LEUCYLNEGAMYCIN, AN ANTIBIOTIC FROM NEGAMYCIN-PRODUCING *STREPTOMYCES*

Sir:

Negamycin was isolated from the culture filtrate of three strains related to *Streptomyces purpeofuscus*,[1] and was shown to be [2-{(3R, 5R)-3, 6-diamino-5-hydroxyhexanoyl}-1-methylhydrazino] acetic acid.[2] In the early phase of growth of a negamycin-producing strain, *Streptomyces* No. M890-C2, the production of another new antibiotic was confirmed by high-voltage paper electrophoresis. This new antibiotic, designated leucylnegamycin, was isolated by resin chromatography and converted to negamycin by leucine aminopeptidase or by washed cells of *Streptomyces* No. M890-C2. Therefore, it is suggested that the antibiotic is an intermediate in the biosynthesis of negamycin. This paper is concerned with the isolation, characterization and structure determination of leucylnegamycin.

By reciprocal shaking culture of strain No. M890-C2 in a medium containing 2.5 % glucose, 2.0 % starch, 2.0 % soybean meal, 0.5 % dry yeast, 0.25 % NaCl, 0.32 % $CaCO_3$, 0.005 % $ZnSO_4 \cdot 7H_2O$, 0.0005 % $CuSO_4 \cdot 5H_2O$ and 0.0005 % $MnCl_2 \cdot 4H_2O$, leucylnegamycin was accumulated for 4~5 days and then gradually disappeared, while negamycin was produced during 5~9 days. The 94-hour cultured broth was filtered and the negamycins in the filtrate (4.6 liters) were adsorbed on a column of Amberlite IRC50 (70 % NH_4^+ form, 230 ml) and eluted with 2 % ammonium hydroxide. The concentrated active eluate was passed through a column of Amberlite CG50 (Type I, NH_4^+ form, 125 ml) and the column was developed with water. Lyophilization of the active eluate yielded 49 mg of pure leucylnegamycin. Then, the column was eluted with 0.1 % ammonium hydroxide and 146 mg of pure negamycin was obtained.

Leucylnegamycin is a colorless powder, m.p. 127~133°C (decomp.), $[\alpha]_D^{26}$ +4.8° (*c* 2, H_2O).

Anal. Calcd. for $C_{15}H_{31}N_5O_5 \cdot {}^1/_2H_2O$:

C 48.63, H 8.71, N 18.91, O 23.76.

Found:

C 48.80, H 8.42, N 18.69, O 23.54.

The molecular formula was derived from the structure studies and mass spectrum of di-N-acetylleucylnegamycin methyl ester (*m/e* 459) which was prepared with acetic anhydride and methanol. Leucylnegamycin shows no ultraviolet absorption except end absorption and the IR spectrum is shown in Fig. 1. The NMR spectrum in D_2O (tetramethylsilane as external standard) (Fig. 2) shows signals corresponding to isopropyl group (two sets of doublets at δ 1.4), 3 protons around δ 2.0 and a proton near δ 4.2, in addition to signals corresponding to the negamycin molecule. Furthermore,

Fig. 1. The IR spectrum of leucylnegamycin (KBr).

