ANTIMETABOLITES PRODUCED BY MICROORGANISMS. X[1]

L-2-AMINO-4-(2-AMINOETHOXY)-*TRANS*-3-BUTENOIC ACID

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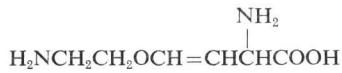
L-2-Amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid was isolated from a fermentation broth of an unidentified species of *Streptomyces*, X-11,085. This substance inhibited the growth of *Streptomyces cellulosae* in a chemically defined minimal medium. The growth inhibition was reversed noncompetitively by L-cysteine and competitively by L-lysine, L-arginine and L-ornithine.

An amino acid antimetabolite which inhibited the growth of *Streptomyces cellulosae* ATCC-3313* on a chemically defined medium[2] was discovered in a fermentation broth of an unidentified *Streptomyces* sp. X-11,085 (deposited with the Northern Regional Research Laboratories under their number NRRL-5331). The growth inhibition was relieved by addition of either L-cysteine, L-arginine, L-lysine or L-ornithine to the medium. The antimetabolite was isolated by a combination of chromatographic techniques. Physical chemical characterization of the pure substance led to the structural proposal, L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid (**I**).



(**I**)

This compound is the subject of U.S. Patent 3,751,459.

Experimental

I. General Methods and Materials

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 Jeolco C-60H instrument and optical rotations were measured with a Perkin-Elmer 141 polarimeter. The purity of preparations was determined 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 and pH 3.2 using a Gelman apparatus.

II. Microbiological Assay

Quantitation of the antimetabolite was achieved by a paper-disc agar diffusion assay employing *Bacillus subtilis* NRRL-558 in the minimal agar medium of DAVIS and MINGIOLI[2] as described previously[3]. The diameter of the inhibition zone was proportional to the log of antimetabolite concentration within the range 5~300 μg/ml. A two-fold increase in the concentration of I

* Also referred to as *Streptomyces griseus*, ATCC-3313.

increased the zone diameter by 4 mm and 20 μg of **I** per ml produced a zone of inhibition about 33 mm in diameter.

III. Fermentation

Spores of *Streptomyces* sp. X-11,085 were inoculated into 6 liter Erlenmeyer flasks containing 2 liters of Trypticase soy broth (Baltimore Biological Laboratories). The flasks were incubated at 28°C for 72 hours on a rotary shaker (240 rpm with a 2-inch stroke). Four liters of this inoculum were then added to 227 liters of fermentation medium containing (in g/liter): Cerelose (Corn Products), 10.0; Bacto-peptone (Difco), 5.0; Bacto-yeast extract (Difco), 3.0; and ferrous ammonium sulfate hexahydrate, 0.03. The pH was adjusted to 6.8 with sodium hydroxide before sterilization. The culture was incubated at 28°C in a 380-liter fermentor, aerated at 85 liters per minute and agitated at 200 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 41 hours the fermentation was filtered by centrifugation through infusorial earth.

IV. Isolation Procedure

The filtered broth from two fermentations, 423 liters, which contained 3.1 kg solids and about 6 g (**I**), was passed through a 12″-diameter column which contained 50 liters Dowex 50 WX4 resin (50~100 mesh in the H^+ form). The resin was successively washed with: 1) 50 liters H_2O; 2) 200 liters 5% aqueous pyridine; 3) 50 liters H_2O. The activity was then eluted with 200 liters 1.0 N NH_4OH. Virtually all of the activity was found in the first 80 liters of eluate obtained after the pH of the eluate rose above 9.0. The active fractions were evaporated at reduced pressure to a 2-liter concentrate which contained 64 g solids. The pH of the concentrate was adjusted to 3.3 with 5 N HCl as 140 g Darco G-60 charcoal was suspended in the concentrate. The suspension was then filtered through a sintered glass funnel containing a Hyflo pad on top of which was layered an additional 70 g charcoal. Essentially all of the activity was contained in a combined volume of 5 liters of filtrate and water wash of the filter cake. After evaporation at reduced pressure to 300 ml, the pH of the concentrate was adjusted to 8.5 by addition of concentrated ammonium hydroxide, then ethanol was added until the solution became slightly turbid (final volume 800 ml). Four such preparations were combined and applied to a column, 57 cm×21 cm i.d., which contained 11 kg Merck silica gel, 0.05~0.2 mm, previously slurried in a solvent consisting of ethanol-H_2O-NH_4OH (75 : 25 : 2). The column was then developed with the same solvent and the activity was found in a 17-liter fraction which appeared after 50 liters of solvent had passed through the column. The active fraction was evaporated at reduced pressure to 100 ml, the pH adjusted to 3.5 with 6 N HCl, and the concentrate applied to a column, 50 cm×2.5 cm i.d., which contained 250 ml Bio-Rad AG 50 WX 4 resin (200~400 mesh in the H^+ form). After washing with water, the resin was eluted with 0.3 M NH_4OH. The active fraction was obtained at an elution volume of 900~1,100 ml. The pH was adjusted to 3.5 with 6 N HCl, the solution concentrated to a syrup, and crystallization was effected from 200 ml methanol-water (19 : 1) to yield 8.7 g of **I**: mp 193~195°C. A second crop, 1.1 g, was crystallized from 10 ml methanol-H_2O (49 : 1). The overall purification was 500-fold and the overall recovery was 40%; ir (KBr) 1650 (COO^-); 1205 cm^{-1} (O—C=C); nmr (20 mg, D_2O, ext TMS); δ 7.25 (d, 1, J=13 Hz, O—C<u>H</u>=CH); 5.44 (d of d, 1, J=10 and 13 Hz, CH=C<u>H</u>—CH);

