ISOLATION AND CHARACTERIZATION OF MANNOPEPTINS, NEW ANTIBIOTICS

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New antibiotics, mannopeptins A and B, were isolated from the fermented broth of *Streptomyces platensis* strain FS-351. Ferrous ion is essential for the antibiotic production, since no productivity was noted with media containing less than 0.11 mM ferrous ion and maximum production was achieved at a concentration of 1.8 mM. The antibiotics are basic glycopeptides with relatively high molecular weight and are similar to ristocetin and vancomycin but can be differentiated from them in view of their chemical composition and chromatographic behavior. The antibiotics were named mannopeptin after the glycopeptide containing mannose.

In our screening for antibacterial antibiotics, new antibiotics mannopeptins A and B were isolated from the fermented broth of *Streptomyces* sp. strain FS-351. The antibiotics are capable of inhibiting the growth of gram-positive and some gram-negative bacteria except for the genera *Pseudomonas* and *Klebsiella*. The producing organism is a typical *Streptomyces* with short aerial mycelium and is an isolate from a soil sample collected at Kawasaki, Japan. The identification revealed that the organism belonged to the *S. hygroscopicus* group and the morphological properties were consistent with those of *S. platensis*[1].

Antibiotic production depends on ferrous ion concentration, because no antibacterial activity was noted with media containing less than 0.11 mM ferrous ion (Table 1), although the growth became more luxuriant as the concentration was diminished. Maximal antibiotic production was observed in a medium containing 1.8 mM of ferrous ion. Ammonium sulfate and yeast extract are also needed for antibiotic production (Table 1). Ordinary nutrients like soybean meal, peptone and corn steep liquor inhibit production.

Table 1. Production of mannopeptins

Media	Mannopeptins (μg/ml)
Basal medium	125
$FeSO_4 \cdot 7H_2O$ reduce to 0.0125%	85
$FeSO_4 \cdot 7H_2O$ reduce to 0.0031%	—
$FeSO_4 \cdot 7H_2O$ omit	—
Starch omit, glucose add	150
$(NH_4)_2SO_4$ omit, NH_4NO_3 (0.2%) add	—
Yeast extract omit	—
Yeast extract omit, dry yeast (0.1 %) add	72
Glucose and corn steep liquor add	—
Soybean meal add	—
Peptone add	—

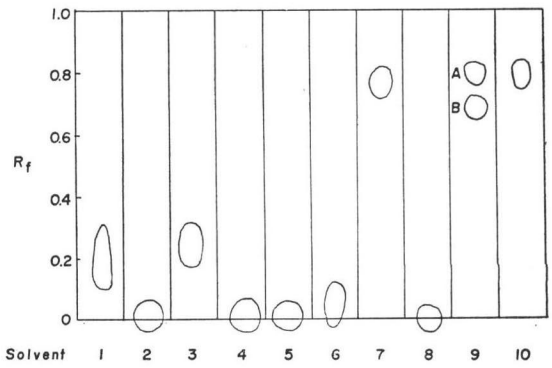
Basal medium was as follows; (w/v, %), glucose 0.2, soluble starch 3.0, yeast extract 0.1, ammonium sulfate 0.2, magnesium sulfate 0.1, potassium biphosphate 0.1, ferrous sulfate heptahydrate 0.05 and calcium carbonate 0.5. Glucose, corn steep liquor, peptone and soybean meal were added to the basal medium at a concentration of 1%.

Mannopeptins are found only in the fermentation filtrate. Although cation-exchange resins such as Amberlite IR-120 and IRC-50 can adsorb the antibiotics, no bioactivity can be recovered by eluting the resins with aqueous acids or ammonia. Activated charcoal can also adsorb the antibiotics, but it is difficult to liberate them by such ordinary procedures

as elution with acidic aqueous methanol or acetone. Quantitative recovery from the adsorbent was achieved by eluting with acidic dimethylformamide or dioxane.

