β-METHYLNORLEUCINE, AN ANTIMETABOLITE PRODUCED BY *SERRATIA MARCESCENS*

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An amino acid was formed by α-aminobutyrate-resistant mutants of *Serratia marcescens* grown in a medium containing norvaline. This amino acid was identified as *erythro*-β-methyl-L-norleucine [(2*S*,3*S*)-2-amino-3-methylhexanoic acid] by instrumental analyses. β-Methylnorleucine inhibited the growth of several bacteria in synthetic medium.

In *Serratia marcescens*, certain amino acids, *i.e.*, norvaline, norleucine, and homoisoleucine have been demonstrated to be formed by the leucine biosynthetic enzymes; moreover, the formation of these unnatural amino acids is regulated by leucine[1,2]. Further, it was shown that these amino acids could be accumulated in the medium when regulatory mutants of *Serratia marcescens* were used[2,3,4]. In the studies relating to the accumulation of norleucine from norvaline by α-aminobutyrate-resistant mutants, an unidentified amino acid was also found to be present in the medium. This paper deals with the isolation, chemical properties, and identification of this compound. Some of its antimicrobial activities are also presented.

Materials and Methods

Bacteria

The microorganisms used in this study were *Serratia marcescens* strain Ar130-1[5], and the other bacteria described in Fig. 4.

Medium and Culture Conditions

The fermentation medium contained 2% glucose, 10% dextrin (Matsutani Chemicals, #3), 1% urea, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.7% corn steep liquor, 1% DL-norvaline and 3% $CaCO_3$, pH 7.0. Glucose and dextrin were autoclaved separately and added to the remaining components of the medium. A loopful of cells that had been grown overnight on a nutrient agar slant was inoculated into 30 ml of the medium in a 500 ml flask. Incubation was carried out at 30°C for 72 hours on a reciprocal shaking machine (140 rpm, 7 cm stroke). Growth inhibition by β-methylnorleucine was determined with a Hitachi automated recording incubator system[6] as follows; organisms were grown in test tubes containing 3 ml of DAVIS and MINGIOLI minimal medium[7] supplemented with glucose 0.5%, biotin 20 μg/liter, thiamine·HCl 300 μg/liter but no citrate. Cultures in late log or early stationary phase were diluted with fresh minimal medium and inoculated at a cell concentration of about 10^6 cells/ml. Incubation was carried out at 30°C with shaking (140 rpm, 4 cm stroke). The growth was measured turbidimetrically at 660 nm.

Paper Chromatography

Paperchromatograms were run using ascending techniques. Solvent systems used were (I) *n*-butanol - acetic acid - water (4: 1: 1) and (II) *tert*-amylalcohol - acetic acid - water (10: 1: 10, upper phase). β-Methylnorleucine was determined quantitatively by ascending chromatography as follows: Samples were applied to Toyo No. 53 paper which was developed with solvent system II for 16 hours. After color development with ninhydrin reagent (0.2% ninhydrin solution in 80% ethanol), the indivi-

dual spots corresponding to the amino acid were cut out and each were extracted with 3 ml of 75% methylcellosolve containing 0.005% $CuSO_4 \cdot 4H_2O$. Extracts were determined spectrophotometrically at 530 nm and the concentration of the amino acid was calculated from a standard curve prepared previously.

Analytical Methods

Melting points were measured with a Yamato melting point apparatus, model MP-21. Infrared (IR) spectra were determined using the Nujol technique with a Shimadzu model IR-27G spectrophotometer. Nuclear magnetic resonance (NMR) was determined in NaOD with a Hitachi model R-20A instrument, and optical rotation was measured with a Perkin-Elmer 141 polarimeter. Mass spectra were determined with a Hitachi RMU-6M spectrometer.

Results

Detection of Compound X_4

An unidentified amino acid (compound X_4) was formed by strain Ar130-1 in the fermentation medium containing 1% DL-norvaline. Rf values of compound X_4 in the two solvent systems employed were higher than those of norleucine and were almost the same as those of homoisoleucine (2-amino-4-methylhexanoic acid) (Table 1).

