ISOLATION OF N-(2,6-DIAMINO-6-HYDROXYMETHYLPIMELYL)-L-ALANINE FROM *MICROMONOSPORA CHALCEA*

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A novel dipeptide, N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine, was isolated from the culture broth of a microorganism identified as *Micromonospora chalcea*. The dipeptide exhibits antimicrobial activity against *Escherichia coli* on a synthetic medium, and the activity is synergistically enhanced by several cell wall synthesis-inhibitors.

In the course of a screening program for new substances which exhibit antimicrobial activity against *Escherichia coli* on a synthetic medium from the genus *Micromonospora*[1,2], a strain numbered PA-3534 and identified as *Micromonospora chalcea,* was found to produce an active substance. It was also found that the activity of the substance was enhanced by penicillin G or some other cell wall synthesis-inhibitors. The active substance was purified and its structure was determined to be N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine.

The fermentation and isolation of the active substance as well as the physico-chemical and biological properties involving the structural evidence and the observation on its synergistic action are presented in this paper.

Taxonomic Studies on the Producing Organism

The strain numbered PA-3534 was isolated from a soil sample collected at Ariake Bay, Fukuoka Prefecture, Japan. The strain showed the following taxonomic characters.

The morphology of the strain was studied on cultures grown on BENNETT's agar at 28°C for 21 days. Vegetative mycelium develops well and is branched. Good growth occurs on BENNETT's agar, but aerial mycelium is not formed. A single spore is formed at the tip of the sporophore branching from the vegetative hypha. Its surface appears smooth or with only minor irregularities when observed by electron microscopy.

The cultural characteristics of the strain on various media are shown in Table 1. The growth is slower than that of *Streptomyces* in general. On synthetic media, the vegetative mycelium is dull orange to dull yellow orange. On organic media, the strain generally exhibits more abundant and folded growth than on synthetic media. Colonies are dull orange, later becoming brownish black to black with moistened to glossy surface when grown on yeast extract-malt extract agar and BENNETT's agar.

Production of melanoid pigment, tyrosinase reaction and liquefaction of gelatin are negative. Coagulation and peptonization of milk and hydrolysis of starch are positive.

According to the method of PRIDHAM and GOTTLIEB[3], L-arabinose, D-xylose, D-glucose, sucrose, raffinose and melibiose are utilized for growth, but inositol, L-rhamnose and D-mannitol are not.

Table 1. Culture characteristics of PA-3534.

Media	Growth	Sporulation	Color of colony	Reverse color	Soluble pigment
Sucrose nitrate agar	Fair	No	Dull yellow orange	Dull yellow orange	None
Glucose asparagine agar	Fair	No	Dull orange	Dull orange	None
Glycerol asparagine agar	Poor	No	—	—	None
Inorganic salts starch agar	Fair	No	Dull orange	Dull orange	None
Tyrosine agar	Fair	No	Pale orange	Pale orange	None
Nutrient agar	Fair	No	Dull orange	Dull orange	None
Yeast extract-malt extract agar	Good	Good	Black	Dull orange	None
Oatmeal agar	Good	No	Dull orange	Dull orange	None
BENNETT's agar	Good	Good	Black	Dull orange	None

From morphological and cultural characteristics, strain PA-3534 is considered to belong to the genus *Micromonospora* ØRSKOV. According to the taxonomic criteria[4~6] of the genus *Micromonospora*, strain PA-3534 is considered to closely resemble *Micromonospora chalcea* (FOULERTON) ØRSKOV.

Comparison of the characteristics of strain PA-3534 with those of the standard strain of *M. chalcea* was carried out and good agreements were obtained except liquefaction of gelatin. Therefore, strain PA-3534 is identified as a strain of *M. chalcea*.

Assay Procedure of Antimicrobial Activity

Escherichia coli NIHJ JC-2 was used as a test organism. The organism was maintained on a slant made of DAVIS medium (K_2HPO_4 0.7%, KH_2PO_4 0.3%, sodium citrate 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $(NH_4)_2SO_4$ 0.1%, glucose 0.2%, pH 7.0) and agar 2.0%. To the above medium (without agar) in a test tube, the organism was inoculated and incubated at 37°C overnight. An assay plate was prepared using the above medium including agar 1.5% seeded with *ca.* 0.02% (v/v) of the above culture by the usual manner for paper disk agar diffusion method. A paper disk (6 mm diameter, for antibiotic examination) was used in this experiment.

