ILEUMYCIN, A NEW ANTIBIOTIC AGAINST *GLOMERELLA CINGULATA*

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A new antifungal antibiotic, named ileumycin, was isolated from culture broth of *Streptomyces* H 698-SY2, which was identified as *S. lavendulae*. The antibiotic was recovered from the culture filtrate by adsorption on Amberlite XAD-II and elution with aqueous methanol and was further purified by ion-exchange column chromatography on SE-cellulose and followed by partition chromatography on silica gel. The antibiotic was named ileumycin, because isoleucine was detected in the acid hydrolyzate of the antibiotic. Ileumycin exhibited antimicrobial activity against only a few species of fungi.

In the course of our screening for new antibiotics against plant pathogenic fungi, *Streptomyces* H 698-SY2 was shown to produce an antifungal antibiotic effective against *Glomerella cingulata*, a plant pathogenic fungus for grapevines, but not against other fungi, yeasts and bacteria. *Streptomyces* H 698-SY2 was isolated from a soil sample collected at Kōchi City, Kōchi Prefecture, and classified as belonging to *S. lavendulae*[1].

The antifungal activity was determined by the cylinder-agar plate method using *Glomerella cingulata* as test microbe on potato sucrose agar. The test microbe, grown for 3 days at 27°C on a potato sucrose agar slant, was suspended in 2 ml of saline to make a mycelial suspension. The mycelial suspension (2 ml) and potato sucrose agar (25 ml) were combined to make the seed agar. The seed agar (5 ml) was poured over a bottom layer of agar (10 ml of potato sucrose agar) to make the assay plate. Inhibition diameter of a test sample was measured after incubation at 27°C for 18~24 hours.

Fermentation

Streptomyces H 698-SY2 was cultured in shake flasks each containing 100 ml of an inoculation medium composed of 1.0% soluble starch and 0.2% yeast extract (pH 7.0); incubation was at 27°C for 24 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute). This inoculum (2%) was used to inoculate shake flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soyameal, 0.5% Ebios (dried yeast, distributed by Tanabe Pharmaceutical Co. Ltd.), 0.25% NaCl, 0.3% $CaCO_3$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $FeSO_4 \cdot 7H_2O$ (pH 7.6 before sterilization). The culture was grown at 27°C for 4 days on the reciprocal shaker.

Extraction and Purification

The antibiotic was recovered from the broth filtrate by adsorption on Amberlite XAD-II polystyrene resin, and elution with 80% aqueous MeOH; the eluate was evaporated to a small volume and lyophilized. The antibiotic was extracted with *n*-BuOH at pH 8 from an aqueous solution, then

Chart 1. Extraction and partial purification of ileumycin

Broth filtrate (37,000 ml, pH 7.2)
↓ stirred with Amberlite XAD-II (2,150 ml) for 2 hours and filtered
Amberlite XAD-II → Filtrate discarded (37,000 ml, total activity 0%)
↓ washed with H_2O (4,340 ml) and eluted thrice with 80% aqueous MeOH
First eluate (5,625 ml, total activity 70.5%)
Second eluate (4,130 ml, total activity 23.1%)
Third eluate (3,510 ml, total activity 4.3%)
↓ the eluates were combined, concentrated *in vacuo* and lyophilized
Crude antibiotic (30,773 mg, total activity 93.6%)
Crude antibiotic (30,000 mg)
↓ dissolved in H_2O (320 ml) and extracted thrice with *n*-BuOH at pH 8.0
n-BuOH layer → H_2O layer discarded (328 ml, total activity 22.7%)
First extract (275 ml, total activity 45.0%)
Second extract (275 ml, total activity 13.6%)
Third extract (275 ml, total activity 3.3%)
↓ extracted with H_2O acidified to pH 2.0 by addition of dilute H_2SO_4
H_2O layer (333 ml, total activity 62.1%) → *n*-BuOH layer discarded (825 ml, total activity 0%)
↓ extracted with ether
H_2O layer (320 ml, total activity 58.0%) → Ether layer discarded (100 ml, total activity 0%)
↓ adjusted to pH 6.0 by addition of aqueous $Ba(OH)_2$; $BaSO_4$ was removed by centrifugation and the supernatant was lyophilized
Partially purified antibiotic (1,767 mg, total activity 63.2%)

transferred from the *n*-BuOH layer to an aqueous layer at pH 2 (Chart 1.). Purification was carried out by ion-exchange column chromatography on SE-cellulose, followed by partition chromatography on silica gel, as summarized in Chart 2. When aqueous sodium chloride is treated with SE-cellulose (H^+-type), Na^+ is adsorbed on SE-cellulose to liberate Cl^-. Thus, the antibiotic on SE-cellulose was recovered as the hydrochloride by elution with a linear gradient concentration of aqueous sodium chloride.