Isolation of Compound X_4

Strain Ar130-1 was cultured for 72 hours on a medium containing 1% DL-norvaline. The fermentation broth (900 ml) was adjusted to pH 2.0 with H_2SO_4 and heated at 100°C for 10 minutes. The cells were removed by filtration using filter aid, Dikalite (Dikalite Orient Co., Tokyo). The filtrate was passed through a column (3×70 cm) of 400 ml of Amberlite IR-120B (H^+ form, Organo, Tokyo). The fractions containing amino acids were eluted with 5% ammonium hydroxide and were concentrated under reduced pressure, yielding 12.2 g of crude amino acid mixture. This mixture was dissolved in 150 ml of 0.2 M sodium citrate buffer (pH 2.2) and adsorbed onto a column (3.6×150 cm) of 1.5 liters of Amberlite CG-120 (Na^+ form) equilibrated with 0.2 M sodium citrate buffer (pH 4.5). Then, the column was eluted with the same sodium citrate buffer (pH 4.5) at a flow rate of 480 ml/hour at 50°C, fractions (28 ml) were collected. The location of amino acids in the eluate fractions was determined by paper chromatography. Fractions No. 105~125 containing only compound X_4 were combined and adsorbed onto a column (3×40 cm) of 200 ml of Amberlite IR-120B (H^+ form). After the column was washed with water, compound X_4 was eluted with 5% ammonium hydroxide. The eluate was concentrated under reduced pressure, decolorized with active charcoal, and recrystallized twice from aqueous ethanol. Compound X_4 was obtained as colorless crystals in a yield of 250 mg.

Table 1. Rf value of compound X_4.

Amino acid	Rf value	
	I	II
Compound X_4	0.78	0.61
Homoisoleucine	0.80	0.63
Norleucine	0.72	0.53
Leucine	0.70	0.47
Isoleucine	0.67	0.39
Norvaline	0.60	0.30
Valine	0.55	0.22

Solvent I; *n*-Butanol - acetic acid - water, 4:1:1 (v/v)
Solvent II; *tert*-Amylalcohol - acetic acid - water, 10:1:10 (v/v, upper phase)

Identification of Compound X_4

The melting point of compound X_4 was 268°C (dec.). Elemental analysis of compound X_4 gave the following results. *Anal.* Found: C, 57.95%; H, 10.31%; N, 9.79%, Calcd. for $C_7H_{15}O_2N$; C, 57.90%; H, 10.41%; N, 9.65%.

Fig. 1. IR spectrum of compound X_4.

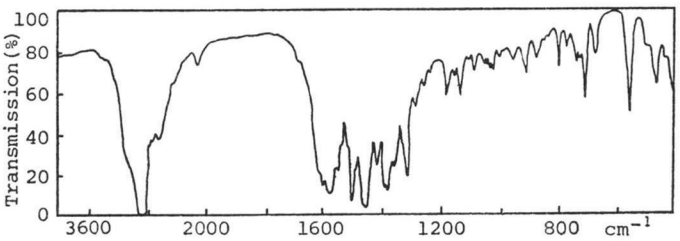


Fig. 2. Mass spectrum of compound X_4.

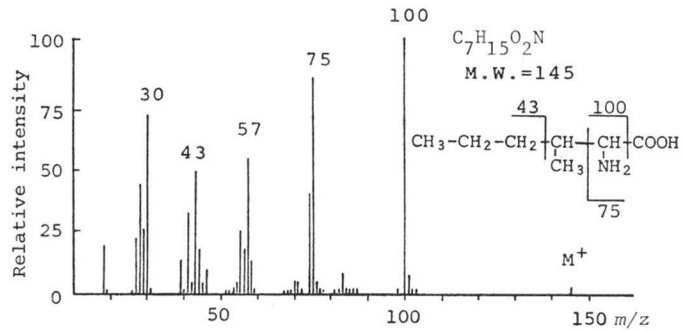
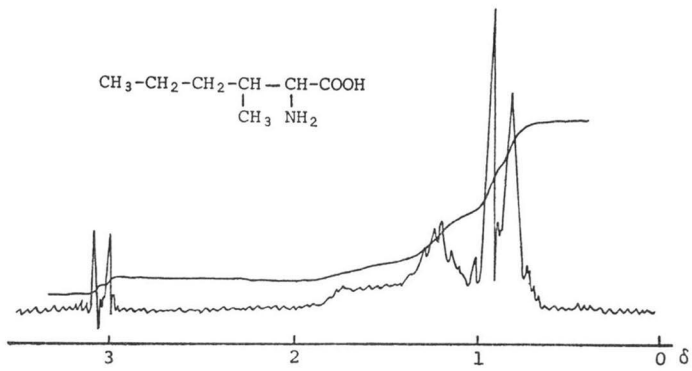