Production and Isolation

Spores of the strain PA-3534 were inoculated into 100 ml of a medium composed of peptone 0.5%, soluble starch 0.5%, glucose 0.5%, beef extract 0.5%, yeast extract 0.25% and NaCl 0.25%, pH 7.0, in a 500-ml Sakaguchi flask, and shake-cultured at 28°C for 3 days. A 4-ml portion of the culture was then transferred to 100 ml of a medium composed of Bacto-Soytone (Difco) 1.5%, corn steep liquor 0.5%, glucose 2.0%, glycerin 0.5%, NaCl 0.3% and $CaCO_3$ 0.3%, pH 7.0, in a 500-ml Sakaguchi flask, which was shake-cultured at 28°C for 4 days.

The culture broth (5 liters) as above was filtered by filter paper and the filtrate was passed through a column of an ion exchange resin, Dowex 1×2 (CH_3COO^-) (0.8 liter) at pH 10.0. The adsorbed materials on the column were eluted with 0.3 N acetic acid. The eluate fractions which exhibited activity by the above assay procedure were collected and decolorized with 12 g of active carbon (Darco G-60). The active substance was then adsorbed on a column of Dowex 50×2 (NH_4^+) (50 ml) at pH 2.0 and eluted with 0.3 N NH_4OH. The active eluate fractions were collected, concentrated and freeze-dried, giving a crude material (3.0 g). The crude material was then subjected to preparative paper

chromatography on thick filter papers (Toyo Roshi No. 515) with *n*-butanol - acetic acid - water (4: 1: 2) by continuous flow developing method in descending manner. A zone of the active substance detected by bioautography and ninhydrin reaction was cut out and extracted with slightly acidified water. The extract was neutralized with IR-4B (OH^-), freeze-dried and then further purified by preparative thin-layer chromatography on silica gel plates (Merck silica gel GF, 750 μ, 20×100 cm) with *i*-propanol - 14% ammoniacal water (2: 1). The zone of the active substance was detected by ninhydrin reaction (Rf *ca.* 0.25) and extracted with 50% aqueous methanol. The extract was concentrated to a nearly aqueous solution, from which the active substance was adsorbed on a small column of Dowex 50×8 (NH_4^+) at pH 2.0 and eluted with 0.3 N NH_4OH. The eluate was freeze-dried, then dissolved in a small amount of water and precipitated by acetone, giving a pure preparation of the active substance (870 mg).

Physico-chemical Properties and Structural Studies

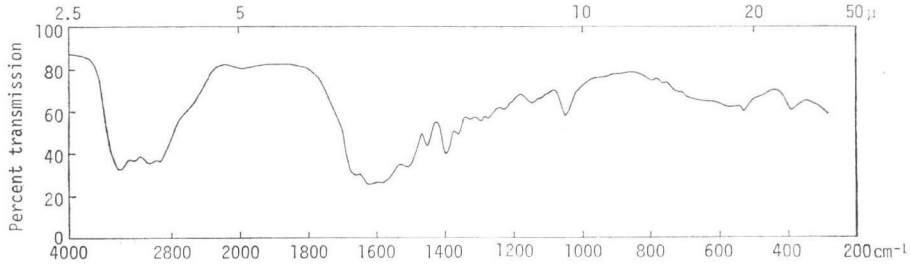
The active substance obtained as above is a colorless amorphous powder, mp 200~210°C (dec.). It gives a single spot on paper chromatograms and thin-layer chromatograms (Table 2). Elemental analysis indicated a molecular formula $C_{11}H_{21}N_3O_6$. *Anal.* Found: C, 44.09; H, 7.41; N, 14.29. Calcd for $C_{11}H_{21}N_3O_6 \cdot \frac{1}{2}H_2O$: C, 43.99; H, 7.38; N, 14.00.

The substance is soluble in water and aqueous alcohols, but insoluble in acetone, ethyl acetate, chloroform and ethyl ether. The ultraviolet absorption spectrum measured in water gives only end absorption. The infrared absorption spectrum in KBr tablet is shown in Fig. 1. The substance is optically active: $[\alpha]_D^{23}$ +8.7±0.5° (*c* 1.035, water). CD: $[\theta]_{200}$ 0, $[\theta]_{212}$ +9530, $[\theta]_{245}$ 0 (*c* 0.0807, 0.5 N HCl).

Table 2. Approximate Rf value of N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine.