Physical and Chemical Properties

Ileumycin was obtained as the hydrochloride. It is an amorphous white powder, decomposing at 230~232°C. The elemental microanalysis gave C, 44.61; H, 6.50; N, 9.74 and Cl, 11.45. No sulfur was detected.

Ileumycin hydrochloride shows ultraviolet absorption maxima at 220 nm ($E^{1\%}_{1cm}$ 192) and 280 nm ($E^{1\%}_{1cm}$ 156) in H_2O; at 222 nm ($E^{1\%}_{1cm}$ 174) and 278 nm ($E^{1\%}_{1cm}$ 164) in 0.1 N HCl; and at 288~290 nm ($E^{1\%}_{1cm}$ 144) in 0.1 N NaOH as shown in Fig. 1. The infrared absorption spectrum of the antibiotic hydrochloride is shown in Fig. 2. Ileumycin was shown to be homogeneous and appeared as a single spot on silica gel G thin-layer plates developed with several solvent systems, using bioautography, ninhydrin reaction or heating at 100°C for 20 minutes after spraying with 40% H_2SO_4 as detection criteria. The chromatography behavior of the antibiotic on paper strips and silica gel thin-layer plates is described in Tables 1 and 2. The antibiotic gave positive ninhydrin (orange), naphthoresorci-

Chart 2. Purification of ileumycin

Partially purified antibiotic (777 mg, total activity 100%)
↓ dissolved in H_2O (800 ml)
SE-Cellulose (H^+-type, 50×3.2 cm diam.)
↓ 1) washed with H_2O (750 ml)
2) eluted with a linear gradient concentration of NaCl, using H_2O (1,500 ml) and 0.1 M NaCl (1,500 ml); 18-ml fractions were collected
Fractions No. 37~54 (pH 3~4)
↓ lyophilized
Residue (368.1 mg, total activity 91.2%)
↓ extracted with MeOH (8.2 ml)
MeOH extract (total activity 63.8%) → Residue discarded (78.0 mg, total activity 0%)
↓ concentrated to 2 ml *in vacuo*
Sephadex LH-20 column (50×1.5 cm diam.)
↓ eluted with MeOH and collected in 9.4-ml fractions each
Fractions No. 7 and 8
↓ evaporated to dryness *in vacuo*
Residue (64.4 mg, total activity 62.8%)
↓ dissolved in 2 ml of *n*-BuOH - acetone - H_2O (4: 1: 1)
Silica gel column (Merck Kieselgel 60, 70~230 mesh, 58×1.8 cm diam.)
↓ developed with *n*-BuOH - acetone - H_2O (4: 1: 1) and collected in 8.8-ml fractions each
Fractions No. 14~18
↓ evaporated to dryness *in vacuo*
Residue (28.4 mg, total activity 61.5%)
↓ dissolved in 2 ml of *n*-BuOH - acetone - H_2O (4: 1: 1)
Silica gel column (Merck Kieselgel 60, 70~230 mesh, 56×1.8 cm diam.)
↓ developed with *n*-BuOH - acetone - H_2O (4: 1: 1) and collected in 10-ml fractions each
Fractions No. 13~16
↓ evaporated to dryness *in vacuo*
Residue (20.9 mg, total activity 60.8%)
↓ dissolved in abs. MeOH (0.5 ml), filtered; abs. ether (8.0 ml) was added to the filtrate
Precipitate → Supernatant discarded (8.5 ml, total activity 3.8%)
↓ dried *in vacuo*
Purified ileumycin (hydrochloride, 16.3 mg, total activity 54.7%)

Table 1. Paper chromatography behavior of ileumycin (hydrochloride)

