DL-N-Acetyl-1-benzyl-5-methoxytryptophan.—A suspension of 7.5 g. (0.0161 mole) of diethyl acetamido(1-benzyl-5-methoxy-3-indolylmethyl)malonate (**3c**) in 32 ml. of 2.5 *N* NaOH was refluxed for 3 hr., clarified with charcoal, cooled to 5°, acidified with 50 ml. of 2 *N* HCl, and refrigerated overnight. The precipitate of acetamido(1-benzyl-5-methoxy-3-indolylmethyl)malonic acid (5.9 g., 92%) melted at 166–168°.

Anal. Calcd. for $C_{29}H_{32}N_2O_6$: N, 6.82. Found: 7.08.

A mixture of 5.5 g. (0.0136) of the acetamidomalonic acid, 100 ml. of ethanol, and 50 ml. of water was refluxed for 4 hr., distilled *in vacuo* to remove alcohol, and refrigerated overnight. The cream-colored precipitate of DL-N-acetyl-1-benzyl-5-methoxytryptophan was filtered off and dried (yield 4.4 g., 89.6%, m.p. 176–177°). A sample, recrystallized from 30% ethanol, melted at 178–179°.

Anal. Calcd. for $C_{21}H_{22}N_2O_4$: C, 68.83; H, 6.05; N, 7.65. Found: C, 69.11; H, 6.35; N, 7.70.

DL-1-Benzyl-2-methyltryptophan (1b).—DL-N-Acetyl-1-benzyl-2-methyltryptophan (2.9 g., 0.0083 mole) was refluxed for 24 hr. with 15 ml. of 2 *N* NaOH solution, treated with charcoal, filtered hot, and acidified to pH 5.5 with acetic acid. The crystalline precipitate was recrystallized from 50% ethanol; yield 0.45 g. (15%), m.p. 237–239°.

Anal. Calcd. for $C_{19}H_{20}N_2O_2$: C, 73.98; H, 6.54; N, 9.09. Found: C, 73.97; H, 6.21; N, 8.84.

DL-N-Acetyl-1-benzyl-2-methyltryptophan (1.2 g., m.p. 213° after recrystallization from ethanol) was recovered from the recrystallization mother liquor.

DL-1-Benzyl-5-methoxytryptophan (1c).—DL-N-Acetyl-1-benzyl-5-methoxytryptophan (3.0 g., 0.0082 mole) was refluxed with 14 ml. of 2 *N* NaOH for 25 hr., filtered hot, cooled, neutralized to pH 5.5 with acetic acid, and stored overnight in the refrigerator. The crystalline precipitate of crude 1c was filtered off and recrystallized from 50% ethanol (yield 0.9 g., 33%, m.p. 232–233°). It melted at 229–230° after a second recrystallization from 50% ethanol.

Anal. Calcd. for $C_{19}H_{20}N_2O_3$: C, 70.35; H, 6.22; N, 8.64. Found: C, 70.18; H, 6.14; N, 8.54.

2-Amino-3-methylthiobutyric Acid, an Isoleucine Antagonist[1]

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2-Amino-3-methylthiobutyric acid, the thia analog of isoleucine, was prepared as a diastereoisomeric mixture by condensing 2-phenyl-4-ethylidene-5-oxazolidone and methanethiol in the presence of sodium methoxide, followed by acid hydrolysis of the resulting intermediate condensation product. 2-Amino-3-methylthiobutyric acid inhibits growth of *Escherichia coli*, *Streptococcus lactis*, *Leuconostoc dextranicum*, *Lactobacillus casei*, and *Leuconostoc mesenteroides* at concentration levels of 6, 6, 60, 60, and 60 γ/ml., respectively. A specific and competitive reversal of 2-amino-3-methylthiobutyric acid toxicity by isoleucine is observed with *E. coli* with an inhibition index of about 30 over a 100-fold range of increasing substrate concentrations.

Substitution of a sulfur atom for a methylene group in certain aliphatic amino acids has been successful in producing thia analogs which act as amino acid antagonists. Among such analogs, S-carbamoyl-L-cysteine (4-thiaglutamine) has been found to inhibit the growth of several lactobacilli by interfering with essential biological functions in which glutamine has a role, but its toxicity is only partially and noncompetitively reversed by glutamine.[2] S-(β-Aminoethyl)-L-cysteine (4-thialysine) has been found to antagonize competitively the utilization of lysine for the growth of *Leuconostoc mesenteroides* P-60 and *Lactobacillus arabinosus* 17-5.[3]

In view of the biological activity observed with these thia analogs, it was anticipated that the introduction of a sulfur in place of the methylene group at the 4-position of isoleucine might produce an effective antagonist of isoleucine in certain microorganisms. In this investigation, 2-amino-3-methylthiobutyric acid was prepared as a diastereoisomeric mixture, and its biological properties were studied in *Escherichia coli* 9723 and several lactobacilli.

