ISOLATION AND CHARACTERIZATION OF HYPEPTIN FROM *PSEUDOMONAS* SP.

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An antibiotic, named hypeptin, was isolated from the culture broth of a strain of *Pseudomonas* sp. The antibiotic was extracted with butanol and purified by chromatography on a Sephadex LH-20 column. The antibiotic is basic in nature. The dihydrochloride is soluble in aqueous alcohols and positive to ninhydrin and SAKAGUCHI's reaction. A molecular formula, $C_{44}H_{71}N_{13}O_{15}\cdot 2HCl$ was indicated by microanalysis and secondary ion MS. The IR spectrum and acid hydrolysis indicated it to be a peptide antibiotic. The stereochemistries of the amino acids produced by hydrolysis were clarified by HPLC using a chiral column. The antibiotic is active *in vitro* against a wide variety of anaerobic bacteria and aerobic Gram-positive bacteria.

In the course of our screening work for new antibiotics from bacteria, a strain of *Pseudomonas* numbered PB-6269 was found to produce an antibiotic dominantly active against anaerobic bacteria. Structural studies, which will be reported in an accompanying paper[1], revealed it to be an octapeptide, in which four β-hydroxyamino acids are contained (Fig. 1). A name hypeptin is given to this antibiotic in relation to the unique amino acid composition.

This paper deals with the taxonomy of the producing organism, the isolation and characterization of the antibiotic.

Taxonomy

The producing organism numbered PB-6269 was isolated from a soil sample collected in Okinawa Prefecture, Japan. The organism is Gram-negative, non-sporulating rods ($0.5\sim0.7\times3.0\sim4.0\,\mu$m) with rounded ends and motile with polar monotrichous flagellation, but occasionally non-motile. On nutrient

Fig. 1. Structure of hypeptin.

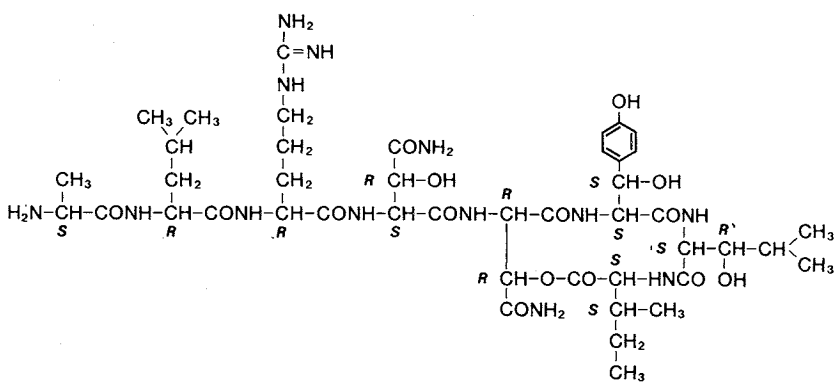


Table 1. Physiological characteristics of strain PB-6269.

	Result	Properties observed	Result
Catalase test	Very weak	Acylamidase test	−
Oxidase test	+	Deoxyribonuclease test	−
OF-test	Oxidative	β-Galactosidase test	+
Peptonization of milk	+	Phenylalanine deaminase test	−
Coagulation of milk	−	Tween-80 esterase test	+
Gelatin liquefaction	+	Urease test	−
Esculin hydrolysis	+	Voges-Proskauer test	−
Starch hydrolysis	−	Methyl red test	−
Indole production	−	Nitrate reduction	−
H_2S production	−	Denitrification	−
Arginine dihydrolase test	−	Citrate utilization	−
Lysine decarboxylase test	−	Fluorescent pigment	−
Ornithine decarboxylase test	−		

agar, it forms circular, entire, convex, wet, opaque and glistening colonies with brownish yellow to orange color. The organism, aerobic, showed good growth at 28°C. Good growth on MacConkey agar. No growth on Trypticase soy agar supplemented respectively by 0.2% (w/v) of cetrimide, 6.5% (w/v) of sodium chloride and 1.0% (w/v) of 2,3,5-triphenyltetrazolium chloride. Accumulation of poly-β-hydroxybutyrate was not observed. Other physiological characteristics are shown in Table 1. On cleavage of carbohydrates, acid formation without gas was observed from glucose, fructose, maltose, lactose and trehalose. No acid formation was observed from arabinose, xylose and sucrose.

From comparison of these characteristics with those of bacteria registered in the Volume 1 of BERGEY's Manual of Systematic Bacteriology[2], the organism should be ascribed to the genus *Pseudomonas*. According to further comparison with the species of *Pseudomonas*, none of the species is identical with the organism.

