ANTIMETABOLITES PRODUCED BY MICROORGANISMS. VIII

N^5-HYDROXY-L-ARGININE, A NEW NATURALLY OCCURRING AMINO ACID[1]

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A new crystalline amino acid was isolated from the fermentation broth of a *Bacillus* species and identified as N^5-hydroxy-L-arginine. This compound possesses antimicrobial activity against several microorganisms, including *Escherichia coli*, which is reversed by L-arginine and related compounds.

The N-hydroxy function is frequently encountered in microbial products, most commonly as part of hydroxamic acids.[2] Naturally occurring hydroxamic acids consist of a hydroxylamine moiety generally supplied by N-hydroxyamino acids such as N^5-hydroxyornithine, and an acyl portion which is usually acetyl or biogenetically derived from acetate. We now report the discovery of N^5-hydroxy-L-arginine (**1**) (Fig. 1) representing a new amino acid and formally resembling hydroxamic acids in that it contains an N-hydroxy function in the form of N^5-hydroxyornithine, but the acyl moiety is replaced by an amidino group.

N^5-Hydroxy-L-arginine is produced by a microorganism isolated from soil collected in Petit Saint Vincent Island, the Grenadines, British West Indies, identified as a member of the genus *Bacillus*, and designated *Bacillus* sp. XB-13248.

Microbiological Assay and Activity

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 MINGIOLI[3] as described previously.[4] The diameter of the inhibition zone was proportional to the log of the antimetabolite concentration within the range of 1~100 μg/ml. A two-fold increase in the concentration of **1** increased the zone diameter by 3 mm.

The antimicrobial activity of **1** was measured in chemically defined medium[3] by the paper-disc agar-diffusion technique. The results are given in Table 1. Selective activity was found against several gram-positive and gram-negative bacteria and a mold. In every case resistance to **1** developed quickly as evidenced by resistant colonies and hazy zones.

L-Arginine was found to reverse the antimicrobial activity of **1** against *Bacillus* sp., *E. coli* and *Pullularia pullulans* when tested by a

Fig. 1. Degradation products of N^5-hydroxy-L-arginine

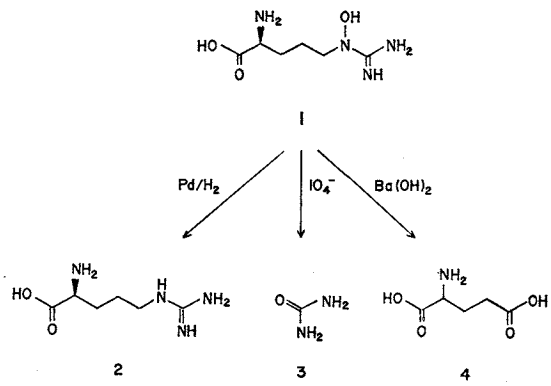


Table 1. Antimicrobial spectrum of N^5-hydroxy-L-arginine

Microorganism	Zone diameter* (mm)	Description of zone
Bacillus cereus ATCC-6464	22	Hazy***
Bacillus subtilis NRRL 558	34	Very hazy***
Bacillus sp. 1283B	50	Resistant colonies
Streptomyces cellulosae ATCC-3313	no zone	
Escherichia coli B	43	Resistant colonies
Serratia sp. 101	27	Very hazy***
Aerobacter aerogenes	25	Hazy ***
Pseudomonas ovalis NRRL-22	no zone	
Pullularia pullulans QM-279C	36	Edge not sharp
Candida albicans NRRL-477**	no zone	

* Paper discs 12.7 mm in diameter were saturated with a solution containing 125 μg of **1** (hydrochloride) per ml and applied to agar surface; each disc contained approximately 15 μg of **1** (hydrochloride).

** Biotin was added to the medium at 100 μg per liter to ensure ample growth.

*** Growth of test organims was not totally inhibited resulting in an inhibition zone containing less than normal growth.

counter-diffusion technique[5]. Reversal could not be definitely determined for the other organisms listed in Table 1 due to haziness of the zones of inhibition.

