MICROBIAL PRODUCTION OF VITAMIN B_{12} ANTIMETABOLITES. I

N^5-HYDROXY-L-ARGININE FROM *BACILLUS CEREUS* 439

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One of the vitamin B_{12} antimetabolites produced by *Bacillus cereus* 439 has been identified as N^5-hydroxy-L-arginine on the basis of its mass-spectrum, ^{13}C-NMR, and mobilities in thin-layer chromatography and paper chromatography in comparison with standard material. The inhibition of growth of *Escherichia coli* (Davis 113-3) by this compound was reversed by vitamin B_{12} and was potentiated by L-lysine and 4-hydroxy-L-lysine. Vitamin B_{12} also partially reversed the inhibition of growth by N^5-hydroxy-L-arginine of *Pseudomonas aeruginosa*, other strains of *E. coli*, and *Klebsiella pneumoniae*. While neither N^5-hydroxy-L-arginine nor L-lysine affected the growth in tissue culture of KB cells, the mixture of these two amino acids was quite inhibitory.

A search for microorganisms producing substances inhibiting vitamin B_{12}-stimulated growth of *Escherichia coli* (Davis 113-3) resulted in selection of a dozen which produced this type of activity[1]. One of these organisms designated as No. 439 (later identified as *Bacillus cereus*) produced two substances with vitamin B_{12} antagonist potency in the *E. coli* (Davis 113-3) system[2]. N^5-Hydroxy-L-arginine, an amino acid previously described as a metabolite from an unidentified *Bacillus* species[3] and from the ascomycete *Nannizzia gypsea*[4], was found to be the most potent of the two inhibitors from *B. cereus* 439. We wish to summarize in this communication our studies on the factors affecting production of this amino acid by *B. cereus* 439 and some of its properties as a vitamin B_{12} antagonist in biological systems.

Materials and Methods

Isolation of culture 439.

A soil sample collected in Madison was diluted with sterile water and plated on nutrient agar. Discrete colonies were transferred to tubes of peptone-meat extract-glucose broth and placed on a shaker in a 30°C-incubator. After several days growth aliquots of the fermented media were centrifuged and the supernatant liquid tested for ability to inhibit growth of *E. coli* (Davis 113-3) (growing on the DAVIS and MINGIOLI medium[5] with added vitamin B_{12}), *E. coli* (growing on nutrient agar), and *Staphylococcus aureus* FDA 209 P (growing on nutrient agar). Those cultures producing broths inhibiting the *E. coli* (Davis 113-3) and not the *E. coli* B or *S. aureus* FDA 209P were selected for further study. One of these was designated as 439 and maintained on slants of nutrient agar and as a suspension in liquid nitrogen.

Identification of culture 439.

Culture 439 was grown in a variety of media mentioned in BERGEY's manual[6] and compared with *Bacillus cereus* ATCC 14579 and *Bacillus mycoides* ATCC 6462 grown under the

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same conditions. On the basis of colonial morphology on glucose agar, culture 439 is closely related to *B. cereus*. Other tests showing identity included: Gram-stain; malachite green stain; crystal violet stain; acid production on glucose agar and no acid production on xylose or mannitol agar; a positive VOGES-PROSKAUER test.

Bioassay of vitamin B_{12} antimetabolites from culture 439.

Samples from media fermented by culture 439 were assayed for presence of vitamin B_{12} antagonists by an agar diffusion method and a turbidimetric method. The agar diffusion assay involved placing 12.7-mm paper discs dipped in the test solutions on the surface of agar plates seeded with *E. coli* (Davis 113-3) and noting the diameter of the inhibition zone after incubation of the agar plates at 37°C for 18 hours. (The plates were prepared as previously described[7] with the addition of 5 mcg of cyanocobalamin to 200 ml of the DAVIS-MINGIOLI medium). The slope of the dose-response curve for a doubling in concentration of the N^5-hydroxy-L-arginine was usually about 3.5 mm, and a solution containing 100 mcg/ml gave a zone with a 22-mm diameter.

The turbidimetric assay used was a modification of that of BURKHOLDER[8] using 25×150 mm test tubes (containing 10 ml of medium) as culture vessels and incubation at 37°C for 17 hours (unless otherwise specified).

Tissue culture cytotoxicity tests.

Cytotoxicity of test preparations was determined by the standard procedures of the National Cancer Institute[9] using KB cells.

Identification of L-lysine and N^5-hydroxy-L-arginine from media fermented by *B. cereus* 439.