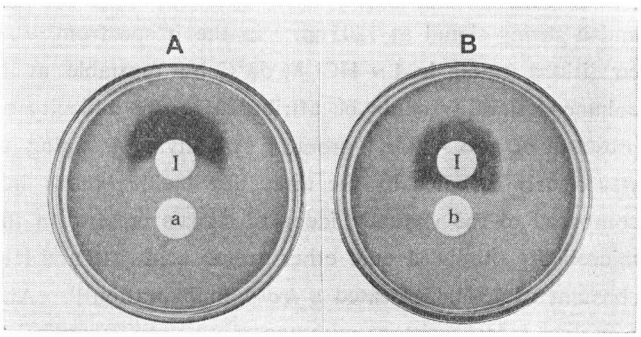
NH_2
|
4.63 (d, 1, J=10 Hz, CH—CH—COOH); 4.52 (t, 2, J=5 Hz, CH_2—CH_2—O); 3.77 (t, 2, J=5 Hz, N—CH_2—CH_2); $[\alpha]_D^{25}$+89.2 (*c* 1, 0.1 M sodium phosphate buffer pH 7); +111.8 (*c* 1, 5 N HCl). Anal. calcd. for $C_6H_{12}N_2O_3 \cdot HCl$: C, 36.65; H, 6.66; N, 14.24. Found: C, 36.50; H, 6.76; N, 13.99.

V. Biological Activity

In a chemically defined medium[2], **I** showed moderate antimicrobial activity against three species of *Bacilli* and *Streptomyces cellulosae* ATCC-3313. Little or no activity was observed against *Escherichia coli*, *Pseudomonas ovalis*, *Corynebacterium glutamicum*, *Candida albicans*, and *Pullularia pullulans*.

The antimicrobial activity of **I** against *S. cellulosae* was found to be reversed by L-cysteine, L-lysine, L-ornithine, and L-arginine. Two general types of reversal were observed and examples are shown in Fig. 1. With cysteine (Fig. IA), a crescent shaped zone of inhibition formed about the disc containing **I**. All points on the boundary between the area of inhibition and the area of reversal are equidistant from the center of disc a from which the reversant diffused. At that distance the concentration of reversant is sufficient to supply the minimum requirement for growth and the concentration of antimetabolite which varies along the boundary is not a factor. This is indicative of noncompetitive reversal. However, with L-lysine (Fig. IB) at every point on the straight line boundary between the area of inhibition and the area of reversal, the ratio of inhibitor to reversant is constant. This is indicative of competitive reversal.* L-Arginine and L-ornithine gave similar results to L-lysine in the counter diffusion test, other amino acids including L-methionine, D,L-homocysteine, L-cystathionine, L-homoserine, and L-serine did not reverse the inhibition against *S. cellulosae*. Neither did the common nucleosides, water-soluble vitamins, or sulfhydryl compounds, dithiothreitol and glutathione.

Fig. 1. Reversal of antimicrobial activity



The photographs show the results of a counter-diffusion antimetabolite bioassay. Petri dishes (100×15 mm) contained 7.5 ml of minimal agar[2] inoculated with *Streptomyces cellulosae* ATCC-3313. Paper discs labeled **I**, a and b contained 12 μg of **I**, L-cysteine and L-lysine, respectively. Photographs were taken after overnight incubation at 37°C.

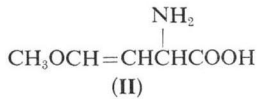
These data are consistent with the theory that **I** is taken up by *S. cellulosae* via a basic amino acid transport system and once inside the cell, blocks the biosynthesis of L-cysteine.

The antimicrobial activity of **I** against *Bacillus subtilis* NRRL-558 was competitively reversed by L-lysine but was only partially reversed by L-cysteine. L-Arginine and L-ornithine did not reverse the activity of **I** against *B. subtilis*.

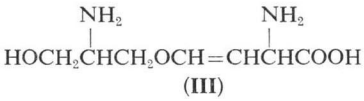
Dr. M. LIEBERMAN at the Post Harvest Plant Physiology Laboratory, United States Depart-

* A similar method distinguishing between competitive and noncompetitive reversal has been previously described by ZÄHNER *et al.*[4,5]

ment of Agriculture, Agricultural Research Service, Beltsville, Maryland has informed us that **I** and the structurally related antimetabolite, **II**[6] inhibit the production of ethylene by plant tissues. This effect is similar to that previously reported[7] for rhizobitoxine (**III**).[8]



(II)



(III)

Discussion

The method of purification, the ir spectrum, the elemental analysis and the electrophoretic mobility (similar to that of lysine) all indicated that **I** was a diamino acid. Additional unsaturation in the molecule was apparent from the elemental analysis, and the unusual orange reaction product with ninhydrin[6,9]. An enol ether was suspected on the basis of rapid iodine uptake[6,10] and a strong signal at 1205 cm^{-1} in the ir spectrum[6,11]. Although **I** was more stable to acid conditions (stable in 1 N HCl at 50°C but unstable at 100°C) than most enol ethers[6,12], the enhanced stability could be attributed to the difficulty of protonating the ether oxygen in the presence of two $-NH_3^+$ groups. An allylic grouping with electron withdrawing substituents was clearly revealed by the three downfield protons in the nmr spectrum particularly when compared to the virtually identical signals present in the spectra of the two already known microbially produced enol ether amino acids, **II**[6] and **III**[8]. As with these substances, a coupling constant of 13 Hz indicated a *trans* configuration[13]. An L-configuration at the optically active α carbon was consistent with the more positive optical rotation in acid solution[14]. The nmr spectrum indicated the presence of an aminoethoxy group in the molecule. Corroboration of the structure of **I** by a synthetic approach will be the subject of a subsequent publication.

Acknowledgements

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