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Microbial Production of Vitamin B_{12} Antimetabolites

II. 2-Amino-4-Keto-3-Methylpentanoic Acids from *Bacillus cereus* 439

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2-Amino-4-keto-3-methylpentanoic acids were isolated as a diastereomeric mixture from *Bacillus cereus* 439 fermentations and found to be vitamin B_{12} antimetabolites in a bioassay system based on the vitamin B_{12}-requiring *Escherichia coli* (Davis 113-3). A similar diastereomeric mixture with bioactivity was synthesized by condensation of 2-bromo-3-butanone with sodio diethyl acetamidomalonate followed by hydrolysis with 6 *N* HCl and purification by ion-exchange chromatography. The growth inhibitory effects of the antimetabolite were reversed by vitamin B_{12}, L-methionine, L-isoleucine, L-leucine, L-valine, and D-alanine.

INTRODUCTION

A search for microorganisms producing substances inhibiting vitamin B_{12}-stimulated growth of *Escherichia coli* (Davis 113-3) resulted in selection of a strain of *Bacillus cereus* which had such ability. Initial studies with this culture (designated as no. 439 in our collection) showed it produced N^5-hydroxy-L-arginine (*1*) and also a second ninhydrin-positive substance. This second substance (designated 439A) differed chemically from the hydroxyarginine and could be separated from it by ion-exchange chromatography. Factor 439A also differed from the hydroxyarginine in biological activity, and optimum conditions for its production differed markedly from those for production of the hydroxyarginine.

B. cereus 439 grows well in a variety of bacteriological media when incubated under aerated conditions at temperatures in the range 20–40°C. Formation of antimetabolite 439A, however, was observed only when the *B. cereus* 439 (and substrains) was incubated in shaken flasks with the incubation temperature between 29 and 31°C, and during the incubation interval between 36 and 40 hr. Increasing the length of the incubation period or changing the incubation temperature resulted in marked decrease in amounts of 439A found in the fermented medium.

The ability of *B. cereus* 439 to produce 439A varied considerably over the 4-year period of experimental studies, and examination of natural variants obtained from the parent organism showed that most were unable to produce measurable amounts of 439A. Several of the more productive strains were selected for large-scale preparation and these were maintained for future use by transfer periodically on agar slants as well as by storage in a liquid N refrigerator.

This paper is dedicated to the memory of Professor S. M. Kupchan.

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The determination of the 439A content of the fermented media was complicated by the proclivity of the *B. cereus* 439 cultures to produce valine, leucine, isoleucine, and other amino acids along with the 439A. Some of these effectively reversed the growth inhibitory effects of 439A on the *Escherichia coli* (Davis 113-3) bioassay (*1*, *2*), and, therefore, the determination of the presence of 439A in fermented media was made by treating the samples with Dowex 50W × 4 resin (H^+ cycle) and eluting the resin with 0.6 *M* pyridine. The pyridine eluates were examined by thin-layer chromatography on silica gel in a methanol–acetone–water (2:2:1) system with ethanolic ninhydrin as detecting spray. As a result of heating the sprayed plate, the 439A could be differentiated from isoleucine, leucine, alanine, glycine, proline, and traces of other amino acids found in the fermentation samples and pyridine eluates, in that 439A gives a bright blue color with ninhydrin in contrast to the purple color obtained with alanine, leucine, isoleucine, and valine and the yellow color obtained with proline.

The availability of this laborious and only semiquantitative analytical method still allowed evaluation of some of the fermentation parameters, including the incubation temperature and the incubation cycle mentioned above. Maximum production of 439A was obtained when the growth medium contained 20 g/liter of glucose and 30 g/liter of soybean meal. Increasing the carbohydrate concentration resulted in significantly lower yields of 439A, while changing the level of the soybean meal (either upward or downward) resulted in lowered yields. The maximum yields obtained in fermentations inoculated with *B. cereus* 439 (and substrains) were of the order of 5 mg/liter of fermented medium. Practical preparation of large amounts of 439A for chemical characterization was accomplished by using shaken flask fermentations (75 ml/250-ml cotton-plugged Erlenmeyer flask); usually 6 liters (80 flasks) were prepared at one time.

