ANTIMETABOLITES PRODUCED BY MICROORGANISMS. II[1]

L-2-AMINO-4-PENTYNOIC ACID

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L-2-Amino-4-pentynoic acid was isolated from a streptomycete fermentation and shown to inhibit the growth of *Bacillus subtilis* in a chemically defined medium. This growth inhibition was reversed by L-methionine or L-leucine.

Recently several laboratories[2~11] have been interested in the search for antimetabolites in fermentation broths. Such compouds are, ordinarily, not observed in conventional antibiotic screens due to the presence of reversing metabolites in the complex media commonly employed. However, by the use of chemically defined minimal media[12] it is possible to detect antimetabolite activities and, by selectively supplementing the assay medium with known substances, to determine which compounds relieve the growth inhibition.

In this paper we report the isolation of an antimetabolite of L-methionine and L-leucine from a fermentation broth produced by an unidentified streptomycete*. The substance which has not been reported in natural sources proved to be L-2-amino-4-pentynoic acid identical with an authentic sample prepared from synthetic racemic material[13] by enzymatic resolution.

Experimental

I. General Methods

All melting points were taken with a Thomas Hoover capillary apparatus and are corrected. IR spectra were obtained in KBr discs with either the Beckman IR-9 or the Perkin-Elmer Model 621 spectrophotometer. NMR spectra were obtained in D_2O using the Varian HA-100 instrument. Ultraviolet spectra were determined with a Beckman DB-G spectrophotometer, mass spectra with a GEC 21-110 mass spectrometer using a direct introduction probe, and optical rotations were taken with a Perkin-Elmer 141 polarimeter. The purity of preparations was checked using the Technicon amino acid analyzer and by means of thin-layer chromatography with Merck cellulose F plates using as developing solvent, butanol - acetic acid - water (12 : 3 : 5). Electrophoresis was carried out at 200 V using the Gelman apparatus. The F & M Model 402 equipment was used for vapor phase chromatography.

* *Streptomyces* sp. # 8-4 (HLR-599A), isolated 1966 from a garden soil of New Brunswick, N. J., kindly supplied by Prof. H. LECHEVALIER, Institute of Microbiology, Rutgers University, The State University of New Jersey.

II. Microbiological Assay

A paper-disc agar-diffusion assay was used to detect the antimetabolite. The chemically-defined minimal-medium of DAVIS and MINGIOLI[14] was used for the bioassay. Five ml portions of inoculated liquefied agar were pipetted into 100×15 mm Petri dishes. After the agar solidified, the dishes were stored at 4°C. For the bioassay of chromatograms, 35.6×16.5 cm pans containing 150 ml of agar were prepared. Dishes and pans were stored for no longer than one week in the cold. Inoculum was prepared with *Bacillus subtilis* NRLL-558 cells grown overnight at 35°C on a rotary shaker (2-inch (5-cm) stroke, 250 r.p.m.) in 500 ml Erlenmeyer flasks containing 100 ml of the inoculum medium described below. Cells were washed three times with water to remove excess nutrients and adjusted to an optical density of 1.2 (wave length, 500 nm; path length, 15.5 mm); 30 ml of the washed cell suspension was added per liter of liquefied agar just prior to distribution.

A paper disc (Schleicher and Schuell, 12.7-mm diameter) was saturated with a solution of metabolite, either L-methionine or L-leucine at 0.1 g/liter, and placed on an assay plate. After thirty minutes, a second disc saturated with sample solution was placed near the first disc (one inch center to center). A third disc was then saturated with sample and placed alone on a separate assay plate. Both plates were incubated at 35°C overnight. A zone of inhibition was present around the disc on the plate without metabolite solution and a crescent-shaped zone was present on the plate with metabolite. Quantitative bioassays were also carried out since the diameter of the zone of inhibition was found to be proportional to the log of the antimetabolite concentration between 1 and 30 μg/ml.

