L-1, 4-CYCLOHEXADIENE-1-ALANINE, AN ANTAGONIST OF PHENYLALANINE, FROM *STREPTOMYCES*

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An antagonist of phenylalanine, L-1, 4-cyclohexadiene-1-alanine, was isolated from the culture broth of a strain of *Streptomyces* designated *Streptomyces diastatochromogenes* var. *sakaii*. It is active against plant-pathogenic fungi, and *Pseudomonas aeruginosa* on synthetic medium. Producing strain, and the production, isolation, identification and biological properties of this antibiotic are described.

In the course of a search for microbial metabolite which would be protective against infection of rice by *Piricularia oryzae* and *Ophiobolus miyabeanus*, an antibiotic was isolated from the culture filtrate of a strain of *Streptomyces*. This antibiotic was found to be an antagonist of phenylalanine and identified as L-1, 4-cyclohexadiene-1-alanine from physico-chemical properties and chromatographic behavior. Although L-1, 4-cyclohexadiene-1-alanine was chemically synthesized by SNOW *et al.*[1] in 1957, its biological production has not been reported.

Producing Organism

The morphological and physiological properties of FN 1636 strain which was isolated from a soil sample collected at Takarazuka are listed in Table 1.

Microscopic observation on aerial mycelium revealed that its sporophores branched and bore little open spirals abundantly and sometimes formed clusters.

Carbon utilization by strain FN 1636 was studied using the method of PRIDHAM and GOTTLIEB[2]. L-Arabinose, D-fructose, D-glucose, rhamnose, sucrose, lactose, trehalose and D-mannitol were well utilized, whereas D-xylose, D-mannose and *i*-inositol were moderately, raffinose and salicin were poorly utilized.

Comparison of this cultural properties and sugar utilization with those for known species revealed that the strain resembles *Streptomyces diastatochromogenes*. However, some minor differences exist between FN 1636 strain and *Streptomyces diastatochromogenes*. For example, the formation of a new antibiotic, L-1,4-cyclohexadiene-1-alanine is noteworthy peculiar character of FN 1636 strain. Thus, we designated the strain as *Streptomyces diastatochromogenes* var. *sakaii*.

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Table 1. Cultural properties of *Streptomyces diastatochromogenes* var. *sakaii*

Media	Growth	Aerial mycelium	Soluble pigment	Remarks
CZAPEK's agar	Greenish brown, flat and spreading	White, powdery	None	
Starch ammonium agar	Light grayish brown, spreading	Light brownish gray, powdery	None	Diastatic action; strong
Glucose asparagine agar	Gray, occasionally dark brown	Gray, powdery	None, occasionally dark brown	
Calcium malate agar	Dark grayish brown	Dark gray, powdery	None, occasionally dark brown	
Tyrosine agar	Brown, flat	None	Dark brown	
Bouillon agar	Dark brown, flat, not spreading	None	Dark brown	
BENNETT's agar	Brown~dark brown, wrinckled	Gray, powdery	Dark brown	Poor growth at 37°C
Gelatin stab	Light gray~dark gray, surface growth	None	Blackish brown	Liquefaction; weak
Glucose CZAPEK's solution	White thin surface growth, abundant growth in the medium	None	None	Nitrite formation; negative
Glucose bouillon	Dark creamy ring growth	None	Dark brown	Slightly acidic
Milk	Grayish white ring growth	None	Grayish brown	Peptonization; poorly. No coagulation
Potato plug	Black wrinckled growth	White powder slightly	Black	
Cellulose	No growth			

Production and Isolation

FN 1636 Strain was inoculated into 500 ml shaking flasks containing 100 ml of medium consisting of 2.0 % glucose, 2.0 % potato strach, 2.0 % Pharmamedia, and 0.3 % $CaCO_3$. Flasks were incubated at 30°C for 4 days on a reciprocal shaker. This antibiotic has fungistatic activity against plant-pathogenic fungi, so assay was carried out against *Ophiobolus miyabeanus* on potato dextrose agar by usual paper-disc method.