The purification of 439A was carried out by the procedure described in Table 1: The fermented medium was adjusted to pH 2 with HCl, and the solids, cells, and residual soybean meal were removed by centrifugation. The clarified supernatant solution was then passed over a column of Dowex 50W × 4 (H^+ cycle), and the column was washed with distilled water and eluted with 0.6 *M* pyridine. The pyridine eluate was concentrated *in vacuo*, and the residue was dissolved in a small amount of water and diluted with methanol. After centrifugation the supernatant solution was evaporated *in vacuo* and then lyophilized. The resulting powder was chromatographed on Avicel with a methanol–acetone–water mixture (2:2:1), and the 439A was separated from most of the leucine, isoleucine, alanine, glycine, and other amino acids. The enriched fractions were lyophilized, and the crude material was chromatographed on Dowex 50W × 4 (equilibrated with pyridine–acetate buffer at pH 3.1) (*3*) using a gradient of pyridine and acetic acid from pH 3.1 to 4.1 to separate the 439A from the remaining amino acids, except for some proline. The purified fraction was then chromatographed on thin-layer plates of silica gel with a methanol–acetone–water–acetic acid system (2:2:1:0.5) to separate the 439A from the proline. The enriched fractions eluted from the tlc plates were rechromatographed on the Dowex 50W × 4 (H^+ cycle) with 0.6 *M* pyridine as eluant. The fractions containing 439A (homogeneous by tlc) were pooled and lyophilized.

An agar diffusion bioassay was used to measure the potency of the fractions obtained during the isolation. This involved placing 12.7-mm filter paper discs dipped in the test solutions on the surface of agar plates seeded with the vitamin B_{12}-requiring *E. coli*

TABLE 1

PURIFICATION OF A VITAMIN B_{12} ANTIMETABOLITE FROM *Bacillus cereus* 439

Purification step	Solids recovered (mg)	Potency by bioassay (U/mg)	Estimated recovery (%)
1 liter of fermented medium adjusted to pH 2 with 6 *N* HCl and centrifuged.		—[a]	—[a]
Supernatant solution passed over Dowex 50×4 (H^+ cycle). Column washed with water and then eluted with 0.6 *M* pyridine. "Active fractions" evaporated to gum, and gum dissolved in water and diluted with MeOH. Supernatant lyophilized.	2500	0.4	100[a]
Chromatography on Avicel using acetone–methanol–water system. Lyophilize "active fractions."	550	1.0	55[b]
Chromatography on Dowex 50×4 (equilibrated with pyridine–acetate, pH 3.1) using a gradient from pH 3.1 to 4.1.	73	10	73[a]
Preparative tlc (acetone–methanol–water system) followed by absorption of Dowex 50×4 (H^+ cycle) and elution with 0.6 *M* pyridine.	3	100	50

[a] Recovery calculations complicated by presence of compound reversing growth inhibitory activity of 439A.

(Davis 113-3) (*2*) and noting the diameter of the inhibition zone after incubation of the plates at 37°C for 18 hr. The slope of the dose–response curve for a doubling in concentration of 439A was usually about 3 mm, and a solution of 439A producing a 22-mm inhibition zone was designated as having a 1-unit/ml concentration. Pure 439A was found to have a potency of about 100 units/mg. This bioassay was adversely influenced by the presence in the samples of a number of amino acids including leucine and isoleucine, all of which were found to reverse the growth inhibition of 439A in this test system. Thus, the overall recovery mentioned in Table 1 is only an estimate since it was not possible to accurately determine the amount of 439A present in the initial fermentation broth samples.