The activity against *E. coli* was reversed by N-acetyl-L-glutamic acid, N^2-acetyl-L-ornithine, L-ornithine, L-citrulline, and L-arginine but not by L-glutamic acid. This suggests that N^5-hydroxy-L-arginine inhibits glutamate acetyltransferase, the first enzyme involved in the biosynthesis of L-arginine from L-glutamic acid[6].

Antimetabolite Production and Isolation

Bacillus sp. XB-13248 was maintained on Trypticase soy agar slants. Growth from a slant was added by a sterile loop to 6-liter Erlenmeyer flasks containing 2 liters of inoculum medium composed of (in g/liter): Trypticase soy broth (BBL) 30, and glycerol 10. The flasks were incubated at 28°C for 72 hours on a rotary shaker. Four liters of inoculum were then added to 225 liters of fermentation medium of pH 7.0, containing (in g/liter): glucose, 10; monosodium glutamate, 10; K_2HPO_4, 4; KH_2PO_4, 2; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.025; $FeCl_3 \cdot 6H_2O$, 0.001; $ZnCl_2$, 0.0005; $CuCl_2 \cdot 6H_2O$, 0.0005; and $MnCl_2 \cdot 4H_2O$, 0.0005. The culture was incubated at 28°C in a 380-liter fermentor, aerated at 0.11 m^3/min. and agitated at 260 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 42 hours the fermentation broth was clarified by centrifugation.

The clarified broth was passed through a column of 30.5 cm diameter containing 50 liters of Dowex 50WX4 (H^+), and the column washed consecutively with 200 liters of water, 200 liters of 5 % aqueous pyridine and 50 liters of water. The activity was then eluted with 1 N ammonium hydroxide solution and was mostly contained in the first 80 liters of ammoniacal effluent which, upon concentration and freeze-drying, gave 30 g of crude antimetabolite of approximately 20 % purity.

Further purification was achieved by dissolving the crude in 400 ml of water, adjusting the pH to 3.5 and passing the filtered solution through a column (70×660 mm) of Dowex 50WX8, 200~400 mesh (Na^+). The column was eluted with 14.7 liters of a buffer[7] prepared by adding a 0.1 M citric

acid solution to a 0.2 M dibasic sodium phosphate solution to pH 6.1. Column development was continued with 3.3 liters of the citrate-phosphate buffer containing an additional 17.53 g (0.3 M) of sodium chloride per liter and finally with the buffer containing 23.38 g (0.4 M) of sodium chloride per liter. The column effluent was collected in 0.5 liter fractions immediately upon commencement of column development with buffer containing 0.4 M sodium chloride; the bulk of the antimetabolite was found in fractions 3~11. These fractions were desalted by passage through a column containing 3 liters of Dowex 50W X4, 50~100 mesh (H^+), followed by a water wash and elution of the biologically active material with 1 N ammonium hydroxide solution. The antimetabolite-containing fractions were concentrated to small volume and brought to dryness, after adjustment to pH 5 with dilute hydrochloric acid, yielding 4 g of solids. Crystallization from aqueous ethanol solution afforded 2.7 g of the hydrochloride of **1** and removed traces of **2**. After one further recrystallization colorless crystals of **1** were obtained; mp 200~210°C (dec.), $[\alpha]_D^{25}+19°$ (*c* 0.9, 5 N HCl).

Anal. Calcd. for $C_6H_{14}N_4O_3 \cdot HCl$: C, 31.79; H, 6.67; N, 24.73; Cl, 15.64.
Found: C, 31.68; H, 6.79; N, 25.00; Cl, 15.61.

