STUDIES ON BLASTICIDIN A

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Blasticidin A ($C_{51\sim52}H_{99\sim101}O_{23}N\ \frac{1}{2}Ca$, m. p. 165.5~166.5°C, λ_{max} 246 mμ and 298 mμ in 50 % methanol, $[\alpha]_D^{24}$ −31° in pyridine) was isolated in crystal form as calcium-chelated compound. The antibiotic shows activity against a variety of microorganisms with relatively less toxicity to mice orally. It seems to have some relationship to polyene macrolide antibiotics, but has marked differences in its properties. The isolation, purification and physicochemical properties of the antibiotic are described.

Blasticidins A, B and C were found by FUKUNAGA and others in 1955[1] in the culture filtrate of the *Streptomyces griseochromogenes* 2A-327. Taxonomic studies of the strain were reported in a previous paper[1]. Their physical and chemical properties were not reported in detail, because they could not be isolated in pure form.

Subsequently, blasticidin S was differentiated from blasticidins A, B and C and isolated in pure form from the culture filtrate of the same strain in 1958[2].

Recently, when *S. griseochromogenes* was cultured in a medium containing calcium carbonate, accumulation of blasticidin A in the mycelium was observed. The antibiotic was extracted from the mycelium with methanol, and purified to the crystalline state as a calcium-chelated compound.

Blasticidin A shows activity against fungi, yeast and some bacteria at low concentrations *in vitro*. Production, isolation, purification procedures, and properties are reported in this paper.

Assay Method of Blasticidin A

A cylinder plate method was used for bioassay of blasticidin A with *Piricularia oryzae* as the test organism. The medium contained 0.2 % yeast extract, 1.0 % sucrose and 1.5 % agar (pH 5.2).

A methanolic solution of the antibiotic was diluted with an equal volume of phosphate buffer at pH 8 for bioassay. Inhibition zone diameters were measured after incubation at 27°C for 42 hours.

A linear relation between the logarithm of the antibiotic concentration and inhibition zone diameters was found for concentrations of 2~5 mcg/ml.

Production of Blasticidin A

The original strain of 2A-327 was compared in two different media with the strain improved for blasticidin S production. They were inoculated into a 500-ml flask containing 100 ml of medium consisting of 3.0 % sucrose, 1.5 % soybean meal, 0.4 % $CaCO_3$ and 0.1 % K_2HPO_4, pH being adjusted to 7.2 (Medium A). The flasks were

Table 1. Production of blasticidins A and S (mcg/ml)

	2A-327				Improved strain			
	filtrate		mycelium		filtrate		mycelium	
	Bla. A	Bla. S	Bla. A	Bla. S	Bla. A	Bla. S	Bla. A	Bla. S
Medium A	2.4	20	294～370	0	±	110	80～124	0
Medium B	±	220	52～68	0	±	540	4.8	0

incubated at 27～28°C on a shaker rotating at 200 r.p.m. for 97 hours.

Conditions developed for blasticidin S production were also tested. The medium consisted of 5% sucrose, 5% soybean meal and 0.1% NaCl, pH being adjusted to 6.5 (Medium B). The flasks were incubated as above at 30°C for 72 hours. The original strain 2A-327 and calcium containing medium were most suitable for production of blasticidin A as shown in Table 1.

When strain 2A-327 was inoculated in Medium A, mycelium weight reached a maximum after 130 hours of fermentation, at this time the pH was 7.5～7.8. Maximum activity was observed when the broth reached pH about 8.1 after 160 hours fermentation. At this time the antibiotic content had increased to 3% of the dry weight of mycelium. The progress of the fermentation of blasticidin A is shown in Fig. 1.

Production of the blasticidin A in jar fermentors was carried out using Medium A. Fifteen litre of medium was sterilized at 120°C for 20 minutes in a stainless steel fermentor of 30-liter volume. Each jar was inoculated with 2% of a broth precultured for 48 hours by shaking in Medium A at 27°C. Air was supplied at a rate of approximately 1/1 volume of broth per minute with agitation of 400 r.p.m. at 27～28°C.

Fig. 1. Fermentation of blasticidin A.