Highly purified samples of 439A were applied to a Beckmann–Spinco amino acid analyzer and were eluted according to the method of Spackman, Stein, and Moore (*4*). The material appeared as a peak between the positions of threonine and serine. The elution time remained unchanged when the sample was previously exposed to conditions used for the hydrolysis of peptides (6 *N* HCl, 110°C, 16 hr). Thus, an amino acid rather than a peptide seemed to be at hand. The ir spectrum of 439A resembled that of isoleucine. No significant uv absorption was found in the range of 210–400 nm. The nmr spectrum of a solution of 439A in D_2O revealed a complex pattern of methyl protons: a pair of doublets centered at 1.16 and 1.21 ppm and two singlets at 2.21 and 2.23 ppm, respectively. For analysis by mass spectra a sample was acetylated with acetic anhydride in acetic acid and then methylated by the addition of an ethereal solution of

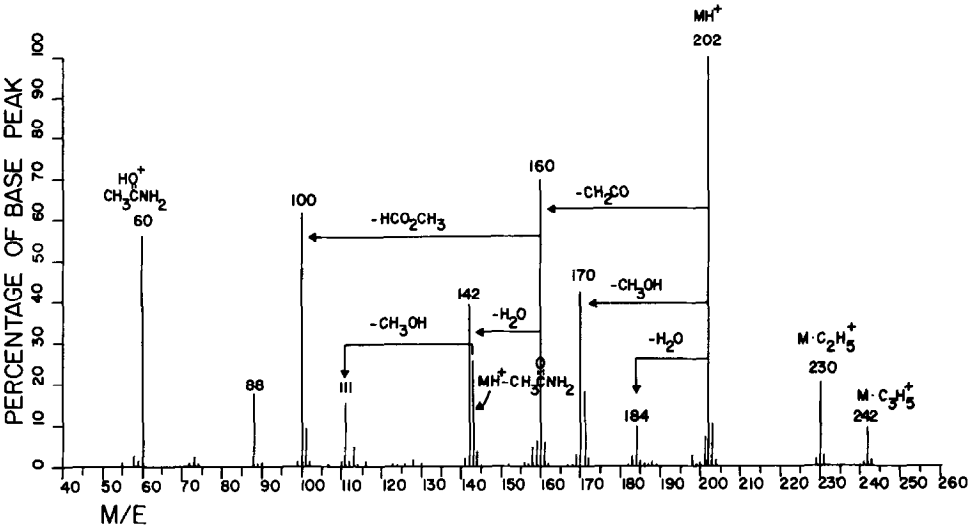
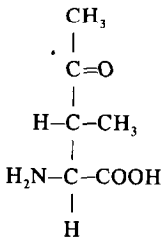


FIG. 1. Mass spectral analysis of acetylated and methylated 439A.

diazomethane to a methanolic solution of the acetyl derivative. The chemical ionization (NH_3) spectrum gave strong evidence for an amino acid derivative with a molecular weight of 201. The most abundant ion corresponded to the protonated ion at *m/e* 202, while the next most abundant ion at *m/e* 170 was consistent with the loss of CH_3OH from MH^+. The high-resolution electron-impact mass spectrum (see Fig. 1) showed a weak peak at *m/e* 201 corresponding to $C_9H_{15}NO_4$. Fragment ions with *m/e* 200 (M^+-OCH_3), 170 (M^+-OCH_3), 164 (M^+-H and 2 H_2O), 159 (M^+-CH_2O), 158 (M^+-CH_3CO), 156 (M^+-COOH), 142 ($M^+-COOCH_3$) and 100 ($M^+-COOCH_3$ and CH_2CO) indicated a *N*-acetylmethyl ester derivative of an amino acid having a molecular weight of 145 and the empirical formula of $C_6H_{11}NO_3$. In a subsequent experiment the *N*-acetylmethyl ester was separated by gas chromatography into two peaks both of which gave *identical* chemical ionization (CH_4). This suggested a pair of diastereoisomers. These data could be best reconciled by assuming the structure of 2-amino-4-keto-3-methylpentanoic acid, **I**, for 439A.

