ANTIMETABOLITES PRODUCED BY MICROORGANISMS. VII[1]*

L-(N^5-PHOSPHONO)METHIONINE-S-SULFOXIMINYL-L-ALANYL-L-ALANINE

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A new antibiotic was isolated from the fermentation broth of an unclassified species of *Streptomyces*. The antibiotic activity against *Serratia* sp. and *Bacillus subtilis*, grown in a chemically defined minimal medium, was reversed by the addition of L-glutamine to the medium. Physical chemical characterization of the antibiotic and of the chemical and enzymatic degradation products revealed the structure to be L-(N^5-phosphono)methionine-S-sulfoximinyl-L-alanyl-L-alanine.

The deliberate search[2~4] for antimetabolites in fermentation broths has, in three instances[5~7], resulted in the isolation of biologically active di- and tripeptides. We now wish to report the occurrence and isolation of a fourth member of this group, L-(N^5-phosphono)methionine-S-sulfoximinyl-L-alanyl-L-alanine, **I**.** This substance is produced by an unclassified *Streptomyces* sp. X-13152. It inhibits the growth of *Serratia* sp. and *Bacillus subtilis* on a chemically defined minimal medium[8] but addition of L-glutamine to the medium relieves this growth inhibition.

Originally[9] the antimetabolite was isolated by an elaborate procedure from broths containing concentrations of about 1 μg/ml. However, a medium improvement and strain selection program increased the fermentation yield over 10-fold and the isolation procedure reported here is suitable only for the high yield broths.

The details of the structure elucidation are being reported separately[9] and hence are only summarized here.

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 and HA-100 instruments 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 Cellulose F plates developed in n-butanol-acetic acid-water (12:3:5). Vapor phase chromatography was carried out with the F & M 402 equipment.

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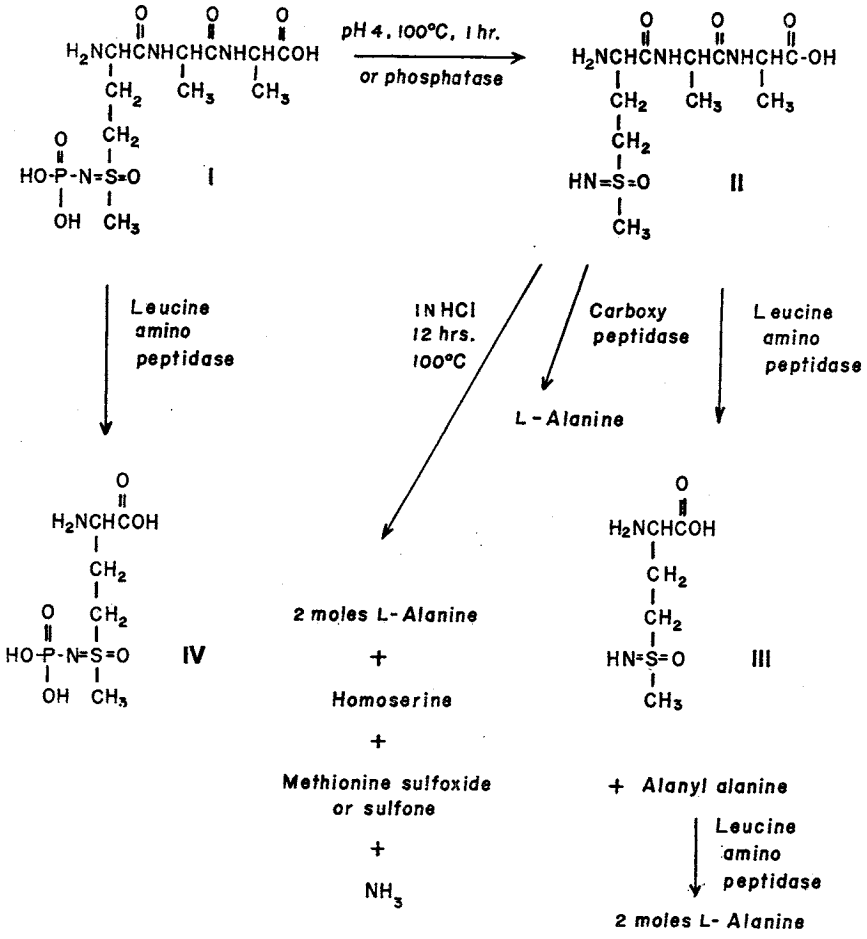
** Roman numerals refer to structures in Fig. 1.