Solvent systems	Rf value
n-BuOH - H_2O (87: 13)	0.47
n-BuOH - H_2O - AcOEt (87: 13: 30)	0.46
n-BuOH - acetone - H_2O (8: 2: 1)	0.41
n-ProOH - AcOEt - H_2O (7: 1: 1)	0.45
sec-BuOH - H_2O - AcOEt (87: 13: 30)	0.31

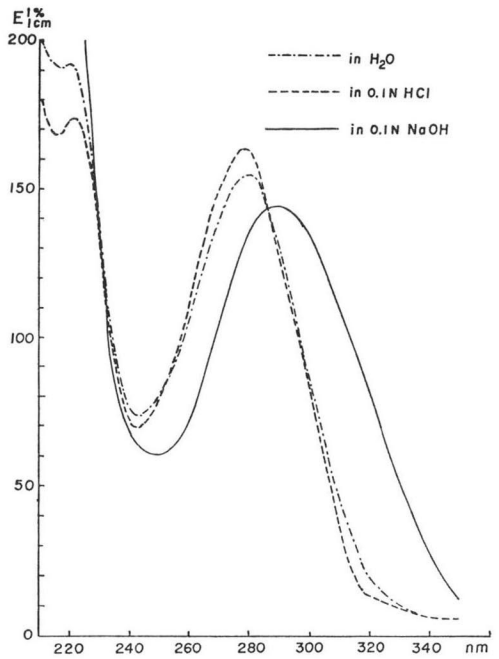
Table 2. Silica gel G thin-layer chromatography behavior of ileumycin (hydrochloride)

Solvent systems	Rf value
n-BuOH - H_2O (87: 13)	0.14
n-BuOH - MeOH - H_2O (4: 1: 1)	0.36
n-ProOH - AcOEt -H_2O (7: 1: 2)	0.68
n-BuOH - acetone - H_2O (4: 1: 1)	0.34
n-BuOH - acetone - H_2O (1: 1: 1)	0.67

nol-H_2SO_4 (yellow), TOLLENS and DRAGENDORFF reactions and decolorized a 1% $KMnO_4$ solution; The ELSON-MORGAN, α-naphthol-phosphate, anthrone-phosphate and SAKAGUCHI reactions were negative. The free base of ileumycin is soluble in H_2O or MeOH, slightly soluble in EtOH, *n*-BuOH

or dioxane, sparingly soluble in acetone, and insoluble in benzene or *n*-hexane. Ileumycin in an aqueous solution was stable when kept at 100°C (pH 2~5) for 5 minutes, but 37% of the activity (pH 7.0) or 85% of the activity (pH 8.0) were lost under the same conditions (100°C, 5 minutes). Ileumycin was hydrolyzed with constant boiling HCl at 110°C for 17 hours and the hydrolyzate gave one ninhydrin-positive spot (Rf 0.78), on paper chromatography developed with *n*-BuOH - AcOH - H_2O (4: 1: 1), which could not be differentiated from those of leucine and isoleucine. The hydrolyzate had the same retention time as isoleucine in the automatic amino acid analyzer.

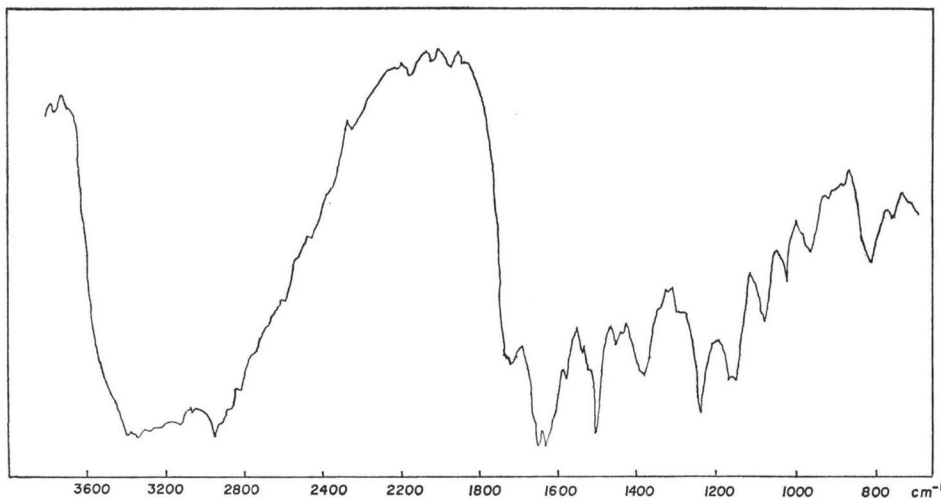
Fig. 1. Ultraviolet absorption spectra of ileumycin (hydrochloride)



Biological Properties

The antimicrobial spectrum of ileumycin is shown in Table 3; the antibiotic shows inhibitory activity only against very few species of plant pathogenic fungi, but not against yeasts and bacteria.

