found to be utilized for growth. Chemical analysis of whole-cell hydrolysate by the procedure of BECKER *et al.*[4] revealed the presence of LL-diaminopimelic acid and glycine.

The taxonomic studies indicated that strain 4181 belongs to the genus *Streptomyces*. The characteristics of strain 4181 were compared with the published description of various *Streptomyces* in the approved lists of bacterial names[5] and subsequent species published validly. It was considered strain 4181 to be closely related to a member of *S. griseus*[6]. Therefore, strain 4181 was classified as a strain of *S. griseus*, named *S. griseus* 4181. This strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Streptomyces* sp. 4181 with accession number of FERM BP-1010.

Fermentation

The antimicrobial activity was determined by the agar well method using *Staphylococcus aureus* FDA 209P and *Micrococcus luteus* ATCC 9341 as test organisms. The organisms were also used for bioautography.

The parent strain 4181 spontaneously decreased in its ability to produce antibiotics after several

times transplantation and thereafter selection of a high-producing strain was examined by monospore isolation. The antibiotic low-producing variant lost simultaneously its ability to produce yellowish brown to dark olive pigment in the fermentation broth. The high-yield variant selected was used throughout the fermentation experiments.

The stock culture of *S. griseus* 4181 was maintained in lyophilized tubes of 10% skim milk and the working cultures were grown on inorganic salts - starch agar slant (ISP medium No. 4). Loopful spores and mycelia from a 2 weeks-old slant were inoculated into 100 ml of the seed medium in a 500-ml Erlenmeyer flask, and incubated at 27°C for 2 days on a rotary shaker at 220 rpm (3.5 cm-stroke). The seed medium consisted of glucose 0.1%, soluble starch 2.4%, Polypeptone 0.1%, beef extract 0.3%, yeast extract 0.5% and $CaCO_3$ 0.3%, pH 7.0. Four ml (4%) of this seed culture were transferred to 100 ml of the fermentation medium in a 500-ml Erlenmeyer flask containing glycerol 4.0%, Pharmamedia 1.0%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.2% and $CaCO_3$ 0.3%, pH 7.0. The fermentation was carried out at 27°C for 4 days on a rotary shaker at 220 rpm (3.5 cm-stroke).

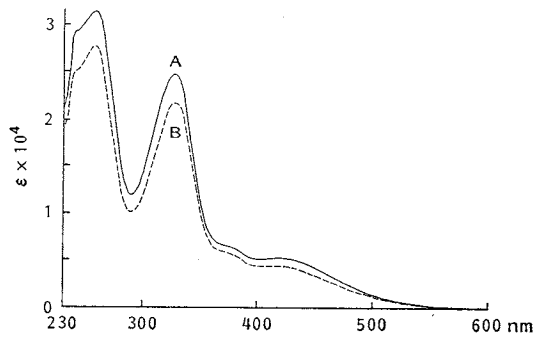
Isolation and Purification

After the fermentation was carried out, the fermentation broth (25 liters) was centrifuged to separate the mycelium from the broth. The mycelial cake was extracted three times with methanol, and the methanol extract was concentrated to a small volume. This concentrate was adjusted to pH 3 and extracted twice with ethyl acetate. The broth filtrate was also adjusted to pH 3 and extracted twice with ethyl acetate. The ethyl acetate layers were combined, washed with water, dried with anhydrous Na_2SO_4 and evaporated to a suitable volume. To the ethyl acetate solution, *n*-hexane was added to obtain the precipitate containing antibiotics. The precipitate was collected by centrifugation, and dried *in vacuo* to yield a dark brown powder (6.32 g). This crude powder was dissolved in chloroform and subjected to silica gel column chromatography. The column was successively developed with chloroform and chloroform - methanol (50 : 1 and 20 : 1).