Fig. 1. Degradation of L- (N^5-phosphono) methionine-S-sulfoximinyl-L-alanyl-L-alanine



II. Microbiological Assay

A paper-disc agar-diffusion microbiological assay was used to detect and quantitatively measure **I**. Inoculum was prepared from *Serratia* sp. 101 grown overnight at 35°C on a rotary shaker in 500-ml Erlenmeyer flasks containing 100 ml of 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); 10 ml of the washed cell suspension was added to one liter of liquefied minimal-agar of DAVIS and MINGIOLI[8] 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 diameter was found to be proportional to the log of the concentration of **I** between 2 and 50 μg per ml. A two-fold increase in the concentration of **I** increased the zone diameter by 3 mm. Four μg of **I** per ml produced an inhibitory zone about 30 mm in diameter.

III. Fermentation

Spores of an unclassified species of *Streptomyces* X-13152, strain 33, were added to a 500-ml Erlenmeyer flask containing 100 ml of inoculum medium containing (in g/liter): tomato pomace (Seaboard Supply), 5; dried distiller's solubles (Soludri, Schenley), 5; cottonseed flour (Proflo, Trader's), 5; protopeptone #366 (Wilson), 5; cornstarch (Anheuser Busch), 10; $CaCO_3$, 1; and K_2HPO_4, 1. The flask was incubated at 28°C for 72 hours on a rotary shaker [250 rpm with a 2 inch (5.08 cm) stroke]. Five ml portions of the resulting inoculum were added to 6 liter Erlenmeyer flasks containing 2 liters of the same medium. Incubation was carried out for 84 hours as described above. Four liters of the resulting inoculum was added to 227 liters of fermentation medium containing (in g/liter): K_2HPO_4, 7; KH_2PO_4, 3; sodium citrate dihydrate, 0.5; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.1; and D-glucose, 2 (autoclaved separately). The culture was incubated at 28°C in a 380-liter fermentor, aerated at 85 liters per minute and agitated at 260 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 36 hours the fermentation broth was filtered through infusorial earth by centrifugation. Two fermentations were run concurrently and the clarified broths pooled.

Production of **I** was observed in a variety of natural and synthetic media. Initially, yields of 1~4 μg/ml were found in shake flask experiments with the inoculum and production media given above; selection of a higher yielding natural variant resulted in an increase to 10 μg/ml. Addition of 100 μg L-methionine-S, R-sulfoximine per ml of fermentation broth at 17 hours increased the yield to between 20 and 30 μg/ml. However, cost considerations prohibited its use in larger scale fermentations.

IV. Isolation Procedure

The clarified broth (400 liters) which contained 3.7 g antimetabolite in 3,600 g solids was applied to 50-liter Dowex 50WX4 resin, 50~100 mesh, in the H^+ form. The resin was washed with 100 liters of distilled water and then eluted with 200 liters of 5 % aqueous pyridine solution. The eluate, which contained 3.45 g antimetabolite in 26.6 g solids, was evaporated at reduced pressure to 2 liters. This concentrate was then applied to 1.25 liter (35 cm ht) Bio-Rad AG50WX4 resin, 50~100 mesh, in the H^+ form and the resin was eluted with 0.2 M HCl. Fractions occurring at an elution volume of 2~9.5 liters were combined and deacidified by readsorption onto 2.5 liters Bio-Rad AG50WX4 resin, 50~100 mesh, in the H^+ form. The resin was washed with 15 liters of distilled water and the antimetabolite eluted with 10 % aqueous pyridine solution. The eluate which contained 3.1 g antimetabolite in 6.2 g solids was concentrated at reduced pressure to 100 ml and the concentrate was applied to a column which contained 3 g "Norit A" charcoal and 3 g Celite filter aid. The pH of the combined effluent and water wash of the column was adjusted to 3.5 with HCl and the solution was evaporated at reduced pressure to 20 ml. While the temperature was maintained at 40°C, methanol was added (180 ml) to a persistent turbidity, the solution was gradually cooled to 0°C and maintained at this temperature for 3 days. The antimetabolite deposited slowly as amorphous spherules. As econd crop was obtained from 100 ml 90 % methanol. The combined crops, 2.7 g, were reprecipitated by the same procedure to obtain 2 g of pure antimetabolite, **I**, m.p. 292°C (dec.): overall recovery, 54 %; overall purification, 975-fold.

Anal. calcd. for $C_{11}H_{28}N_4O_8PS$: C, 32.90; H, 5.77; N, 13.93; P, 7.70; S, 7.96.

Found: C, 32.85; H, 5.95; N, 13.93; P, 7.47; S, 7.79.

