Biological Specificities of 4,5-Dehydro Analogues of Isoleucine and Alloisoleucine*

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A number of unsaturated analogues have been found to be effective competitive antagonists of natural amino acids (1, 2). Among such analogues, 2-amino-3-methyl-4-pentynoic acid has recently been prepared in the form of a mixture of the diastereoisomers, \( \omega \)-dehydroisoleucine and \( \omega \)-dehydroalloisoleucine (3). Several attempts to separate this isomeric mixture with methods which were successful for the separation of isoleucine and alloisoleucine failed to yield the desired products. In view of the potent inhibitory activity of the mixture of diastereoisomers, the preparation of each of the pure isomers by an indirect synthesis was accordingly undertaken.

The synthesis of 2-amino-3-methyl-4-pentynoic acid with a configuration corresponding to alloisoleucine has been reported (4), and a partial hydrogenation of this compound would afford a method of obtaining \( \omega \)-dehydroaloisoleucine. However, in the method of synthesis of 2-amino-3-methyl-4-pentynoic acid herein reported, a mixture of both of the diastereoisomeric forms of the acetylenic derivatives was obtained. Subsequent fractional recrystallization of the mixture separated the isomers into the two racemic forms. Partial hydrogenation of the appropriate triple bond compound produced either \( \omega \)-dehydroisoleucine or \( \omega \)-dehydroalloisoleucine, and complete hydrogenation yielded the anticipated DL-isoleucine or DL-alloisoleucine. The ethylenic analogues are more effective growth inhibitors than the acetylenic derivatives for *Escherichia coli*, but for *Lactobacillus arabinosus* the relative inhibitory activity depends upon whether the *L. arabinosus* assays were incubated at 30° and those with *E. coli* and *S. faecalis* at 37°. Each of the assays was incubated for approximately 16 hours.

In all assays the analogues were dissolved in sterile water and added aseptically to the sterile assay tubes without being heated. The amount of growth was determined turbidimetrically in terms of galvanometer readings adjusted such that distilled water reads 0 and an opaque object, 100.

**Organic Syntheses**

**Organic Intermediates—3-Butyn-2-ol** was purchased from Farchan Research Laboratories, and the ethyl formamidomalonate was obtained from Southeastern Biochemicals, Inc. 2-Bromo-3-butyne was prepared by adding, over a 2-hour period, a mixture of 46 g of 3-butyne-2-ol and 3 ml of pyridine to an ice-cold sample of 75 g of phosphorous tribromide containing a few milligrams of hydroquinone. The reaction mixture was then placed in a refrigerator for approximately 6 days during which time the originally homogenous solution yielded two phases. The product was finally twice distilled to yield 41 g of material; b.p., 51° (180 mm). The refractive index was in agreement with the literature value (9, 10).

Ethyl 2-formamido-2-(1-methyl-2-propynyl)malonate was prepared by adding 15 g of ethyl formamidomalonate to a reaction mixture of 1.9 g of sodium in 50 ml of ethanol followed by a dropwise addition of 15 g of 2-bromo-3-butyne. The resulting solution was heated under reflux for approximately 15 hours and cooled; the sodium bromide was removed by filtration, and the clear filtrate was evaporated to dryness in a vacuum. The residue was dissolved in chloroform and filtered, and the solvent was again removed in a vacuum. The residue was then dissolved in approximately 40 ml of hot ethanol and allowed to stand in a refrigerator overnight. There were recovered 4 g of

**Experimental Procedure**

**Microbial Assays**

For the growth assays with *Escherichia coli* 9723, a previously described inorganic salts-glucose medium was employed (5), and the procedure has been reported in detail elsewhere (6). For the growth inhibition and metabolite reversal studies with *Lactobacillus arabinosus* 17-5, a previously described amino acid medium (7) was modified by adjusting the concentration of DL-isoleucine or DL-valine or both, as indicated in the tables. Calcium pantothenate was present at a concentration of 100 µg per ml in the vitamin supplement. The differential growth assays for isoleucine and alloisoleucine were carried out with both *Streptococcus faecalis* 8043, which responds only to isoleucine (8), and *L. arabinosus*, which responds to both isoleucine and alloisoleucine in the above described medium containing the normal concentration of valine. The *L. arabinosus* assays were incubated at 30° and those with *E. coli* and *S. faecalis* at 37°. Each of the assays was incubated for approximately 16 hours.

In all assays the analogues were dissolved in sterile water and added aseptically to the sterile assay tubes without being heated. The amount of growth was determined turbidimetrically in terms of galvanometer readings adjusted such that distilled water reads 0 and an opaque object, 100.

