

ANTIMETABOLITES PRODUCED BY MICROORGANISMS. XII¹⁾

(S)-ALANYL-3-[α -(S)-CHLORO-3-(S)-HYDROXY-2-OXO-3-AZETIDINYLMETHYL]-(S)-ALANINE, A NEW β -LACTAM CONTAINING NATURAL PRODUCT

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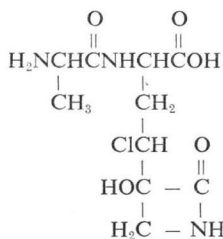
(S)-Alanyl-3-[α -(S)-chloro-3-(S)-hydroxy-2-oxo-3-azetidylmethyl]-(S)-alanine was isolated from a fermentation broth of an unidentified *Streptomyces* species 372 A. The structure was determined by single crystal X-ray diffraction analysis. The substance inhibits the growth of several strains of gram-positive and gram-negative bacteria in a chemically defined medium but growth inhibition is relieved by addition of L-glutamine to the medium.

Several di- and tripeptides with antimetabolite activity have recently been isolated from fermentation broths.¹⁻⁷⁾ We wish to report here the isolation and structure elucidation of another member of this series, (S)-alanyl-3-[α -(S)-chloro-3-(S)-hydroxy-2-oxo-3-azetidylmethyl]-(S)-alanine, (I). This substance is produced by an unidentified species of *Streptomyces* and inhibits the growth of several strains of bacteria on a chemically defined minimal medium.⁸⁾ The growth inhibition is relieved by the addition of L-glutamine to the medium. The structure of I, which was determined by X-ray diffraction analysis, is remarkably similar to that of wildfire toxin⁹⁾ (II). The latter compound, now called tabtoxin,¹⁰⁾ is produced by several *Pseudomonas* species^{10,11)} and like I possesses a biological activity reversed by L-glutamine.¹²⁾ Aside from these dipeptides, the penicillins and cephalosporins, the only other compounds found in nature known to possess β -lactam rings are the pachystermines,¹³⁾ bleomycins¹⁴⁾ and phleomycins.¹⁴⁾

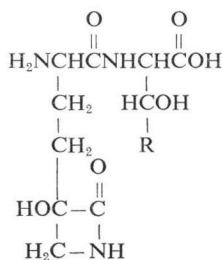
Experimental

I General Methods

IR spectra were obtained from KBr discs with a Perkin-Elmer Model 621 spectrophotometer. NMR spectra were obtained using a Varian A-60 instrument and optical rotations were measured with a Perkin-Elmer 141 polarimeter. The purity of preparations was deter-



I



II R=CH₃ or H

mined using the Technicon amino acid analyzer and by thin-layer chromatography with Merck silica gel F-254 plates developed in acetone-methanol-water (2 : 2 : 1) and Merck cellulose F plates developed in ethanol-water (7 : 3).

II Microbiological Assay

Detection and quantitation of the antimetabolite was achieved by a paper-disc agar-diffusion assay employing *Escherichia coli* B in the minimal agar medium of DAVIS and MINGIOLI⁹⁾ as described previously.¹⁰⁾ The diameter of the zone of inhibition was proportional to the log of the antimetabolite concentration within the range of 0.1~10.0 $\mu\text{g/ml}$. A two fold increase in the concentration of I increased the zone diameter by 2 mm.

III Fermentation

Spores of an unidentified species of *Streptomyces** were added to a 6-liter Erlenmeyer flask containing 2 liters of inoculum medium composed of (in g/liter): Bacto-peptone (Difco), 6.0; N-Z Amine type A (Sheffield), 4.0; yeast autolyzate (Natural Yeast Products), 3.0; beef extract (Wilson), 1.5; and dextrose, 1.0. The flasks were incubated at 28°C for 96 hours on a rotary shaker [250 rpm with a 2-inch throw]. Four liters of inoculum was then added to 230 liters of fermentation medium, containing (in g/liter): tomato paste (Contadina), 20; glycerol, 20; and calcium carbonate, 5. The pH was adjusted to 7 before sterilization. The culture was incubated in a 380-liter fermentor, aerated at 113 liters per minute and agitated at 280 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing and the pH was maintained between 7.0 and 7.5 by addition of 5N sulfuric acid. After 72 hours the fermentation was filtered through infusorial earth by centrifugation.

Production of I was observed in many media containing a variety of natural constituents. However, yields never exceeded about 10 mg per liter. During the course of our work, the productive capacity of the culture dropped and a program of natural variant selection was required to obtain a strain possessing the original biosynthetic capacity.

IV Isolation procedure

The low potency of broth, the instability of the activity, and the presence of closely related compounds, possibly degradation products, all caused difficulties during the purification of I. Although many forms of partition and adsorption chromatography were investigated the most efficient procedure was repetitive use of cation-exchange chromatography with a final partition chromatography step. There was no evidence for the presence of active compounds other than I.