Before eluting the antibiotics, the charcoal was successively washed with acidic aqueous methanol and acetone. Then, the antibiotics were eluted with acidic dimethylformamide and the eluate, after neutralizing with Amberlite IR-4B (OH^-), was concentrated to 1/3 volume *in vacuo*. The crude mannopeptins were purified by carbon column chromatography and further purification was accomplished by CM-cellulose column chromatography. The antibiotics were eluted at pH 3 from CM-cellulose column by using a concentration gradient between water and 0.1 N sulfuric acid. The eluate, neutralized with Amberlite IR-4B (OH^-), was concentrated to small volume *in vacuo* and the resulting syrup was lyophilized yielding crude mannopeptin as pale green powder.

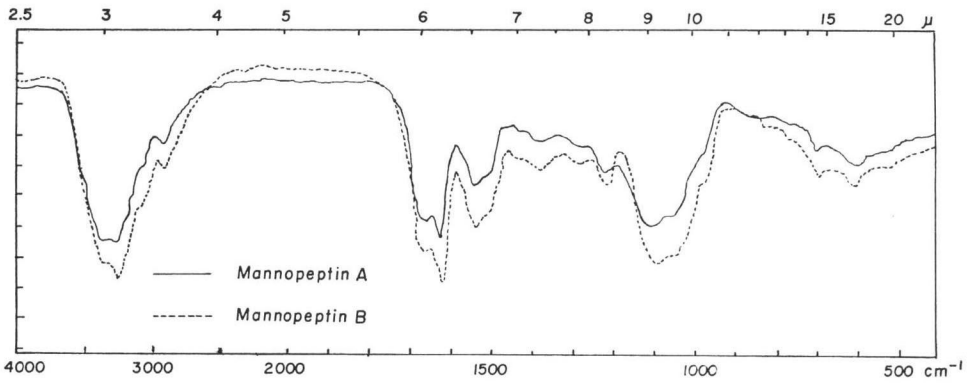
Fig. 1. Paper chromatogram of mannopeptins A and B.
Solvent
1. H_2O 2. Butanol (H_2O-saturated) 3. Butanol acetic acid - H_2O (4:1:2) 4. Butanol - pyridine - acetic acid - H_2O (15:10:3:12) 5. 80% Aqueous phenol 6. 50% Aqueous methanol 7. 50% Aqueous propanol 8. 50% Aqueous acetone 9. 0.1 N H_2SO_4 - acetone (1:1) 10. 0.1 N H_2SO_4-dimethylformamide (1:1)



Bioautograms of the crude preparation (Fig. 1) showed the presence of two components, which were separated by Bio Gel P-4 column chromatography. Mannopeptin A was eluted from the column with distilled water and then, mannopeptin B was eluted with dilute sulfuric acid. Both antibiotics crystallized as colorless prisms by keeping the concentrates in the cold.

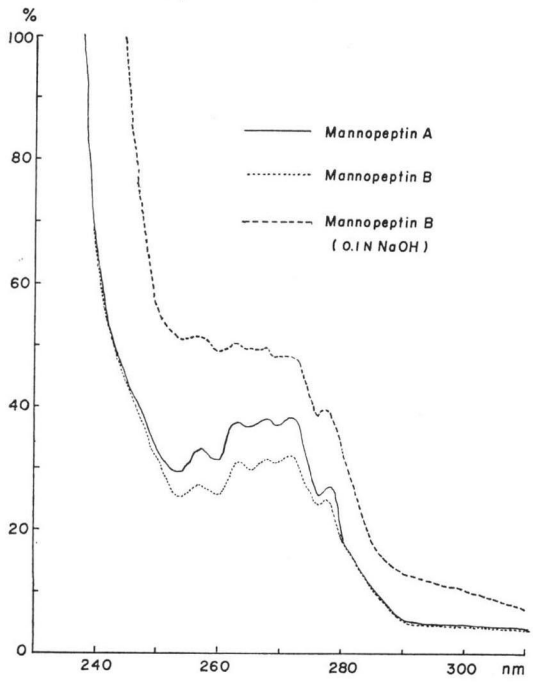
The antibiotics show no definite melting points; they turned brown at 200°C and charred above 250°C. They are soluble in water, dioxane and dimethylformamide, but insoluble in most organic solvents such as alcohols, ether, chloroform, benzene and *n*-hexane. The IR spectra are very similar to those of ristocetin[2], vancomycin[3] and LL-AV 290[4]; which are

