ARANOFLAVIN, A NEW ANTIBIOTIC

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(Received for publication September 16, 1970)

A new antibiotic, named aranoflavin, which inhibits growth of bacteria, protozoa, and tumor cells *in vivo*, has been isolated from *Arachniotus flavoluteus*. The antibiotic was separated into related two components designated as aranoflavin A ($C_{23}H_{33}NO_6$), and aranoflavin B ($C_{26}H_{39}NO_9$).

During our screening for new antibiotics from fungi, a new antibiotic, aranoflavin, has been isolated from the culture broth of *Arachniotus flavoluteus*[1,2] NHL 2271. The production, isolation and properties of the antibiotic are presented in this paper.

Production and Isolation

The organism was cultured at 26°C with shaking in a medium containing 2% glucose, 10% potato extracts which extracted from 300 g potato slices in 1 liter of water for 1 hour at 100°C, 0.5% Pharma media (Traders Oil Mill Co., Ltd.), 0.5% KH_2PO_4 and 0.25% $MgSO_4$ at pH 6.5.

The antibiotic produced was determined by paper disc method using *Staphylococcus aureus* FDA 209P as a test organism. The cultured broth was harvested after 72-hour fermentation.

Aranoflavin is a solvent-extractable antibiotic complex, which is obtained mainly from mycelium but also in smaller amounts of it from culture filtrate.

The mycelial cake obtained by filtration of the harvested broth (5 liters, pH 6.2) was extracted twice with 1 liter of acetone. The extracts were concentrated under reduced pressure and the remaining aqueous solution was extracted with 1 liter of butyl acetate.

The antibiotic in the broth filtrate (4 liters, 60 mcg/ml) was extracted twice with 1.5 liters of butyl acetate. Both the extracts from mycelium and filtrate were combined and concentrated *in vacuo* to an oily syrup. Two active spots were detected at Rf 0.7 and 0.45 with bioautography of thin-layer chromatography (TLC) using silicic acid (Merck, Kieselgel G, solvent system : ethyl acetate).

In order to separate aranoflavin complex, the syrup in 10 ml of ethyl acetate was applied on silicic acid column (Mallinckrodt, 100 mesh) and developed with benzene – ethyl acetate (4 : 1).

Two main active eluates were colleted and evaporated to dryness. These two fractions were named aranoflavin A (2.5 g) and aranoflavin B (1.0 g), respectively.

Further purification of each component was carried out by silicic acid column

chromatography. When the column was developed with benzene-ethyl acetate in the ratio of 2:1 and 1:1, 1.0 g aranoflavin A and 0.5 g aranoflavin B were recovered.

Physico-chemical Properties

Pure aranoflavins A and B are colorless powders. The antibiotics were obtained in crystalline form from benzene, however, crystallinity was lost upon drying. The physico-chemical properties of the antibiotics were summarized in Table 1.

As shown in Table 1 and Fig. 1, the components A and B exhibiting same ultraviolet absorption maxima are suggested to have chemically close relationship with each other. The infrared spectra are shown in Fig. 2. The NMR spectra in $CDCl_3$ measured at 60 MHz are indicated in Fig. 3.

The antibiotic is relatively stable at neutral and acidic pH but very labile at alkaline pH.

Table 1. Physico-chemical properties of aranoflavin

	Aranoflavin A	Aranoflavin B
M. p.	123~125°C	93~95°C
$[\alpha]_D^{23}$	−10° (*c* 0.5, $CHCl_3$)	inactive
M. w. (Mass spectrometry)	419	477
Anal : Found	C 65.74	C 64.88
	H 7.80	H 8.43
	N 3.36	N 2.90
	no S & halogen	no S & halogen
Calcd.	C 65.85	C 65.38
	H 7.93	H 8.23
	N 3.34	N 2.93
Formula	$C_{23}H_{33}NO_6$	$C_{26}H_{39}NO_7$
UV λ_{max}^{MeOH}	266 mμ ($E_{1cm}^{1\%}$ 800)	266 mμ ($E_{1cm}^{1\%}$ 690)
Solubility soluble :	lower alcohols acetone, $CHCl_3$, esters	lower alcohols acetone, $CHCl_3$, esters
insoluble :	H_2O, CCl_4, petroleum ether	H_2O, CCl_4, petroleum ether
Color reaction positive :	TOLLENS, permanganate	TOLLENS, permanganate
negative :	$FeCl_3$, ninhydrin, EHRLICH	$FeCl_3$, ninhydrin, EHRLICH
TLC : Rf (Merck, Kieselgel G, Ethyl acetate)	0.70	0.45

Biological Properties

The antibacterial and antiprotozoal activities of aranoflavin determined by agar dilution method are given in Table 2.