	Rf
P.C. (Toyo Roshi No. 51)	
n-BuOH - AcOH - H_2O (4 : 1 : 2)	0.15
n-PrOH - Pyridine - AcOH - H_2O (15 : 10 : 3 : 12)	0.28
TLC (Merck precoated silica gel 60F)	
$CHCl_3$ - EtOH - 14% NH_4OH (4 : 7 : 2)	0.02
n-BuOH - AcOH - H_2O (4 : 1 : 2)	0.07
i-PrOH - 14% NH_4OH (2 : 1)	0.28

Fig. 1. Infrared absorption spectrum of N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine (KBr).



When the substance was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours and the hydrolyzate was analyzed by an automatic amino acid analyzer, the presence of two amino acids (alanine and an unknown amino acid) was indicated. Further, when the substance was dinitrophenylated in the usual manner and then hydrolyzed, a mono-DNP-derivative of the unknown amino acid (ninhydrin positive) and alanine was detected by amino acid analysis and thin-layer chromatographic

experiments. Thus, the active substance was assumed to be a dipeptide composed of the unknown amino acid and alanine.

To obtain both constituent amino acids, some 100 mg of the active substance was hydrolyzed in the same manner. After concentration to dryness, the hydrolyzate was subjected to preparative paper chromatography on Toyo Roshi No. 51 with *n*-butanol - acetic acid - water (4: 1: 2). Two separated ninhydrin-positive zones (Rf: 0.31 and 0.07) were extracted with water and purified by adsorption on a Dowex 50×8 (NH_4^+) column and elution with 0.3 N NH_4OH. Freeze-drying of both eluates gave alanine (23 mg) and the unknown amino acid (63 mg).

The alanine is proved to be in L-configuration: $[\alpha]_D^{26}$ +13.0±0.8° (*c* 0.631, 5 N HCl). ORD: $[\phi]_{215}$ +830, $[\phi]_{224}$ +1610, $[\phi]_{250}$ +480 (*c* 0.342, 0.5 N HCl). The unknown amino acid is obtained as a colorless amorphous powder, mp 240~250°C (dec.). $[\alpha]_D^{26.0}$ +8.1 ±1.0° (*c* 0.506, 5 N HCl), ORD: $[\phi]_{215}$ +590, $[\phi]_{225}$ +970, $[\phi]_{250}$ +260 (*c* 0.264, 0.5 N HCl). Elemental analysis indicated a molecular formula $C_8H_{16}N_2O_5$: *Anal.* Found: C, 43.42; H, 7.52; N, 12.53. Calcd for $C_{18}H_{16}N_2O_5$: C, 43.63; H, 7.32; N, 12.72. From the ^{1}H-NMR and ^{13}C-NMR spectra (Tables 3 and 4), this amino acid was suggested to be 2,6-diamino-6-hydroxymethylpimelic acid (**II** in Scheme 1). The validity of this suggested structure was proved

Table 3. Chemical shifts and coupling constants in ^{1}H-NMR spectra.

Proton	δ ppm (*J*, Hz)	
	I	II
11-CH_3	1.35 d (7.3) 3 H	
4-CH_2	1.4~1.8 m 2 H	1.4~1.8 m 2 H
3-CH_2 and 5-CH_2	1.7~2.0 m 4 H	1.7~2.0 m 4 H
8-CH_2	3.69 d (12.0) 1 H 3.91 d (12.0) 1 H	3.70 d (12.1) 1 H 3.93 d (12.1) 1 H
2-CH	3.96 t (6.5) 1 H	3.71 t (6.5) 1 H
10-CH	4.17 q (7.3) 1 H	

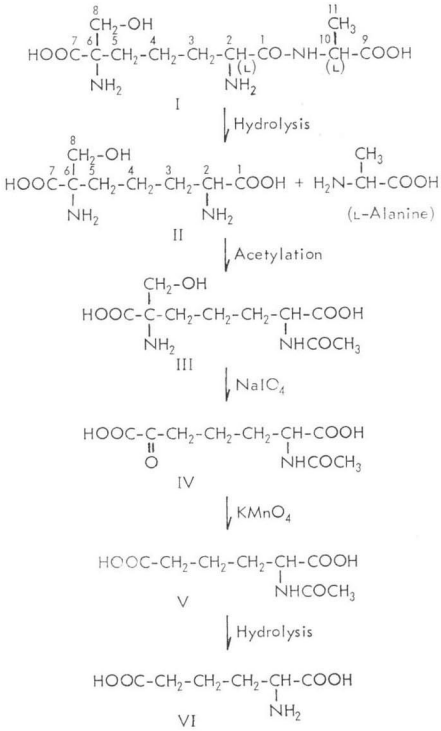
The spectra were recorded on a Varian A-60A spectrometer (60-MHz), in D_2O using DSS as an internal reference. Accuracy of δ_H is within 0.02 ppm.