Melting points were determined in a Thomas-Hoover capillary apparatus (and are not corrected). Optical rotations were determined in a Bendix 1100 polarimeter. IR spectra were obtained in KBr discs using a Perkin Elmer model 257 spectrophotometer. NMR spectra were run in D_2O using a Bruker Spectrospin (model HX-90E) with TMS as external reference. ^{13}C-NMR spectra were run in D_2O-H_2O (1:4) solution using the Bruker instrument with dioxane as internal standard (the chemical shift values are given relative to TMS).

Mass spectra were recorded on a Finnigan 1015 quadrupole apparatus using a direct insertion probe and operating at 70 eV ionization energy and 250 microamperes electron energy. Trimethylsilyl derivatives of the compounds were prepared by reaction of 0.5 ml bistrimethylsilyltrifluoroacetamide (BSTFA) and 1.0 mg amino acid in 0.2 ml acetonitrile at 125°C for 15 minutes.

Micro-analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

The purity of the compounds was checked by means of thin-layer chromatography with Macherey and Nagel precoated silica gel plates. Ionophoresis was performed using a Savant apparatus (56 volts/cm for 40 minutes) and sheets of Whatman No. 1 paper.

Isolation of N^5-hydroxy-L-arginine from media fermented by *Bacillus cereus* 439.

Eight liters of the Casamino acids®-glucose medium (fermented by *B. cereus* 439 for 3 days at 30°C) with a potency of 120 mg N^5-hydroxy-L-arginine per liter (by bioassay) was adjusted with 6 N HCl to pH 2.0 and then filtered with the aid of acid-washed Celite (Hyflo Super Cel®). The clarified broth was then treated with 80 g of Darco G-60, stirred for 1 hour at room temperature and filtered. The filtrate was passed through a 4.7×57 cm column of Dowex 50W×2 resin (100~200 mesh, H^+ form). The column was washed consecutively with 2 liters of water, 4 liters of 5% aqueous pyridine, and 2 liters of water. The N^5-hydroxy-L-arginine was then eluted with 3 liters of 1 N NH_4OH. The bioactive fractions were combined and lyophilized to give 7.5 g of approximately 5% purity. This was dissolved in 40 ml of pH 9.4 alkaline buffer (N-ethylmorpholine—α picoline—pyridine—CO_2 free distilled water (60:80:40:3820) which was adjusted with acetic acid to pH 9.4) and the solution applied to a 5.3×64 cm column of Dowex AG 1×2 resin (200~400 mesh, Na^+ form). The column was eluted with 4 liters of the alkaline pH 9.4 buffer. Those fractions containing ninhydrin-positive material were combined and

concentrated *in vacuo* to a small volume. This was then acidified with 6 N HCl and evaporated to dryness. Water was then added and the evaporation repeated (to remove excess HCl). Solution of the syrupy residue in methanol followed by neutralization with pyridine and addition of excess ethanol yielded 2.37 g of crystals. Recrystallization from hot water-ethanol 1:10 afforded 1.82 g product, m.p. 200~205°C (dec.), $[\alpha]_D^{25}+30°$ (*c* 1, 5 N HCl).

Anal. Calcd. for $C_6H_{14}N_2O_2 \cdot HCl$: C, 39.45; H, 8.28; N, 15.34; Cl, 19.41.
Found: C, 39.57; H, 8.12; N, 15.37; Cl, 19.52.

These data together with spectroscopic evidence showed that the compound was L-lysine monohydrochloride.

The antimetabolite was eluted from the Dowex AG 1×2 column with 3 liters of the same buffer of pH 8.4, obtained by addition of more acetic acid. The antimetabolite-containing fractions were combined, concentrated to a small volume and brought to dryness after adjustment to pH 5.0 with diluted HCl yielding an oil, which crystallized upon addition of ethanol-ether (1:4). Recrystallization from aqueous ethanol solution afforded 305 mg of a pure compound: m.p. 202~206°C (dec.); $[\alpha]_D^{25}+19°$ (*c* 1, 5 N HCl): IR (KBr), 3380, 3360, 3300, 3057, 2956, 1490 (NH and OH), 1680, 1595 (C=N, guanidium I and II) 1610, 1415 (COO^-) cm^-; *m/e* of the pentatrimethylsilyl derivative 550; NMR (D_2O) δ 2.35 (m, $J \cong 6$ cps, β CH_2 and γ $-CH_2$), 4.19 (t, $J=6$ cps, δ—CH_2); 4.27 (t, $J=5.6$ cps, α—CH).