Fig. 2. Infrared absorption spectrum of ileumycin (hydrochloride) in KBr



Discussion

The physico-chemical and biological properties of ileumycin are similar to those of nucleoside antibiotics[2]. Ezomycin A_1, a nucleoside antibiotic, is also active against only a few species of plant pathogenic fungi, including *Glomerella cingulata*, while ezomycin A_2 is inactive against *Glomerella cingulata*[3]. Nevertheless, ezomycin A_1 shows ultraviolet absorption maxima at 278 nm ($E^{1\%}_{1cm}$ 139) in 0.1 N HCl, and at 271 nm ($E^{1\%}_{1cm}$ 105) in 0.1 N NaOH[3]. Ileumycin can be differentiated from ezo-

Table 3. Antimicrobial spectrum of ileumycin

Test organisms	MIC (mcg/ml)	Test organisms	MIC (mcg/ml)
Staphylococcus aureus FDA 209 P	>100	*Candida albicans* Yu 1200	>100
Bacillus subtilis PCI 219	>100	*Candida krusei*	>100
Corynebacterium bovis	>100	*Saccharomyces cerevisiae*	>100
Micrococcus flavus	>100	*Colletotrichum lagenarium*	0.78
Micrococcus lysodeikticus IFO 333	>100	*Colletotrichum gloeosporioides* Penzig	0.19
Pseudomonas phaseolicola	>100	*Glomerella cingulata*	0.05
Pseudomonas solanacearum	>100	*Aspergillus niger* F-16	>100
Escherichia coli NIHJ	>100	*Alternaria kikuchiana*	>100
Erwinia carotovora	>100	*Gloeosporium laeticolor*	>100
Agrobacterium tumefaciens	>100	*Helminthosporium oryzae*	>100
Mycobacterium smegmatis ATCC 607	>100	*Trichophyton mentagrophytes* (833)	>100
Candida tropicalis NI 7495	>100	*Trichophyton asteroides* 429	>100
Candida pseudotropicalis NI 7494	>100	*Elsinoë fawcetti* BITANCOURT *et* JENKINS	>100
Candida albicans 3147	>100		

Agar dilution method on potato sucrose agar

mycin A_1 by paper chromatography developed with *n*-BuOH - AcOH - H_2O (2: 1: 2): Rf value of ezomycin A_1 is 0.36, whereas that of the free base of ileumycin is 0.87.

Ablastmycin[4], bulgerin[5] and SF-1508[6] have also reported to be active against a limited number of species of plant pathogenic fungi and to show ultraviolet absorption spectra similar to those of ileumycin. Ablastmycin shows ultraviolet absorption maxima at 274 nm ($E^{1\%}_{1cm}$ 270) in 0.1 N HCl, and at 235 nm ($E^{1\%}_{1cm}$ 225) and 290 nm ($E^{1\%}_{1cm}$ 185) in 0.1 N NaOH. Bulgerin shows maxima at 230 nm ($E^{1\%}_{1cm}$ 147, shoulder) and 289 nm ($E^{1\%}_{1cm}$ 410) in 0.05 N HCl, and 242 nm ($E^{1\%}_{1cm}$ 336) and 306 nm ($E^{1\%}_{1cm}$ 263) in 0.05 N NaOH. SF-1508 shows maxima at 295 nm ($E^{1\%}_{1cm}$ 235) in 0.1 N HCl, and 243 nm ($E^{1\%}_{1cm}$ 282) and 312 nm ($E^{1\%}_{1cm}$ 180) in 0.1 N NaOH.

Thus, ileumycin can be differentiated from the above antibiotics and can be considered to be new.

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