Table 4. Chemical shifts in ^{13}C-NMR spectra.

Carbon	δ (ppm)		
	I		II
9	180.3	s	
7	174.9*	s	175.4*
1	169.9*	s	174.9*
6	66.7	s	66.7
8	65.0	t	64.9
2	53.9**	d	55.4
10	52.2**	d	
3	32.7***	t	32.8**
5	31.8***	t	31.5**
4	19.8	t	20.3
11	18.0	q	

*,**,*** Assignments within any vertical column may be reversed. The spectra were recorded on a Varian NV-14-FT-NMR spectrometer (15.087 MHz) in D_2O using DSS as an internal reference. Accuracy of δ_C is within 0.1 ppm.

Scheme 1.



as follows: The amino acid was acetylated with acetic anhydride in a dilute sodium bicarbonate solution, affording 2-acetylamino-6-amino-6-hydroxymethylpimelic acid (**III** in Scheme 1). It was treated with $NaIO_4$ in a dilute alkaline solution and followed by oxidation with $KMnO_4$. Finally, the N-acetyl group was removed by acid hydrolysis. The product of these sequential reactions illustrated in Scheme 1 was identified with L-α-aminoadipic acid (**VI** in Scheme 1) by direct comparison with an authentic specimen in automatic amino acid analysis and measurement of its CD spectrum: CD: $[\theta]_{240}$ 0, $[\theta]_{210}$ +4200, $[\theta]_{200}$ +2510 (*c* 0.1286, 0.5 N HCl).

To elucidate the structure of the active substance (dipeptide) it was necessary to determine which side of the carboxyl group of 2,6-diamino-6-hydroxymethylpimelic acid was linked to the amino group of alanine. SHEINBLATT had reported a method for determination of sequence of amino acid residues in di- and tripeptides by ^{1}H-NMR techniques[7]. The method is based on the following facts: A neutral peptide molecule in aqueous solution exists as a zwitterion having positively charged groups (NH_3^+) and negatively charged groups (COO^-). Addition of base to an aqueous solution of a peptide neutralizes the NH_3^+ group. Consequently the spectrum of the adjacent group should shift toward higher field. On the other hand, the addition of acid results in the protonation of the ionized carboxyl group and consequently the spectral line of the adjacent group should shift toward lower field. The principle of this method was employed to resolve the above problem.

The zwitterionic form of the proposed structure of the dipeptide is illustrated in Scheme 2 and the shifts of spectral lines by addition of acid or base in ^{1}H-NMR spectra are listed in Table 5.

Scheme 2.

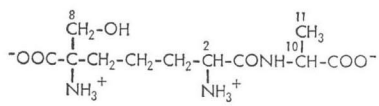


Table 5. Shifts of spectral lines in the ^{1}H-NMR of N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine by addition of acid or base.

	Shift (in ppm)*			
	8-CH_2	2-CH	11-CH_3	10-CH
Acid	+0.05	+0.09	+0.11	+0.25
Base	−0.17	−0.60	−0.02	−0.11

* Plus sign denote a downfield shift. pH values of acidic, basic, and the reference solutions were 0.5, 11.0 and 5.0, respectively. The conditions for recording the spectra were the same as in Table 3.

The spectral line of 10-CH and to a smaller extent the spectral line of 11-CH_3 shifted downfield in the acidic solution, indicating the presence of a neighboring carboxyl group. The spectral line of 8-CH_2, which is present in the β-position from both negative charged and positive charged centers, shifted in a smaller magnitude at both acidic and basic pHs. With respect to the spectral line of 2-CH, a much greater upfield shift by addition of base was observed, as compared with a small downfield shift by addition of acid. This indicated the presence of an amino group but the absence of a carboxyl group in the neighborhood of 2-CH.

Thus, the structure of the dipeptide was determined except for the configuration at 6-C as **I** in Scheme 1, called N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine.

Biological Properties

Antimicrobial activity was assayed by paper-disk agar diffusion method (Table 6). The dipeptide was active against *E. coli* NIHJ JC-2 and *E. coli* EC-14, but not against *E. coli* K-12 W3110 on DAVIS medium. However, when assayed on nutrient medium, no activity was shown.

Table 6. Antimicrobial activity of N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine.