III. Fermentation

Spores of an unidentified streptomycete* were added to 6-liter Erlenmeyer flasks containing 2 liters of inoculum medium composed of (in g/liter): Bacto-peptone (Difco), 6; N-Z-Amine type A (Sheffield), 4; yeast autolyzate (National Yeast Products), 3; beef extract (Wilson), 1.5; and glucose, 1.0. The flasks were incubated at 28°C for 96 hours on a rotary shaker (220 r.p.m. with a 2-inch (5-cm) stroke). Four liters of inoculum were added to 300 liters of fermentation medium containing (in g/liter): NaCl, 13; Cerelose (Corn Products) 11; $MgSO_4 \cdot 7H_2O$, 6.35; Bacto-peptone (Difco), 5.0; Bacto-yeast extract (Difco), 3.0; KCl, 0.72; $CaCl_2$, 0.50; $FeSO_4 \cdot 6H_2O$, 0.25. The culture was incubated at 28°C in a 300-liter fermentor, aerated at 170 liters/min. and agitated at 200 r.p.m. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 68 hours of incubation the fermentation broth was filtered through infusorial earth by centrifugation.

IV. Preliminary Characterization

The antimetabolite activity in the filtered broth was shown to be stable at pH values 2, 7 and 10 for one hour at 50°C. It was not adsorbed onto charcoal nor extracted into organic solvents. During electrophoresis at pH 3 and 10 it moved as a cation and anion respectively. Thus the good pH stability and ionic nature of the compound permitted full use of ion-exchange resins.

V. Isolation Procedure

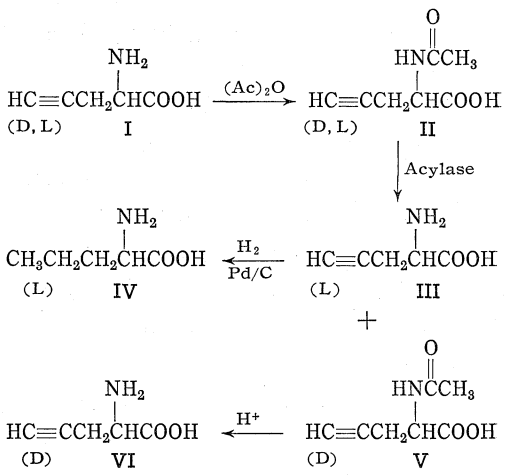
The filtered broth (180 liters) containing 25 g/liter solids and 9.7 mg/liter antimetabolite was treated with 10 g/liter of Darco G-60. After filtration with Celite, the filtrate and wash contained 100 % of the activity.

The filtrate was passed through a 12 inch diameter column containing 50 liter Dowex 50W×4 resin (50~100 mesh, in the hydrogen ion form). The activity was then eluted from the resin with 5 % aqueous pyridine solution. From 75 liters of eluate, 197 g of solids were obtained after concentration and lyophilization. Recovery, 90 %;

purification, 20 fold.

A column, 6 cm in diameter, filled to a height of 60 cm with 2 liters of AG 1×8 (200~400 mesh anion-exchange resin in the bicarbonate form), was charged with 60 g of solids obtained above, dissolved in 400 ml water and adjusted to pH 8.9 with 25 % trimethylamine solution. The resin was eluted with 0.2 M trimethylammonium bicarbonate buffer[15]* adjusted to pH 9.3 with 25 % trimethylamine solution. The activity was present in 2 liters of eluate after 13 liters had passed through the column. After evaporation at reduced pressure, 2.6 g of solids were obtained. Recovery, 85 %; purification, 20 fold.