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, 31.55; H, 6.81; N, 24.54; Cl, 15.76.

Results and Discussion

Factors Affecting N^5-Hydroxy-L-arginine Production by *Bacillus cereus* 439

Preliminary study showed that antimetabolite production by *B. cereus* 439 was independent of growth of the organism: Although good growth was obtained over incubation temperature range 20°~40°C in a number of media, in either static or shaken culture, optimal conditions for antimetabolite production were high aeration (achieved in shaken flasks; 25 ml medium per 250 ml Erlenmeyer flask) and incubation at 30°C.

Some of the data collected in a study of the effect of composition of the growth medium on antimetabolite production are summarized in Table 1. (The agar diffusion bioassay was used in this study with 200 mg/liter of L-lysine·HCl added to the DAVIS-MINGIOLI agar medium). Addition of L-arginine·HCl periodically to growing cultures of inclusion of this amino acid at a level of 100 mg/liter of the four media listed in Table 1 did not result in increased antime-

Table 1. Effect of fermentation medium composition on production of N^5-hydroxy-L-arginine by *Bacillus cereus* 439

Composition of medium g/liter	Production of N^5-hydroxy-L-arginine (mg/liter)			Growth (O.D.)		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Casamino acids®, 3 g glucose, 10 g	85.0	90.0	120.0	2.7	2.9	3.5
Casamino acids®, 36 g glucose, 10 g	2.5	3.5	3.0	8.4	9.1	9.7
Difco nutrient broth, 8 g glucose, 10 g	1.5	1.9	3.1	6.9	7.3	8.3
Difco yeast extract, 5 g glucose, 10 g	1.7	2.5	3.5	6.8	7.3	11.0

Fermentation condition: 25 ml medium per 250 ml cotton plugged Erlenmeyer flask placed on rotary shaker (280 rpm, 1-inch displacement) in a 30°C incubator

tabolite production as determined by the agar diffusion bioassay.

B. cereus 439 produces a second vitamin B_{12} antimetabolite (identified by MATTHEWS[3] as a peptide and denoted as "Factor A"). The optimal conditions for production of this material differ markedly from those for N^5-hydroxy-L-arginine, with maximum yields being obtained when *B. cereus* 439 was grown in nutrient broth, and poor production obtained when the organism was grown in Casamino acids® containing media.

Identification of *B. cereus* 439 Metabolite as N^5-Hydroxy-L-arginine

The *B. cereus* 439 metabolite was identified as N^5-hydroxy-L-arginine on the basis of the following characteristics:

1) Melting point: 202~206° (dec.). (MAEHR *et al.*[3] report 200~210°C (dec.)
2) Rotation: $[\alpha]_D^{25}+19$ (*c* 1, 5 N HCl)·(MAEHR *et al.*[3] report $[\alpha]_D^{25}+19$ (*c* 0.9, 5 N HCl)
3) When the compound was heated in 0.5 N $Ba(OH)_2$ at 100°C for 2 days, glutamic acid was formed (identified by tlc), and hydrogenation with Pd on carbon yielded L-arginine. (Both of these conversions were previously reported for N^5-hydroxy-L-arginine by MAEHR *et al.*[3])
4) Analysis: calculated for $C_6H_{14}N_4O_3 \cdot HCl$: C, 31.79; H, 6.67; N, 24.73; Cl, 15.64; found: C, 31.55; H, 6.81; N, 24.24; Cl, 15.76.
5) Mobility in thin chromatography and paper ionophoresis is summarized in Table 2. The R_f values obtained in these solvents are in line with those reported by MAEHR *et al.*[3]

Table 2. Mobility of N^5-hydroxy-L-arginine and related compounds in thin-layer chromatography and paper ionophoresis

Amino acid	R_f in thin-layer chromatography			Mobility in ionophoresis at pH 1.9
	System A	System B	System C	
L-N^5-Hydroxy-N^5-hydroxy-L-arginine	0.32	0.62	0.62	18.3 cm
L-Arginine	0.18	0.38	0.43	21.2
L-Lysine	0.09	0.26	0.48	23.6
L-Ornithine	0.14	0.32	0.46	23.3

Composition of solvent systems:
system A: ethanol-water-conc. NH_4OH, 49:49:2
system B: ethanol-water-conc. NH_4OH, 40:50:10
system C: chloroform-methanol-NH_4OH-water, 10:40:10:20
pH 1.9 buffer: acetic-formic acid-water, 20:2:78

