ACRYLAMIDINE, AN ANTI-FUNGAL SUBSTANCE PRODUCED BY A STREPTOMYCES

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An antibiotic weakly active against *Candida* was isolated as a colorless crystalline hydrochloride from a culture of a streptomyces which showed some resemblances with *Streptomyces eurythermus*. This antibiotic was unstable to acid and alkali, but was recovered in good yield by elution with 0.5 M ammonium chloride solution from the ion-exchange resin, Amberlite IRC-50 (Na^+ type). It was determined to be acrylamidine by spectroscopic data, degradation and direct comparison with a synthetic sample. The LD_{50} (mice) of acrylamidine was 44 mg/kg intravenously.

In the course of screening studies for anti-*Candida* substances, a streptomyces, designated strain No. D 274-2 in the authors' laboratory, produced an antibiotic weakly active against a limited number of fungi. The antibiotic was labile in acidic and alkaline solutions, and was determined to be acrylamidine. In this report, characteristics of the producing strain, the processes of production, isolation, and the identification of the antibiotic are described.

Characters of the Strain No. D 274-2

The strain was isolated from a soil sample collected at Izu, Shizuoka Prefecture. Colonies on a synthetic agar plate were examined microscopically. Long flexuous aerial mycelia develop from fine branched substrate mycelia. The surface of the spore is smooth under electron miroscopy, as shown in Plate 1. As shown by the characteristics on various media summarized in Tables 1 and 2, strain D 274-2 belongs to *Streptomyces* and to the chromogenic type. It forms no whorl but spirals. The surface of the spores is smooth. The growth on various media is pale yellow to brownish with white to gray aerial mycelium. Soluble brown pigment is formed in most of the media employed. The proteolytic action is fairly strong and it hydrolyzes starch. Among known species, *Streptomyces eurythermus* has many characteristics in common with strain D 274-2. However, several differences are found between the strain D 274-2 and *S. eurythermus* as shown in Table 3. Since

Plate 1. Aerial mycelium of the strain No. D 274-2 under electron microscope.

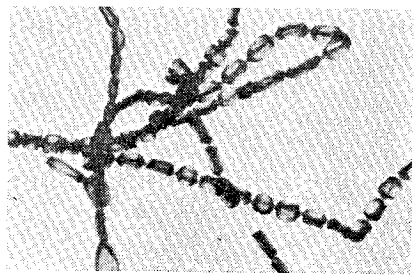

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Table 1. Characters of strain D 274-2 on various media (1)

	Growth	Aerial mycelium	Soluble pigment
Glycerol nitrate agar 27°C	colorless～brownish gray～brown olive *(Mustard Brown 2_{pl})	grayish white～bluish gray *(Aqua Gray 19_{fe})	yellowish brown
Glucose-asparagine agar 27°C	colorless dark brown	brownish white～brownish gray *((Rose Beige 4_{eg}) Gray (Covert Gray 2_{fe}))	greenish yellow
Calcium malate agar 27°C	spreading deep into the medium, colorless～pale yellow	white～grayish white～gray	none
Peptone solution (containing 1.0 % of $NaNO_3$) 27°C	colorless	white	blackish
Starch agar 27°C	colorless～brownish	white～bluish gray～gray	none at first, later becoming brownish
Tyrosine agar 27°C	colorless or grayish?	thin, white	black
Potato plug 27°C	colorless～pale yellow ～light brown	abundant, powdery, grayish white	grayish brown
Nutrient agar 27°C	colorless	white	brown
37°C	colorless	white	brownish
LOEFFLER's serum 37°C	colorless～olive gray	white	brown
Gelatin stab 20°C	colorless～pale yellow	white	greenish dark brown
Skimmed milk 37°C	colorless～dark brown	white	brown

* Color Harmony Manual. (Container Corporation of America).

Table 2. Characters of strain D 274-2 on various media (2)

Solubilization of Ca-malate	(卄) 3 days, strong
Nitrate reduction	(−)
Hydrolysis of starch	(±) 5 days, (+) 10 days, (卄) 14 days
Tyrosinase reaction	(卅) chromogenic
Liquefaction of gelatin	(+) 3 days, (卅) 22 days
Milk	coagul. (−), pepton. (+) 5 days
Liquefaction of serum	(−)
Cellulose	(−)
Utilization of carbohydrates*	(卄): inositol, lactose, mannitol, glycerol, fructose, sucrose (+): starch, dextrin, raffinose, rhamnose, galactose, glucose, xylose, maltose, mannose (±): salicin (∓): inulin (−): sorbitol, dulcitol, arabinose

* Basal medium : PRIDHAM-GOTTLIEB medium

Table 3. Differences between strain D 274-2 and *S. eurythermus*

	D 274-2	*S. eurythermus*
Spiral	+	− ?
Hydrolysis of starch	medium	rapid
Antibiotic substance	acrylamidine	angolamycin
Utilization of carbohydrates		
arabinose	−	+
rhamnose	+	−

there is no description of spirals of *S. eurythermus*[1,2], the authors assumed that *S. eurythermus* formed no spirals and it is indicated as "−?" in Table 3.