Most of antibiotic 4181-A was eluted as a major component with chloroform. The active fractions containing antibiotic 4181-A were combined, concentrated to dryness, and further purified by recrystallization from a mixture of chloroform and acetone to give an orange powder (733 mg). The fractions of antibiotic 4181-B, eluted with a mixture of chloroform and methanol (20 : 1) as a minor component, were concentrated to dryness and subsequently obtained as a reddish orange powder (218 mg) after recrystallization from a mixture of chloroform and methanol.

The preparation of antibiotics 4181-A and B were analyzed by HPLC to determine their purity under the following conditions: Column, μBondapak CN (150×3.9 mm i.d.); mobile phase, (a) *n*-hexane - acetic acid - chloroform (195 : 5 : 100) and (b) (95 : 5 : 100); flow rate, 1.5 ml/minute; detector, UV at 254 nm. The respective retention times of antibiotics 4181-A and B were 4.6 and *ca.* 44 minutes with mobile phase (a) and were 1.7 and 6.5 minutes with mobile phase (b).

Fig. 1. UV spectra of 4181-A and B (in $CHCl_3$).



Physico-chemical Properties

Both of the antibiotics 4181-A and B were

acidic and slightly soluble in chloroform, methanol, ethyl acetate and dimethylsulfoxide, and insoluble in water, *n*-hexane and ethyl ether. The molecular formula of antibiotics 4181-A and B were determined as $C_{29}H_{21}NO_9$ and $C_{28}H_{19}NO_9$ from elemental analysis and the molecular weight determination by fast atom bombardment mass spectrometry (FAB-MS). The ions observed in FAB-MS of these antibiotics are probably the *quasi*-molecular ions of $(M+1)^+$ for respective hydroquinone forms[7]. The UV and IR spectra of antibiotics 4181-A and B are shown in Figs. 1 and 2, respectively. The characteristic ketone absorption observed at 1686 cm^{-1} (or 1680 cm^{-1}) in IR spectra suggests that

Fig. 2. IR spectra of 4181-A and B (KBr).