Production and Isolation

A cell suspension of strain PB-6269 was inoculated into 100 ml of a medium consisting of soluble starch 2.0%, glycerol 0.5%, soy bean meal 1.5%, corn steep liquor 0.5%, NaCl 0.3% and $CaCO_3$ 0.3% (pH 7.0) in a 500-ml Erlenmeyer flask. It was cultured in the usual shaking manner for 2 days at 23°C.

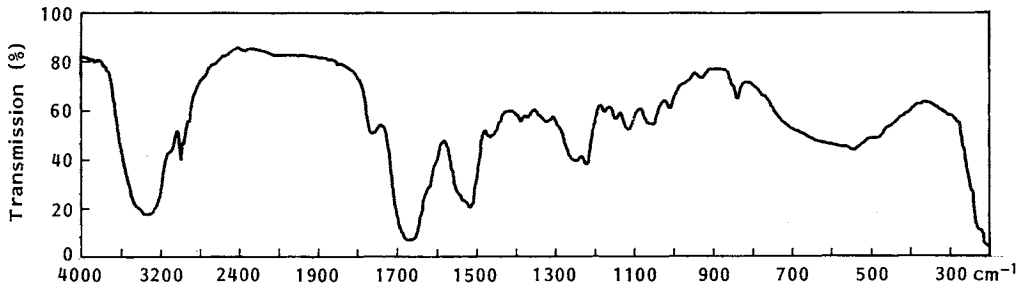
The culture broth (5 liters) was adjusted to pH 2.5 with HCl. It was mixed with the same volume of a mixture of BuOH-MeOH (1:1) and filtered. The filtrate was evaporated under reduced pressure at pH 7.0 and then repeatedly extracted with BuOH at pH 8.0. The BuOH extract, after washing with water, was adjusted to pH 2.0 and concentrated under reduced pressure to an oily residue, which was triturated with acetone to give a crude powder (4.5 g).

The crude powder (1.0 g) was purified by column chromatography on a Sephadex LH-20 column (3.6×88 cm) developed with MeOH. The active eluate fraction was concentrated and trituration of the residue with acetone afforded a semi-pure preparation of the dihydrochloride. Repetition of the procedure gave a purified preparation as a colorless powder. From 4.5 g of the crude powder, 185 mg of the purified preparation was obtained.

Chemical Characterization

Hypeptin is basic in nature, and the hydrochloric acid salt is obtained as a colorless amorphous powder, mp 220~228°C (dec), soluble in methanol, aq ethanol, aq butanol and dimethyl sulfoxide, and substantially insoluble in acetone, ethyl acetate, chloroform and water. It gives positive reactions with

Fig. 2. IR spectrum of hypeptin dihydrochloride (KBr).



ninhydrin and SAKAGUCHI's reagents.

The antibiotic gave a single peak on HPLC using a Nucleosil 5 C_{18} column (4.6 × 150 mm) with 26% acetonitrile containing 0.1% trifluoroacetic acid, showing a retention volume of 5.06 ml.

A molecular formula, $C_{44}H_{71}N_{13}O_{15}$, is indicated by microanalysis and liquid secondary ion (LSI)-MS.

Anal Calcd for $C_{44}H_{71}N_{13}O_{15}\cdot 2HCl$: C 48.26, H 6.58, N 16.63, Cl 6.49.
Found: C 47.99, H 7.06, N 15.88, Cl 6.16.

LSI-MS: *m/z* 1,022 $(M+H)^+$, 1,044 $(M+Na)^+$.

Dominant adsorptions at 1670 and 1515 cm^{-1} in the IR spectrum (Fig. 2) indicated this antibiotic to be a peptide. An absorption at 1760 cm^{-1} suggests the presence of a lactone linkage. For the cause of a slight shift to higher wave number than that of a usual lactone linkage, a possibility is considered to be due to the presence of any electron negative group adjacent to the hydroxy group involved in the lactone linkage.

In the UV spectrum, the following absorption maxima are observed: λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 226 (113), 276 (18), 280 (sh, 16); $\lambda_{max}^{dil\ HCl\text{-}MeOH}$ nm ($E_{1cm}^{1\%}$) 225 (111), 275 (17), 280 (sh, 16), $\lambda_{max}^{dil\ NaOH\text{-}MeOH}$ nm ($E_{1cm}^{1\%}$) 247 (182), 289 (76). These absorptions resemble those of tyrosine, suggesting the presence of tyrosine or tyrosine analogue in the molecule.

