ANTIMETABOLITES PRODUCED BY MICROORGANISMS. XI[1]. 1-(S)-HYDROXY-2-(S, S)-VALYLAMIDO-CYCLOBUTANE-1-ACETIC ACID

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1-(S)-Hydroxy-2-(S, S)-valylamido-cyclobutane-1-acetic acid was isolated from a fermentation broth of an unidentified *Streptomyces* species X-1092. The structure was determined by single crystal X-ray diffraction analysis of the *p*-bromophenylcarbamyl derivative. The substance inhibits the growth of gram-positive microorganisms in a chemically defined medium but growth inhibition is partially reversed by L-cysteine.

In several instances[2~6] the deliberate search for amino acid antimetabolites in fermentation broths has resulted in the isolation of biologically active di- or tripeptides containing at least one unusual amino acid. We wish to report the occurrence of another such compound, 1-(S)-hydroxy-2-(S, S)-valylamido-cyclobutane-1-acetic acid (**I**). This substance, produced by an unidentified *Streptomyces* species X-1092, inhibits the growth of several gram-positive organisms in a chemically defined medium[7] but addition of L-cysteine to the medium partially relieves this growth inhibition. The active compound was isolated in crystalline form from the fermentation broth and possessed physical chemical properties consistent with the given structure. The structure was proved by single crystal X-ray diffraction analysis of the *p*-bromophenylcarbamyl derivative (**II**).

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 using the Varian A-60 and HA-100 instruments. Thin-layer chromatography was performed with E. Merck cellulose F plates developed with *n*-butanol-acetic acid-water (12:3:5) or ethanol-water (80:20) and with E. Merck Silica Gel 60 F-254 plates developed with ethanol-water-ammonium hydroxide (49:49:2). Vapor phase chromatography was carried out with the F & M 402 equipment and a 6′-column of 5% UCW 98 on Gas Chrom Q support.

II. Microbiological Assay

The paper-disc agar-diffusion microbiological assay *versus Bacillus subtilis* previously described[8] was used to measure **I** quantitatively. The zone diameter was found to be proportional to the log of the concentration between 20 and 500 μg per ml. A two-fold increase in

the concentration increased the zone diameter by 4 mm and 100 μg per ml gave approximately a 34 mm zone.

III. Fermentation

Spores of an unidentified *Streptomyces* species X-1092, were added to 6-liter Erlenmeyer flasks containing 2 liters of Trypticase soy broth (Baltimore Biological Laboratories). The flasks were incubated at 28°C for 76 hours on a rotary shaker [240 rpm with a 2-inch (5.08 cm) throw]. Four liters of inoculum was added to 200 liters of fermentation medium containing (in g/liter): K_2HPO_4, 7.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; sodium citrate dihydrate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; and D-glucose, 2.0 (autoclaved separately). The culture was incubated at 28°C in a 380-liter fermentor, aerated at 113 liters per minute and agitated at 260 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 66 hours of incubation, the fermentation broth was filtered through infusorial earth.

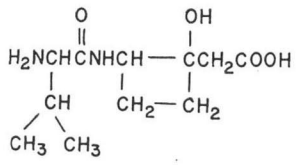
IV. Isolation Procedure

The clarified broth from two 200-liter fermentations was applied to 50 liters Dowex 50WX-4 resin, 50~100 mesh, in the H^+ form. After washing with 200 liters distilled water, the resin was eluted with 400 liters 5% aqueous pyridine solution. The eluate which contained 33 g solids was evaporated under reduced pressure to 2 liters, the pH adjusted to 2.5 by addition of 5 N HCl and the solution applied to a column (70 cm ht) containing 2.5 liters Bio-Rad AG50WX-4 resin, 100~200 mesh, in the Na^+ form, which had been equilibrated with 0.2 M sodium phosphate-citrate buffer,[9] pH 4.2. The resin was then eluted with the same buffer and the activity was obtained at an elution volume of 11~13 liters. This fraction was desalted by readsorption of the antimetabolite onto 1.2 liters Bio-Rad AG50WX-4 resin, 50~100 mesh, in the H^+ form followed by elution with 10% aqueous pyridine solution. The eluate was evaporated under reduced pressure to a small volume, the concentrate was treated with charcoal, the filtrate from the charcoal step was again evaporated and **I** was crystallized from ethanol-water (9:1): yield, 1.5 g; mp. 247~250°C; $[\alpha]_D^{25}$ +8.4 (*c* 1, H_2O), −31.2 (*c* 1, 5 N HCl); pK_1 3.8, pK_2 7.5; ir (KBr disk) 3365 and 3240 (OH and amide NH), 1660 and 1520 (sec amide), 1615 and 1390 cm^{-1} (carboxylate); nmr (D_2O, 20 mg, ext TMS), δ 4.61 (m, 1, N-C<u>H</u>(C)(C)), 4.23(d, 1, J=6Hz, N-C<u>H</u>(-C=O)-CH), 2.92 (3.18 with DCl) (s, 2, C-C<u>H</u>$_2$-C(=O)-O-), 2.30~2.80 (m, 5), 1.47 (d, 6, J=6.5Hz, C<u>H</u>$_3$C<u>H</u>$_3$>CH).