Fig. 2. Infrared spectra of mannopeptins A and B in KBr



glycopeptide antibiotics with high molecular weights (Fig. 2). The spectra indicate the presence of peptide bonds (1627, 1548 cm^{-1}) and probably carbohydrate residues (3300, 1180 cm^{-1}); the presence of the latter was supported by a positive MOLISCH test. The antibiotics moved toward the anode on paper electrophoresis, indicating that they are basic substances. UV absorption spectra showed five peaks around 270 nm (257, 263, 267, 271 and 278 nm). In alkaline condition increases in UV absorptivity are less than those of known glycopeptide antibiotics and no bathochromic shifts were noted, thus showing that mannopeptins are different from these glycopeptide antibiotics (Fig. 3).

Fig. 3. Ultraviolet spectra af mannopeptins A and B (500 μg/ml solution)



Vigorous acid hydrolysis of mannopeptins yielded six ninhydrin-positive substances (Table 2); serine, tyrosine, β-methylphenylalanine[5], unidentified basic amino acid I and unidentified basic amino acid II for mannopeptin A and in the hydrolyzate of mannopeptin B, L-phenylalanine was present instead of β-methylphenylalanine. The amino acid compositions are different from that of vancomycin. Acid

Table 2. Amino acid composition of mannopeptins

Amino acids	Retention time (min)	Molar ratio	
		Mnnnopeptin A	Mannopeptin B
Serine	91	3	3
Glycine	140	3	3
Tyrosine	244	2	2
Phenylalanine	260	0	2
β-Methylphenylalanine	285	2	0
Unidentified basic amino acid I	60	2	2
Unidentified basic amino acid II	72	2	2

Molar ratios of unidentified basic amino acids were calculated on the assumption that their molecular weights are 158.

hydrolysis of vancomycin has shown that it yields N-methyl-D-leucine, L-aspartic acid, D-glucose and vancomycinic acid[6].

Mild acid hydrolysis of mannopeptins liberated a carbohydrate that shows the same Rf value as D-mannose on paper-chromatograms, but neither amino sugar nor pentose was detected in the hydrolyzates. Retention time analyses on gas chromatography[7] also demonstrates that the carbohydrate portion from the antibiotics is mannose. Therefore, mannopeptins differentiate from ristocetins whose carbohydrates composition is glucose, mannose, D-arabinose and rham-

nose[8]. Although the structures have not yet been clarified, mannopeptins are clearly new antibiotics in view of their physical and chemical properties in addition to their unique antibacterial properties[9].

Experiments

Antibiotic production was measured by a microbiological disc plate assay procedure with spore suspension of *Bacillus subtilis* PCI 219, growing on a nutrient agar plate as the test organism. The molten nutrient agar was seeded with the spore suspension and allowed to solidify. The fermented broth or preparation to be tested was applied onto 8-mm paper disc and these were placed on the surface of the seeded agar and incubated at 37°C for 18 hours. The bio-unit was determined by the growth inhibitory zone.