Experimental[4]

Organic Syntheses. 2-Benzamido-3-methylthiobutyric Acid.—To a solution of 4.8 g. of methanethiol in 100 ml. of absolute methanol, in which 0.5 g. of sodium had reacted, was added slowly a solution of 18.5 g. of 2-phenyl-4-ethylidene-5-oxazolidone[5] in 100 ml. of benzene at 5–10° with constant stirring. After addition was complete, the reaction mixture was allowed to stand at 40° for 72 hr. The reaction mixture was acidified to pH 2 by the addition of 6 *N* HCl and then taken to dryness by removal of the solvents under reduced pressure. The residual solid was extracted with 50 ml. of boiling ethanol, and the insoluble material was removed by filtration. After chilling the alcoholic filtrate in a refrigerator overnight, there was obtained 20.1 g. of white crystals, m.p. 72–74°. A sample recrystallized from hot ethanol melted at 74–75°.

Anal. Calcd. for $C_{12}H_{15}NO_3S$: C, 56.89; H, 5.97. Found: C, 56.97; H, 6.30.

2-Amino-3-methylthiobutyric Acid (4-Thiaisoleucine) Hydrochloride Monohydrate.—A solution of 2 g. of 2-benzamido-3-methylthiobutyric acid in 200 ml. of 4 *N* HCl was heated at reflux for 2 hr. The reaction mixture was extracted twice with 50-ml. portions of ether to remove the benzoic acid. The aqueous phase was taken to dryness *in vacuo*, and the residual oil was treated with 100 ml. of benzene. The resulting mixture was allowed to stand several days at room temperature in order to effect crystallization of the desired product. The solid was collected on a filter, washed with benzene, and dried in a desiccator under vacuum ($CaCl_2$). There was obtained 0.9 g. of chromatographically pure product which softened to a gelatinous solid at 65–67° and melted at 122–132° dec.

Anal. Calcd. for $C_5H_{11}NO_2S \cdot HCl \cdot H_2O$: C, 29.48; H, 6.92; N, 6.87; S, 15.74. Found: C, 29.71; H, 6.91; N, 7.20; S, 15.98.

The R_f values in 1-butanol–acetic acid–water (3:1:1), 65% pyridine, and 95% methanol were 0.50, 0.67, and 0.48, respec-

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(4) All melting points are corrected. The microanalyses were performed by International Chemical and Nuclear Corp., City of Industry, Calif. All R_f data were determined using the ascending technique of paper chromatography in the solvents indicated, and ninhydrin reagent was used for the development of the spots. The infrared spectrum was determined on a Beckman Instruments, Inc., Model IR-8 spectrophotometer using the potassium bromide pellet technique and at a concentration of 0.5%.

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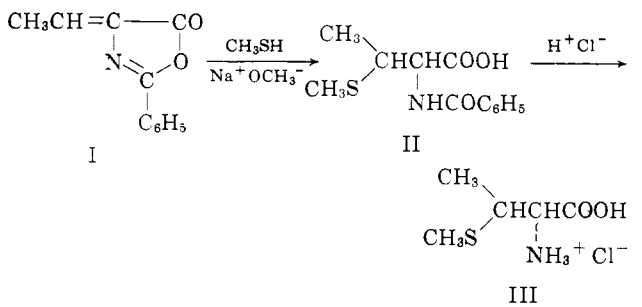
tively. An infrared spectrum showed strong absorption peaks at 3.4, 5.8, 6.3, 5.75, 7.1, and 8.3 μ.