All melting points are uncorrected. The authors are deeply indebted to Dr. J. M. Ravel and Mrs. Jean Humphreys for assistance with the microbial assays and to Charles Hedgecoth and J. D. Class for the elemental analyses. All of the paper chromatographic data were obtained with the ascending technique in the solvents indicated, and the chromatograms were developed with ninhydrin reagent. The ninhydrin used for the quantitative
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Erythro-\(\text{-}\)-Amino-3-methyl-4-pentynoic Acid (Isoleucine Analogue)—The three forms isolated above were the least soluble fraction in the ethanol-water recrystallization scheme presented in Fig. 1. The more soluble fractions were recovered by dissolving the various residues in the minimal amount of water and adding ethanol to induce crystallization. The compositions of the various crystalline fractions were subsequently determined through catalytic reduction to isoleucine or alloisoleucine or both followed by microbial assay. After about fraction \(6\), no further separation of the erythro form could be accomplished with ethanol water, and the solvent system was then changed to dioxane-water as indicated in Fig. 1. The ethanol-water fractions, \(\theta\), \(6\), \(6a\), \(6b\), \(6d\), and \(7\), were recrystallized from dioxane-water after combining the various residues in the manner indicated in Fig. 1. There was ultimately recovered in fraction \(8\) 0.66 g of product (m.p., 206–209°; decomposition point) which upon catalytic hydrogenation produced only \(\text{n}\)-isoleucine as indicated by microbial assays with \(L.\) arabinosus and \(S.\) faecalis.

\(\text{C}_9\text{H}_9\text{NO}_2\)

Calculated: C 56.08, H 7.13, N 11.02

Found: C 56.05, H 7.23, N 11.05

Erythro-\(\text{-}\)-Amino-3-methyl-4-pentynoic Acid (\(\omega\)-Dehydroisoleucine)—With palladium on calcium carbonate which had been treated with lead as a catalyst (12), 450 mg of erythro-2-amino-3-methyl-4-pentynoic acid were treated with hydrogen in the presence of 100 ml of methanol at room temperature and atmospheric pressure until 1 mole equivalent had been absorbed. The catalyst was filtered and washed with methanol, and the combined filtrates were evaporated to dryness in a vacuum to yield 430 mg of material. Paper chromatographic analysis indicated that this reaction product was contaminated with the fully hydrogenated product, isoleucine, as well as with some unhydrogenated starting material. Fractional recrystallization from ethanol-water removed the acetylenic compound, but column chromatography was required to separate \(\omega\)-dehydroisoleucine from the isoleucine contaminant.

A 53-mg sample of the mixture dissolved in 1 ml of ammonium formate, pH 3.1, was placed on a \(9 \times 760\) mm column prepared from 60 g of Dowex 50-2X resin which had been thoroughly washed with 0.2 m ammonium hydroxide and then equilibrated with 0.2 m ammonium formate buffer (13). The column was then eluted with ammonium formate, pH 3.1, at a flow rate of 12 to 16 ml per hr, and 2- to 4-ml fractions were collected. The ninhydrin-positive fractions were taken to dryness in a vacuum and assayed quantitatively by the method of Moore and Stein (14). The \(\omega\)-dehydroisoleucine was eluted from the column in the fractions between 90 and 120 ml of eluate, and the isoleucine was obtained in the fractions between 130 and 160 ml of eluate. After reduction to dryness, the residue containing \(\omega\)-dehydroisoleucine was heated in a vacuum at 55° for approximately 12 hours to remove the ammonium formate. There were obtained 28 mg of product which were recrystallized from ethanol-water for elemental analysis; m.p., 217–218° (decomposition point).
Catalytic reduction of a sample of the above amino acid followed by microbial assays with *S. faecalis* indicated that the hydrogenated product was DL-isoleucine.

**dl-α-Amino-3-methyl-4-pentenoic Acid (ω-Dehydroalloisoleucine)—** This isomer was prepared by the technique described above with 350 mg of *threo*-2-amino-3-methyl-4-pentenoic acid. After 1 mole equivalent of hydrogen had been absorbed the catalyst was removed, and the filtrate was reduced to dryness in a vacuum to yield 330 mg of material, which was recrystallized twice from water-ethanol. The material was nonhomogeneous as indicated by paper chromatography, and the desired product was ultimately obtained through ion exchange chromatography.

With the use of the same chromatographic procedure as indicated above for ω-dehydroisoleucine, 78 mg of material were charged to the column, and ω-dehydroalloisoleucine was recovered between 90 and 115 ml of eluate; the alloisoleucine contaminant was recovered between 120 and approximately 150 ml of eluate. The combined fractions containing the ω-dehydroalloisoleucine were reduced to dryness and the residue was heated in a vacuum at 55° for approximately 12 hours to yield 39 mg of crystalline material, which, after recrystallization from ethanol-water, had a melting point of 221–222° (decomposition point).