Fig. 1. Resolution and reactions of 2-amino-4-pentynoic acid



Cation-exchange chromatography was then carried out by dissolving 2.0 g of the solids in 15 ml of distilled water and applying the solution onto 750 ml AG 50W×4, (50~100 mesh, hydrogen ion form; colum dimensions, 42 cm×4.8 cm). After washing the material onto the resin with 100 ml distilled water the column was eluted with 1 N aqueous HCl. After 2.05 liters of eluate had been collected, the active material was eluted in 370 ml. This eluate was evaporated to dryness and the residue was taken up in 10 ml distilled water and applied to 15 ml AG 50W×8 (50~100 mesh cation exchange resin in the hydrogen ion form). After washing with 50 ml distilled water, the resin was eluted with 10 % aqueous pyridine solution. The pyridine eluate was evaporated to dryness and the residue, 296 mg, (recovery 85 %, purification 5.8 fold) was crystallized from aqueous ethanol (yield, 159 mg; recovery, 70 %; 1.1 fold). (Overall recovery 45 %, overall purification 3,000 fold). After recrystallization from aqueous ethanol, colorless needles of L-2-amino-4-pentynoic acid, **III** (Fig. 1) were obtained, m.p. 241~242° (dec.). IR (KBr): 3300 (H-C≡), 1600 (carboxylate C=O) cm^{-1}; NMR (D_2O, 8 mg ext TMS) δ=4.34 (t, 1, J=5.5 Hz, CH_2-C<u>H</u>-), 3.28 (d of d, 2, J=5.5 and 2.5 Hz, ≡C-C<u>H_2</u>-), 2.97 (t, 1, J=2.5 Hz, <u>H</u>-C≡); mass spectrum, m/e at 74, 68, 41 and 39. $[\alpha]_D^{25}$ −31.1° (*c* 1, H_2O); −5.5° (*c* 1, 5 N HCl).

Anal. calcd for $C_5H_7NO_2$: C 53.09, H 6.24, N 12.38.
Found: C 53.02, H 6.46, N 12.40.

VI. Reduction of **III** to L-norvaline, **IV**

A solution of 2 mg of **III** in 0.2 ml H_2O and 1 ml ethanol containing 2 mg 5 % Pd on charcoal slurried in 10 ml ethanol was reduced in the Brown[2] hydrogenator. After 45 minutes of vigorous stirring in the hydrogen atmosphere, the reaction was terminated and the reduction product was separated from the catalyst by filtration. Thin layer chromatography on cellulose with ethanol-wather (4:1) developing solvent indicated that **III** had been converted entirely to a compound with the same Rf as

* This exceptionally volatile buffer is completely removed by a single evaporation at 40°C and 25 mm.

norvaline. Vapor phase chromatography of the N-trifluoroacetylprolyl derivatives[16] of the methyl esters of both the reduction product and an authentic sample of L-norvaline showed that both had the same retention times, thus indicating an L-configuration.

VII. Resolution of D, L-2-Amino-4-Pentynoic Acid, I

A. Acetylation of I to II: To a suspension of 0.09 mole (10.2 g) I[13] in 620 ml boiling acetic acid, 13.6 g acetic anhydride was added. After 10 minutes at reflux the solution was allowed to cool to 25°C and evaporated at reduced pressure to an oil. After addition and evaporation of 3×10 ml distilled water, the residue was crystallized from acetone to yield 10.9 g of N-acetyl-D, L-2-amino-4-pentynoic acid (80 %, m.p. 137 ~139°C). IR (KBr): 3330 (amide NH); 3300 (H-C≡H): 1745 (carboxylic C=O); 1620 (amide C=O) cm^{-1}.

Anal. calcd for $C_7H_9NO_3$: C 54.19, H 5.85, N 9.03.
Found: C 54.38, H 6.06, N 9.08.

B. Enzymatic resolution of II: The pH of a solution of 10 g of II in 550 ml deionized water was adjusted to 7.7 with 2 N lithium hydroxide. Hog kidney acylase* 80 mg, 4,400 units, dissolved in 20 ml deionized water was added to the substrate solution and the reaction was followed by quantitative ninhydrin assay. After 4 hours at 37°C, the reaction was 94 % complete and incubation was continued an additional 12 hours.