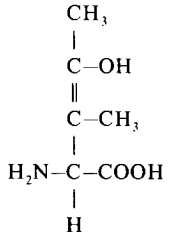


I

Firm evidence for the correctness of this assumption was secured by synthesis. The sodio derivative of diethyl acetamidomalonate was alkylated wtih 2-bromo-3-butanone, and the product was hydrolyzed and decarboxylated. The purified keto acid, a mixture of four isomers, was indistinguishable by tlc and by nmr spectra from 439A isolated from cultures of *B. cereus* 439. On the amino acid analyzer the synthetic sample

emerged with the same elution time as the natural one, and their mixture gave a single peak. Last, but not least, the synthetic product inhibited the growth of the B_{12}-dependent *E. coli* (Davis 113-3).

Questions about the inhibitory properties of the four individual isomers will be answered after their separation, for which experiments are in progress. Treatment of the purified 439A and the chemically synthesized mixture with L-amino acid oxidase resulted in complete loss of bioactivity (as measured by the agar diffusion bioassay). The characteristic blue spot on tlc was absent in the enzyme treated microbial material but was present in the similarly treated synthetic product. The presence of two diastereoisomers probably both having the L-configuration in the fermentation product requires further investigation. They might have been formed directly by a biosynthetic mechanism or could be the result of subsequent racemization, e.g., through the enol **II** in which the chiral center at the β-carbon is lost.



II

The purified 439A was studied for its ability to inhibit the growth of various microorganisms. When the organisms were grown in a chemically defined medium significant

TABLE 2

ANTIMICROBIAL ACTIVITY OF 2-AMINO-4-KETO-3-METHYLPENTANOIC ACID

Test organism	Minimal inhibitory concentration (μg/ml)	
	Chemically defined medium[a]	Medium containing peptones or other natural products[b]
Escherichia coli Davis 113-3	20	>1000
Escherichia coli B	15	>1000
Staphylococcus aureus 209P	5	>1000
Sarcina lutea	10	>1000
Bacillus subtilis Marburg	5	>1000
Pseudomonas aeruginosa 24	80	>1000
Eagle's KB cells	n.d.	>100
L-1210 cells	n.d.	>100

[a] The chemically defined medium contained per liter: KH_2PO_4, 3 g; K_2HPO_4, 7 g; $(NH_4)_2SO_4$, 1 g; Na_3citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; agar, 15 g; distilled water to 1 liter. Glucose (sterilized separately), 10 g.

[b] The Difco medium No. 11 was used for the bacteria for the peptone-containing medium. The mammalian cell cultures were grown in the medium described by Geran et al. (*6*) which contains serum, amino acids, vitamins, and salts.

potency was obtained in experiments with Gram-positive and Gram-negative bacteria. Some of the data collected are summarized in Table 2. No inhibitory activity was noted when the organisms were grown in a peptone–yeast extract medium, presumably because the methionine, valine, leucine, and isoleucine present reversed the inhibition of the growth of the test organisms.

The ability of various amino acids and vitamin B_{12} to reverse the inhibition of growth of the test organisms by 2-amino-4-keto-3-methylpentanoic acid was tested by Smith's counterdiffusion method (*5*) and by a modified gradient plate method where the reversant was incorporated into both layers of the agar. In Smith's counterdiffusion test using *E. coli* (Davis 113-3) as test organism, the inhibition of growth was competitively reversed by vitamin B_{12}, L-methionine, L-leucine, L-isoleucine, L-valine, D-alanine, and D-cycloserine. It was noncompetitively reversed by alanosine. No reversal of inhibition was noted when the following were tested in the counterdiffusion method: L-alanine; D-methionine; glycine; β-L-alanine; L-phenylalanine; L-aminobutyric acid; sarcosine; and L-threonine.

