ANTIMETABOLITES PRODUCED BY MICROORGANISMS. VI[1] L-N^5-(1-IMINOETHYL) ORNITHINE

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A new antimetabolite of arginine and metabolically related compounds was isolated from a fermentation broth produced by an unclassified Streptomycete. The structure was shown to be L-N^5-(1-iminoethyl) ornithine and the compound was synthesized by reaction of the copper complex of L-ornithine with ethyl acetimidate. Mild base hydrolysis of the compound gave the expected products, L-ornithine and L-N^5-acetylornithine and, in addition, a rearranged product, L-N^2-acetylornithine. Arginase hydrolyzed the antimetabolite to L-ornithine.

In previous papers[1,2] a general discussion of the method employed in our search for antimetabolites in fermentation broths was presented. In this paper we wish to report the isolation of an antimetabolite of L-arginine which was produced by an unidentified streptomycete. Physical chemical characterization of the compound resulted in a structural proposal, L-N^5-(1-iminoethyl) ornithine, I*, which was confirmed by syntheses from both L-N^2-acetylornithine[3], V, and the copper complex of L-ornithine[4], III. The compound is a new chemical entity.

Experimental

I. General Methods

All melting points were taken with a Thomas Hoover capillary apparatus and are corrected. IR spectra were obtained from KBr discs with the Perkin-Elmer Model 621 spectrophotometer. NMR spectra were obtained in D_2O using the Varian A-60 instrument and optical rotations were measured with a Perkin-Elmer 141 polarimeter. The purity of preparations was determined using the Technicon amino acid analyzer and by thin-layer chromatography with Merck silica gel F-254 plates developed in ethanol - water - ammonia (49 : 49 : 2). Electrophoresis was performed at 200 V using a Gelman apparatus. Vapor phase chromatography was carried out with the F & M 402 equipment.

II. Microbiological Assay

A paper-disc agar-diffusion microbiological assay was used to detect and quantitatively measure I. Inoculum was prepared from *Escherichia coli* B grown overnight at 35°C on a rotary shaker in 500-ml Erlenmeyer flasks containing 100 ml of

* Roman numerals refer to the structures in Fig. 1.

medium composed of (in g/liter): Bacto-peptone (Difco), 6.0; N-Z Amine type A (Sheffield), 4.0; yeast autolyzate (National Yeast Products), 3.0; beef extract (Wilson), 1.5; and glucose, 1.0. The cells were washed three times with water to remove excess nutrients and adjusted to an optical density of 1.0 (wave length, 500 nm; path length, 15.5 mm); 5 ml of the washed cell suspension was added to one liter of liquefied minimal-agar of DAVIS and MINGIOLI[5] just prior to distribution. Five ml portions of this inoculated medium were pipetted into Petri dishes (100×15 mm). After the agar solidified, the dishes were stored at 4°C and used within a week. Samples to be assayed were applied to paper discs which were then placed on the agar. After incubation overnight at 35°C, the diameters of the zones of inhibition were measured; the zone diameters were found to be proportional to the log of the concentrations between 10 and 200 μg per ml of I. A two fold increase in the concentration of I increased the zone diameter by 3 mm.

III. Fermentation

Spores of an unidentified Streptomycete* were added to 6-liter Erlenmeyer flasks containing 2 liters of the inoculum medium described above. The flasks were incubated at 28°C for 96 hours on a rotary shaker (250 rpm with a 2 inch (5.08 cm) stroke). Six liters of inoculum was added to 227 liters of fermentation medium containing (in g/liter): Cerelose (Corn products), 11.0; Bacto-Yeast extract (Difco), 2.0; asparagine, 0.5; and K_2HPO_4, 0.5. The culture was incubated at 28°C in a 380-liter fermentor, aerated at 140 liters per minute and agitated at 180 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 70 hours of incubation the fermentation broth was filtered through infusorial earth by centrifugation.

