BIOSYNTHETIC PATHWAY OF β-METHYLNORLEUCINE, AN ANTIMETABOLITE PRODUCED BY *SERRATIA MARCESCENS*

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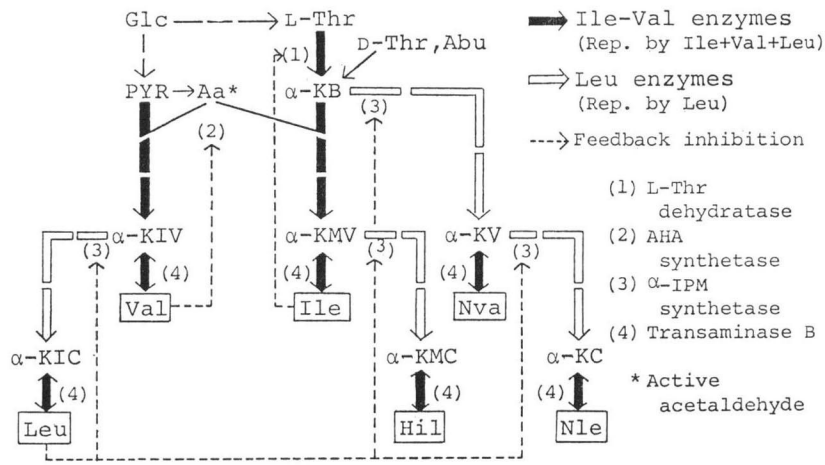
β-Methylnorleucine biosynthesis was examined in *Serratia marcescens* using regulatory mutants of branched-chain amino acid biosynthesis. The accumulation of β-methylnorleucine from norvaline in the wild-type strain was inhibited by the simultaneous additions of isoleucine, valine and leucine, although its accumulation in the derepressed mutant of isoleucine, valine and leucine biosynthesis was markedly increased and was not inhibited by additions of these amino acid. Accumulation of this compound was not observed in an isoleucine-valine auxotroph, although its accumulation was not affected in an isoleucine or leucine auxotroph. Transaminase B catalyzed the conversion of α-keto-β-methylcaproate to β-methylnorleucine. These results suggest that β-methylnorleucine is formed from α-ketovalerate, α-ketoacid corresponding to norvaline, by enzymes of the isoleucine-valine biosynthetic pathway.

We reported that an unidentified amino acid was accumulated by a regulatory mutant of *Serratia marcescens* in a medium containing norvaline[1]. This amino acid was isolated from fermentation broth and was identified as *erythro*-β-methyl-L-norleucine [(2*S*,3*S*)-2-amino-3-methylhexanoic acid]. β-Methylnorleucine had not been described previously as a natural product, although it had been synthesized chemically[2,3]. β-Methylnorleucine inhibited the growth of several bacteria in a chemically defined medium.

We have isolated some other unusual amino acids, *i.e*, norvaline, norleucine and homoisoleucine, from the fermentation broth and shown that these amino acids were synthesized from α-ketobutyrate, α-ketovalerate and α-keto-β-methylvalerate, respectively, by the leucine biosynthetic enzymes due to their rather broad substrate specificities[4,5]. The biosynthetic pathway of β-methylnorleucine, a branched-chain amino acid, was also presumed to be closely linked to the biosynthesis of the natural branched-chain amino acid, *i.e*, isoleucine, valine and leucine.

The regulatory mechanisms of isoleucine, valine and leucine biosynthesis in *Serratia marcescens* have been clarified as follows. The isoleucine-valine biosynthetic enzymes, *i.e*, L-threonine dehydratase, α-acetohydroxyacid synthetase and transaminase B, are multivalently repressed by isoleucine, valine and leucine[8]. The last two enzymes are common to the biosynthesis of isoleucine and valine, and transaminase B also functions in the biosynthesis of leucine. L-Threonine dehydratase and α-acetohydroxyacid synthetase are feedback-inhibited by isoleucine and valine, respectively. The leucine biosynthetic enzymes are repressed by leucine, and α-isopropylmalate synthetase is feedback-inhibited by leucine[6]. These mechanisms are summarized in Fig. 1. On the bases of these regulatory mechanisms of branched-chain amino acid biosynthesis, investigations relating to the biosynthetic pathway of β-methylnorleucine are presented in this paper.