Assay of the Antibiotic

Candida albicans 3147 is cultured at 37°C for 48 hours on a slant of SABROURD agar medium, and the mycelium is sus-

pended in 10 ml of sterilized distilled water. It is added to the seed layer of the assay plate at 0.3 % concentration. The assay procedure for the antibiotic is the ordinary cylinder plate method. Solutions of acrylamidine containing 40 and 20 mcg/ml used as standards show inhibition zones of 24~25 and 18~20 mm diameter respectively after incubation at 37°C overnight.

Production of the Antibiotic

Strain D 274-2 was inoculated into 100 ml of medium in a 500-ml flask, and cultured at 27~28°C on a reciprocating shaking machine (120 strokes/min., 8 cm amplitude). The pH, amount of production, and period of the shaking culture for maximum production in various media are shown in Tables 4, 5 and 6.

For the production of the antibiotic, glucose is the most suitable carbon source,

Table 4. The effect of various carbon sources on antibiotic production

Sugars	Maximum production in					
	Medium-1			Medium-2		
	pH	mcg/ml	days	pH	mcg/ml	days
Starch	7.7	24	3~4	6.1	46	4~5
Glucose	6.2	35	4	5.0	81	5
Glycerol	8.1	25	4	5.2	75	5
Dextrin	9.0	0	2~3	7.0	52	5
Lactose	9.0	0	2~3	5.6	47	5
Sucrose	8.7	17	2~3	6.1	48	5

Medium-1: 3 % sugars, 1 % Polypeptone, 0.2 % yeast extract, 0.6 % $CaCO_3$, pH 6.6.
Medium-2: 3 % sugars, 1.5 % soybean meal, 0.2 % $MgSO_4 \cdot 7H_2O$, 0.1 % K_2HPO_4, pH 6.6.

Table 6. The influence of soybean meal and Polypeptone on antibiotic production

Media	pH	Maximum production	
		mcg/ml	days
Medium-2	6.4	92	5
Medium-2-1	6.4	150	5
Medium-2-2	6.4	88	5

Basal medium: 3 % Glucose, 0.2 % $MgSO_4 \cdot 7H_2O$, 0.1 % K_2HPO_4.
Medium-2 : Basal medium + 1.5 % soybean meal.
Medium-2-1: Basal medium + 1.5 % soybean meal + 0.3 % Polypeptone.
Medium-2-2: Basal medium + 1.5 % soybean meal + 0.5 % Polypeptone.

Table 5. The effect of various nitrogen sources on antibiotic production

Nitrogen sources	%	Maximum production		
		pH	mcg/ml	day
Polypeptone	0.5	6.2	27	3
	1.0	6.0	51	3~4
Meat extract	0.5	6.4	20	3~4
	1.0	6.2	26	3~4
Yeast extract	0.5	5.6	23	3
	1.0	5.2	31	3
N-Z-amine	0.5	6.0	19	3
	1.0	5.4	20	3
Soybean meal	0.5	5.6	48	4~5
	1.0	6.0	77	4~5
Corn steep liquor	0.5	6.2	7	3~4
	1.0	5.8	0	3~4
$NaNO_3$	0.5	6.8	0	2~5
	1.0	6.8	0	2~5
KNO_3	0.5	6.6	0	2~5
	1.0	6.6	0	2~5
NH_4Cl	0.5	6.6	0	2~5
	1.0	6.4	0	2~5
$(NH_4)_2SO_4$	0.5	6.6	0	2~5
	1.0	6.6	0	2~5
$(NH_4)_2HPO_4$	0.5	5.4	0	2~5
	1.0	5.0	0	2~5
None	—	6.4	0	2~5
	—	6.4	0	2~5

Basal medium: 3 % Glucose, 0.2 % $MgSO_4 \cdot 7H_2O$, 0.1 % K_2HPO_4.

Table 7. The course of the fermentation in jar fermenter.