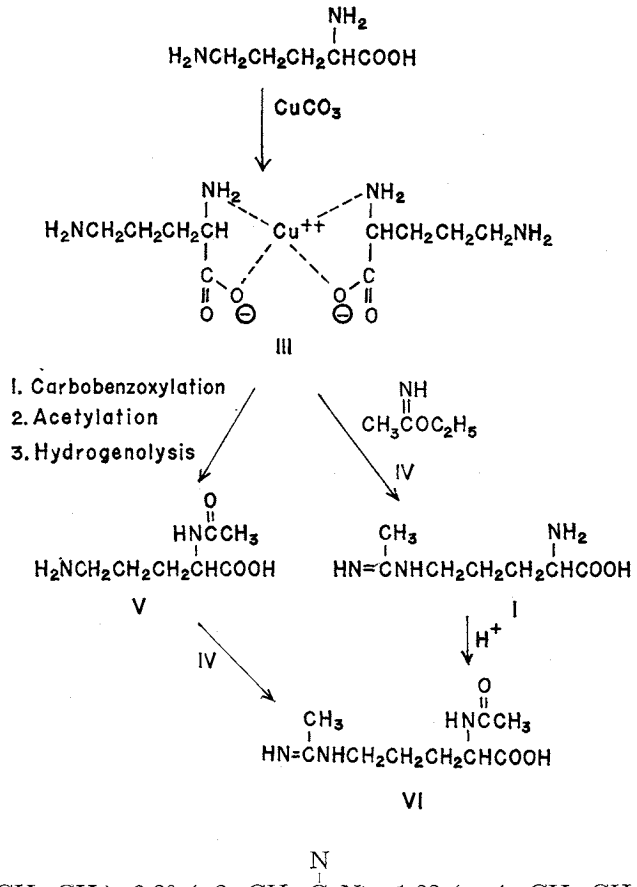
IV. Isolation Procedure

The clarified broth (200 liters) containing 7 g/liter solid and 8 mg/liter antimetabolite was applied immediately to 50-liter Dowexs 50WX4 resin, 50~100 mesh, in the H^+ form. (The activity rapidly disappeared from the clarified broth during storage, presumably due to arginase activity.) The column was successively washed with 80 liters distilled water, 120 liters 10% aqueous pyridine solution, and 80 liters distilled water. The activity was then eluted with 180 liters 1 M ammonium hydroxide solution. Active fractions were combined, concentrated at reduced pressure and lyophilized to give 9.8 g solids, >90% recovery, 138 fold purification.

Solids, 8.0 g, were dissolved in 40 ml distilled water and the pH was adjusted to 6.1 by the addition of 1 M citric acid. The solution was applied to a column, 56 cm ×5.3 cm inside diameter, containing 1,250 ml AG50WX8 resin, 200~400 mesh, in the Na^+ form. The column was eluted with 6 liters of a linear gradient of 0.3 M to 1.0 M NaCl in 0.15 sodium citrate-phosphate buffer[6], pH 6.1. Active fractions which appeared at an elution volume of 3.0~3.7 liters were combined and desalted by adsorption onto 1 liter AG50WX4 resin, 50~100 mesh, in the H^+ from. The column was successively washed with 500 ml distilled water, 3 liters 20% aqueous

* *Streptomyces* sp. #7-25 (HLR-586 A) kindly supplied by Prof. H. LECHEVALIER, Institute of Microbiology, Rutgers University, The State University of New Jersey.

Fig. 1. Synthesis of L-N^5-(1-iminoethyl)ornithine



pyridine solution and 500 ml distilled water. The activity was then eluted with 2 liters 1 M ammonium hydroxide solution. Active fractions were concentrated at reduced pressure and then lyophilized to give 1.2 g solids: recovery 84 %; purification 5.6-fold for the two step procedure. The hydrochloride of I, 910 mg, crystallized from a 6 % solution in 85 % aqueous ethanol after adjustment to pH 6.2 by addition of one molar equivalent of HCl: overall recovery 57 %; overall purification, 875-fold. Recrystallization from the same solvent yielded colorless needles: m.p. 226~229°C; pK_1 1.97, pK_2 8.86, pK_3 11.83; ir 1690 cm^{-1} (C=N); nmr (D_2O, 20 mg, int TMS), δ 3.86 (t 1, CH_2-$C\underline{H}(NH_2)COOH$), 3.39 (t 2, N-$C\underline{H}_2$-CH_2), 2.30 (s 3, $C\underline{H}_3$-C=N), 1.93 (m 4, CH_2-$C\underline{H}_2$-$C\underline{H}_2$-CH); $[\alpha]_D^{25}$ +20.6 (c 1, 5 N HCl).

Anal. calcd. for $C_7H_{16}ClN_3O_2$: C 40.10, H 7.69, N 20.04.
Found: C 40.11, H 7.55, N 19.85.