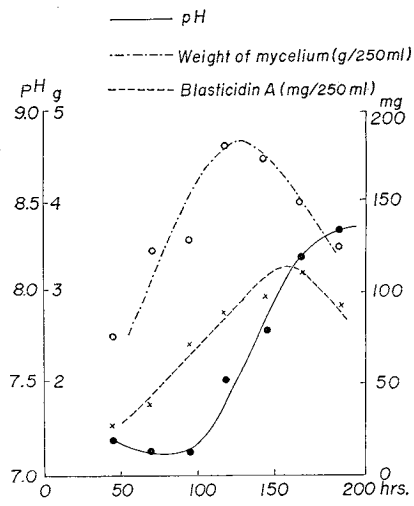


Chart 1. Extraction and purification of blasticidin A

Culture broth (pH 8.0, 15 liters×3)
↓ filtration
→ Filtrate
Mycelium
↓ extracted with hot MeOH (5 liters×3)
MeOH (15 liters)
↓ MeOH removed *in vacuo*
extracted with BuOH (5 liters)
→ Water layer
BuOH layer
↓ concd. *in vacuo* (200 ml)
cooled
Precipitate 31 g (yield 69%)
↓ dissolved in hot MeOH (15 liters)
stood at 30°C for 5～7 days
Crystalline powder 9.2 g m.p. 165～167°C (yield 41%)
↓ recrystallization from hot MeOH
Crystals 7.3 g (yield 33%) m.p. 165.5～166.5°C

Isolation and Purification of Blasticidin A

After fermentation was completed, 12 liters of the culture broth was filtered to collect the mycelium. Roughly 97~98 % of the total activity was found in the mycelium cake.

The antibiotic was extracted from the mycelium cake with 5 liters portions of hot methanol at 60°C. The methanol extract was concentrated under reduced pressure to an aqueous slurry. The slurry was extracted with 5 liters of butanol. The butanol extract was concentrated *in vacuo* to about 200 ml and the solution was allowed to stand at 4°C. Thirty one g of active amorphous precipitate were obtained (yield 69 %). The precipitate was filtered off, washed with a small volume of cold methanol, and then dissolved in hot methanol at a concentration of 1,000~2,000 mcg/ml. The methanol solution was kept in the dark at 30°C after 5~7 days, 9.29 g of colorless crystalline blasticidin A was obtained (yield 41 %). Recrystallization from hot methanol yielded 7.3 g of pure blasticidin A as colorless micro needles for an overall yield of 33 % of the original potency. Extraction and purification of blasticidin A are shown in Chart 1.

Physical and Chemical Properties of Blasticidin A

Blasticidin A is a colorless micro-needle crystal, melting at 165.5~166.5°C. The antibiotic is stable at 100°C at pH 2.0~7.0, but unstable on the alkaline side. The stability of blasticidin A against ultraviolet irradiation in methanol was examined with the results shown in the Table 2.

Blasticidin A is easily soluble in dimethyl sulfoxide; soluble in methanol, 80 % aqueous ethanol, wet butanol, methylcellosolve, dimethylformamide, pyridine, acetic acid and alkaline water; slightly soluble in ethanol, butanol and water; insoluble in acetone, ethylacetate, benzene, chloroform, ether and carbon tetrachloride.

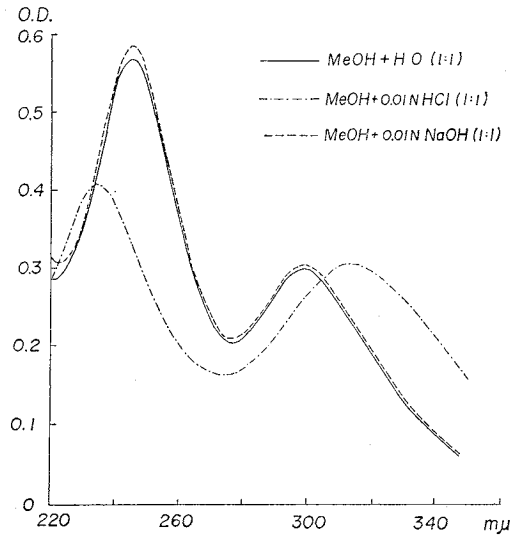
Blasticidin A shows positive EHRLICH, LIEBERMANN-BURCHARD, CARR-PRICE, ferric chloride, LEMIEUX and conc. H_2SO_4 (reddish brown) reactions, and negative reactions ninhydrin, carbylamine, mustard oil, SIMON, sodium nitroprusside, FEHLING, MILLON, and citric acid-aceticanhydride (test for tertiary amines) tests.