This antibiotic is optically active, CD $[\theta]_{215}-12{,}500$, $[\theta]_{230}-1{,}590$, $[\theta]_{235}-2{,}120$, $[\theta]_{247}-1{,}370$, $[\theta]_{283}0$, $[\theta]_{305}+430$, $[\theta]_{350}0$ (*c* 0.416, phosphate buffer, pH 7.0).

By acid hydrolysis, hypeptin produced the following amino acids: β-Hydroxyaspartic acid (hereafter, abbreviated as HyAsp) (2), β-hydroxyleucine (abbreviated as HyLeu) (1), Ala (1), Ile (1), Leu (1) and Arg (1). β-Hydroxytyrosine, whose presence was indicated by NMR studies[1], was not found in the hydrolysate, probably because of complete degradation of the amino acid during hydrolysis.

These amino acids were isolated from the hydrolysate by paper chromatography. The stereochemistries of these amino acids were examined by HPLC using a chiral column in comparing with respective authentic specimens[3]. Consequently, the HyAsp is revealed to be a mixture of D-*threo* and L-*erythro* forms, HyLeu to be L-*threo* form, Ala to be L-form, Ile to be L-form, Leu to be D-form and Arg to be D-form, respectively.

We have previously isolated L-*threo*-β-hydroxyaspartic acid and L-*threo*-β-hydroxyleucine from katanosins A and B[4]. This is the first reported instance of the isolation of D-*threo*-β-hydroxyaspartic acid and L-*erythro*-β-hydroxyaspartic acid as natural products. That these diastereoisomers are part of a peptide is of interest from the viewpoint of biosynthesis.

Table 2. Antibacterial spectrum of hypeptin against anaerobic bacteria.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Peptococcus asaccharolyticus ATCC 14963	0.78	*C. difficile* ATCC 17857	0.78
P. prevotii ATCC 9321	0.2	*Veillonella parvula* ATCC 10790	12.5
Peptostreptococcus micros VPI 5464-1	6.25	*Bacteroides fragilis* GM 7000	50
Streptococcus constellatus ATCC 27823	1.56	*B. fragilis* ATCC 25285	50
Eubacterium limosum ATCC 8486	0.2	*B. thetaiotaomicron* WAL 3304	50
E. aerofaciens ATCC 25986	1.56	*B. vulgatus* ATCC 29327	6.25
Propionibacterium acnes ATCC 11827	0.78	*B. melaninogenicus* GAI 0413	3.13
Bifidobacterium adolescentis JCM 1250	0.2	*Fusobacterim varium* ATCC 8501	50
B. bifidum JCM 1122	1.56	*F. necrophorum* ATCC 25286	12.5
B. longum ATCC 15707	0.78	*F. nucleatum* ATCC 25586	12.5
Clostridium perfringens ATCC 13124	0.2	*F. mortiferum* ATCC 9817	12.5

Inoculum size: One loopful of 10^6 cfu/ml.
Medium: GAM Agar (Nissui).

Antibacterial Properties

Hypeptin is active against anaerobic Gram-positive bacteria and weakly against anaerobic Gram-negative bacteria. It is also active against aerobic Gram-positive bacteria as shown in Tables 2 and 3.

Table 3. Antibacterial spectrum of hypeptin against aerobic bacteria.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA JC-1	0.78
S. aureus Smith	1.56
Streptococcus pyogenes C-203	0.78
S. pneumoniae Type 1	0.78
Escherichia coli NIHJ JC-2	50
E. coli EC-14	100
Klebsiella pneumoniae SR1	50
Proteus vulgaris CN-329	>100
Enterobacter cloacae ATCC 13047	>100
Serratia marcescens ATCC 13880	>100
Pseudomonas aeruginosa ATCC 25619	>100

Experimental

The UV absorption spectrum was measured with a Hitachi 323 spectrometer, IR absorption spectrum with a Jasco DS-403G spectrometer, CD spectrum with a Jasco J-40 C automatic recording spectropolarimeter and LSI-MS with a Hitachi M-90 mass spectrometer. Amino acid analysis was carried out with a Hitach amino acid autoanalyzer 835.

Acid Hydrolysis

A few mg of hypeptin dihydrochloride was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours in a vacuum sealed tube and the hydrolysate was analyzed by an automatic amino acid analyzer. The following amino acids were found (μmol/mg): HyAsp (1.13), HyLeu (0.56), Ala (0.81), Ile (0.71), Leu (0.79) and Arg (0.73). β-Hydroxyamino acids were usually produced in slightly decreased amounts by acid hydrolysis from peptides because of a β-elimination reaction. Therefore, the molar ratio described in the text were considered to be reasonable.