Fig. 1. Regulatory mechanisms of biosynthesis of branched-chain amino acids and their analogs in *Serratia marcescens*.



Materials and Methods

Bacteria

Serratia marcescens No. 1[7] and its mutants, listed in Table 1, were used. Strain Ar130-1 was a derepressed mutant of the isoleucine, valine and leucine biosynthetic enzymes[6,8]. In strain S-11, α-isopropylmalate synthetase was insensitive to leucine[6]. Other auxotrophs were derived from strain Ar130-1 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment according to the procedure of ADELBERG *et al.*[9].

Table 1. *Serratia marcescens* mutants.

Strain	Parent	Phenotypes	References
1		wild	7
Ar130-1	1	abu-r[a]	6, 8, 13
149	Ar130-1	abu-r, Ile⁻	6
179	Ar130-1	abu-r, Leu⁻	6
180	Ar130-1	abu-r, Ile·Val⁻	
183	Ar130-1	abu-r, Ile·Val⁻	
S-11	149	abu-r, Ile±	5, 6

[a] α-Aminobutyrate-resistant.

Media and Culture Conditions

The fermentation medium and conditions employed were the same as described in the previous paper[1]. Isoleucine, valine, leucine and methionine were added at levels of 0.5~2 mg per ml, when necessary.

Chemicals

β-Methylnorleucine was isolated from the fermentation broth as described previously[1]. Norvaline was purchased from Sigma Chemical Co. All amino acids used were of L-configuration otherwise noted. Other chemicals were reagent grade.

Assay of Transaminase B Activity

A strain of *Serratia marcescens* was grown in a 500 ml flask containing 150 ml of DAVIS-MINGIOLI[10] minimal medium (with glucose 0.5% and without sodium citrate) at 30°C with shaking. The cells were harvested in the exponential phase of growth and washed twice with 0.05 M potassium phosphate buffer (pH 8.0). The cells were suspended in the same buffer containing 30% glycerol and were disrupted by sonic treatment for 5 minutes below 5°C. After centrifugation at 27,000×*g* for 30 minutes at 5°C the supernatant fluid was decanted and used for enzyme assays. Protein was determined by the method of LOWRY *et al.*[11]. Transaminase B activity was assayed as follows: The reaction mixture contained 100 μmole potassium phosphate buffer (pH 8.0), 20 μmole α-ketoglutarate, 20 μmole amino acid, 0.4 μmole pyridoxal phosphate and enzyme (0.3~1.0 mg of protein) in a total volume of 1.0 ml. The mixture was preincubated for 5 minutes at 30°C, and then the reaction was initiated by addition of enzyme. After 15 minutes, the reaction was terminated by boiling for 5 minutes at 100°C. The amount of L-

glutamic acid formed was determined microbiologically, using *Leuconostoc mesenteroides* P-60[12]. One unit of enzyme activity catalyses the formation of 1 μmole of glutamic acid per minute under these conditions.

Other Analytical Methods

β-Methylnorleucine and norvaline were determined by paper chromatography as described previously[1]. Valine was determined microbiologically using *Leuconostoc mesenteroides* P-60[12]. Growth was estimated by measuring the optical density at 660 nm of fermentation broth diluted with 0.1 N HCl and expressed as dry cell weight calculated from a standard curve that an optical density of 0.10 corresponded to 160 μg of dry cells per ml.