Optical rotation of the blasticidin A was $[\alpha]_D^{24}$ −31° (*c* 0.9, pyridine). Molecular weight of blasticidin A estimated by the titration equivalent using 80 % methylcello-

Table 2. Stability of blasticidin A (in methanol)

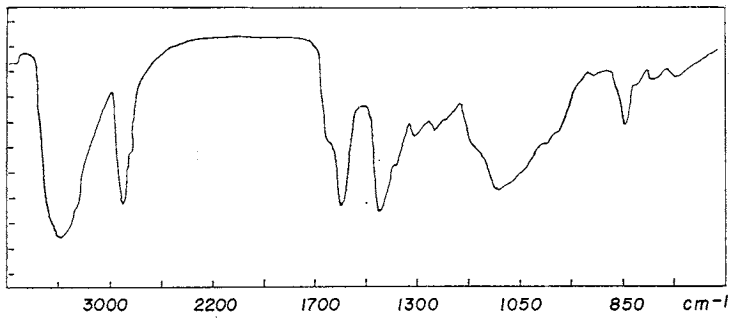
Ultraviolet irradiation hours	Activity remaining	
	Blasticidin A	Trichomycin
0	100 %	100 %
44	60~70 //	10~20 //
70	50~60 //	2~5 //

Fig. 2. U. V. Spectra of blasticidin A.



solve as solvent was 1,120, and pK_{mcs} was 3.7. Elemental analysis of blasticidin A gave values of C 54.92, H 8.82, O 32.54, N 1.24 and Ca 1.89. Atomic absorption spectrometry analysis for calcium gave a value of 1.84. From these data an empirical formula of $C_{51\sim52}H_{99\sim101}O_{23}N\frac{1}{2}Ca$ can be proposed. The ultraviolet absorption spectrum is shown in Fig. 2, with absorption maxima at 246 mμ ($E^{1\%}_{1cm}$ 113) and 298 mμ ($E^{1\%}_{1cm}$ 60) in 50 % aqueous methanol, 236 mμ ($E^{1\%}_{1cm}$ 81) and 314 mμ ($E^{1\%}_{1cm}$ 61) in acidic aqueous methanol (MeOH 1 : 0.01 N HCl 1). In basic aqueous methanol the same spectrum as in neutral aqueous methanol is observed. The infrared absorption spectrum of blasticidin A is shown in Fig. 3.

Fig. 3. I. R. Spectra of blasticidin A (KBr).



Preparation and Properties of Calcium-Free Blasticidin A

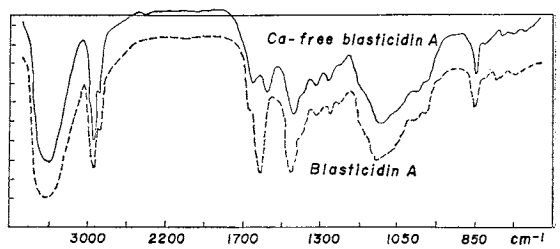
As mentioned above blasticidin A was isolated as a calcium chelate. When calcium was removed the antibiotic could not be crystallized, and turned slightly reddish colored. The chelated blasticidin A was dissolved in water-saturated butanol and was well mixed with 1 N sulfuric acid. The butanol layer was washed with distilled water and concentrated *in vacuo* to give a slightly reddish powder. This material was chromatographed on nitric acid-washed crystalline cellulose (Avicel) from water-saturated butanol. The active eluate was concentrated *in vacuo* to yield the antibiotic free of calcium. The infrared absorption spectrum of calcium-free blasticidin A is shown in Fig. 4. The band at 1600 cm^{-1} observed in the calcium chelate had disappeared, and new bands are found at 1570 and 1650 cm^{-1}.

The calcium-free blasticidin A can be reconverted to calcium-containing material by the addition of calcium chloride to a methanolic solution of the antibiotic. The product gives the same infrared spectrum as the original.

The ultraviolet absorption spectrum of calcium-free blasticidin A in methanol shows peaks at 235 and 308 mμ, while the rechelated compound gave the same spectrum as the original.

The biological activity of calcium-free blasticidin A is about half the activity of the original antibiotic,

Fig. 4. I. R. Spectrum of Ca-free blasticidin A (KBr).



probably due to the unstability of the calcium-free material.

Biological Properties of Blasticidin A

Biological activities of blasticidin A were examined by the agar streak dilution method. The results are shown in Tables 3 and 4. Blasticidin A inhibits a variety of microorganisms. Among fungi, *Alternaria*, *Botrytis*, *Corticium*, *Gloeosporium*, *Glomerella*, *Macrosporium*, *Ophiobolus*, *Penicillium* and *Piricularia* are sensitive to blasticidin A at low concentration. Some strains of *Candida* and *Rodotorula* are also sensitive to blasticidin A. The antibiotic is also active against specific bacteria *e. g. Bacillus subtilis*, *Corynebacterium xerosis*, *Pseudomonas solanacearam*, *Proteus vulgaris*, *Shigella sonnei* and *Staphylococcus aureus* at low concentration.