V. Structure Elucidation

The antimetabolite, **I**, was dephosphorylated either chemically by incubating an aqueous solution at 100°C and pH 4 for one hour ($^1/_2$ life 10 min.) or enzymatically with calf intestinal or *E. coli* alkaline phosphatase. However, wheat germ acid phosphatase was not effective. The dephospho compound **II** was obtained crystalline and analytically pure. The nmr spectrum revealed the presence of two CH_3—CH groups, a CH_2—CH_2—CH system and a CH_3 group bound to S or O. Strong acid hydrolysis of either **I** or **II** liberated 2 molar equivalents of L-alanine and, depending on experimental conditions, varying amounts of homoserine, and methionine sulfoxide or sulfone, and ammonia. These compounds had previously[10] been detected in acid hydrolysates of methionine sulfoximine. Small amounts of alanylalanine were detected during the course of the reaction. Enzymatic hydrolysis of **II** with leucine amino peptidase gave two molar equivalents of L-alanine and one molar equivalent of L-methionine-S-sulfoximine, **III**. (Identified by comparison of m.p. and $[\alpha]_D$ with those of the resolved[11] optical isomer). During the enzymatic hydrolysis substantial amounts of alanylalanine appeared as an intermediate. Treatment of **II** with carboxypeptidase liberated L-alanine from the carboxy terminal position. The amino terminal amino acid was confirmed as methionine sulfoximine by dansylation followed by hydrolysis.[12] Thus, the amino acid sequence of **II** was proved and a cyclic peptide was excluded. Polymeric structures were also ruled out by the absence of required intermediates during enzymatic or chemical hydrolysis.

The position of the phosphate in the antibiotic was determined by treatment of **I** with leucine amino peptidase and isolation of **IV** from the hydrolyzate. This compound had essentially the same ir and was equally susceptible to chemical and enzymatic hydrolysis as synthetic[13] **IV** (racemic at S).

VI. Antimicrobial Activity

The antimicrobial spectrum of **I** in defined medium[8] is presented in Table I. Growth inhibition against gram-positive and gram-negative bacteria was observed. No inhibition was observed against fungi. A clear cut reversal of inhibition by L-glutamine was observed only with *Bacillus subtilis* and *Serratia* sp.

Table 1. Antimicrobial spectrum of L-(N^5-phosphono)methionine-S-sulfoximinyl-L-alanyl-L-alanine

Test organism	Inhibition zone diameter (mm) with 2.5 μg/ml[a]
Bacillus subtilis NRRL-558	25
Micrococcus glutamicus ATCC-13761[b]	28
Streptomyces cellulosae ATCC-3313	29
Escherichia coli B	30
Serratia sp.	28
Aerobacter aerogenes	26
Pseudomonas ovalis NRRL-22	26
Candida albicans NRRL-477[b]	0
Aspergillus niger NCIM-612	0
Pullularia pullulans QM-279C	0

a) Paper disc agar diffusion assays were performed with 12.7 mm discs on minimal agar medium as described in Section II.
b) Biotin was added to the medium at 100 μg per liter to insure ample growth of these test organisms.

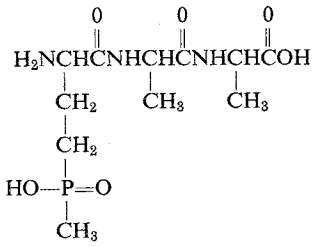
Phosphomethionine sulfoximine, **IV**, was inactive at 1 mg/ml when tested against *Serratia* sp. and *Streptomyces cellulosae*. Against *Bacillus subtilis* and *Escherichia coli*, **IV** was, respectively, 1/10 and 1/100 as active as **I**.

Discussion

It is known[14] that glutamine synthetase catalyzes the phosphorylation of methionine sulfoxi-

mine by ATP to form **IV**. The product, **IV**, is a very effective inhibitor of glutamine synthetase[13,14]. Dr. ALTON MEISTER (personal communication) has found that **I** inhibits sheep brain glutamine synthetase almost as effectively as does **IV**. Thus, the reversal of the antibiotic activity of **I** by glutamine is not surprising.

Glutamine also reverses the activity of phosphinothricyl-L-alanyl-L-alanine[7], **V**, an antibiotic with a structure closely related to **I**. The tripeptide, **V**, has much greater antibacterial activity than the constituent amino acid, phosphinothricin. However, only the amino acid effectively inhibits *Escherichia coli* glutamine synthetase[7]. It has been suggested[7] that the tripeptide is more easily transported into the cell where it is hydrolyzed to the amino acid which then inhibits the enzyme. A similar transport effect may account for the greater antibacterial activity of **I** compared to **IV**.



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Acknowledgements

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