V. Synthesis of I from L-Ornithine, II

A solution of the copper complex of L-ornithine, III[4], (prepared from 40 g L-ornithine hydrochloride, and 15 g $CuCO_3$ in 800 ml H_2O) was adjusted with 2 N NaOH to pH 10.0. The pH was maintained at 9.5~10.0 by occasional addition of 2 N NaOH while adding 33 g ethylacetimidate hydrochloride[8], III, to the well-stirred solution. After 1 hour at 25°C, 2 N HCl was added to pH 7.5 and the solution kept for 16 hours at 25°C to ensure complete hydrolysis of the excess imidate, then poured onto 1 liter AG50WX4 resin (50~100 mesh in the H^+ form). The resin was washed with 500 ml distilled water, 2.5 liters 10 % aqueous pyridine solution and 1 liter distilled water and the activity then eluted with 2 liters 1 M ammonium hydroxide solution. After evaporation under reduced pressure to a small volume, the pH of the eluate was adjusted to 6.2 by addition of 2 N HCl and 37 g of crude I (74 % yield) crystallized as described above. This material contained about 5 % each of ornithine and a reaction by-product of unknown structure. These impurities were reduced to less

than 0.5% by displacement chromatography[7]. Crude I, 26 g, dissolved in 300 ml distilled water, was adsorbed onto a column 24×4.4 cm, which contained 350 ml AG50WX4 resin (200~400 mesh in the H^+ form). After washing with 150 ml distilled water, the column was eluted with 0.1 M ammonium hydroxide solution. As the pH of the eluate rose above 9.5, 25 ml fractions were collected and assayed by thin-layer chromatography. Both impurities were present chiefly in the first 3 fractions while the next 16 fractions contained most of the product. These latter fractions were evaporated under reduced pressure, and I crystallized as the hydrochloride as described above. The yield of recrystallized I was 20 g, 77%, m.p. 222~225°C; $[\alpha]_D$ +20.9 (*c* 1, 5 N HCl); ir and bioactivity were identical to that of isolated material. The product contained less than 0.5% impurities as determined by thin-layer chromatography.

VI. Synthesis of I from L-N^2-Acetyl Ornithine, V

V[8] was synthesized by acetylation of L-N^5-carbobenzoxyornithine followed by hydrogenolysis of the carbobenzoxy group. After several recrystallizations, chromatographically pure V was obtained; m.p. 216~218°C; $[\alpha]_D^{25}$ −8.1 (*c* 2, 5 N HCl).

Anal. calcd. for $C_7H_{14}N_2O_3$: C 48.27, H 8.10, N 16.08.
Found: C 48.43, H 8.42, N 16.29.

A 50-ml aqueous solution of 3.0 g of V and 2.4 g of IV[8] was maintained for 40 minutes at pH 10.0 by addition of 2 N NaOH solution. The pH was then adjusted to 6.5 with 2 N HCl and the reaction mixture maintained at 25°C for 16 hours. The solution was applied to 100 ml AG50WX4 resin (50~100 mesh in th H^+ form) and, after washing the resin with 300 ml distilled water, the product was eluted with 300 ml 10% aqueous pyridine solution. After solvent removal under reduced pressure, the product was crystallized with difficulty from boiling ethanol to give, in two crops, 650 mg VI (m.p. 243~245°C).

Anal. calcd. for $C_9H_{17}N_3O_3$: C 50.22, H 7.96, N 19.52.
Found: C 49.40, 49.61, H 8.0, 7.93, N 18.88, 18.91.

The analysis was not improved by recrystallization; however, VI was sufficiently pure for conversion to I.

Crude VI, 300 mg, was refluxed for 2 hours in 15 ml 2 N HCl. After solvent removal at reduced pressure, the residue was redissolved in 10 ml distilled water and applied to 10 ml AG50WX4 resin (50~100 mesh in the H^+ form). The resin was washed with 100 ml distilled water and 100 ml 10% aqueous pyridine solution after which the product was eluted with 100 ml 1 M ammonium hydroxide solution. After partial removal of solvent under reduced pressure, the pH of the solution was adjusted to 6.2 with 2 N HCl and, after removal of additional solvent under reduced pressure, crystallization from 90% aqueous ethanol gave 211 mg I (yield 78%). After recrystallization the product melted at 228~230°C; $[\alpha]_D^{25}$ +19.9 (*c* 1, 5 N HCl). Bioactivity and ir were identical to the isolated material.

Anal. calcd. for $C_7H_{16}ClN_3O_2$: C 40.10, H 7.69, N 20.04.
Found: C 39.80, H 7.88, N 19.74.