Filtered broth, 230 liters, was applied to a column which contained 50 liters of Dowex 50W \times 4 resin, 50~100 mesh (H⁺). The column was washed with 100 liters of distilled water and the activity was then eluted with 200 liters of 5% aqueous pyridine solution adjusted to pH 5.7 with 2.3 liters of glacial acetic acid. After evaporation under reduced pressure 73 g of solids, 0.3% pure, were obtained. This material was taken up in 350 ml of distilled water, adjusted to pH. 3.2 and applied to a column, of Bio-Rad AG50W \times 4 resin, 100~200 mesh (Na⁺, 92 \times 1,000 mm). The column was eluted with 0.2M sodium citrate-phosphate buffer,¹⁰⁾ pH 3.85. The activity appeared in a 5-liter fraction at an elution volume of 30 liters. The active fraction was desalted by reabsorption onto 2 liters Bio-Rad AG50W \times 4 resin, 50~100

* *Streptomyces* sp. #B-5b (HLR-372A) kindly supplied by Prof. H. LECHEVALIER, Institute of Microbiology, Rutgers University, The State University of New Jersey.

mesh (H^+) followed by elution with 10% aqueous pyridine solution. The eluate was evaporated under reduced pressure to 4.3 g solids, 5% pure. Material obtained from four 230-liter fermentations, purified as described above, was combined, taken up in 60 ml water, adjusted to pH 3.0, and applied to a column of Bio-Rad AG50W \times 4 200~400 mesh (Na^+ , $41\times 1,900$ mm). The resin was eluted with 0.2M sodium citrate-phosphate buffer,¹⁶⁾ pH 3.5. The activity appeared in a 3.3-liter fraction at an elution volume of 7.5 liters. This fraction was desalted as described above on a 1-liter column of AG50W \times 4 cation-exchange resin, 50~100 mesh (H^+), and evaporated under reduced pressure to give 4.2 g of 19% pure material. This was taken up in 20 ml of *n*-butanol-acetic acid-water (12:3:5), and applied to a dry column of Bio-Rad cellulose MX (36 \times 500 mm). The cellulose was eluted with the same solvent and the activity appeared in a 300-ml fraction at an elution volume of 240 ml. This was partially evaporated under reduced pressure, diluted with water to a homogeneous solution and applied to a 40-ml column of Bio-Rad AG50W \times 4, 50~100 mesh resin (H^+). The activity was eluted with 15% aqueous pyridine solution, the eluate concentrated under reduced pressure, and **I** was crystallized in two crops from aqueous methanol to give 434 mg single-spot material. The mother liquors from the original crystallization were rechromatographed on another cellulose column (18 \times 800 mm). After repetition of the above described treatment an additional 82 mg (**I**) crystallized.

This was combined with the previously obtained material and recrystallized to give 439 mg (**I**); *Anal.* calcd. for $C_{10}H_{10}ClN_2O_3$; C 40.89, H 5.49, N 14.31. Found: C 41.07, H 5.57, N 14.11, mp 220~225°C (dec. with release of NH_4Cl); overall purification, 3,200 fold; overall recovery, 44%; ir 1745 (β -lactam), 1710, 1680 (amide), 1635 cm^{-1} (carboxylate); nmr (D_2O , DCl , 15 mg, ext TMS), δ 5.33 (dd, 1, $J=6.5$ and 8 Hz, $Cl-CH-CH_2$), 4.90 (dd, 1, $J=6.5$ and 9 Hz, $CH_2-\overset{\overset{N}{|}}{\underset{\underset{O}{||}}{C}}$), 4.78 (q, 1, $J=7$ Hz, $CH-CH_3$), 4.09 (d, 1, J gem=13.5 Hz, $C-CH_2-N$), 3.87 (d, 1, J gem=13.5 Hz, $C-CH_2-N$), 2.92 (m, 2, $CH-CH_2-CH$), 2.13 (d, 3, $J=7$ Hz, $CH-CH_3$).

V X-Ray Diffraction Analysis of **I**

The small acicular crystals of **I** are orthorhombic, space group $P 2_12_1$, with $a=5.51$ (1), $b=13.27$ (2), $c=17.39$ (3) Å and $d_{calc}=1.53$ for $Z=4$. The diffraction data were measured on a HILGER-WATTS four-circle diffractometer. Because of the small size of the crystal (0.025 \times 0.025 \times 0.10 mm) a peak-top scan technique was used. An empirical correction was applied to convert the peak-top data to integrated scan data. Intensity data were measured in two octants of reciprocal space (hkl data for which $\theta < 57^\circ$ and $h\bar{k}l$ data for which $\theta < 43^\circ$). Of the 1534 reflections measured, only 720 had intensities significantly greater than background, and these data were used in the structure analysis.