Test organism	Medium	Inhibitory, diameter (mm) 1 mg/ml
E. coli NIHJ JC-2	D	16.0
E. coli EC-14	D	19.0
E. coli K-12 W3110	D	0
E. coli NIHJ JC-2	N	0
E. coli EC-14	N	0

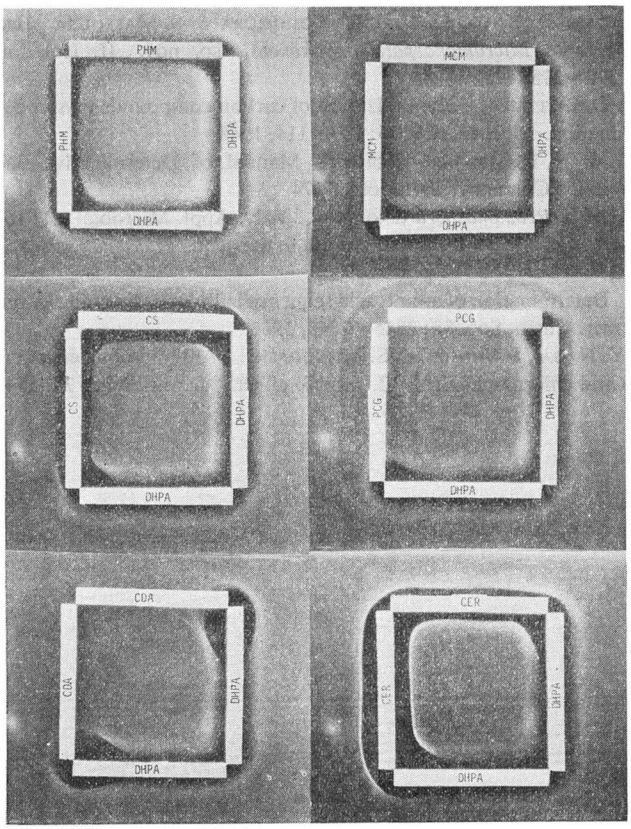
Method: Paper-disk agar diffusion method.
Medium: D (DAVIS medium), N (Nutrient medium).

The antimicrobial activity shown against the above strains of *E. coli* on DAVIS medium was synergistically enhanced by phosphonomycin (PHM), cycloserine (CS), chloro-D-alanine(CDA), macarbomycin (MCM), penicillin G (PCG) and cephaloridine (CER). These are well-known inhibitors of cell wall synthesis. The qualitative observations of synergism between the dipeptide (abbreviated as DHPA) and these antibiotics are shown in Fig. 2, which were made by the crossed paper strip method reported by KAWAKAMI *et al.*[8] However, other cell wall synthesis-inhibitors such as O-carbamyl-D-serine, vancomycin (these are inactive against the *E. coli* strains) and

Fig. 2. Synergistic action of N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine.

DHPA: N-(2,6-Diamino-6-hydroxymethylpimelyl)-L-alanine, 500 mcg/ml
PHM: Phosphonomycin, 2,000 mcg/ml
CS: Cycloserine, 50 mcg/ml
CDA: Chloro-D-alanine, 2,000 mcg/ml
MCM: Macarbomycin, 4,000 mcg/ml
PCG: Penicillin G, 200 mcg/ml
CER: Cephaloridine, 200 mcg/ml

Filter paper strips (Toyo Roshi No. 50, 5×50 mm) were dipped in solutions of the above drugs and placed as shown on agar plates made of DAVIS medium seeded with *E. coli* NIHJ JC-2. The plates were incubated at 28°C for 16 hours.



bacitracin did not show synergistic action with the dipeptide. Several antibiotics of other action mechanisms, such as neomycin, erythromycin, chloramphenicol, polymyxin E and actinomycin D, also did not show any synergistic action with the dipeptide.

Subcutaneous administration of the dipeptide at dose of 100 mg/kg×2 to mice infected with *E. coli* EC-14 did not show any therapeutic effect, and intraperitoneal injection of 100 mg/kg did not show any toxic symptoms.

Consideration

A novel dipeptide, N-(2,6-diamino-6-hydroxymethylpimelyl)-L-alanine, was isolated from *Micromonospora chalcea*. This dipeptide contains a derivative of 2,6-diaminopimelic acid, which is well known to be a cell wall constituent of almost all Gram-negative bacteria and several kinds of Gram-positive bacteria. A very limited antimicrobial activity is observed with the dipeptide; *i.e.* against some strains of *E. coli* on a synthetic medium, and this activity is synergistically enhanced by several cell wall synthesis-inhibitors.

It may be considered that the action mechanism of this dipeptide is concerned with the cell wall synthesis of the *E. coli* strains. However, we have no clear evidence in support of this hypothesis at present by observation of the morphological change of the *E. coli* cells affected by this dipeptide.

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