A gradient plate method useful in quantitating the potency of these reversants was also used and some of the data collected are summarized in Fig. 2. Those compounds

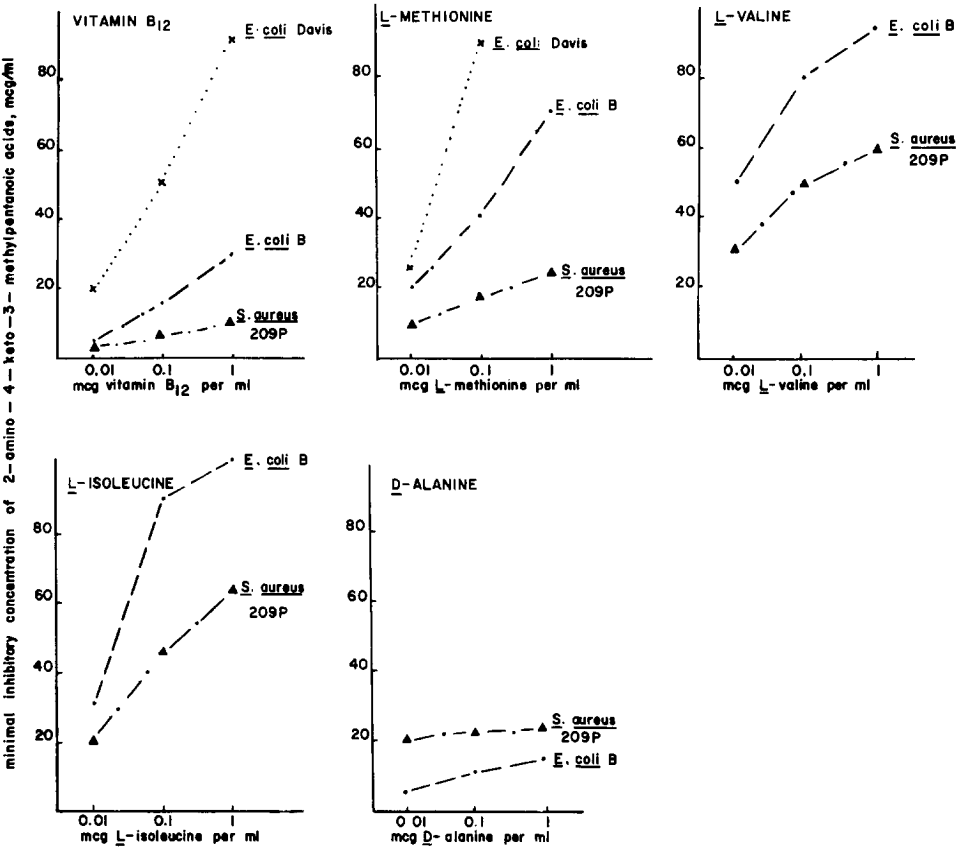


FIG. 2. Dose–response curves obtained in studies of amino acid reversal of growth inhibition by 2-amino-4-keto-3-methylpentanoic acid.

where the slope of the change in minimal inhibitory concentration was significant are considered to be competitive reversants while those where the slope is very low are considered to be noncompetitive reversants.

EXPERIMENTAL

Preparation of 439A by Fermentation

Bacillus cereus 439 was maintained on agar slants and by storage in liquid nitrogen refrigerators. Fermentations were started by transferring cells to flasks of a sterile medium containing 3% soybean mean and 2% glucose with 100 ml of medium/250-ml cotton-plugged Erlenmeyer flask. These flasks were placed on a 300-rpm rotary shaker at 30°C. After 2 days of incubation, 5 ml of the vegetative growth was transferred to additional flasks of the soybean meal–glucose medium, and the newly inoculated flasks were placed on the rotary shaker. Incubation was continued for 36 to 40 hr, at which time the pH of the fermented medium had dropped to pH 5.3–5.5 and most of the cells had sporulated. The contents of the flasks were pooled for the isolation of the 439A.