6) I.R. (in KBr pellet): 3380, 3360, 3300, 3057, 2956, 1490 (NH and OH), 1680, 1505 (C=N, guanidium I and II), 1610, 1415 (COO^-) cm^{-1}. The presence of a guanidine group is supported by the 1680 and 1595 bands in the IR spectrum. Furthermore, the position of these guanidinium bands suggests that the guanidine group is disubstituted rather than monosubstituted as in arginine[10].
7) Mass spectrum: *m/e* of the pentatrimethylsilyl derivative, 550. The mass spectrum is shown in Fig. 1. The fragmentation pattern is similar to that observed by FISCHER *et al.*[4] The easily recognizable molecular ion and M^+-15 $(R\text{-}O{=}SiMe_2)^+$ pair of peaks at *m/e* 550 and 535 are consistent with the presence of 5 trimethylsilyl groups. Ions of no structural utility or ions arising from residual silylating reagent include *m/e* 59 $(MeCO\text{-}NH_2)^+$, 73 $(Me_3Si)^+$, 75 $(Me_2SiOH)^+$, and 147 $(Me_3SiO{=}SiMe_2)^+$. Some of the fragment ions observed in the high mass region of various trimethylsilylated amino acids by BARKER *et al.*[11] are found at 478 (M^+-Me_3SiH), 463 (M^+-Me-(Me_3SiH), and 446 (M^+-Me-(Me_3SiO). Characteristic abundant ions at *m/e* 364 and 187 are explained by the loss of the $Me_3SiN{=}C{=}NSiMe_3$

Fig. 1. Mass spectrum of trimethylsilyl derivative of N^5-hydroxy-L-arginine

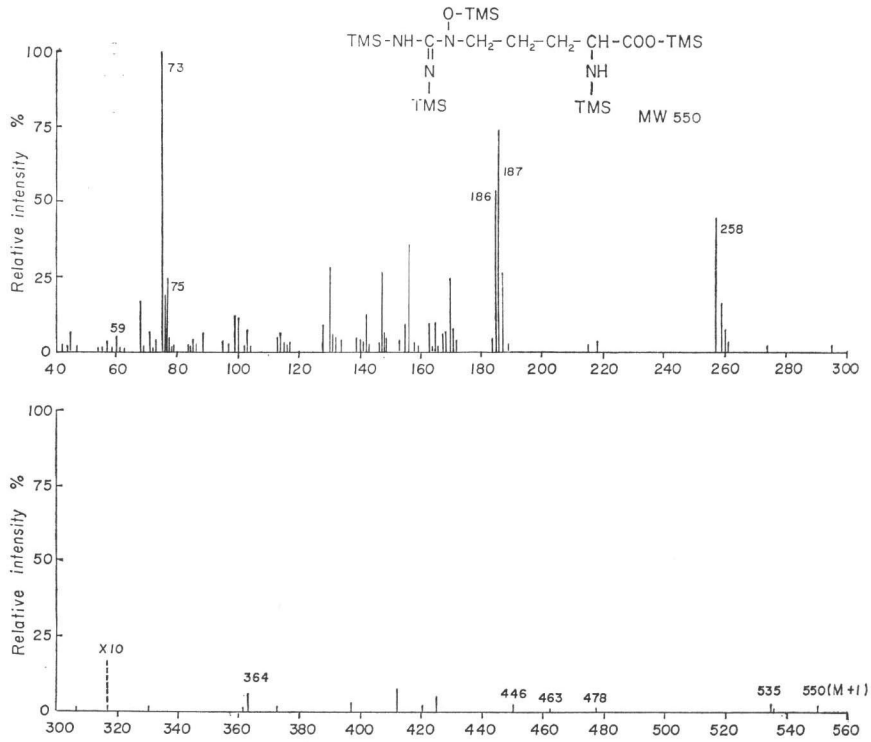


Table 3. ^{13}C NMR Spectrum of N^5-hydroxy-L-arginine and related compounds

Compound	COO^-	C=N	$\alpha-C$	$\delta-C$	$\beta-C$	δ_1-C
N^5-Hydroxy-L-arginine	175.1	158.8	55.4	51.5	28.2	22.5
L-Arginine	175.1	157.8	55.3	41.5	28.5	24.8
L-Lysine	175.3	40.2	55.5	30.8	17.4	22.3

Samples dissolved in D_2O-H_2O(1:4) with results reported relative to TMS

fragment and the formation of the corresponding $Me_3SiNH^+=C=NSiMe_3$ ion (which indicates that the hydroxyl group is not located on one of the ω-nitrogens).