Blasticidin A was suspended in sterilized water containing 0.25% carboxymethyl cellulose and the suspension was given to mice intraperitoneally or orally to determine its toxicity. The LD_{50} was 26 mg/kg intraperitoneally and 800~1,000 mg/kg orally.

Table 3. Antifungal spectrum of blasticidin A

Test organism	M. I. C. mcg/ml
Alternaria kikuchiana IAM 5005	0.39
Aspergillus oryzae	>100
Botrytis cinerea IAM 5127	3.13
Botrytis fabae IAM 5125	0.78
Cladosporium fulvum	12.5
Corticium sasakii	0.78
Fusarium lini	>100
Gibberella fujikuroi	>100
Gibberella saubinetii	100
Gloeosporium kaki	0.39
Glomerella cingulata IAM 8050	0.78
Glomerella lagenarium	0.39
Helminthosporium sesamum	3.13
Helminthosporium sigmoideum	3.13
Macrosporium bataticola IAM 5014	0.78
Mucor ramannianus IAM 6128	12.5
Ophiobolus miyabeanus	0.39
Penicillium chrysogenum Q176	0.39
Piricularia oryzae	0.1
Trichophyton mentagrophytes NIHJ 640	0.78
Candida utilis IAM 4215	0.78
Cryptococcus neoformans IAM 4514	3.13
Rhodotorula glutinis IAM 4757	0.1
Saccharomyces cerevisiae NIHJ F-130	1.56

Table 4. Antibacterial spectrum of blasticidin A

Test organism	M. I. C. mcg/ml
Aerobacter aerogenes IAM 1063	>100
Agrobacterium tumefaciens B6	>100
Bacillus subtilis PCI 219	0.2
Corynebacterium xerosis IID	0.39
Escherichia coli	>100
Mycobacterium phlei IID Timothee	1.56
Mycobacterium smegmatis ATCC 607	100
Mycobacterium tuberculosis var. *hominis* IID $H_{37}Rv$	6.25
Pseudomonas fluorescens IAM 1201	>100
Pseudomonas solanacearum NIAS	0.39
Pseudomonas tabaci	>100
Proteus vulgaris OX-19	0.2
Sarcina lutea NIHJ	6.25
Serratia marcescens IAM 1021	>100
Serratia marcescens var. *kilensis* ATCC 9986	>100
Shigella sonnei IID	0.39
Staphylococcus aureus 209P	0.39
Xanthomonas oryzae NIAS	>100

Differentiation of Blasticidin A from Other Antibiotics

Blasticidin A is clearly distinguished from other similar antibiotics such as folimycin[3], scopamycin[4], PA-128[5], 1-81-d-S[6], humidin[7] and ikutamycin[8] by the chemical and physical properties and also by the biological properties as described above.

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References

1) FUKUNAGA, K.; T. MISATO, I. ISHII & M. ASAKAWA : Blasticidin, a new anti-phytopathogenic fungal substance. I. Bull. Agr. Chem. Soc. Jap. 19 : 181～188, 1955
2) TAKEUCHI, S.; K. HIRAYAMA, K. UEDA, H. SAKAI & H. YONEHARA : Blasticidin S, a new antibiotic. J. Antibiotics, Ser. A 11 : 1～5, 1958
3) YAMAMOTO, H.; K. NAKAZAWA, S. HORII & A. MIYAKE : Studies on agricultual antibiotic. Folimycin, a new antifungal antibiotic produced by *Streptomyces neyagawaensis* nov. sp. (in Japanese). J. Agr. Chem. Soc. Jap. 34 : 268～272, 1960
4) HÜTTER, R.; W. KELLER-SCHIERLEIN, J. NÜESCH & H. ZÄHNER : Stoffwechselprodukte von Mikroorganismen. 48 Mitteil. Scopamycine. Arch. Mikrobiol. 51 : 1～8, 1965
5) RAO, K. V. & J. E. LYNCH : PA 128, a new antiprotozoal antibiotic. Antibiot. & Chemoth. 8 : 437～440, 1958
6) HERMANN, E. C.; L. M. LARSON & W. A. PRICE, Jr. : Antibiotic and production thereof. U. S. Pat. 2,805,185. Sept. 3, 1957
7) NAKAZAWA, K.; H. YAMAMOTO, M. SHIBATA, T. KANZAKI, F. HIGASHIDE, A. MIYAKE, H. HITOMI & S. HORII : Humidin, a new antibiotic substance. Jap. Pat. 35-11,600. Aug. 20, 1960.
8) SAKAGAMI, Y.; A. UEDA & S. YAMABAYASHI : Studies on ikutamycin, a new antifungal antibiotic. I. Isolation and properties of ikutamycin. J. Antibiotics, Ser. A 20(6) : 299～303, 1967.