Fermentation: *Streptomyces platensis* strain FS-351 was grown in the following medium (w/v, %) glucose 0.2, soluble starch 3.0, yeast 0.1, $(NH_4)_2SO_4$ 0.2, $MgSO_4 \cdot 7H_2O$ 0.1, KH_2PO_4 0.1, $FeSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 0.5. The seed was cultured at 27°C for 72 hours and then 600 ml volume was transferred into a 100-liter tank containing the same medium as described above (60 liters). The tank was agitated at 250 rpm and 27°C under aeration of 30 liters/min. After fermentation had been completed, the clear culture filtrate was obtained by filtering with the aid of Celite. Activated charcoal (2 %) was added to the filtrate. The charcoal collected by filtration was washed thoroughly with water and then 50 % aqueous methanol containing 0.05 N HCl. The antibiotics were eluted with acidic aqueous dimethylformamide (0.2 N sulfuric acid 1 part : dimethylformamide 3 parts) and the eluate was concentrated to 1/3 volumn *in vacuo*. The concentrate was passed over a carbon column and the adsorbed antibiotics were eluted with acidic aqueous dioxane (0.2 N sulfuric acid 1 part : dioxane 1 part). The eluate was neutralized with Amberlite IR-4B (OH^-) and concentrated *in vacuo* to 100 ml. Further purification was accomplished by column chromatography with CM-cellulose (H^+) (300 ml). Gradient elution technique between water (1,000 ml) and 0.1 N sulfuric acid (1,000 ml) was used for elution. The bioactivity was eluted as a colorless solution at pH 3, and combined active fractions were neutralized with Amberlite IR-4B (OH^-). The neutralized eluate was concentrated to a small volume *in vacuo* and the resulting syrup was lyophilized yielding crude mannopeptins as a pale green powder (20 g).

Separation of mannopeptins A and B: The crude mannopeptins (2.9 g, purity 80 %) was dissolved in a small amount of water and the resulting solution was applied to the top of a column packed with BiO Gel P-4 (200~400 mesh, 500 ml). Mannopeptin A was eluted from the column with distilled water and then mannopeptin B was eluted with 0.02 % aqueous sulfuric acid. Sulfuric acid was removed from the combined active fractions with Amberlite IR-4B (OH^-). Both active fractions were concentrated to small volume *in vacuo* and the resulting syrups were lyophilized yielding mannopeptin free bases as amorphous powder. The free bases crystallized as minute prisms after keeping the syrups in a refrigerator for several days.

Mannopeptin A has empirical formula $C_{75}H_{107}N_{15}O_{52}$, calculated C 42.59%, H 5.20%, N 10.21 %; found C 42.53%, H 5.39%, N 10.39%. Mannopeptin B has empirical formula $C_{73}H_{107}N_{15}O_{52}$ C 42.59%, H 5.20%, N 10.21%; found 42.78 %, H 5.24 %, N 10.45 %; chlorine, sulfur and iron were not detected. The antibiotics show positive MOLISCH and negative ELSON-MORGAN and ninhydrin tests.

Stability of mannopeptins: Mannopeptins are stable to ultraviolet and visible light. No bioactivity loss was observed on heating them in a boiling water bath at pH 3 or 7 for 60 minutes, but they completely lost bioactivity at pH 10 under the same conditions.

Paper chromatography of mannopeptins: The spots of mannopeptins were detected by the bioautographic technique using *Staphylococcus aureus* FDA 209 P as a test organism. The antibiotics remained at origin when developed with the following solvent systems: water-saturated butanol, 80% aqueous phenol, 50% aqueous methanol, 50 % aqueous acetone, and *n*-butanol-

pyridine-acetic acid-water (15 : 10 : 3 : 12). They gave Rf 0.1~0.25 (tailing) when developed with either water or *n*-butanol-acetic acid-water (4:1:2), and Rf 0.8 with 0.1 N sulfuric acid-dimethylfgrmamide (1:1). The mannopeptin A is 0.67 and that of B is 0.80 with the solvent system of 0.1 N sulfuric acid-acetone (1:1).

Amino acid composition: Two mg of mannopeptin A/B were dissolved in 2 ml of 6 N hydrochloric acid and the mixture was heated in sealed tube at 110°C for 20 hours. Amino acid analyzer (JLC-50AH, No. 7 type) was used for amino acid analysis of the hydrolyzate and each peak was identified from the retention time in the chromatograms.