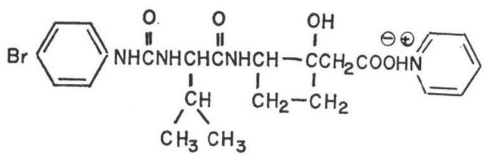
Anal. Calcd. for $C_{11}H_{20}N_2O_4$: C 54.03, H 8.25, N 11.47.
Found: C 54.11, H 8.40, N 11.57.

V. Synthesis of II

A well-stirred suspension of 244 mg (1 mmole) of finely ground **I** and 396 mg (2 mmoles) of *p*-bromophenylisocyanate in 20 ml pyridine was maintained at 25° for 1 hour. After removal of 34 mg of unreacted (I) by filtration, the filtrate was evaporated under reduced pressure to 5 ml and 2 volumes of water was added to convert excess isocyanate to *p*-bromophenylurea.



I



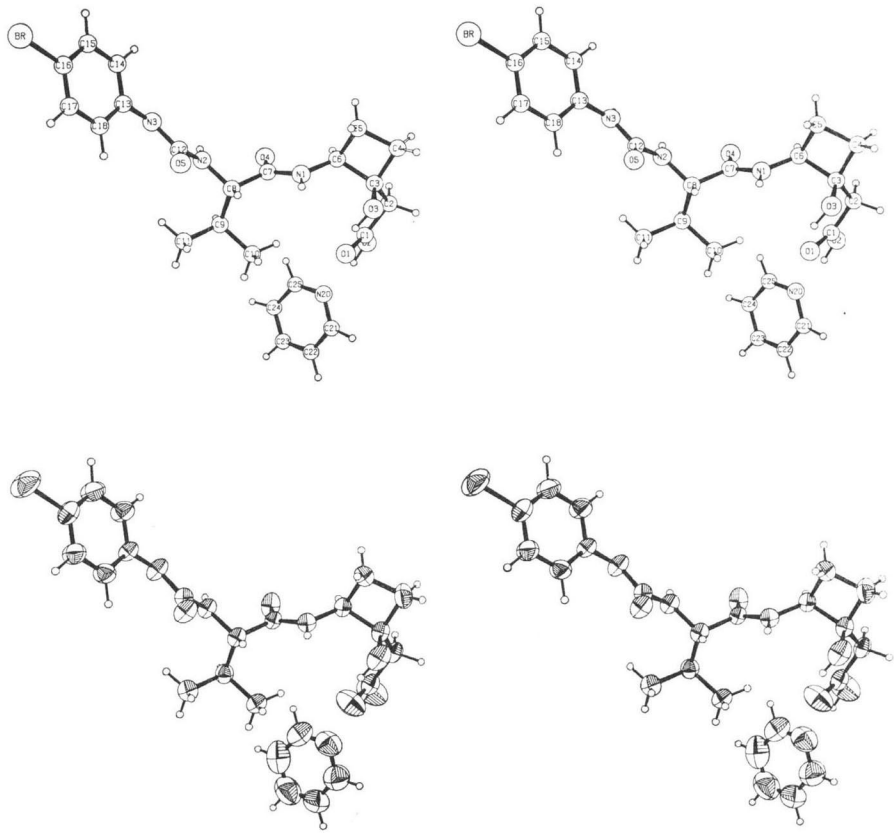
II

This was removed by filtration and the filtrate evaporated under reduced pressure to a residue of 379 mg. From this, 323 mg of **II** (yield 76 %) was crystallized in two crops from ethanol-ethyl acetate: mp. 193°; ir (KBr) no urethane group frequency at 1720 cm^{-1}. *Anal.* calcd. for $C_{23}H_{29}BrN_4O_5$: C 52.98, H 5.61, N 10.75. Found: C 52.91, H 5.24, N 10.56. A crystal suitable for X-ray analysis was obtained after recrystallization from ethanol.

VI. X-Ray Diffraction Analysis of II

The crystals of **II** are monoclinic, space group $P2_1$, with two formula units in a unit cell of dimensions a=4.862(2), b=23.443(10), c=10.958(4) Å, β=99.35(2)°. Intensity data were collected on a Hilger-Watts four circle diffractometer from a crystal approximately 0.1×0.1×0.5 mm in size. Nickel-filtered Cu Kα radiation was used and the data were corrected for absorption (μ=28.5 cm^{-1}). Of the 2143 unique reflections with $2\theta<140°$ that were measured, 1818

Fig. 1. Conformation and absolute stereochemistry of **II**



had intensities significantly greater than background and these data were used in the structure analysis.