Fig. 3. NMR spectrum of compound X_4.



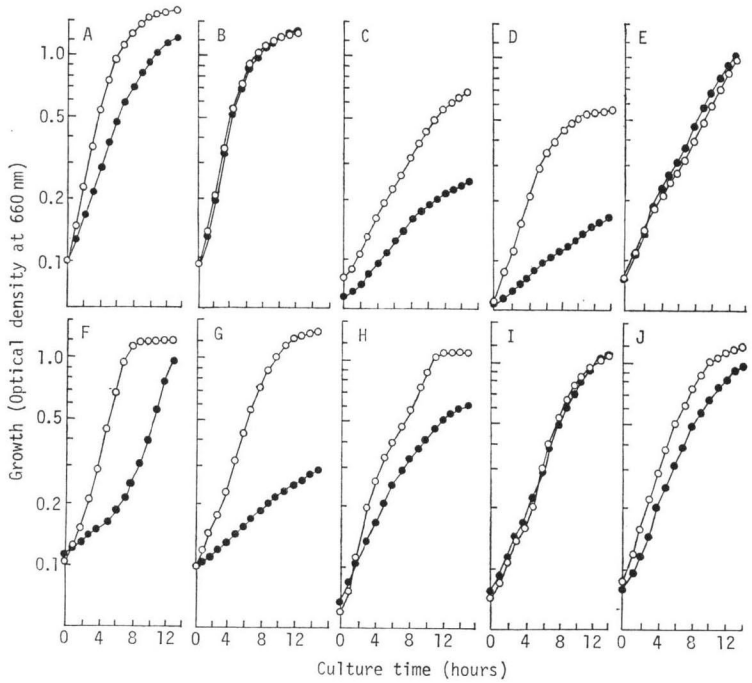
These results suggest that compound X_4 is a saturated amino acid containing seven carbons. The IR spectrum of compound X_4 showed absorption bands at 2100, 1610, 1580, 1510, 1460 and 1415 cm^{-1}, typical bands for a zwitterionic amino acid (Fig. 1).

The mass spectrum of compound X_4 showed significant peaks at m/z 145 (M^+), 100 (M^+-45), 75 (M^+-70), 57 ($M^+-45-43$), 43 (M^+-102) (Fig. 2). The peaks at m/z 100 and 75 indicate that compound X_4 is an α-amino acid. The peak at m/z 43 indicates that the propyl group may be branched in the structure of compound X_4.

The NMR spectrum of compound X_4 showed absorption peaks at δ 3.01, 1.3~1.7, and 0.9 due to one, five, and six protons, respectively (Fig. 3). The doublet peak at δ 3.01 provides evidence that the proton of the β-carbon is methine.

Fig. 4. Antimicrobial activity of β-methylnorleucine.

Growth was determined in the absence (○) or presence (●) of 10^{-3} M β-methylnorleucine. Test organisms: (A) *Achromobacter butyri* OUT 8004; (B) *Aerobacter aerogenes* OUT 8017; (C) *Arthrobacter ureafaciens* IAM 1658; (D) *Bacillus subtilis* OUT 8109; (E) *Brevibacterium helvolum* IAM 1645; (F) *Escherichia coli* B ATCC 11303; (G) *Escherichia coli* K-12 ATCC 14948; (H) *Pseudomonas aeruginosa* OUT 8252; (I) *Pseudomonas fluorescens* IFO 3081; (J) *Serratia marcescens* OUT 8259.