The structure was solved by PATTERSON and FOURIER methods. All refinements were carried out by full-matrix least squares. During the initial refinement (Cl anisotropic, lighter atoms isotropic, no hydrogen) the imaginary part of the anomalous dispersion correction was set to zero. Structure factors were then calculated for the two enantiomers taking into account both the real and imaginary parts of the anomalous dispersion corrections. The absolute configuration was taken as that of the enantiomer corresponding to the lower weighted R value (0.176 compared to 0.179). (Although this result is significant at the 0.995 level

Fig. 1. Stereodrawing showing the absolute configuration (see text) of **I** and its conformation in the crystalline state

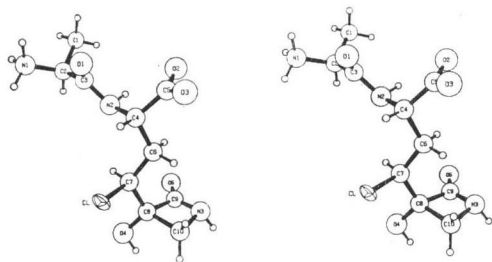


Table 1. Antimicrobial spectrum

Microorganism	Inhibition zone diameter* (mm)
<i>Bacillus cereus</i> ATCC-6464	66
<i>Bacillus subtilis</i> NRRL-558	22
<i>Bacillus</i> . sp. ATCC-27860	17
<i>Streptomyces cellulosa</i> ATCC-3313	40
<i>Escherichia coli</i> B	60
<i>Klebsiella pneumoniae</i> ATCC-27858	44
<i>Serratia</i> sp.	37
<i>Pullularia pullulans</i> QM-279C	0

* Paper discs (12.7-mm diameter) containing approximately 12 μ g of **I** were placed on agar surfaces previously seeded with the test organisms.

according to the test described by HAMILTON,¹⁷ the relatively poor quality of the diffraction data in this case makes one cautious in accepting the result). In the final cycles of refinement the hydrogen atoms were included in the structure factor calculations but their parameters were not refined. The final discrepancy index is $R=0.113$ for the 720 observed reflections (Cl anisotropic, all other atoms isotropic). A stereodrawing of **I** is presented in Fig. 1.

VI Biological Activity

The antimicrobial activity of **I** was measured in chemically defined medium⁹⁾ by the paper-disc agar-diffusion technique.¹⁵⁾ The results are given in Table 1. Antibiotic activity against both gram-positive and gram-negative bacteria was observed. In all cases growth inhibition was relieved by the addition of L-glutamine to the agar.

The antibacterial activity of **I** was not lost by incubation with *Bacillus cereus* penicillinase (Calbiochem). Incubations were carried out for 90 minutes at 25°C at pH 7 with two levels of penicillinase, 2,000 and 200,000 units per ml and two concentrations of **I**, 1.7 and 17 mM. In addition **I** did not appear to be an inhibitor of penicillinase when tested by the method of O'CALLAGHAN *et al.*¹⁸⁾ For this study the substrate concentration was 0.1 mM. The enzyme activity, expressed as μ moles substrate destroyed per minute per ml of enzyme solution, was 0.1 and the concentration of **I** was 1.1 mM.

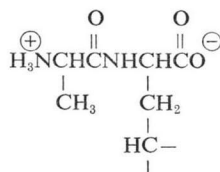
Discussion

When subjected to strong acid hydrolysis (5 N HCl, 100°C, 1 hour) one molar equivalent of alanine was released (amino acid analyzer and VPC of the TMS derivative). The alanine was shown to be of the L-configuration by the VPC retention time of the diastereoisomeric trifluoroacetylpropyl¹⁹⁾ derivative. The only other identifiable product of acid hydrolysis was one molar equivalent of ammonia detected and quantitated by reaction with NESSLER'S reagent and by the amino acid analyzer. Although **I** was not hydrolyzed by leucine amino peptidase even when a great excess of enzyme was used the L-alanine was shown to be in the N-terminal position by the dansylation method²⁰⁾ (detection by TLC). Several derivatives of **I** were submitted to mass spectrometric analysis but no interpretable results were obtained.

Chloride ion was released from an aqueous solution of **I** under the following conditions: (1) 5 minutes, 100°C, pH 7; (2) 15 minutes, 25°C, pH 11; (3) 1 minute, 50°C, pH 7, in the presence of Ag⁺. This type of instability is characteristic of chlorine bound to a secondary

carbon atom.²¹⁾

Thus, assuming a dipeptide zwitterionic system, based on ir evidence and the method of purification, the above-mentioned properties and the nmr spectrum led to the following partial structure.



The undetermined portion, $\text{C}_3\text{H}_4\text{ClNO}_2$, contained a $\text{C}=\text{O}$ group with ir absorption at 1745 cm^{-1} (urethane, β -lactam, ester), acid labile nitrogen, a methylene probably adjacent to an optically active carbon but not coupled to other protons in the nmr spectrum, a chlorine atom on a secondary carbon atom and one other degree of unsaturation, probably a small ring system since no olefinic protons were detectable in the nmr.

For complete structural analysis an X-ray diffraction study was undertaken which permitted the assignment of I. This structure is consistent with the spectral and chemical properties summarized above. The release of ammonia during acid hydrolysis has been noted with other vicinal amino alcohols, *eg.* serine and threonine.²²⁾ The assignment of absolute configuration does not rest on X-ray diffraction analysis alone. The relative configuration, determined by X-ray analysis, plus the chemical determination of L-alanine confirm the absolute configuration of the molecule.

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