The structure was solved by the heavy atom method. The absolute configuration was established by computing structure factors for both enantiomers after a preliminary refinement in which anomalous dispersion had been ignored. The absolute configuration was taken as the one corresponding to the lower R value (R=0.1156 and 0.1184). A difference FOURIER calculated after anisotropic refinement of the non-hydrogen atoms revealed most of the hydrogen atoms in the anion molecule but none of the pyridinium hydrogens. The final refinement was carried out by full matrix least squares with anisotropic thermal parameters for all atoms except the hydrogens. These were included at their calculated positions but were not refined. The final discrepancy index is R=0.044.

The conformation and absolute configuration of **II** are shown in Fig. 1. The drawing also shows the spacial relationship between the pyridinium moiety and the carboxylate group to which it is hydrogen bonded. Although the proton is shown bonded to the carboxylic group, it is possible that it is on the pyridine nitrogen.

VII. Biological Activity

The antimicrobial spectrum of **I** was measured in a chemically defined medium[7] by the paper disc agar-diffusion technique. The results are given in Table 1. The activity was limited to gram-positive organisms.

The antimetabolite activity of **I** was determined by the counter diffusion method previously described.[10] Against *Streptomyces cellulosae*, the activity of **I** was noncompetitively[1] reversed by the addition of either L-cysteine or L-cystine to the medium. Partial reversals were observed with either L-methionine or D, L-homocysteine. Other common amino acids, nucleosides and water-soluble vitamins did not reverse the activity of **I**. In addition glutathione and dithiothreitol did not reverse the inhibition; so it can be concluded that the reversal by cysteine is probably not due to chemical inactivation by thiols.

Only slight reversal of inhibition against the three species of *Bacilli* and *Micrococcus glutamicus* was observed with L-methionine, L-cystine and L-cysteine; no reversal was observed with other common amino acids, nucleosides and water-soluble vitamins.

Table 1. Antimicrobial spectrum

Test organism	Inhibition zone diameter* (mm)
Bacillus cereus ATCC-6464	60
Bacillus sp. ATCC-27860	25
Bacillus subtilis NRRL-558	34
Streptomyces cellulosae ATCC-3313	34
Micrococcus glutamicus ATCC-13761**	20
Escherichia coli B	0
Pseudomonas ovalis NRRL-22	0
Candida albicans NRRL-477**	0
Pullularia pullulans QM-279c	0

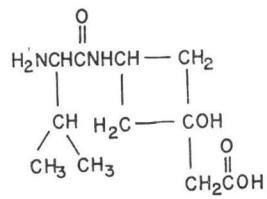
* Paper-disc agar-diffusion assays were performed with 12.7 mm discs each containing 12 μg of **I**.

** Biotin was added to the medium at 100 μg per liter to insure ample growth of these test organisms.

Discussion

Both acid hydrolysis and leucine amino peptidase digestion of **I** give rise to one molar equivalent of L-valine (isolated crystalline and analytically pure) and varying amounts of at least 3 other compounds which were not purified or characterized. The valine was shown to

be in the N-terminal position by dansylation followed by hydrolysis[11] (TLC and electrophoretic identification). The carboxy function of the valine was in an amide linkage (ir and enzyme specificity), and the remaining portion of the molecule possessed a carboxylate group (ir, pK and method of purification) next to a methylene group (nmr) which appeared as a sharp singlet and thus was adjacent to a tetra-substituted carbon. This left a C_4H_6O fragment with one degree of unsaturation to be accounted for. There was no evidence for C=C (nmr, uv, Br_2 uptake), for an additional carbonyl group (ir) nor for an ether (ir and nmr). The oxygen appeared (ir) to be an alcohol which was presumed to be tertiary since excess *p*-bromophenylisocyanate did not react to give a urethane nor were there any unassigned carbon bound protons with appropriate chemical shifts in the nmr spectrum. The evidence thus indicated that the C_4 fragment contained a small carbocyclic ring. Since the nmr spectrum ruled out a cyclopropyl ring only a cyclobutyl system remained and the substitution requirements allowed only two possibilities exclusive of stereoisomers. The structure, **III**, with a plane of symmetry was favored by the nmr evidence since the exocyclic methylene might be expected to have magnetically nonequivalent protons were it adjacent to an optically active carbon as in **I**. However, **III** would possess only one chiral center, the α carbon of valine, and thus should have molar optical rotations in water and acid analogous to those of a simple carboxy substituted L-valine. However, the $[M]_D$ values for L-valylglycine[12] in water and 10 % hydrochloric acid are +160 and +68.5 respectively, while the corresponding values for **I** are +20.5 and −76.1. An X-ray diffraction analysis of the most suitable crystal available, the pyridinium salt of the *p*-bromophenylcarbamyl derivative **II**, established the structure of **I** and permitted the assignment of absolute configuration for the chiral centers as all S. It was coincidental that both protons of the exocyclic methylene group had the same chemical shift.



III

The multiplicity of degradation products of **I** is probably due to the ease of dehydration of the tertiary alcohol and perhaps to the formation of a γ-lactam as well.

The reversal of antibiotic activity of **I** by cysteine and methionine in several gram-positive microorganisms suggests that **I** may interfere with sulfur amino acid biosynthesis.

Acknowledgements

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