Microbiological Assays.—For *E. coli* 9723, a previously described inorganic salts–glucose medium[6] was used, and the organism was incubated at 37° for 15 hr. For the lactic acid bacteria, a previously reported amino acid medium[7] was modified by the addition of calcium pantothenate (0.2 g./ml.), by decreasing the amount of isoleucine (4 γ/ml.), valine (15 γ/ml.), and leucine (15 γ/ml.), and with additional modifications noted for each organism. For *L. dextranicum* 8086 and *L. mesenteroides* 8293, 0.02 γ/ml. of pantethine was added under aseptic conditions, and the phosphate concentration was increased fourfold. For *S. lactis* 8039 and *L. casei* 7469, 4 γ/ml. of L-glutamine was added under aseptic conditions. The latter organism was incubated at 37° for about 24 hr., and the other lactic acid organisms were incubated at 30° for 30 hr. In all assays the amount of growth was determined photometrically at 660 mμ with a Bausch and Lomb Spectronic 20 spectrophotometer in terms of absorbance readings of the turbid culture medium against a blank of uninoculated medium set at 0 absorbance. The amino acid analog was dissolved in sterile water and added aseptically to the previously autoclaved tubes.

Results and Discussion

As indicated in the accompanying equations, the synthesis of 2-amino-3-methylthiobutyric acid (III) was adapted from the procedure for the preparation of 2-amino-3-benzylthiobutyric acid[8] using methanethiol in



place of benzyl mercaptan as reactant with 2-phenyl-4-ethylidene-5-oxazolidone (I) in the presence of sodium methoxide. The course of this reaction should result in a mixture of two diastereoisomeric forms of 2-benzamido-3-methylthiobutyric acid (II); however, several attempts to separate these diastereoisomers by fractional recrystallization techniques were unsuccessful, even though this technique was satisfactory for the separation of the diastereoisomeric mixture of 2-benzamido-3-benzylthiobutyric acid.[8] In view of the difficulty in separating these diastereoisomers through recrystallizations, the resulting preparation of 2-benzamido-3-methylthiobutyric acid (II) was converted by acid hydrolysis to the desired amino acid analog (III). Paper chromatograms of the isolated material from the reaction hydrolysis in several solvent systems showed the presence of only one ninhydrin-positive component.

This preparation of 2-amino-3-methylthiobutyric acid inhibits the growth of several microorganisms, and subsequent preparations did not vary appreciably in their biological activities. Under the testing conditions previously described, the concentration levels of 2-amino-3-methylthiobutyric acid (III) required for complete inhibition of growth with five different organisms are recorded in Table I. These inhibitory levels vary from 6 γ/ml. for *E. coli* and *S. lactis* to 60 γ/ml. for the other microorganisms.

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Table I

Inhibition of Microbial Growth by 2-Amino-3-methylthiobutyric Acid (III)

Microorganism[a]	Inhibitory level of III, γ/ml.[b]
E. coli 9723	6
S. lactis 8039	6
L. dextranicum 8086	60
L. casei 7469	60
L. mesenteroides 8293	60

[a] In the presence of 4 γ/ml. of isoleucine and 15 γ/ml. of valine which are required for growth, except for *E. coli*. [b] Amount required for complete inhibition of growth.

Table II

Reversal of Toxicities of 2-Amino-3-methylthiobutyric Acid (III) for *E. coli* 9723[a] by Amino Acid Supplements

Amino acid supplement, γ/ml.	Minimal concentration of analog for complete inhibition of growth, γ/ml.
None	6
DL-Isoleucine	
2	60
6	200
20	600
DL-Valine	
2	6
6	6
20	20
DL-Leucine	
2	6
6	6
20	6
DL-Isoleucine + DL-valine	
2 + 2	60
6 + 6	200
20 + 20	600
Threonine	
0.6	20

[a] Incubated at 37° for 15 hr.

Table III

Reversal of 2-Amino-3-methylthiobutyric Acid (III) by DL-Isoleucine in *E. coli* 9723[a]

	DL-Isoleucine, γ/ml.					
III, γ/ml.	0	0.2	0.6	2.0	6.0	20
	Absorbance readings[b]					
0	0.75	0.73	0.72	0.75	0.75	0.72
0.6	0.70					
2	0.21	0.68	0.68			
6	0.06	0.07	0.60	0.73		
20			0.15	0.61	0.74	
60				0.12	0.61	0.71
200					0.00	0.61
600						0.00

[a] Incubated at 37° for 15 hr. [b] A measure of culture turbidity in which absorbance readings of 0.52, 0.30, 0.15, and 0.05 are equivalent to 0.76, 0.43, 0.215, and 0.06 mg. of dry weight of cells/ml. of culture, respectively.

