L-1,4-CYCLOHEXADIENE-1-ALANINE, AN ANTIMETABOLITE OF L-PHENYLALANINE PRODUCED BY A *PSEUDOMONAS*

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L-1,4-Cyclohexadiene-1-alanine, an antimetabolite of L-phenylalanine, was isolated from the culture broth of a bacterium belonging to the genus *Pseudomonas*. Although this antimetabolite has been already isolated from culture broths of several species of Streptomycetes,[1,2,3] its production by bacteria has not been reported, thus far.

The L-1,4-cyclohexadiene-1-alanine producing organism, designated *Pseudomonas* sp. strain I-30, was isolated from a soil sample collected in Matsuo Mura, Iwate, Japan. Strain I-30 was found to belong to the genus *Pseudomonas* from the following taxonomical characteristics: rod with rounded end, $0.6\times2.0\sim3.0$ μm in size, motile by polar flagella, Gram-negative, oxidative but not fermentative, catalase positive, oxidase positive, reduces nitrate and easily grows in synthetic media. An electronmicrograph of strain I-30 is shown in Fig. 1.

The strain I-30 produces a strong growth inhibitory substance against *Neurospora crassa*, when this bacterium was grown in a modified Czapeck-Dox medium containing 3% sucrose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4\cdot7H_2O$, 0.001% $FeSO_4\cdot7H_2O$, pH 7.0. This substance was purified by a modified method of YAMASHITA *et al.*[1] The culture broth was centrifuged (10 minutes, $10,000\times g$), and the active principle in the supernatant was adsorbed to an anion-exchange resin (Amberlite IRA-45, OH^- form) at pH 4.5 and eluted with 50% aqueous acetone. The eluate was concentrated *in vacuo*, spotted on a filter paper (Toyo Roshi No. 527 40×40 cm), and was subjected to ascending paper chromatography with water saturated *n*-butanol. After the paper was dried, the active fraction (Rf $0.50\sim0.63$) was cut off and eluted with water and the eluate was concentrated *in vacuo* to dryness. The dried sample was then dissolved in a small amount of water saturated *n*-butanol and centrifuged (5 minutes, $400\times g$) to remove the insoluble material. The supernatant was kept at 20°C for several days to yield crystals. Recrystallization from water saturated *n*-butanol resulted in colorless crystals.

Elemental analysis of the compound; Found: C 64.36, H 7.79, N 8.32. *Anal.* Calcd. for $C_9H_{13}NO_2$: C 64.70, H 7.84, N 8.38. The mass spectrum with peaks at *m/z* 167, 93, 74, contained the molecular ion *m/z* 167, and M–74 (loss of $H_2NCHCOOH$). The ^1H NMR and ^{13}C NMR spectra (D_2O, DSS) are shown in Figs. 2 and 3, respectively. In Fig. 2 there are three olefinic protons, δ 5.77 (s 2) and δ 5.69 (s 1), and six allylic protons, δ 2.67. In Fig. 3 there are

Fig. 1. Electronmicrograph of the strain I-30 (Bar indicates 1 μm).

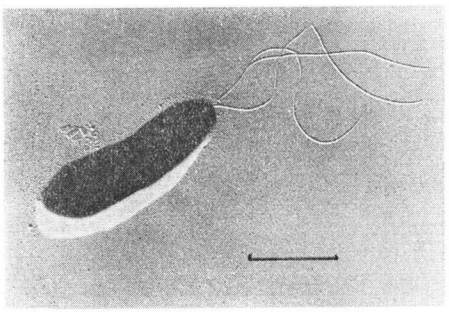


Fig. 2. ^1H NMR spectrum of the substance in D_2O at 100 MHz.

DSS: 3-(Trimethylsilyl)propanesulfonic acid, sodium salt. ($C_6H_{15}NaSSiO_3$).

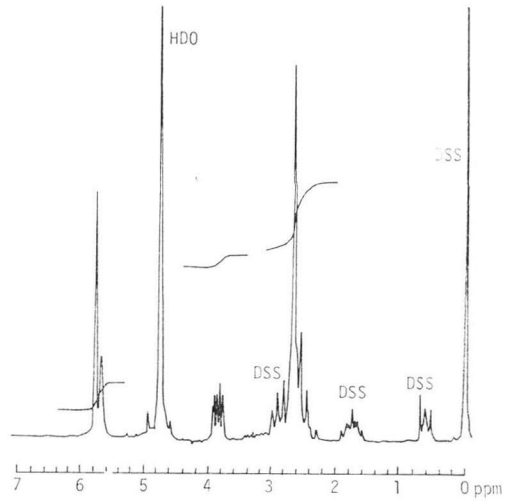
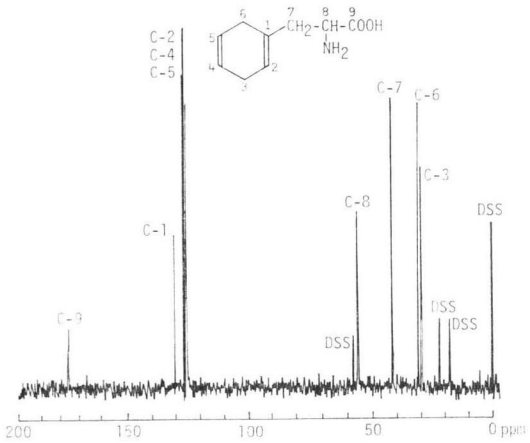


Fig. 3. ^{13}C NMR spectrum of the substance in D_2O.



nine carbons (signals δ 28.8, 30.2, 41.3, 55.2, 126.8, 127.0, 127.3, 131.9 and 177.3). No ultraviolet absorption was seen at wave length higher than 230 nm. L-Amino acid oxidase can abolish the antibiotic activity of this substance but D-amino acid oxidase can not. From these results, this substance was concluded to be L-1,4-cyclohexadiene-1-alanine.

This compound, L-1,4-cyclohexadiene-1-alanine, inhibited the growth of *Neurospora crassa* on Czapeck-Dox agar at lower concentrations than 10 μg/ml (paper disc and dilution methods were used), but the growth inhibition was relieved by the addition of L-phenylalanine to the medium. This compound is positive in ninhydrin reaction (brick orange) and is not stable in the solid state, and is partially converted to L-phenylalanine on exposure to the air. When each of the aromatic amino acids such as L-Phe, L-Tyr, L-Trp or phenylpyruvate (each 1 mg/ml) was added to the culture medium, the strain I-30 did not produce this compound while addition of other amino acids, L-Ala or L-Arg, did not inhibit its production at concentrations higher than 10 mg/ml. These results suggest that L-1,4-cyclohexadiene-1-alanine is synthesized through the route of aromatic amino acids pathway and a feed-back regulation operates in the synthesis of this compound. In this respect, the mechanism of accumulation of this anti-metabolite (100 mg/liter in culture broth at 48 hours) under feed-back regulation seems to be interesting.

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