Isolation of 439A from Fermented Media

Six liters of fermented medium were adjusted to pH 2.1 using 6 *N* HCl, and the acidified suspension was agitated for 20 min with a magnetic stirrer. The suspension was then centrifuged at 5000 rpm, and the supernatant liquid was collected. Next, 5400 ml of the acidified liquid was passed over a 4 × 30-cm column of Dowex 50W × 4 (H^+ cycle). The column was washed with 2 liters of distilled water, eluted with 2 liters of 0.6 *M* pyridine, and 500-ml fractions were collected. The first two fractions were pooled and evaporated *in vacuo* at 40–45°C, the residue was dissolved in 60 ml of water, and 600 ml of methanol was added with stirring. A brown gum separated. The supernatant was removed, the gum was washed with 50 ml of methanol, the combined methanol solutions were evaporated *in vacuo*, and the water was removed by lyophilization to leave 15 g of a light foam (as powder). Five grams of this powder was applied to a 45-g column (2.3 × 51 cm) of Avicel microcrystalline cellulose. The Avicel was first suspended in methanol–acetone–water mixture (2:2:1) before packing. The column was developed with the methanol–acetone–water mixture, and 12-ml fractions were collected. The desired compound appeared in tubes 12 and 13. These fractions were pooled and evaporated to dryness *in vacuo*. The solid obtained was dissolved in a small amount of pyridine–acetic acid solution (pH 3.1) and applied to a column of Dowex 50W × 4 (200–400 mesh) which had been equilibrated with this solution. A 70-ml, 2 × 30-cm column was used for 1 g of sample, and the desired compounds were separated by a gradient elution (pH 3.1 to 4.1) with 8-ml fractions being collected. A total of 64 fractions were collected. Each was concentrated *in vacuo* and analyzed for 439A using the tlc system. Fractions 6–11 contained 439A and a substance giving a yellow color with the ninhydrin reagent (proline ?). They were pooled and evaporated and finally lyophilized to give 134 mg of a light tan powder.

Further purification was obtained by dissolving this powder in methanol and by thin-layer chromatography using the methanol–acetone–water–acetic acid system (2:2:1:0.5). The material eluted from the tlc plates was purified by absorption on the

Dowex 50W × 4 column (H^+ cycle) and eluted with 0.6 *M* pyridine. The fractions containing the 439A were concentrated *in vacuo* and lyophilized to give about 20 mg of a light tan powder.

Bioassay Procedures for Fermentation Samples and Concentrates

The biopotency of the crude materials was checked by an agar diffusion bioassay with *Escherichia coli* (Davis 113-3) as a test organism. The growth medium contained per liter: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; Na_3citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; glucose, 10 g (sterilized separately); agar, 15 g; and distilled water q.s. 1 liter. To the molten sterile agar 50 μg of sterile vitamin B_{12} was added just before inoculation with washed cells of the *E. coli* (Davis 113-3). Next, 200 ml of the seeded agar was poured into sterile 3-quart Pyrex baking dishes (covered with aluminum covers). The samples dissolved in water were applied to the surface of the solidified seeded agar using 12.7-mm filter paper discs. The plates were incubated overnight at 37°C and the diameters of the zones of inhibition were measured. A 22-mm zone was considered to be 1 unit of activity. Discs containing neomycin were placed on each plate as a control compound; the 6.7-mm discs containing 30 μg of neomycin gave a 19-mm zone under these conditions.

The minimal inhibitory concentrations of the 2-amin-4-keto-3-methylpentanoic acids for a series of bacteria were determined with the above agar medium and Difco medium No. 11 with washed cells of a 24-hr-old culture as inoculum. The potency of various compounds as reversants for the inhibition caused by the 2-amino-4-keto-3-methylpentanoic acids was determined by Smith's counterdiffusion method (5) and by a gradient plate method (Perlman, in press, 1977) where the reversant was included in both layers of the gradient.