C. Isolation of V: The enzymatic digest solution was concentrated to 180 ml and passed through 300 ml of a cation-exchange resin, AG 50W×8 (50~100 mesh in the hydrogen ion form) in a column, 6.0-cm diameter, filled to a height of 10 cm. The effluent and 2 liters of distilled water wash of the column were combined and concentrated to a thick syrup which was taken up in acetone. Upon cooling 2.45 g of V crystallized. An additional 0.7 g of material was obtained by concentrating the mother liquors (total yield, 62 %). Recrystallization from a mixture of acetone and petroleum ether (B.R. 30~60), gave pure N-acetyl-D-2-amino-4-pentynoic acid (m.p. 126~127°C). IR (KBr): essentially the same as that for II.

Anal. calcd for $C_7H_9NO_3$: C 54.19, H 5.85, N 9.03.
Found: C 54.38, H 5.82, N 9.00.

$[\alpha]_D^{25}$ −26.1° (*c* 1, H_2O); −37.8 (*c* 1, 5 N HCl).

D. Isolation of III: The column, after elution of V, was then eluted with 10 % aqueous pyridine solution and fractions containing significant amounts of ninhydrin positive material were combined and concentrated. Crystalline III (2.72 g, 75 % yield) was obtained from aqueous ethanol. Recrystallization gave pure L-2-amino-4-pentynoic acid, m.p. 248~249°C (dec.). IR (KBr): the same as the material isolated from the fermentation broth. $[\alpha]_D^{25}$ −32.6° (*c* 1, H_2O); −5.0° (*c* 1, 5 N HCl).

Anal. calcd for $C_5H_7NO_2$: C 53.09, H 6.24, N 12.38.
Found: C 53.17, H 6.18, N 12.53.

E. Hydrolysis of V to VI: A solution of 2.4 g of V in 30 ml 2 N HCl was refluxed for 2 hours. After evaporation at reduced pressure and addition and evapora-

* Hog kidney acylase. Purchased from Mann Research Laboratories, New York, N. Y.

tion of 20 ml distilled water 3 times, the residue was taken up in water and the pH adjusted to 6.0 with NH_4OH. Solvent was removed at reduced pressure and the residue crystallized from aqueous ethanol to give D-2-amino-4-pentynoic acid (0.91 g, 52 %). Additional crystalline material was obtained from mother liquors but extensive work up was required to remove the last traces of ammonium chloride from the product. Twice recrystallized material melted at 245~248°C (dec.). The IR was essentially the same as that for I. $[\alpha]_D^{25}$ +32.9° (*c* 1, H_2O); +6.0° (*c* 1, 5 N HCl).

Anal. calcd for $C_5H_7NO_2$: C 53.09, H 6.24, N 12.38.
Found: C 52.81, H 6.23, N 12.26.

Discussion

The physical chemical properties of III, in particular the nuclear magnetic resonance spectrum permitted the structural proposal, 2-amino-4-pentynoic acid. The infrared spectrum showed the compound to be an α amino acid and a sharp band at 3300 cm^{-1} indicated the probable presence of a terminal acetylene group. The four non-exchangeable protons in the nmr spectrum could be assigned as follows: $HC{\equiv}CCH_2CH$, with long distance coupling of the methylene protons to the acetylenic proton. The structure was strongly supported by the fragmentation pattern found in mass spectrometry which contained, in addition to the decarboxylation fragment, other fragments consistent with cleavage between the α and β carbon atoms. Vapor phase chromatography analysis of the methyl ester of the trifluoroacetylprolyl derivative indicated an L-configuration. Further confirmation was obtained by reduction to L-norvaline.

Finally the synthetic racemic mixture[13], was resolved enzymatically after conversion to the N-acetyl derivative and the L-isomer was shown to be the compound obtained by reduction of the antimetabolite by comparison of the optical rotations.

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