Gas-chromatographic identification of mannose[7]: Mannopeptin A/B (5 mg) was dissolved in 2.5 N aqueous trifluoroacetic acid and the mixture was heated in a sealed tube at 100°C for 7 hours. To remove basic substances, the hydrolyzate was treated with an Amberlite CG-50 (H^+) column and effluent was again treated with an Amberlite CG-4B column (acetate form). The combined effluents and washing were concentrated to dryness *in vacuo*. The residue was dissolved in 0.5 ml water and 0.5 ml of 1% aqueous $NaBH_4$ was added. After 30 minutes at 30°C, 1 ml of aqueous 10% acetic acid was added to stop the reduction. The reaction mixture was passed through an Amberlite CG-120 (H^+) column and the effluent was concentrated *in vacuo* to dryness. Boric acid was completely removed by repeated addition of methanol. To each residue, 0.1 ml of ethylacetate and trifluoroacetic anhydride was added. The reaction mixture was used directly for gas chromatographic analysis using 2% XF-1105 in a glass column (4mm×2m) at 180°C. The carbohydrate portion was identified as mannose by its retention time. Amino sugars, if present, were adsorbed by an Amberlite CG-120 column, however, no amino sugar was detected in the eluate with 2 N HCl.

Identification of β-methylphenylalanine: Fifty mg of mannopeptin A was hydrolyzed in 5 ml of 6 N hydrochloric acid at 110°C for 20 hours. Separation of amino acids was carried out by ion-exchange chromatography according to MOORE and STEIN[10]. An aromatic amino acid corresponding to phenylalanine in mannopeptin B was isolated in crystalline form (5 mg) and identified as β-methylphenylalanine from its pmr and mass spectrum. The molecular weight of the ethyl ester was confirmed by a M^+ ion at *m/e* 207. The mass spectrum showed peaks at *m/e* 134 ($M-COOC_2H_5$), *m/e* 105 ($M-CH(NH_2)COOC_2H_5$), and *m/e* 91 (C_6H_5-CH). The pmr spectrum in D_2O showed signals at δ 1.5 (d, 3H), 3.4 (s, 1 H), 4.6 (m, 1 H) and 7.4(s, 5 H).

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References

1) WAKSMAN, S.A.: The Actinomycetes. Vol. 2, pp. 258~259. The Williams and Wilkins Company, 1961
2) PHILIP, J.E.; J.R. SCHENK & M.P. HARGIE: Ristocetins A and B, two new antibiotics. Antibiot. Ann. 1956/1957: 699~705, 1957
3) HIGGINS, H.M.; W.H. HARRISON, G.M. WILD, H.R. BUNGAY & M.H. MCCORMICK: Vancomycin, a new antibiotic. Antibiot. Ann. 1957/1958: 906~914, 1958
4) KUNSTMANN, M.P.; L.A. MITSCHER, J.N. PORTER, A.J. SHAY & M.A. DARKEN: LL-AV 290, a new antibiotic. I. Fermentation, isolation and characterization. Antimicr. Agents & Chemoth. 1968: 243~245, 1969
5) WAISVISZ, J.M.; M.G. VANDERHOEVEN & B.T. NIJENHUIS: The structure of sulfur-containing moiety of bottromycin. J. Am. Chem. Soc. 79: 4524~4527, 1957
6) MARSHALL, F.J.: Structure studies on vancomycin. J. Med. Chem. 8: 18~22, 1965
7) IMANARI, T.; Y. ARAKAWA & Z. TAMURA: Gas chromatographic analysis of sugar. Proc. Symp. on Chem. Physiol. & Pathol. Vol. 7: 38~41, 1968
8) BRAZHNIKOVA, M.G.; N.N. LOMAKINA, M.F. LAVROVA, I.V. TOLSTYKH, M.S. YURINA & L.M. KLYUEVA: Isolation and properties of ristocetin. Antibiotiki 8: 392~396, 1963
9) HARADA, Y.; T. NEHASHI, T. HAYASHI & K. ANDO: Antimicrobial property of mannopeptins J. Antibiotics 28: 508~513, 1975