Result

Accumulation of β-Methylnorleucine by an α-Aminobutyrate-resistant Mutant

Accumulation of α-methylnorleucine was examined using strains No. 1 and Ar130-1 (Table 2). In wild-type strain No. 1, addition of norvaline to the medium resulted in an inhibition of growth. However, the addition of leucine concomitantly overcame this inhibition and resulted in β-methylnorleucine accumulation. On the other hand, an α-aminobutyrate-resistant mutant, strain Ar130-1, was not subjected to any significant growth inhibition by norvaline, and accumulated at a 4.4 fold greater amount of β-methylnorleucine. As reported previously[6,8], the α-aminobutyrate-resistant mutant was derepressed in the isoleucine, valine and leucine biosynthetic enzymes. Therefore, biosynthesis of β-methylnorleucine appeared to be correlated with either the biosynthesis of isoleucine-valine or leucine.

Table 2. β-Methylnorleucine formation by wild strain and α-aminobutyrate-resistant mutant.

Strain	Addition of medium (mg/ml)		Growth (mg/ml)	Amino acid formed (mg/ml)		
	Nva	Leu		Mnl	Val	Nva[a]
1 (wild)	0	0	14.9	0	0.5	0
	10	0	5.2	0	0	8
	10	0.5	18.4	0.5	0.8	7
Ar130-1	0	0	16.4	0	6.2	0
	10	0	14.3	2.2	4.5	6

[a] Residual norvaline.
Abbreviation: Nva, norvaline; Leu, leucine; Mnl, β-methyl-norleucine; Val, valine.
Incubation was carried out for 72 hours.

Accumulation of β-Methylnorleucine in Regulatory Mutants of Leucine Biosynthesis

In strain Ar130-1, α-isopropylmalate synthetase is feedback-inhibited by leucine. On the other hand, in strain S-11, α-isopropylmalate synthetase is released from leucine inhibition. The leucine biosynthetic enzymes were derepressed in both strains[6]. To examine whether the leucine biosynthetic enzymes participate in β-methylnorleucine synthesis or not, the accumulation of the latter compound was examined in these strains (Table 3). Accumulation of β-methylnorleucine was not inhibited by leucine in strain Ar130-1. In strain S-11, addition of norvaline resulted in an markded inhibition of growth. Since strain S-11 was released from the feedback inhibition of α-isopropylmalate synthetase, it accumulated leucine in the medium. Therefore this inhibition of growth was not responsible to norvaline, an antagonist of leucine[13]. As reported previously[5], norleucine was formed from α-ketovalerate, α-ketoacid of norvaline, by the leucine biosynthetic enzymes owing to those broad substrate specificities. Because norleucine was a strong antagonist of methionine[14], the growth inhibition of strain S-11 was due to the small amount of norleucine accumulated. Addition of methionine reversed the growth inhibition and accumulation of norleucine increased. However, the accumulation of β-methylnorleucine in strain S-11 did not increase in comparison to that observed in the study with strain Ar130-1. These

Table 3. Participation of the leucine biosynthesis in β-methylnorleucine formation.

Strain	Addition to medium (mg/ml)			Growth (mg/ml)	Amino acid formed (mg/ml)				
	Nva	Leu	Met		Mnl	Val	Leu	Nle	Nva[a]
Ar130-1	10	0	0	16.4	2.2	4.5	0	0	6
	10	2	0	14.4	2.1	4.5	0	0	6
	10	2	0.5	16.4	2.2	4.3	0	0	6
S-11	0	0	0	14.0	0	0	13.8	0	0
	10	0	0	3.3	0	0	0.5	0.2	8
	10	0	0.5	17.6	2.2	0	4.5	1.5	5
	10	2	0.5	18.4	2.3	0	6.3	1.8	5

[a] Residual norvaline.
Abbreviations: Nle, norleucine; Met, methionine; Others were the same as described in Table 2.

Table 4. Inhibition of β-methylnorleucine formation by isoleucine, valine and leucine.