**Results and Discussion**

The syntheses of the 4,α-dehydrogenated analogues corresponding to DL-isoleucine and DL-alloisoleucine containing either an acetylenic or an ethylenic grouping were carried out in the indicated fashion.2

\[
\begin{align*}
&\text{H–C≡C–CH–Br} \rightarrow \text{H–C≡C–CH–C–NHCHO (V)} \\
1. \text{Hydrolysis and decarboxylation} & \text{H–C≡C} \\
2. \text{Fractional crystallization} & \text{H–C≡C} \\
& \text{CH}_2 \text{CH}_2 \text{COOC}_2 \text{H} \\
& \text{CH}_2 \text{CH}_2 \text{COOC}_2 \text{H}
\end{align*}
\]

Under the experimental conditions herein described, the hydrolysis and decarboxylation of the formamidomalonic ester intermediate gave a mixture of approximately equal amounts of the two diastereoisomers corresponding to isoleucine and alloisoleucine; whereas, a previously reported procedure yielded a reaction hydrolyzate in which only the isomer corresponding in configuration to alloisoleucine was detected (4). Subjection of this mixture of diastereoisomers to systematic fractional crystallization resulted in the separation of both the *erythro* (I) and the *threo* (II) forms of 2-amino-3-methyl-4-pentenoic acid.2 The identity of the diastereoisomers was established by conversion of the isomers to the corresponding saturated amino acids by catalytic hydrogenation and subsequent microbiological assays of the hydrogenated products with *L. arabinosus,* which responds to either isoleucine or alloisoleucine, and *S. faecalis,* which responds to isoleucine but not to alloisoleucine (8).

The corresponding 2-amino-3-methyl-4-pentenoic acids were prepared by hydrogenation of the appropriate acetylenic analogue in the presence of a lead-treated palladium catalyst. Although only 1 molecular equivalent of hydrogen was absorbed, the reaction mixtures from both isomers contained not only the anticipated pentenoic acid but also some unreacted pentynoic acid as well as some of the completely saturated product. The desired olefinic amino acids, however, could easily be separated from the other materials by crystallization and ion exchange chromatography on Dowex 50 resin. ω-Dehydroisoleucine (III) and ω-dehydroalloisoleucine (IV) were isolated in this manner and recrystallized, and their configurations were confirmed by complete catalytic hydrogenation to the saturated amino acids and subsequent microbiological assays for isoleucine and alloisoleucine.

In a study of the biological properties of these unsaturated amino acids, the two diastereoisomer forms of 2-amino-3-methyl-4-pentenoic acid were found to be about equally effective in inhibiting the growth of *E. coli* in an inorganic salts-glucose medium, i.e., they were toxic at approximately 1 to 2 μg per ml. In contrast, the isomers of 2-amino-3-methyl-4-pentynoic acid are appreciably less effective as growth inhibitors; the *erythro* form prevents growth of *E. coli* at a concentration of approximately 10 μg per ml, and the *threo* form is toxic only at a level of 50 μg per ml.

The effects of supplements of isoleucine, valine, and a mixture of the two natural amino acids upon the amount of ω-dehydroisoleucine and ω-dehydroalloisoleucine necessary for inhibition of growth of *E. coli* were determined, and the results are indicated in Fig. 2. Although neither isoleucine nor valine alone reverses the toxicity of the diastereoisomers in a competitive manner, a mixture of isoleucine and valine do competitively reverse both ω-dehydroisoleucine and ω-dehydroalloisoleucine with inhibition indices (ratio of analogue to substrate just necessary for inhibition of growth) of approximately 2 over a 50-fold range in concentrations. Thus, it is apparent that either diastereoisomer is an antagonist of both isoleucine and valine. In the presence of high concentrations of isoleucine, the toxicities observed would be

\[ \text{C}_6\text{H}_{11}\text{NO}_3 \]

**Catalytic reduction of the product followed by microbial assays with *L. arabinosus* and *S. faecalis* indicated that the hydrogenated material was DL-alloisoleucine.**

**References**

1. E. D. Parker, C. G. Skinner, and W. Shive 3269

2. The stereochemical structures of the four products, I, II, III, and IV, were established through catalytic hydrogenation of the appropriate unsaturated analogue to either isoleucine or alloisoleucine. The α-amino and β-methyl group of isoleucine are reported to be in an *erythro* configuration (15, 16); and thus, the stereochemistry of the corresponding alloisoleucine analogues may be designated as *threo* configurations. These stereochemical conventions are used throughout this article for the corresponding unsaturated analogues.
primarily due to an inhibition of valine utilization; thus, as indicated in Fig. 2, the two isomers are about equally effective as valine antagonists. However, in the presence of high concentrations of valine, which would cause the isoleucine antagonism to be the primary effect, \( \omega \)-dehydroisoleucine is appreciably more inhibitory than \( \omega \)-dehydroalloisoleucine; this is an indication that the former analogue is the more effective isoleucine antagonist.