The results shown in Table 2 demonstrated that aranoflavin A is more active than B. The antibiotic

Fig. 1. Ultraviolet spectra of aranoflavin.

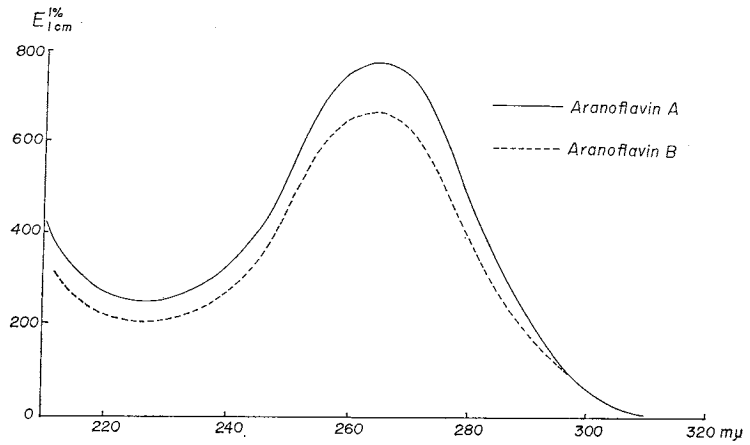
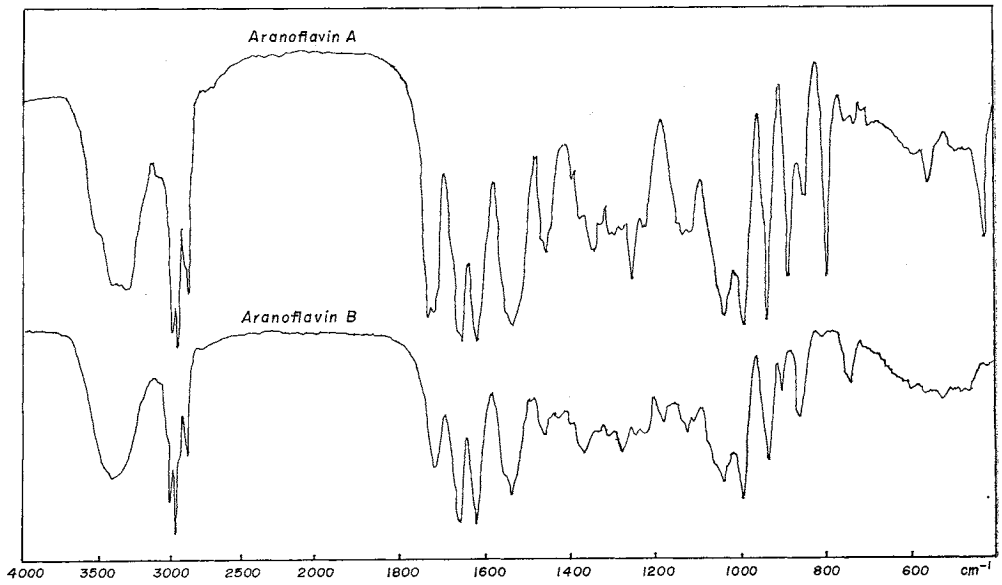


Fig. 2. Infrared spectra of aranoflavin (KBr)



has no antifungal activity.

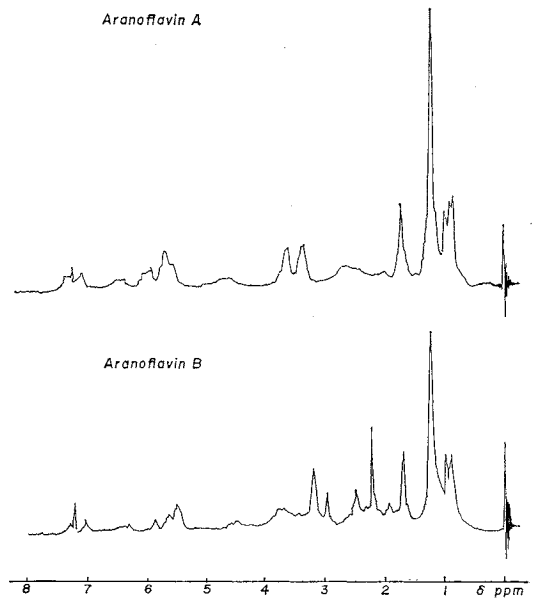
Aranoflavin A showed cytotoxic effect at 1.0 mcg/ml, and cytostatic effect at 0.5 mcg/ml on HeLa cells, and also showed similar effect on L cells, CEF cells, and HEL cells in tissue culture.