Hours	pH	Activity as acrylamidine (mcg/ml)
0	6.8	—
12	6.6	—
24	6.4	—
30	6.2~6.4	5
42	5.6	36
48	5.6~5.8	58
65	5.6	107

Stainless steel jar fermenter 30-liter volume.
Medium: 15 liters of the medium-2-1.
Sterilization: 120°C, 20 minutes.
Inoculum: 200 ml of 48-hour shaking cultured broth.
Stirring: 250 r.p.m. Aeration: 14 liters/min.

and soybean meal and Polypeptone are suitable nitrogen sources. In the medium 2-1 containing 3 % glucose, 0.2 % $MgSO_4 \cdot 7H_2O$, 0.1 % K_2HPO_4, 1.5 % soybean meal and 0.3 % Polypeptone and adjusted to pH 6.6, 150 mcg/ml of the antibiotic was produced after 5 days' fermentation.

In a jar fermenter containing 15 liters of the medium 2-1, strain D 274-2 gave the results shown in Table 7.

Isolation of the Antibiotic

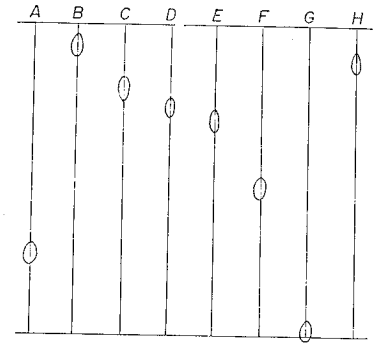
The cultured broth from two jar fermenters was adjusted to pH 6.6 and filtered, yielding 23 liters of the filtrate containing 2.2 g of the antibiotic. In a preliminary experiment, Amberlite IRC-50 (Na^+ type), completely adsorbed the activity if 8 % of the filtrate volume was used. Therefore, the filtrate above was passed through a column containing 2.3 liters of the resin. Since the activity was decreased by elution with 0.5 N hydrochloric acid, the antibiotic on the column was eluted with 0.5 M ammonium chloride. Three liters of active eluate were collected and lyophilized. The residual white powder was extracted with methanol - acetone (1 : 5), and filtered to remove 93 g of inactive solid. The filtrate (1,150 ml) was concentrated to dryness under reduced pressure, and the residue was washed with ether to yield 3.28 g of slightly yellow brownish powder. The suspension of the crude powder in 10 ml of methanol was treated with 140 ml of acetone and filtered to remove 1.59 g of inactive solid. The filtrate was concentrated to dryness under reduced pressure yielding 1,311 mg of crude crystalline hydrochloride of 90 % purity. The overall yield was 54 %. It was recrystallized from 1-butanol - acetone or 1-propanol - acetone (1 : 5~1 : 10).

Properties of the Antibiotic

The hydrochloride forms hygroscopic colorless crystals. It is easily soluble in water and lower alcohols, fairly soluble in acetone or chloroform containing water and lower alcohols, and insoluble in ethyl acetate, acetone, chloroform, ether and *n*-hexane. It melts at 138.5~139.5°C. It is optically inactive. It decolorizes potassium permanganate solution and bromine water, and gives an orange color with nitroprusside reagent[9] (10 % NaOH - 10 % $Na_2[Fe(CN)_5]NO \cdot 2H_2O$ - 10 % $K_3Fe(CN)_6$ - H_2O (1 : 1 : 1 : 3)), but negative ninhydrin, SAKAGUCHI, ferric chloride, BENEDICT and TOLLENS reactions. It is stable in aqueous solution at pH 2.0 to 6.0, but unstable in more acid or alkaline solutions. In alkaline solution it liberates ammonia. The summarized papergram detected by bioautography is shown in Fig. 1. The molecular weight determined by vapor pressure osmometer using water as solvent was 96. Anal. Found: C 33.38, H 7.00, N 24.22,

Fig. 1. Summarized papergrams of acrylamidine

Solvent systems
- A : Wet butanol
- B : 20 % Ammonium chloride
- C : 75 % Phenol
- D : 50 % Acetone
- E : Butanol, methanol, water (4 : 1 : 2), 1.5 % methyl orange
- F : Butanol, methanol, water (4 : 1 : 2)
- G : Benzene, methanol (4 : 1)
- H : Water



Cl 32.94. From the elemental analysis, a molecular formula, $C_3H_7N_2Cl$ (Calcd.: C 33.81, H 6.63, N 26.29, Cl 33.27), is possible for the antibiotic, although the content of nitrogen is low because of its hygroscopic and labile characters. As shown in Fig. 2, the antibiotic has only an end absorption in water and 0.1 N HCl, but a shoulder at 230 to 250 mμ in 0.1 N NaOH. The infrared spectrum of the antibiotic pelleted with potassium bromide is shown in Fig. 3. A strong band at 970 cm^{-1} is attributed to an out-