Cultured broth from flasks was filtered with the aid of Celite 545. The filtrate was absorbed on an anion-exchange resin (Amberlite IR-45) in OH^- form at pH 4.5 and eluted with 50 % aqueous acetone. The eluate was concentrated *in vacuo* and added into two volume of acetone, yielding powdery precipitate. The precipitate, after washing with acetone, was further purified on a cellulose column by elution with water-saturated *n*-butanol. Active fractions were combined and kept for several days at room temperature to yield coloress plate crystals (FN 1636 substance). Thus, 500 mg of crystals was obtained from 10 liters of culture broth.

Chemical and Physical Properties

Chemical and physical properties of the isolated crystalline substance are listed in Table 2 comparing with that of authentic L-1, 4-cyclohexadiene-1-alanine. The most characteristic property of the new crystalline substance is to give a brick-orange color with ninhydrin reagent on paper or cellulose layer. Chemically synthesized L-1, 4-cyclohexadiene-1-alanine also gives similar color with ninhydrin. The fermentation

Table 2. Physico-chemical properties of FN 1636 substance and L-1, 4-cyclohexadiene-1-alanine

	FN 1636 substance	L-1, 4-Cyclohexadiene-1-alanine[1)]
Appearance	colorless plate	colorless plate
M. W. (mass)	167	167
Elementary analysis (%)	C 64.84, H 7.79, N 8.40 %	C 65.0, H 7.86, N 8.53 %*
Optical rotation	$[\alpha]_D^{23}$ −60.3° (*c* 0.8, H_2O)	$[\alpha]_D^{25}$ −61.6° (*c* 0.74, H_2O)
Stability	unstable in solid state	unstable in solid state
Color reaction	ninhydrin, brick-orange	ninhydrin, brick-orange
Thin-layer chromatography	water-saturated *n*-butanol	water-saturated *n*-butanol
	Rf 0.59	Rf 0.59
(MN cellulose)	*n*-BuOH - Pyridine - AcOH - H_2O	*n*-BuOH - Pyridine - AcOH - H_2O
	(30 : 20 : 6 : 24)	(30 : 20 : 6 : 24)
	Rf 0.71	Rf 0.71

* Calcd. for $C_9H_{13}NO_2$: C 64.70, H 7.84, N 8.38 %

Fig. 1. Infrared spectra of FN 1636 substance and L-1, 4-cyclohexadiene-1-alanine (Nujol mull)

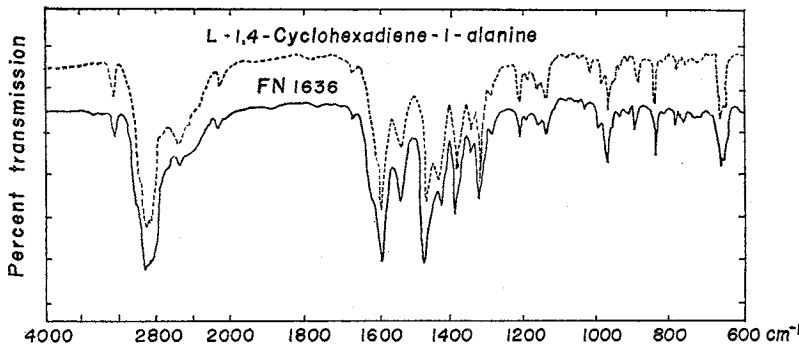


Fig. 2. NMR spectrum of FN 1636 substance in D_2O

L-1,4-Cyclohexadiene-1-alanine
(L-2,5-Dihydrophenylalanine)

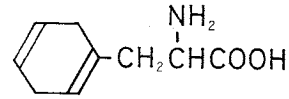
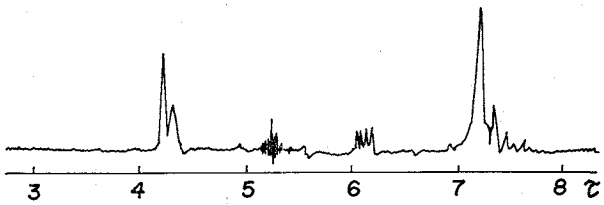