The antimycoplasma activities were measured by broth dilution method (Table 3).

Moderate prolongation in the survival period of mice inoculated with lymphatic leukemia L-1210 was observed by intraperitoneal administration of aranoflavin A. At daily doses of 5 mcg per mouse for 3 days, prolongation rate of the survival period was 150 %.

The antitumor activity of aranoflavin A was also observed on mouse ascites tumor with sarcoma 180, and the results are given in Table 4.

Fig. 3. NMR spectra of aranoflavin (60 MHz in $CDCl_3$, internal reference : TMS)



The intraperitoneal LD_{50} of aranoflavin A in mice was 4.6 mg/kg body weight, and no toxicity was observed at 100 mg/kg of aranoflavin B.

Discussion

Two active components aranoflavins A and B, were obtained from the cultured broth of *Arachniotus flavoluteus*. Aranoflavins A and B are thought to be related as suggested

Table 2. Antimicrobial activites of aranoflavin

Test organism	Minimum inhibitory concentration (mcg/ml)	
	Aranoflavin A	Aranoflavin B
Pseudomonas aeruginosa	>100	>100
Escherichia coli NIHJ	25	>100
〃 〃 B	>100	>100
Klebsiella pneumoniae PCI 602	12.5	>100
Proteus vulgaris OX 19	12.5	>100
Salmonella paratyphi A	12.5	>100
〃 〃 B	12.5	>100
〃 *enteritidis*	>100	>100
Shigella dysenteriae E-1	25	>100
〃 *flexneri*	50	>100
〃 *sonnei* E-33	>100	>100
Bacillus subtilis PCI 219	<0.2	3.2
Staphylococcus aureus FDA 209P	0.8	25
〃 *albus*	0.8	25
〃 *citreus*	0.8	25
Micrococcus flavus	0.8	0.8
Sarcina lutea	0.8	<0.2
Vibrio A	6.3	>100
〃 B	1.6	>100
Mycobacterium 607	>100	50
Trichomonas vaginalis TV-1099	3.13～1.56	50

Table 3. Antimycoplasma activities of aranoflavin A

Test organism	Minimum inhibitory concentration (mcg/ml)
Mycoplasma pneumoniae	0.6
M. gallisepticum	0.6
M. hominis	5.0
M. laidlowii	0.05

Table 4. Antitumor activity of aranoflavin A on mouse ascites tumor (sarcoma 180)

Dose (mg/kg)	No. of mice	Death/Treated	Ave. of tumor weight (g)
2.0	4	2/4	0.00
1.5	4	1/4	0.00
0.5	4	0/4	0.00
0.25	4	0/4	1.37
Saline (control)	10	0/10	5.38

Treatment was started 24 hours after tumor inoculation and made once daily intraperitoneally for 6 days.

by their ultraviolet and infrared spectra; however, it is very interesting that differences were observed in the biological tests. Aranoflavin A is more active and more toxic than aranoflavin B.

Compared with known antibiotics exhibiting ultraviolet absorption maximum at 266 mμ, no similar antibiotics were found in literatures except Rhi-12-648[3], which was easily differentiated from aranoflavin by the presence of chlorine atom in the molecule and antifungal activity.

Other species of *Arachniotus* have been reported to produce another antibiotic, aranotin[4], which can be distinguished from aranoflavin since aranotin has sulphur atoms in the molecule.

Acknowledgements

The authors wish to express their sincere thanks to Dr. S. UDAGAWA, National Institute of Hygienic Science, for kind supply of the strain, *Arachniotus flavoluteus* NHL 2271, and to Prof. N. ISHIDA, Tohoku University, for measurement of antitumor activities.

They also indebted to Dr. H. HORIE, Institute of Microbial Chemistry, for antiprotozoal assay, and Dr. S. OHMURA of Kitasato Institute for the measurement of mass spectra.

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