Strain	Addition to medium (mg/ml)					Growth (mg/ml)	Amino acid formed (mg/ml)				
	Nva	Ile	Val	Leu	Met		Mnl	Val	Leu	Nle	Nva[a]
1	10	0	0	0.5	0	18.4	0.5	0.8	0	0	7
	10	1	1	1	0	20.3	0	0.2	0	0	7
Ar130-1	10	0	0	0	0	14.3	2.2	4.5	0	0	6
	10	0	0	0.5	0	14.6	2.2	4.4	0	0	6
	10	1	1	1	0	14.3	2.0	4.3	0	0	6
S-11	10	0	0	0	0.5	17.6	2.2	0	4.5	1.5	5
	10	0	0	0.5	0.5	17.6	2.1	0	4.8	1.5	5
	10	1	1	1	0.5	17.2	2.0	0	4.8	1.2	5

[a] Residual norvaline.
Abbreviations: Ile, isoleucine; Others were the same as described in Table 3.

results indicate that β-methylnorleucine is not formed by the leucine biosynthetic enzymes.

Participation of the Isoleucine-Valine Biosynthetic Enzymes in β-Methylnorleucine Synthesis

Isoleucine-valine biosynthetic enzymes are multivalently repressed by isoleucine, valine and leucine[8]. To examine whether β-methylnorleucine accumulation was repressed or not, these amino acids were added to the medium (Table 4). In wild-type strain No. 1, accumulation of β-methylnorleucine was inhibited by the simultaneous additions of isoleucine, valine and leucine. On the other hand, inhibition of β-methylnorleucine synthesis was not observed in strains Ar130-1 and S-11. This result supports the view that β-methylnorleucine is formed by the isoleucine-valine biosynthetic enzymes.

Accumulation of β-Methylnorleucine by Branched-chain Amino Acid Auxotrophs

To provide additional evidence for the biosynthetic pathway of β-methylnorleucine, its accumulation was investigated in the branched-chain amino acid auxotrophs derived from strain Ar130-1 (Table 5). The leucine auxotroph, strain 179, the isoleucine auxotroph (L-threonine dehydratase deficient),

Table 5. Effects of branched-chain amino acid auxotrophies on β-methylnorleucine formation.

Strain (auxotrophy)	Growth (mg/ml)	Amino acid formed (mg/ml)		
		Mnl	Val	Nva[a)]
Ar130-1 (parent)	14.3	2.2	4.5	6
149 (Ile⁻)	16.4	2.5	2.9	6
179 (Leu⁻)	16.8	2.2	2.2	6
180 (Ile·Val⁻)	16.0	0	0	8
183 (Ile·Val⁻)	17.2	0	0	8

Isoleucine, valine or leucine was added at 0.5 mg/ml to the medium containing 10 mg/ml of norvaline.
a) Residual norvaline.
Abbreviations were the same as described in Table 4.

Table 6. Transamination between α-ketoglutarate and branched-chain amino acids catalyzed by extracts of wild strain and α-aminobutyrate-resistant mutant.

Amino acid	Enzyme activity (units/mg of protein)	
	1 (wild)	Ar130-1
β-Methylnorleucine	0.01	0.06
Isoleucine	0.04	0.17
Valine	0.05	0.24
Leucine	0.09	0.20
Norvaline	0.06	0.28
Norleucine	0.03	0.10

strain 149, as well as strain Ar130-1 accumulated β-methylnorleucine. On the other hand, the isoleucine-valine auxotrophs, strain 180 and 183, did not accumulate this compound. These results suggest that β-methylnorleucine is synthesized by the four enzymes common to the biosynthesis of isoleucine and valine.