Fig. 2. Ultraviolet absorption spectrum of acrylamidine

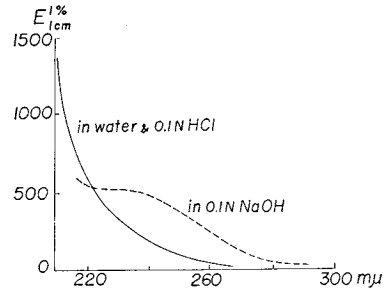
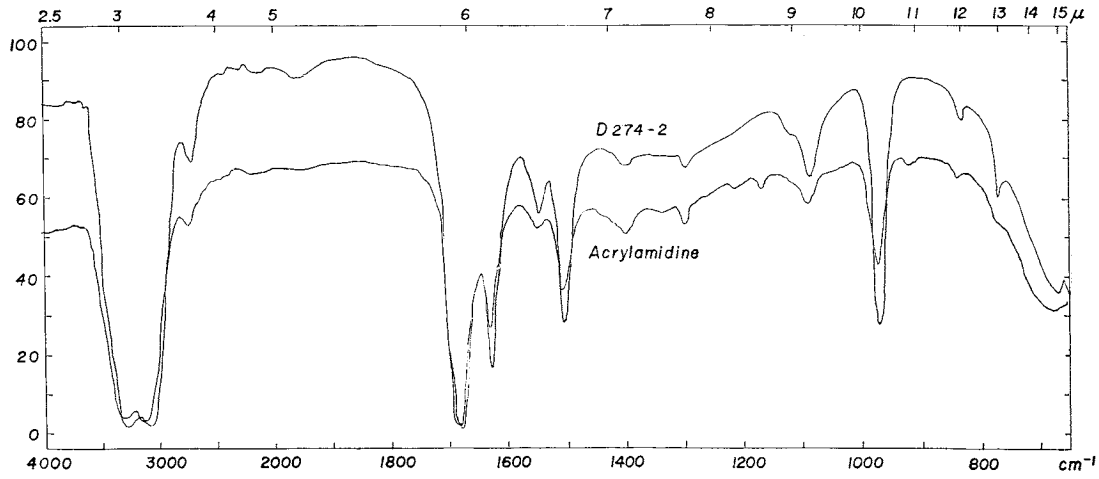


Fig. 3. Infrared absorption spectra of D 274-2 substance and acrylamidine



of-plane CH deformation vibration of a terminal vinyl group. The n.m.r. spectrum of its solution in deuterium oxide taken at 60 Mc by A-60 Varian spectrometer is shown in Fig. 4 and shows a splitting pattern at δ 5.8~6.8 p.p.m. corresponding to three protons. As shown in Fig. 4, the 100 Mc spectrum taken with a Type JMN 4H-100 spectrometer of Japan Electron Optics Laboratory gives a typical ABC-pattern which is commonly observed in the spectra of derivatives of acrylic acid.

The foregoing results, that is, its molecular formula, infrared and n.m.r. spectra, liberation of ammonia by the treatment with alkaline solution, and positive nitroprusside reaction[2] which is characteristic of compounds having -N-C-N- grouping,

Fig. 4. NMR spectrum of D 274-2 substannce in deuterium oxide at 60 Mc and 100 Mc