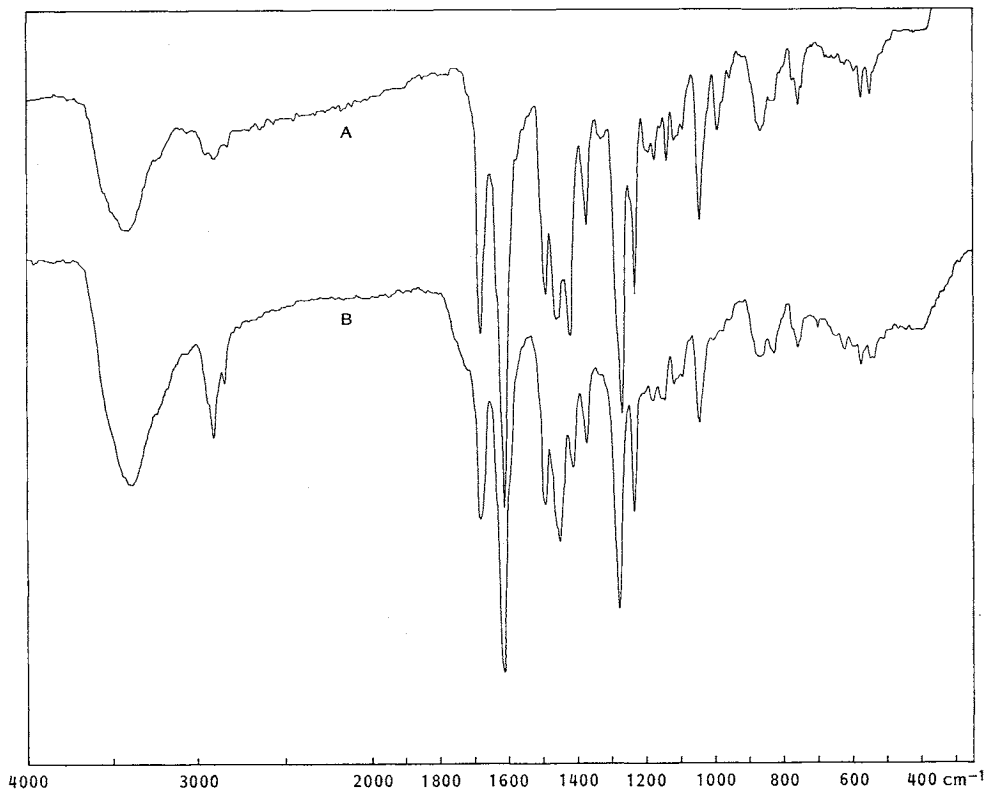


Table 3. Physico-chemical properties of 4181-A and B.

	4181-A	4181-B
Appearance	Orange powder	Reddish orange powder
MP (°C, dec)	>300	>260
Molecular formula	$C_{29}H_{21}NO_9$	$C_{28}H_{19}NO_9$
Elemental analysis	C 65.48, H 4.36, N 2.58.	C 63.45, H 5.57, N 1.91.
FAB-MS (*m/z*)	530.147 $(MHQ+1)^+$ (calcd 530.149)	516.134 $(MHQ+1)^+$ (calcd 516.139)
UV $\lambda_{max}^{CHCl_3}$ nm (ε)	258 (31,000), 327 (24,800), 376 (sh, 6,650), 418 (5,330)	258 (22,700), 327 (18,000), 377 (sh, 5,220), 418 (3,770)
IR ν_{max}^{KBr} cm^{-1}	3430, 1686, 1621, 1468, 1461, 1425, 1278, 1238	3400, 1680, 1612, 1489, 1450, 1411, 1278, 1231
Rf value*	0.39	0.07

* Silica gel TLC: $CHCl_3$ - MeOH (40 : 1).

these compounds belong to quinone antibiotics. The ^{1}H NMR spectrum of antibiotic 4181-A, shown in Table 4, indicates the presence of five aromatic protons (δ 7.4~8.2), two methoxy groups (δ 3.92 and 3.99) and one aromatic hydroxyl group (δ 14.00), together with three methylene groups and a tertiary methyl group. Antibiotic 4181-B is closely related to 4181-A, except for an aromatic hydroxyl group instead of a methoxy group in the ^{1}H NMR spectrum. These results as well as other physico-chemical properties of antibiotics 4181-A and B are summarized in Table 3.

Table 4. ^{1}H NMR data of 4181-A and B (in DMSO-d_6, 100 MHz).

	4181-A	4181-B
Aromatic OH	14.00 (1H, s)	13.98 (1H, s), 10.14 (1H, s)
Aromatic H	8.15 (2H, s), 7.46 (3H, s)	8.13 (2H, s), 7.43 (1H, s), 7.42 (1H, s), 7.40 (1H, s)
OCH_3	3.99 (3H, s), 3.92 (3H, s)	3.98 (3H, s)
CH_2	4.24 (2H, br t), 4.0~3.6 (2H, m), 3.37 (2H, br s)	4.23 (2H, br t), 3.9~3.6 (2H, m), 3.35 (2H, br s)
CH_3	1.35 (3H, s)	1.35 (3H, s)

Biological Properties

Antimicrobial activity was determined by serial agar-dilution method using Nutrient agar for bacteria and Sabouraud agar for yeast and filamentous fungi. MICs were exhibited in terms of μg/ml after overnight-incubation at 37°C for bacteria and after incubation for 48~72 hours

Table 5. Antimicrobial spectra of 4181-A and B.