Characterization and Structure Determination

The hydrochloride of **1** was readily converted to the free amine by charging an aqueous solution of the salt onto a column of Dowex 50 (H^+), washing with water and eluting with 1 N ammonium hydroxide solution. Concentration of the ammoniacal effluent gave a thin syrup from which the free amine crystallized upon addition of ethanol; mp 206~212°C (dec.), $[\alpha]_D+21°$ (*c* 1.0, 5 N HCl).

Anal. Calcd. for $C_6H_{14}N_4O_3 \cdot 0.25H_2O$: C, 37.01; H, 7.51; N, 28.78.
Found: C, 37.13; H, 7.53; N, 29.02.

Other salts of **1** were readily prepared by adjusting an aqueous solution of the free amine to pH 5 with the appropriate acid. Thus, the 2-chloro-5-nitrobenzenesulfonic acid salt was obtained as slightly tan needles, mp 202~205°C (dec.).

Anal. Calcd. for $C_6H_{14}N_4O_3 \cdot C_6H_4ClNO_5S$: C, 33.69; H, 4.24; N, 16.37.
Found: C, 33.44; H, 4.28; N, 16.22.

The hydrobromide salt was prepared in analogous fashion; mp 209~212°C (dec.), $[\alpha]_D^{25}+15°$ (*c* 1.0, 5 N aq. HCl).

Anal. Calcd. for $C_6H_{14}N_4O_3 \cdot HBr$: C, 26.58; H, 5.58; N, 20.67; Br, 29.47.
Found: C, 26.68; H, 5.70; N, 20.78; Br, 29.31.

N^5-Hydroxy-L-arginine and its salts give positive ninhydrin tests but only the free amine exhibits intense brown-red coloration with ferric chloride solution. The similarity of **1** to arginine and ornithine is evident by comparison of ^1H nmr spectra (Varian HA-100, D_2O, TMS as external

Table 2. Some physico- chemical properties of N^5-hydroxyarginine and related amino acids

Compound	^1H Chem. shifts $\left(\delta_{TMS}^{D_2O}\right)$			R_f in solvent system		
	CH	$N-CH_2$	$(CH_2)_2$	A	B	C
Ornithine HCl	4.22	3.48	2.32	0.21	0.56	0.18
Arginine HCl	4.22	3.70	2.28	0.11	0.34	0.11
N^5-Hydroxyarginine HCl	4.25	4.19	2.34	0.26	0.70	0.55

System A: chloroform - methanol - conc. ammonium hydroxide soln., 2:2:1, v/v.
System B: chloroform - methanol - conc. ammonium hydroxide soln., water, 1:4:2:1, v/v.
System C: abs. ethanol - water - conc. ammonium hydroxide soln., 49:49:2, v/v.

reference) and thin-layer chromatographic behavior (Silica Gel G, E. Merck, Darmstadt) shown in Table 2. Analogous to hydroxamic acids[8], **1** is oxidized by periodate as demonstrated by the formation of urea and disappearance of the substrate. In contrast to **2**, heating **1** in 0.6 M barium hydroxide solution at 100°C for 40 hours yielded glutamic acid (**4**), as determined by amino acid analysis and comparison of ir spectra[9].

Hydrogenolysis of a 1% solution of the hydrochloride of **1** in methanol-water, 1 : 1, with Pd on carbon at 3.4 atm in a Parr apparatus for 48 hours at room temperature, yielded the hydrochloride of L-arginine (**2**) in approximately 50% yield. Separation from unreacted **1** was achieved by liquid chromatography (silica gel, solvent system B).

Anal. Calcd. for $C_6H_{14}N_4O_2 \cdot HCl$: C, 34.21; H, 7.18; N, 26.60. Found: C, 34.12; H, 7.28; N, 26.55; $[\alpha]_D$+23° (*c* 0.5, 5 N HCl).

The absolute stereochemistry of **1** at C-2 was thus established. Chemical degradations of **1** are summarized in Fig. 1. The total structure of **1** was elucidated by crystal structure analysis of the hydrobromide salt.