Stereochemistries of the Amino Acids Produced by Hydrolysis

Some 100 mg of hypeptin dihydrochloride was hydrolyzed by the same manner. The hydrolysate was separated by paper chromatography on two papers (Toyo Roshi No. 51, 60×60 cm) with BuOH - AcOH - H_2O (4 : 1 : 2) by the descending manner. Five ninhydrin positive zones which separated were each extracted with water and the extracts were concentrated to residues.

The 1st fraction from the zone of the lowest Rf, Rf 0.12, was purified by adsorption on a Dowex 50X2 (H^+) column and elution with 0.1 N NH_4OH. The eluate was concentrated to dryness to give HyAsp as a colorless powder (13 mg). CD measurement with this preparation showed substantially no optical activity. An HPLC examination using a chiral column, whose detail will be described later, revealed it to be a mixture of D-*threo* and L-*erythro* forms of HyAsp.

The 2nd fraction from the zone of Rf 0.21 was purified by adsorption on a Dowex 50X2 (NH_4^+) and

elution with 0.3 N NH_4OH. The eluate was concentrated to dryness, which was dissolved into dil HCl and freeze-dried to give arginine hydrochloride (11.5 mg). CD $[\theta]_{210}$ −4,220, $[\theta]_{215}$ −2,890, $[\theta]_{245}$0 (*c* 3.309 mM, 0.5 N HCl). By the HPLC examination, it coincided with D-Arg.

The 3rd fraction from the zone of Rf 0.45 was purified by adsorption on a Dowex 50X2 (H^+) column and elution with 0.3 N NH_4OH. The eluate was concentrated to dryness to give Ala as a colorless powder (5.5 mg). CD $[\theta]_{210}$ +2,290, $[\theta]_{215}$ +1,590, $[\theta]_{245}$0 (*c* 5.197 mM, 0.5 N HCl). The HPLC examination showed it to be L-alanine.

The 4th fraction from the zone of Rf 0.61 was worked up similarly to give HyLeu as a colorless powder (8 mg). CD $[\theta]_{210}$ +4,800, $[\theta]_{245}$0 (*c* 3.077 mM, 0.5 N HCl). By the HPLC examination, it coincided with L-*threo*-β-hydroxyleucine.

The 5th fraction from the zone of Rf 0.71 was worked up similarly to give a mixture of Ile and Leu as a colorless powder (19 mg). The HPLC examination revealed it to be a mixture of L-isoleucine and D-leucine.

HPLC Examination Using a Chiral Column

A chiral column MIC GEL CRS 10W (4.6 × 50 mm) (Mitsubishi Chemical Industries Limited) was used. The mobile phase used was: I: 0.1 mM $CuSO_4$, II: 0.25 mM $CuSO_4$, III: 2.0 mM $CuSO_4$ or IV:CH_3CN-2.0 mM$CuSO_4$ (1:9). HPLC was carried out under a flow rate of 0.45 ml/minute and monitored by optical density at 254 nm. The reference amino acids used in the above experiment were clearly distinguished in this condition. The retention volumes are listed in the Table 4.

Table 4. Retention volumes of amino acids used as references.

Mobile phase	Amino acid	Retention volume (ml)
I	D-Alanine	4.05
	L-Alanine	5.30
II	D-Arginine	1.45
	L-Arginine	1.90
III	D-*threo*-β-Hydroxyleucine	2.90
	L-*threo*-β-Hydroxyleucine	3.55
	D-*erythro*-β-Hydroxyleucine	7.20[a]
	L-*erythro*-β-Hydroxyleucine	8.65[a]
III	D-Isoleucine	7.74
	L-Isoleucine	14.60
III	D-Leucine	7.43
	L-Leucine	12.50
IV	D-*threo*-β-Hydroxyaspartic acid	8.60
	D-*erythro*-β-Hydroxyaspartic acid	9.80
	L-*threo*-β-Hydroxyaspartic acid	11.80
	L-*erytrho*-β-Hydroxyaspartic acid	13.50

[a] Tentative assignment based on general rule for elution order of D and L forms.

Synthesized specimens of D,L-*threo* and D,L-*erythro*-β-hydroxyleucine and a specimen of L-*threo*-β-hydroxyleucine which was isolated from katanosins A and B[4] were used as reference compounds. Assignment of the peaks of D and L forms of *erythro*-β-hydroxyleucine was tentative and based solely on a general rule that D-form is eluted faster than L-form in this procedure[3]. As references β-hydroxyaspartic acids, synthesized specimens of D,L-*threo*, L-*threo*, D-*erythro* and L-*erythro* forms were used.

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