The effects of supplements of isoleucine, valine, leucine, a mixture of isoleucine and valine, and threonine upon the amount of 2-amino-3-methylthiobutyric acid necessary for growth of *E. coli* were determined, and the results are indicated in Table II. In the presence of 2-, 6-, and 20-γ/ml. supplements of DL-isoleucine, the inhibitory level required for complete inhibition is increased to 60, 200, and 600 γ/ml., respectively.

Growth toxicity of this amino acid analog is only slightly affected by higher concentrations of DL-valine (*i.e.*, in the presence of 20 γ/ml. of DL-valine, the minimal concentration of antagonist required for complete inhibition is increased from 6 to 20 γ/ml.) and is not affected to any appreciable extent by DL-leucine. Equal concentrations of a mixture of isoleucine and valine are no more effective than the isoleucine concentrations alone in preventing the inhibitory effects of 2-amino-3-methylthiobutyric acid. However, threonine does prevent the inhibitory effects of the antagonist appreciably.

Threonine is known to serve as a precursor of isoleucine in *E. coli*,[9,10] and the reversal by threonine of an isoleucine antagonist has been previously studied in detail.[11]

The inhibitory effects of 2-amino-3-methylthiobutyric acid and its reversal by isoleucine have been studied most extensively with *E. coli*, as indicated in Table III. The growth inhibitions produced by this analog were competitively reversed by increasing concentrations of isoleucine over approximately a 100-fold range with an inhibition index (ratio of inhibitor to substrate necessary for complete inhibition of growth) of about 30. From these results, it is apparent that 2-amino-3-methylthiobutyric acid is a specific and effective antagonist of isoleucine in the microorganisms studied.

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Synthesis of Some Pyrimidine Amino Acids by the Rhodanine Method and Tests *vs.* the Ehrlich Ascites Carcinoma[1]

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Some pyrimidine amino acids and intermediates (α-keto acids, α-thioketo acids, and α-oximino acids) have been synthesized by the rhodanine method from 2-mercapto-6-oxo-pyrimidine-4-carboxaldehyde and various 1- and 5-methyl and ethyl derivatives thereof. The reduction of the oximino acids to the amino acids was performed with sodium amalgam in alkaline solution followed by neutralization with a weakly acidic ion-exchange resin. Representative examples of these compounds were tested as inhibitors of growth and protein synthesis in Ehrlich ascites carcinoma in mice.

Granacher[4] has shown the applicability of rhodanine to organic syntheses. Its active methylene group permits reactions with aldehydes yielding stable condensation products.[5] These are easily cleaved with alkali to thioketo acids which are believed to exist in equilibrium with the tautomeric sulfhydryl forms since they give a deep green color with ferric chloride.[4] Oximino acids, produced by the action of hydroxylamine on the thioketo acids, can be converted with sodium amalgam to amino acids.[4] The oximino acids also can be converted with hydrochloric acid in the presence of formaldehyde to the corresponding keto acid.[6] This synthetic sequence, if applicable to the pyrimidinecaldehydes, was considered to be of unusual interest since the intermediates as well as the amino acids might be expected to show some antitumor activity in view of their structural relationship to various metabolites.

The pyrimidine nucleosides and nucleotides are important as coenzymes and as metabolic precursors of the nucleic acids. Consequently, it is not surprising that a number of bacteriostatic and carcinostatic drugs are derivatives of pyrimidines. We have reported[7] recently that various derivatives of pyrimidine-4-carboxaldehydes inhibit growth of the Ehrlich ascites carcinoma in mice. It seemed to be of interest to prepare various α-amino acids, α-keto acids, and α-thioketo acids with pyrimidine substituents in the β-position for testing as possible inhibitors of tumor growth. It was found that these compounds could be prepared from the rhodanine derivative as outlined previously. The rhodanine derivative itself was of some interest since it can be considered to be a thio analog of a γ-lactone, and certain lactones[8,9] have been reported[10,11] to inhibit tumor growth. Substitution of pyrimidines in the β-position of the amino acid alanine might yield a potential inhibitor of protein synthesis as well as nucleic acid synthesis, and the corresponding α-oximino acid would be of interest in view of a previous report[12] of antitumor activity in this series. Substitution of pyrimidines in the β-position of pyruvic acid and thiopyruvic acid might yield an inhibitor of lactic dehydrogenase as well as a pyrimidine antimetabolite. This might be of special interest in the inhibition of tumors in which there is particular dependence upon glycolysis[13] for which lactic dehydrogenase is an essential enzyme.

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