Intensity data were collected on a Hilger-Watts model Y290 diffractometer by θ—2θ scans. Because of the small size of the crystal (0.02 × 0.07 × 0.11 mm), there were only 1069 observed reflections ($I > 2.5\sigma(I)$) in the hemisphere with $\theta < 70°$ and $h \geqslant 0$. The intensity data were corrected for absorption. The structure was solved by the heavy atom method. The N and

Table 3. Crystal data of N^5-hydroxy-L-arginine hydrobromide

Empirical formula Formula weight	$C_6H_{14}N_4O_3 \cdot HBr$ 271.12	
Space group	$P2_1$	
a	5.192 (1)	Å
b	7.673 (3)	Å
c	13.585 (3)	Å
β	101.65 (2)	degrees
Z	2	
$d_{obs}(CHBr_2CHBr_2/C_5H_{11}Br)$	1.67	g cm^{-3}
d_{calc}	1.698	g cm^{-3}
$\mu(CuK\alpha)$	58.1 cm^{-1}	

Table. 4. Intermolecular hydrogen bond distances*

O(3) - - - H · · · O(2)i	2.65 Å	O(2) · · · H - - - O(3)ii	2.65 Å
N(1) - - - H · · · O(1)ii	2.81 Å	O(1) · · · H - - - N(1)i	2.81 Å
N(1) - - - H · · · O(1)iii	2.96 Å	O(1) · · · H - - - N(1)v	2.96 Å
N(1) - - - H · · · O(3)iv	3.12 Å	O(3) · · · H - - - N(1)vi	3.12 Å

* Superscripts denote atoms whose coordinates are related to those in the basic molecule by the following transformations:

i	x	0.5+y	z
ii	x	−0.5+y	z
iii	1.0+x	y	z
iv	x	−1.0+y	z
v	−1.0+x	y	z
vi	x	1.0+y	z

Fig. 2. Stereodrawing of conformation of the cation in a crystal of N^5-hydroxy-L-arginine hydrobromide

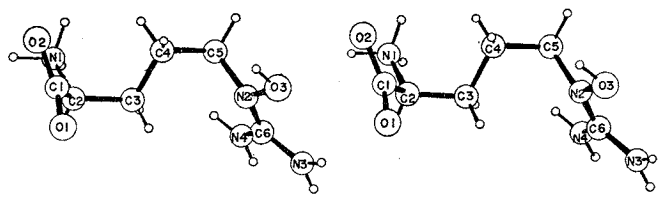
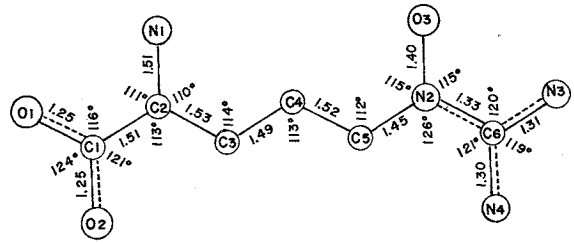


Fig. 3. Bond length and bond angles in N^5-hydroxy-L-arginine hydrobromide



O atoms were identified on the basis of the temperature factors and molecular geometry found following the initial refinement in which all C, N and O atoms were treated as C atoms. After additional refinement, structure factors were calculated for both enantiomers. The absolute configuration was taken to be the one with the lower R value (R_2=0.083 and 0.091). For the final cycles of least squares refinement, the Br^- ion had anisotropic thermal parameters, the C, N and O atoms had individual isotropic temperature factors, and the hydrogens were held fixed at their calculated positions. The final discrepancy index was R=5.5 %.

The crystal data are summarized in Table 3. A stereodrawing, showing the conformation of the cation in the crystal, is presented in Fig. 2. The bond lengths and angles are shown in Fig. 3 and the intermolecular hydrogen bond distances are given in Table 4.

Acknowledgements

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Note added in proof: N^5-Hydroxyarginine has also been isolated by Drs. W. KELLER-SCHIERLEIN and H. ZÄHNER (personal communication).