The optical rotation was $[\alpha]_D^{25}$ +29.0° (*c* 1.5, 6 N HCl). This value indicates that compound X_4 is of the L-*erythro*-configuration[9,10]. On the basis of these experimental data, the structure of compound X_4 was determined to be *erythro*-β-methyl-L-norleucine [(2*S*,3*S*)-2-amino-3-methylhexanoic acid].

Antimicrobial Activity of β-Methylnorleucine

Since β-methylnorleucine is an analog of the natural amino acid, its effect on the growth of some bacteria was examined. First, the antimicrobial activity of β-methylnorleucine was measured in nutrient medium by the paper-disk agar-diffusion technique. Under these conditions, β-methylnorleucine did not exhibit an inhibitory effect (data not shown). The antimicrobial activity of the compound was then examined in a minimal medium (Fig. 4). β-Methylnorleucine showed a marked inhibitory effect upon *Bacillus subtilis* (D) and *Escherichia coli* K-12 (G). The growth of *Achromobacter butyri* (A), *Arthrobacter ureafaciens* (C), *Escherichia coli* B (F) and *Pseudomonas aeruginosa* (H) were slightly inhibited by β-methylnorleucine. By contrast, *Aerobacter aerogenes* (B), *Brevibacterium helvolum* (E), *Pseudomonas fluorescens* (I) and *Serratia marcescens* (J) were not affected by β-methylnorleucine.

Discussion

An amino acid (compound X_4) produced by *Serratia marcescens* from norvaline was isolated by ion-exchange chromatography as a colorless crystalline material having the molecular formula of $C_7H_{15}O_2N$. IR spectrum showed absorption bands typical for a zwitterionic amino acid structure. Mass spectrum

exhibited absorption peaks characteristic of an α-amino acid branched with a propyl group. NMR spectrum indicated that the β-carbon of compound X_4 was methine. From these results, we determined that compound X_4 was β-methylnorleucine. Since β-methylnorleucine possesses two asymmetric carbon atoms, there are four stereoisomers. The specific optical rotation of the β-methylnorleucine was $[\alpha]_D^{25}+29.0°$ (*c* 1.5, 6 N HCl). OKUBO and IZUMI have synthesized DL-β-methylnorleucine and resolved the racemate into four stereoisomers[8,9]. The specific optical rotations (*c* 1.5, 6 N HCl) of the four isomers were L-*erythro*, +30.4°; L-*threo*, +46.7°; D-*threo*, −32.2°; D-*erythro*, −45.3°. Therefore, our β-methylnorleucine is probably of the L-*erythro*-configuration. This is supported by the observations that β-methylnorleucine appears to be synthesized by the isoleucine biosynthetic enzymes and that isoleucine produced by *Serratia marcescens* is all of the L-*erythro*-configuration.

β-Methylnorleucine has not been shown previously to be a naturally occurring amino acid, although it has been obtained as a synthetic product[8,10,11]. This is the first report concerning the isolation of β-methylnorleucine from natural sources. β-Methylnorleucine inhibited the growth of *Bacillus subtilis* and *Escherichia coli* K-12 in a synthetic medium. The antagonism of β-methylnorleucine activity by L-isoleucine will be presented in a separated report.

β-Methylleucine (2-amino-3,4-dimethylpentanoic acid) was known as a precursor of *N*,β-dimethylleucine, an important component of etamycin produced by *Streptomyces* sp.[12]. The biosynthetic pathways of unnatural β-methylamino acids are of interest in relation to the biosynthesis of antibiotics. In a previous report[2], we found that homoisoleucine (2-amino-4-methylhexanoic acid) was synthesized from α-keto-β-methylvalerate by the leucine biosynthetic enzymes. On the other hand, β-methylnorleucine was shown to be synthesized by the isoleucine-valine biosynthetic enzymes. These results will be presented in the subsequent report.

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