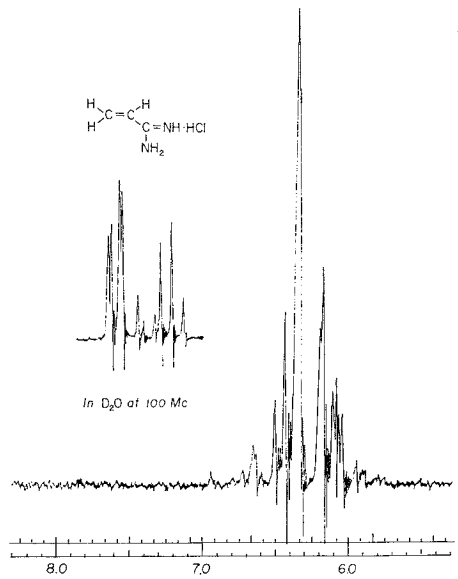


Table 8. The bacteriostatic effects of acrylamidine tested by agar streak method

	Organisms	Minimum inhibitory concentration mcg/ml		Organisms	Minimum inhibitory concentration mcg/ml
Medium 1*	*Staphylococcus aureus* 209P	>100	Medium 3*	*Penicillium chrysogenum*	>100
	Staphylococcus aureus Terajima	>100		*Aspergillus niger*	>100
	Staphylococcus aureus Smith	>100		*Trichophyton mentagrophytes*	75
	Bacillus megaterium	>100		*Saccharomyces sake*	75
	Bacillus anthracis	>100		*Saccharomyces cerevisiae*	75
	Bacillus subtilis NRRL B-558	>100		*Hansenula anomola*	100
	Bacillus subtilis PCI 219	>100		*Torula utilis*	100
	Sarcina lutea 1001	>100		*Candida albicans*	50~75
	Micrococcus flavus M-16	>100		*Candida stellatoidea*	50~75
	Escherichia coli	>100		*Candida tropicalis*	50~75
	Klebsiella pneumoniae PCI 602	>100		*Cryptococcus neoformans*	50
	Salmonella typhimurium 1406	>100		*Histoplasma capsulatum*	>100
	Salmonella paratyphi A	>100		*Hormodendrum pedrosoi*	50~75
	Shigella dysenteriae	>100		*Microsoporum audouini*	75
	Shigella flexneri	>100		*Fusarium lini*	100
	Shigella sonnei	>100	Medium 4*	*Pyricularia oryzae*	>100
	Pseudomonas tabaci	>100		*Xanthomonas oryzae*	>100
	Pseudomonas aeruginosa	>100		*Fusarium oxysporum*	>100
Medium 2*	*Mycobacterium tuberculosis* 607	>100		*Gibberella saubinetii*	>100
	Mycobacterium phlei	>100			

* Medium 1: Bouillon agar, pH 6.8~7.0 (37°C)
2: 1.5 % Glycerol, 0.75 % polypeptone, 0.75 % meat extract, 0.2 % NaCl, 1.8 % agar, pH 6.8~7.0 (37°C)
3: 1.5 % Glucose, 1.0 % polypeptone, 1.8 % agar, pH 5.6~6.0 (27°C)
4: 2.0 % Sucrose, 0.5 % polypeptone, 0.2 % glutamic acid, 0.2 % yeast extract, 1.8 % agar (27°C)

suggest that the antibiotic is acrylamidine. The hydrolysis of the antibiotic with N NaOH at 70°C for 5 minutes gives acrylamide as shown by paper chromatography using Toyo Filter Paper #51 and 1-butanol - methanol - water (4 : 1 : 2) and by thin-layer chromatography using Eastman Chromatogram Sheet 6061 and 1-butanol - glacial acetic acid - water (4 : 1 : 5). The antibiotic is detected at Rf 0.43 on the paper and at Rf 0.56 on the thin-layer chromatogram by iodine, nitroprusside reagent, potassium permanganate or ultraviolet light, and acrylamide and the hydrolysis product are detected at Rf 0.70 on the paper chromatogram and at Rf 0.88 on the thin-layer chromatogram.

Acrylamidine was synthesized, starting from acrylonitrile by the method reported by MARTINEZ[4]. The identity of the antibiotic with acrylamidine synthesized was proved by paper and thin-layer chromatography described above and by the infrared spectra as shown in Fig. 3.

The minimum inhibitory concentration of acrylamidine against various micro-organisms was measured by agar streak method as shown in Table 8. Acrylamidine hydrochloride inhibits the growth of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Cryptococcus neoformans*, *Hormodendrum pedrosoi*, *Trichophyton mentagrophytes*, *Saccharomyces sake*, *Saccharomyces cerevisiae* and *Microsporum audouini* at 50~75 mcg/ml, but does not inhibit *Piricularia oryzae*, *Xanthomonas oryzae*, Gram-

positive and negative bacteria and *Mycobacteria* even at 100 mcg/ml. The LD_{50} to mice of acrylamidine hydrochloride was 38 mg/kg subcutaneously and intraperitoneally, and 44 mg/kg intravenously. No curative effect was observed, when 0.6 mg/mouse of acrylamidine hydrochloride was intraperitoneally injected to mice infected with *Candida albicans*.

In tissue culture, acrylamidine hydrochloride caused the denaturation of HeLa cells at 50 mcg/ml, and 82.3 % and 3.6 % inhibition of growth of YOSHIDA rat sarcoma cells at 100 and 20 mcg/ml, respectively, were observed. However, acrylamidine hydrochloride showed no therapeutic effect on ascites type or solid type EHRLICH carcinoma, sarcoma 180, mouse leukemia SN-36 and L 1210.

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