The isomeric 2-amino-3-methyl-4-pentynoic acids also require indices as determined with equal amounts of isoleucine and valine for a competitive reversal of growth inhibition of \( \text{E. coli} \) as indicated in Table I. The inhibition indices as determined with equal amounts of isoleucine and valine are approximately 10 for the \( \text{erythro} \) form and 20 for the \( \text{threo} \) isomer. Since isoleucine alone is more effective in reversing the
toxicity of either analogue than is valine, the primary effect appears to be that of isoleucine antagonism. As an isoleucine antagonist, the erythro isomer, with a configuration similar to that of isoleucine, is approximately 5 times as effective as the threo isomer, whose configuration corresponds to alloisoleucine. The erythro isomer is only approximately twice as effective as a valine antagonist, as indicated by the relative inhibitory activities in the presence of supplements of isoleucine.

For comparative purposes, the inhibitory properties of these four unsaturated diastereoisomers were studied with respect to their relative effects in antagonizing isoleucine and valine utilization in *Lactobacillus arabinosus* 17-5, an organism which requires both isoleucine and valine for growth (17). As indicated in Table II, \( \omega \)-dehydroisoleucine is an effective antagonist of isoleucine with an inhibition index of 2 at the lower concentrations and approximately 5 at the higher concentrations of isoleucine. This enhanced reversal by isoleucine at the higher concentration levels was also noted with all of the other 4,5-dehydrogenated isoleucine analogues in this organism. \( \omega \)-Dehydroalloisoleucine is only approximately one-third as effective as \( \omega \)-dehydroisoleucine in antagonizing isoleucine utilization, which suggests that some steric specificity exists for optimal enzymic binding. The acetylenic analogue of isoleucine, erythro-2-amino-3-methyl-4-pentynoic acid, is only approximately one-half as effective as an \( \omega \)-dehydroisoleucine in this organism, this is an indication that steric hindrance results from the restriction of the terminal carbon-carbon triple bond and the \( \beta \)-carbon to a linear position. Unless the presence of the triple bond in the molecule interferes with binding on the surface due to electrostatic effects. Changing the configuration on the \( \beta \)-carbon of this acetylenic derivative to form the isomeric threo-2-amino-3-methyl-4-pentynoic acid decreases the enzymic binding ability 10 fold (Table II). Although none of these changes in structure of the analogues prevent enzymic binding completely, the introduction of a threo configuration on the \( \beta \)-carbon in addition to the presence of the triple bond results in an appreciable decrease in binding ability with the enzymic site essential for isoleucine utilization.

The abilities of these four unsaturated analogues to antagonize valine utilization in *L. arabinosus* are compared with the antagonistic activities of isoleucine and alloisoleucine in Table II. The most effective antagonist is \( \omega \)-dehydroalloisoleucine, which inhibits valine utilization with an inhibition index of 5 to 10. \( \omega \)-Dehydroisoleucine is only approximately one-fourth to one-half as effective, which suggests that in this organism the enzymic site occupied by the isopropyl group of valine is not symmetrical. In addition, since both erythro configurations of the unsaturated amino acid analogues are equivalent in activity (and are less active than the corresponding threo derivatives in both instances), it appears that the site of attachment of the methyl group in valine corresponding in configuration to the methyl group of isoleucine is less sterically restricted than the other methyl grouping of valine. These comparative inhibitory effects are more apparent in the ethylenic analogues in which the angle between the ethylenic grouping and the \( \beta \)-carbon lies in between that for the corresponding angle in the saturated derivatives (isoleucine and alloisoleucine) and the 180° angle in the acetylenic analogues. The saturated analogues are appreciably less effective than any of the four unsaturated derivatives in competing with the enzymic site associated with valine utilization.

The relative biological activities of these analogues give evidence of the geometry of the enzymic site associated with the utilization of valine.

### SUMMARY

Both diastereoisomeric forms of 2-amino-3-methyl-4-pentynoic acid were obtained through a fractional recrystallization of the racemic amino acid mixture formed from alkaline hydrolysis of the condensation product from 2-bromo-3-butyne and ethyl formamidomalonate. Controlled catalytic hydrogenation of these isomers produced the corresponding ethylenic amino acids, \( \omega \)-dehydroisoleucine and \( \omega \)-dehydroalloisoleucine. With *Escherichia coli* 9723 and *Lactobacillus arabinosus* 17-5 as the assay organisms, the relative activities of these analogues as isoleucine and valine antagonists were determined.

### REFERENCES

Biological Specificities of 4,5-Dehydro Analogues of Isoleucine and Alloisoleucine
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