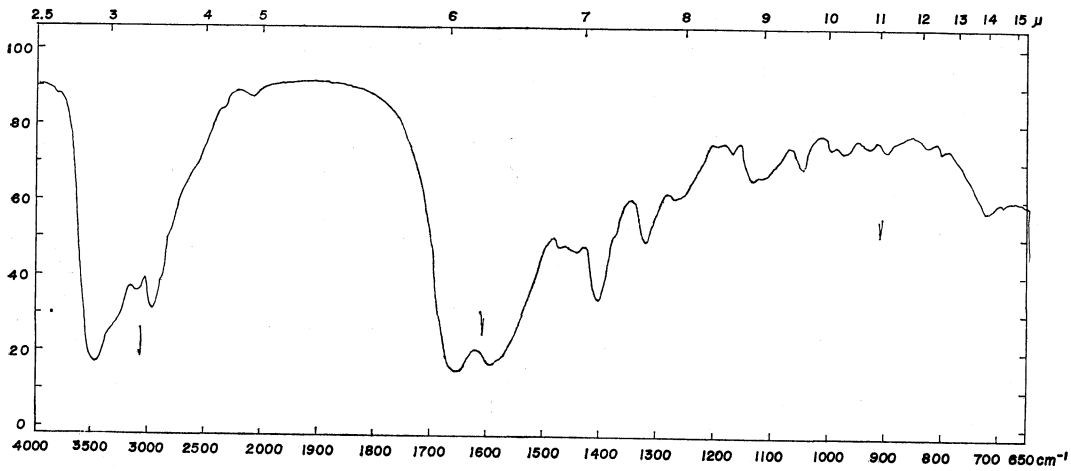
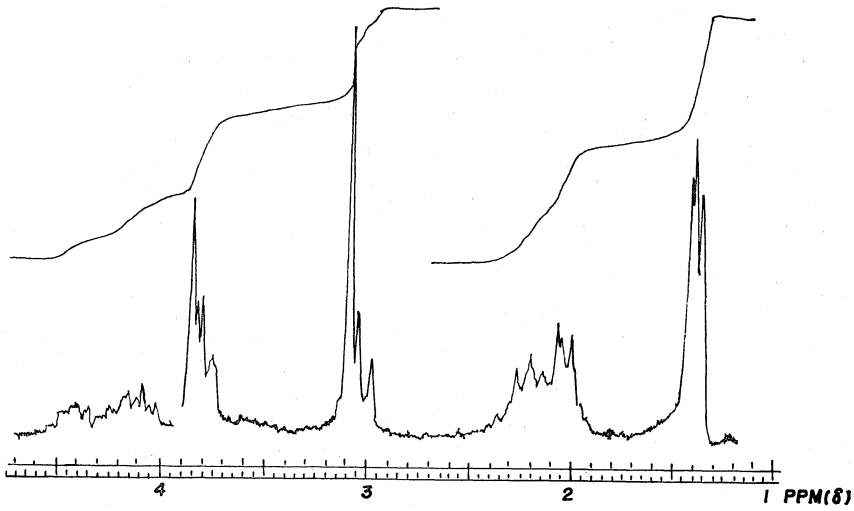


Fig. 2. The NMR spectrum of leucylnegamycin in D_2O (100 MHz, tetramethylsilane as external standard).



signals corresponding to ε-methylene protons of δ-hydroxy-β-lysine ((3R, 5R)-3, 6-diamino-5-hydroxyhexanoic acid)[2] are shifted to a lower-field (δ 3.8) than those of negamycin. Leucylnegamycin gives positive ninhydrin, red tetrazolium and RYDON-SMITH reactions. Under high-voltage paper electrophoresis, 3,500 V for 20 minutes in formic acid - acetic acid - water (25 : 75 : 900 in volume), it moves 14.3 cm to cathode with Rm (relative mobility against alanine) 1.24.

The antimicrobial spectrum of leucylnegamycin by the agar dilution method is shown in Table 1 and is weaker than that of negamycin. Mice tolerated intravenous injection of up to 500 mg/kg.

By high-voltage paper electrophoresis, it was shown that mild hydrolysis of leucylnegamycin by refluxing with 0.5 N HCl for 3 hours yielded 6~7 ninhydrin-positive products: 1-methylhydrazinoacetic acid (Rm 0.78), leucine (Rm 0.83), an unknown product (Rm 1.36), trace of negamycin (Rm 1.53), δ-hydroxy-β-lysine (Rm 1.80), 1,2-dimethylhydrazine (Rm 1.80), and a trace of δ-hydroxy-β-lysine lactone (Rm 2.21). These hydrolysis products except leucine and the unknown product were identical with the hydrolysis products of negamycin.[2] The unknown product was separated from the hydrolysate (0.5 N HCl, 100°C, 2 hours) by the column chromatography of Amberlite CG50 (Type I, NH_4^+ form) as a colorless powder, m.p. 82~90°C.