Fig. 3. NMR spectrum of L-1, 4-cyclohexadiene-1-alanine in D_2O



substance is rather heat labile and after having been kept for 3 hours at 110°C about one third of it was oxidized to L-phenylalanine. A methanolic solution showed weak end absorption in ultraviolet absorption spectrum. The infrared absorption spectra of the fermentation substance and chemically prepared L-1, 4-cyclohexadiene-1-alanine

are shown in Fig. 1, and their NMR spectra in Figs. 2 and 3 respectively. From these results, it seems reasonable to decide that the fermentation material is identical with L-1, 4-cyclohexadiene-1-alanine.

Biological Activities

The antimicrobial spectrum of the fermentation substance was tested by the agar dilution streak method and the results are shown in Table 3. Inhibition of plant-pathogenic fungi was noted, and growth of *Trichophyton asteroides*, *Trichophyton rubrum*, *Sclerotinia arachidis*, *Helminthosporium sativum*, *Ophiobolus miyabeanus* and *Piricularia oryzae* was partially inhibited at the concentration of 25~50 mcg/ml. The growth of *Pseudomonas aeruginosa* IAM 1052 is inhibited at the concentration of 5 mcg/ml only on synthetic medium. In studies using this bacterial strain the reversal by amino acids of the antibacterial activity of the fermentation substance was examined. Only phenylalanine among 19 amino acids tested reversed the inhibition of growth as shown in Table 4. This suggests that the fermentation substance is an antagonist of phenylalanine. Also this antibiotic has the same antibacterial activity against *Ps. aeruginosa* as authentic sample of L-1, 4-cyclohexadiene-1-alanine; inhibitory zones of 28 mm at the concentration of 200 mcg/ml by paper-disc assay.

Table 3. Antimicrobial spectrum of FN 1636 substance

Media	Organisms	M.I.C. (mcg/ml)
A	*Microsporum audouinii*	6.3
	Ustilago zeae	100
	Botrytis cinerea	100
	Trichophyton interdigitale	200
	Alternaria kikuchiana	>200
	Helminthosporium sativum	>200
	Ophiobolus miyabeanus	>200
	Piricularia oryzae	>200
	Sclerotinia arachidis	>200
	Trichophyton asteroides	>200
	Trichophyton rubrum	>200
B and C	*Bacillus megatherium*	>200
	Bacillus subtilis	>200
	Escherichia coli	>200
	Pseudomonas aeruginosa	>200
	Staphylococcus aureus FDA 209P	>200
D	*Pseudomonas aeruginosa* IAM 1052	5
E and F	*Aspergillus oryzae*	>200
	Penicillium chrysogenum	>200
G and H	*Candida albicans*	>200
	Saccharomyces cerevisiae	>200
	Torula utilis	>200

Media (A) Potato dextrose agar
(B) Nutrient agar
(C) STEPHENSON-WHETHAM medium
(D) glucose 1%, Na-glutamate 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, agar 1.5%
(E) Malt extract agar
(F) CZAPEK medium
(G) Malt extract agar
(H) HAYDUCK medium

Table 4. Reversal of inhibition of FN 1636 substance by L-phenylalanine in *Pseudomonas aeruginosa* IAM 1052

L-Phenylalanine (mcg/ml)	FN 1636 substance	
	200 mcg/ml	100 mcg/ml
0	28 mm	23 mm
10		22.5
20		21
50	28	15
100	26	10
200	17	0
400	0	

The antibacterial activities of test solutions containing various concentration of FN 1636 substance and L-phenylalanine was examined using *Pseudomonas aeruginosa* IAM 1052 on the medium D described in Table 3 by paper-disc assay.

Mice survived more than 10 days after intravenous administration of 500 mg/kg.

Thus, from physico-chemical properties and antimicrobial activity the fermentation substance was identified as L-1, 4-cyclohexadiene-1-alanine and shown to be an antagonist of phenylalanine.

Acknowledgements

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