Synthesis of 2-Amino-4-Keto-3-Methyl Pentanoic Acids

To a solution of 12 g of diethyl acetamidomalonate dissolved in 50 ml of dry dimethylformamide was added 2.4 g of sodium hydride (in 50% oil suspension) over a 15-min period in small portions (with stirring and occasional cooling). The mixture was stirred for an additional 15 min at the end of which a gray solution was obtained. Then 7.5 g of 2-bromo-3-butanone was added slowly with stirring and cooling. The mixture was stirred overnight at room temperature and then evaporated under reduced pressure. The crude residue was refluxed with 120 ml of 6 *N* HCl for 5 hr. The dark solution was decolorized with charcoal, and the filtrate was evaporated under reduced pressure. The residue was dissolved in 30 ml of H_2O and re-evaporated. This was repeated twice to eliminate the excess HCl. The residue was dissolved in a small amount of H_2O and applied to a 40 × 5-cm column of Dowex 50W × 4 (100–200 mesh, H^+ cycle). The column was washed with deionized water until the effluent was free of Cl^- ions and then the 2-amino-4-keto-3-methylpentanoic acid was eluted with 0.6 *M* aqueous pyridine. The eluate was evaporated under reduced pressure. Then 200 ml of ethanol was added and evaporated. After a second addition of the ethanol the product crystallized. The precipitate was filtered with suction, washed with cold ethanol, and dried to give 3.7 g of cream white product.

The ethanolic mother liquor, after evaporation and lyophilization, gave an additional 0.7 g of material which was much more soluble in ethanol than the material precipitated from the ethanol.

The ethanol-soluble product gave a single, ninhydrin-positive spot when analyzed by tlc on silica gel using the following systems: methanol–acetone–water (2:2:1); *n*-butanol–acetic acid–water (4:1:1); and ethanol–water (4:1).

The material precipitated from the ethanol solution contained some glycine (as determined by the tlc studies) and was purified by the gradient elution technique on the Dowex 50W × 4 column as described above for the 439A from the fermented medium. Both purified products were identical in mobility in the tlc systems.

Anal. Calcd. for $C_6H_{11}NO_3$: C, 49.65; H, 7.64; N, 9.65.

Found: C, 49.62; H, 7.56; N, 9.48.

The synthetic preparation was active in the agar diffusion bioassay using *E. coli* (Davis 113-3) as test organism; A solution containing 6 mg/ml gave a 24-mm inhibition zone, and the inhibition was reversed by L-methionine, L-isoleucine, L-leucine, L-valine, and vitamin B_{12}.

Treatment of 439A with L-*Amino Acid Oxidase*

In 5 ml of H_2O, 100 mg of synthetic 2-amino-4-keto-3-methylpentanoic acid was dissolved and adjusted to pH 7.6 with NaOH. Then 50 mg of cobra venom L-amino acid oxidase (0.25 units/mg) obtained from Sigma Chemical Company was added, and the solution was shaken for 24 hr at 37°C. Bioassays showed that the material had lost all of its growth inhibitory activity for *E. coli* (Davis 113-3), while the tlc analyses showed a ninhydrin-positive material with an R_f equal to the starting material and giving the same blue color as the starting material. Treatment of the 439A isolated from the fermentations lead to the loss of bioactivity and also loss of the characteristic spot on tlc of the active material.

Note added in proof. Since this paper was submitted for publication, a series of experiments were carried out attempting the determination of the configuration of the isomer of 2-amino-4-keto-3-methylpentanoic acid produced by fermentation. High pressure liquid chromatography in the system methylene chloride–methanol–water (65:35:10) of the synthetic material led to separation of two racemates that could be distinguished by their different NMR spectra and thin layer chromatography in the system 2-butanone–pyridine–H_2O–acetic acid (70:15:15:2). Yet, on standing at room temperature, epimerization occurred. Therefore, the problem was not pursued further.

ACKNOWLEDGMENTS

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