VII. Base Hydrolysis of I

A solution of 1 g I and 500 mg KOH in 50 ml water was maintained at 90°C for

10 minutes. After rapid cooling, the solution was poured onto a column containing 20 ml AG50WX4 resin (50~100 mesh in the H^+ form). After washing with water, the column was eluted succcessively with aqueous pyridine and 1 M ammonium hydroxide solution. The pyridine eluate was concentrated at reduced pressure to yield 160 mg residue. Thin-layer chromatography revealed the presence of two compounds with Rf values identical to those of N^2-acetyl ornithine and N^5-acetyl ornithine. VPC analysis of the trimethylsilyl derivatives indicated that the ratio of acetyl ornithine isomers was $N^2/N^5=1/4$. It was possible to separate the two isomers by crystallization from aqueous ethanol; L-N^5-acetylornithine crystallized first as colorless needles [107 mg; m.p. 235°C; $[\alpha]_D^{25}$ +23.4 (*c* 1, 5 N HCl) (Lit[8] +24.0]. The ir was identical with that of authentic material.

Anal. calcd. for $C_7H_{14}N_2O_3$: C 48.26, H 8.10, N 16.08.
Found: C 48.51, H 8.43, N 16.27.

L-N^2-acetylornithine was crystallized from the mother liquor, [20 mg; m.p. 219°C; $[\alpha]_D^{25}$ −8.1 (*c* 1, 5 N HCl) (Lit[8] −7.5)]. The ir was identical with that of authentic material.

Anal.: C 48.00, H 8.20, N 16.08.

The ammonium hydroxide eluate from the column described above was concentrated at reduced pressure to yield 600 mg residue. L-Ornithine (485 mg) was crystallized as the hydrochloride from aqueous ethanol [m.p. 236°C (dec.), $[\alpha]_D^{25}$ +22.5 (*c* 1, 5 N HCl) (Lit[9] +22.3)]. The ir was identical with that of authentic material.

Anal. calcd. for $C_5H_{13}ClN_2O_2$: C 35.61, H 7.77, N 16.60.
Found: C 35.34, H 7.78, N 16.55.

VIII. Enzymatic Hydrolysis of I

A solution of 1 mg arginase (PL Biochemicals) in 0.5 ml 0.05 M manganese (II) maleate buffer, pH 7.0, after preincubation for 4 hours at 37°C, was added to a solution of 115 mg I in 5 ml water. The pH was adjusted to 9.0 with 1 N NaOH and the temperature was maintained at 37°C. After 24 hours another 0.5 ml preincubated enzyme solution was added and after an additional 24 hours the reaction had gone to completion. L-Ornithine was isolated from the enzyme digest by the cation exchange method described in the preceding section and the hydrochloride was crystallized from aqueous ethanol [m.p. 238°C (dec.); $[\alpha]_D^{25}$ +22.5 (*c* 1, 5 N HCl)]. The ir was identical with that of authentic material.

Anal: C 35.36, H 7.78, N 16.36.

Discussion

The NMR spectrum of I was almost identical to that of arginine except that the antimetabolite had an additional peak at δ 2.30 which was attributed to an uncoupled methyl group. This plus the elemental analysis, $C_7H_{15}N_3O_2 \cdot HCl$, and the fact that the compound was hydrolyzed by either base or arginase to L-ornithine led to the structural assignment L-N^5-(1-iminoethyl) ornithine. The synthesis of I was accomplished by reacting either L-N^2-acetyl-ornithine or the copper complex of L-ornithine with ethyl acetimidate. This reaction took place in aqueous solution without serious competing hydrolysis of the ethyl acetimidate[10].

Since aminolysis of amidines is a well known reaction[11], we may presume the

formation of L-N^2-acetyl ornithine during basic hydrolysis to be due to intramolecular aminolysis. A control experiment showed that L-N^5-acetyl ornithine does not rearrange to L-N^2 acetyl ornithine under these conditions.

By the use of the agar-diffusion method previously described[1], the inhibitory activity of **I** against *E. coli* was found to be reversed by L-arginine, L-ornithine, L-citrulline and L-N^2-acetyl-ornithine; no reversal was found with L-glutamic acid or L-N-acetylglutamic acid. These facts suggest that **I** interferes with one or more of the intermediate steps in arginine biosynthesis, *viz.* those catalyzed by N-acetyl-α-glutamokinase, N-acetylglutamate semialdehyde dehydrogenase, and N-acetylornithine δ-aminotransferase.

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