Transaminase B Activity for β-Methylnorleucine Synthesis

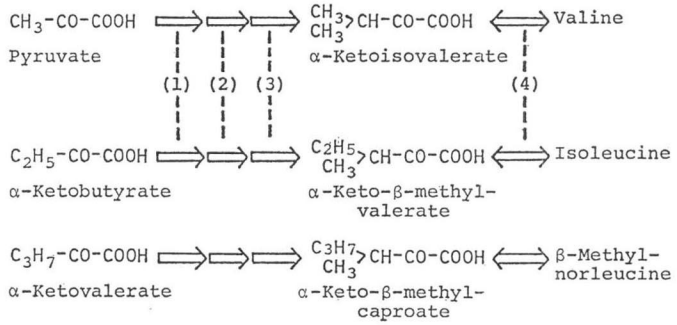
If β-methylnorleucine is synthesized by the isoleucine-valine biosynthetic enzymes, it is likely that transaminase B catalyzes the conversion of α-keto-β-methylcaproate to β-methylnorleucine. Therefore, transaminating activities between α-ketoglutarate and β-methylnorleucine were compared in strains No. 1 and Ar130-1 (Table 6). The activity for β-methylnorleucine was lower than that for other original substrates. Its activity in strain Ar130-1 increased about 5-fold of that in strain No. 1, in the same manner as the activities for other substrates. These results suggest that transaminase B participates in the synthesis of β-methylnorleucine.

Discussion

To determine the metabolic pathway of β-methylnorleucine biosynthesis, we examined its accumulation from norvaline using various regulatory mutants of isoleucine, valine and leucine biosynthesis. β-Methylnorleucine accumulation by strain No. 1 was inhibited by the simultaneous addition of isoleucine, valine and leucine to the medium (Table 4). On the other hand, its accumulation by the α-aminobutyrate-resistant mutant Ar130-1 (the derepressed mutant of isoleucine, valine and leucine biosynthesis) was markedly increased and was not affected by additions of these amino acids. These results indicate that β-methylnorleucine might be formed *via* the isoleucine-valine or the leucine biosynthetic pathway. However β-methylnorleucine accumulation did not increase in the strain S-11 that accumulated leucine. A leucine auxotroph and an isoleucine auxotroph both formed β-methylnorleucine. Therefore, it was concluded that β-methylnorleucine is formed by neither enzymes of the leucine biosynthetic pathway nor L-threonine dehydratase. On the other hand, an isoleucine-valine auxotroph defective in one of the four enzymes common to isoleucine-valine biosynthesis did not synthesize β-methylnorleucine. These results provide evidence compatible with the view that β-methylnorleucine is synthesized *via* the isoleucine-valine biosynthetic pathway. Conceivably, α-acetohydroxyacid synthetase catalyze the condensation of α-ketovalerate, the α-ketoacid of norvaline, with active acetaldehyde and the product of this reaction is then converted to β-methylnorleucine *via* α-keto-β-methylcaproate (Fig. 2).

This hypothesis is supported by the finding that transaminase B, one of the isoleucine-valine bio-

Fig. 2. Biosynthetic pathway of β-methylnorleucine.
(1) α-Acetohydroxyacid synthetase; (2) Dihydroxyacid reductoisomerase; (3) α,β-Dihydroxyacid dehydratase; (4) Transaminase B (Branched-chain amino acid transaminase).



synthetic enzymes, catalyzes the last step of β-methylnorleucine synthesis. These results indicate that the enzymes of isoleucine-valine biosynthesis as well as those of the leucine pathway have broad substrate specificities. If α-ketoacids other than pyruvate, α-ketobutyrate or α-ketovalerate are metabolized by the isoleucine-valine biosynthetic enzymes, other novel amino acid analogs may be synthesized. For example, α-ketocaproate corresponding to norleucine is metabolized, β-methylhomonorleucine may be synthesized. However, unusual amino acid analogs other than β-methylnorleucine were not detected in the medium. The affinities of α-ketocaproate for isoleucine-valine biosynthetic enzymes may be much lower than that of α-ketovalerate.

In recent years, various unusual amino acids have been found as secondary metabolites produced by microorganisms, especially actinomycetes[15]. However, little is known about the biosynthetic pathways of those amino acids. The biosynthesis of β-methylnorleucine is one example that some secondary metabolites are synthesized by use of the biosynthetic enzymes of the primary metabolites. We have found that β-methylnorleucine inhibits the growth of some microorganisms[1] and acts as an *isoleucine* antagonist. Detailed work on the mode of the action will be reported in a separated paper.

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