Table 1. The antimicrobial spectrum of leucylnegamycin

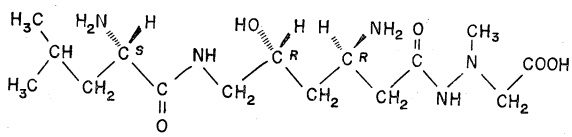
Organisms	Minimum inhibitory concentration (mcg/ml)	
	Nutrient agar	Peptone agar
Staphylococcus aureus FDA 209 P	>100	>100
Staphylococcus aureus Smith	100	25
Micrococcus flavus 16	>100	>100
Sarcina lutea PCI 1001	>100	100
Bacillus subtilis NRRL B-558	>100	>100
Escherichia coli NIHJ	25	6.25
Escherichia coli K-12	50	6.25
Escherichia coli K-12 ML 1629	50	6.25
Shigella sonnei 191-66	50	12.5
Salmonella typhosa	25	1.56
Serratia marcescens	100	25
Klebsiella pneumoniae PCI 602	50	25
Proteus vulgaris OX 19	25	1.56
Proteus rettgeri GN 311	25	12.5
Proteus rettgeri GN 466	50	6.25
Pseudomonas fluorescens	12.5	1.56
Pseudomonas aeruginosa A3	100	50
Pseudomonas aeruginosa No. 12	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100
Candida albicans 3147	>100	>100

Anal. Calcd. for $C_{12}H_{25}N_3O_4 \cdot H_2O$:
C 49.13, H 9.28, N 14.33.
Found: C 48.60, H 8.90, N 14.07.

It gives positive ninhydrin and RYDON-SMITH

reactions, and negative red tetrazolium reaction. Complete acid hydrolysis of the product by refluxing with 6 N HCl for 3 hours yielded leucine and δ-hydroxy-β-lysine. It does not consume periodate during 24 hours by the method of RAMMLER and RABINOWITZ.[3] The NMR spectrum in D_2O is very similar to that of leucylnegamycin except for signals corresponding to the N-CH_3 (δ 3.10) and N-CH_2CO (δ 3.85). The IR spectrum in KBr shows amide bands (1650 and 1585 cm^{-1}) and no absorption at ester carbonyl resion. Therefore, the structures of the dipeptide and the antibiotic are N^{ε}-leucyl-δ-hydroxy-β-lysine and leucylnegamycin, respectively. These structures were also confirmed by high-resolution mass spectroscopy of di-N-acetylleucylnegamycin methyl ester, which showed peaks corresponding to 1-methylhydrazinoacetic acid fragment (calcd. for $C_4H_{10}N_2O_2$, 118.074; found *m/e* 118.070±0.005) and leucyl-δ-hydroxy-β-lysine fragment (calcd. for $C_{16}H_{28}N_3O_5$, 342.203; found, *m/e* 342.208±0.005).

The determination of the configuration of leucine has been approached enzymatically.[4] An acid hydrolysate of the dipeptide was subjected to the action of hog kidney D-amino acid oxidase (Worthington Biochemical Corp.) and snake venom L-amino acid oxidase (Tokyo Kasei Kogyo Co., Ltd.). Thin-layer chromatography using Silica Gel G (BuOH - AcOH - H_2O, 4 : 1 : 2 in volume) showed that leucine was absent in the sample treated with L-amino acid oxidase for 30 minutes at 37°C, but present both in samples treated with D-amino acid oxidase and untreated. Consequently the structure of leucylnegamycin is [2-{3(R)-amino-5(R)-hydroxy-6-L-leucylaminohexanoyl}-1-methylhydrazino] acetic acid.



Leucylnegamycin was easily hydrolysed into leucine and negamycin by the action of hog kidney leucine aminopeptidase (Miles Laboratories, Inc., 37°C, 2 hours)[5] or the washed cells of *Streptomyces* No. M890-C2 (27°C, 21 hours). These reaction products were detected by high-voltage paper electrophoresis with ninhydrin and bioautography (*E. coli*).

The strain No. M890-C2 was cultured on a reciprocal shaker by the above described method. After 72 hours, L-leucine-(U)-^{14}C was added to the culture and the fermentation was continued. From the 90-hour cultured broth, negamycin and leucylnegamycin were extracted by a resin column. The crude powder containing negamycin and leucylnegamycin was examined by high-voltage paper electrophoresis with ninhydrin reaction and radioassay. The result showed that L-leucine-(U)-^{14}C was incorporated only into leucylnegamycin.

From the foregoing results, it was confirmed that leucylnegamycin was a direct metabolic intermediate in the biosynthesis of negamycin, similar to the case of leucylblasticidin S.[6]

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