Test organisms	MIC (μg/ml)	
	4181-A	4181-B
Staphylococcus aureus FDA 209P	6.25	<0.2
Micrococcus luteus ATCC 10240	25	<0.2
M. luteus ATCC 9341	100	<0.2
Bacillus subtilis ATCC 6633	6.25	<0.2
Streptococcus feacalis subsp.	3.13	<0.2
Mycobacterium smegmatis ATCC 607	100	100
Escherichia coli B IFO 13168	>100	1.56
Proteus vulgaris IFO 3849	>100	1.56
Klebsiella pneumoniae ATCC 10031	>100	3.13
Salmonella typhimurium	>100	6.25
Serratia marcescens IFO 12648	>100	3.13
Pseudomonas aeruginosa IFO 13275	>100	6.25
Citrobacter freundii IFO 12681	>100	6.25
Enterobacter cloacae IFO 13535	>100	6.25
Acinetobacter calcoaceticus IFO 12552	>100	3.13
Flavobacterium meningosepticum IFO 12235	>100	0.39
Candida albicans IFO 1061	0.78	0.39
C. utilis IAM 4220	0.78	0.78
C. krusei IFO 0011	3.13	3.13
Saccharomyces delbrueckii IAM 12236	0.78	1.56
Rhodotorula rubra	1.56	1.56
Pichia farinosa IFO 0193	3.13	3.13
Aspergillus niger IFO 6341	>100	3.13
Penicillium notatum IFO 4640	>100	1.56
Trichophyton mentagrophytes IFO 5466	100	0.78
Sclerotinia sclerotiorum IFO 4876	100	1.56
Mucor racemosus Fresenius f. *racemosus* IFO 4581	100	0.78
Arthroderma tuberculatum IFO 8165	50	1.56

at 27°C for yeast and filamentous fungi. The antimicrobial spectra of 4181-A and B are shown in Table 5. As shown in the table, the antibiotic 4181-A has activity against Gram-positive bacteria, but not against Gram-negative bacteria. Antibiotic 4181-B showed strong antibacterial activity against a wide variety of Gram-positive and Gram-negative bacteria, and higher activity against Gram-positive bacteria compared to antibiotic 4181-A. Antibiotic 4181-B showed strong antifungal activity, but antibiotic 4181-A was less active against filamentous fungi.

The cytocidal activity of antibiotics 4181-A and B was examined on KB cells *in vitro*. When the cells were exposed to the antibiotic for 3 days, the ED_{50} values of antibiotics 4181-A and B were 0.003 μg/ml and 0.005 μg/ml, respectively.

Antitumor activity of antibiotics 4181-A and B was evaluated by prolongation of median survival time of mice bearing P388 leukemia. Antibiotics were administered intraperitoneally on day 1 and on day 1, 5 and 9 in BDF_1 mice. The antibiotics exhibited antitumor activity at a dose range of 5 to 40 mg/kg/day for 4181-A and 0.625 to 5 mg/kg/day for 4181-B. After intraperitoneal administration of 40 mg/kg of these antibiotics, the *dd*Y mice did not show any symptoms of toxicity.

Discussion

The physico-chemical and biological properties of antibiotics 4181-A and B were compared with those of known antibiotics. Recently, ŌMURA *et al.*[8,9] reported the antibacterial and anti-mycoplasmal antibiotics, cervinomycins. The spectral data of antibiotic 4181-A were very similar to those of cervinomycin A_2. Cervinomycin A_2 was reported to determine the molecular formula by high-resolution electron impact mass spectrometry (EI-MS), whereas the EI-MS of antibiotic 4181-A gave no characteristic ion peaks because of its less volatility.

The molecular formula of antibiotic 4181-B ($C_{28}H_{19}NO_9$) was found to differ only by CH_2 with that of 4181-A ($C_{29}H_{21}NO_9$). The ^1H NMR spectra showed antibiotic 4181-B to have one methoxy and two aromatic hydroxyl groups, while 4181-A showed two methoxy and one aromatic hydroxyl groups in their structures. Cervinomycin A_2 has been reported to be inactive against fungi and to have no antitumor activity, but antibiotics 4181-A and B to show antibacterial, antifungal and antitumor activity. Their biological properties differ from those of cervinomycins. Therefore, antibiotics 4181-A and B is considered to be new antibiotics.

Detailed studies on the structures and antitumor activity of these antibiotics will be reported elsewhere.

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