8) ^1H NMR (in D_2O): δ2.35 (m, J≅6 cps, $\beta-CH_2$ and $\gamma-CH_2$); 4.19 (t, J=6 cps, $\delta-CH_2$); 4.27 (t, J=5, 6 cps, $\alpha-CH$).

9) ^{13}C NMR (in D_2O-H_2O, 1:4): δ 175.1 (COO^-), 158.8 (C=N), 55.4 ($\alpha-C$), 51.5 ($\delta-C$), 28.2 ($\beta-C$), 22.5 ($\gamma-C$). The ^{13}C NMR of N^5-hydroxy-L-arginine is compared with L-arginine and lysine in Table 3.

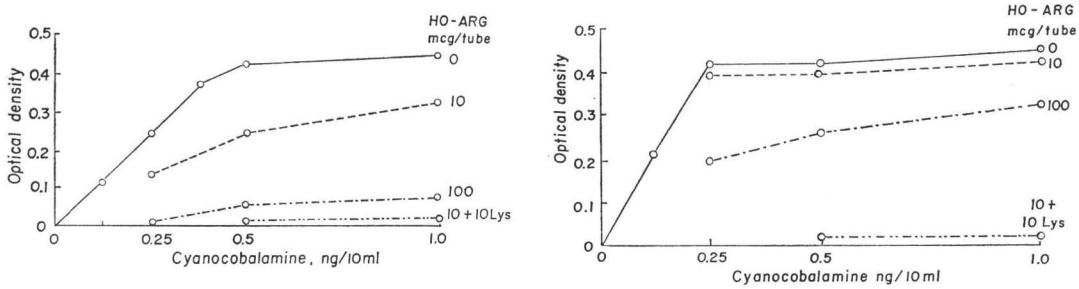
N^5-Hydroxy-L-arginine as a Vitamin B_{12} Antimetabolite in *Escherichia coli* (Davis 113-3)

Examination of the growth-inhibitory properties of N^5-hydroxy-L-arginine for *E. coli* (Davis 113-3) in the turbidimetric assay showed that decreased growth inhibition occurred when the incubation period was extended. Some of the data collected are summarized in Fig. 2. If the incubation period was extended to 40 hours, no inhibition of the *E. coli* (Davis 113-3) was observed with levels of N^5-hydroxy-L-arginine up to 200 mcg/tube (10 ml).

Fig. 2. Effect of incubation period on cyancobalamin reversal of N^5-hydroxy-L-arginine growth inhibition of *Escherichia coli* (Davis 113-3)

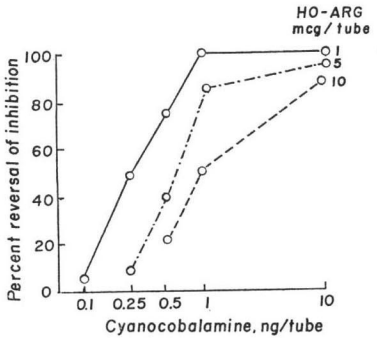
A: Incubated at 37°C for 18 hours

B: Incubated at 37°C for 24 hours



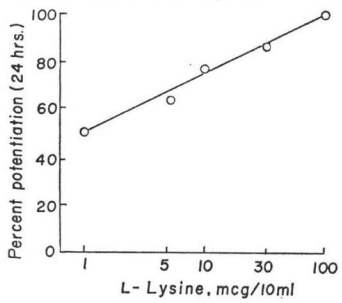
A study of the relationship of concentration of cyanobalamin to N^5-hydroxy-L-arginine is summarized in Fig. 3. A definite reduction in sensitivity of the bacteria to the amino acid was noted with increasing concentration of cyanocobalamin. The inhibition index for these components ranged from 500 to 1,000 *e.g.* 1 ng cyanocobalamin reversed inhibition by 1 mcg of the amino acid.

Fig. 3. Cyancobalamin reversal of growth inhibition of *Escherichia coli* (Davis 113-3) by N^5-hydroxy-L-asginine



As mentioned above, L-lysine is also found in these fermentations and was discovered to potentiate the growth inhibition caused by addition of N^5-hydroxy-L-arginine to the *E. coli* (Davis 113-3). (This was independently noted by FISCHER *et al.*[4] using a cross-strip agar diffusion assay). Some of the data collected in the study of L-lysine potentiation are summarized in Fig. 2 and 4. The mechanism of this potentiation is not known, but 4-hydroxy-L-lysine also has this effect in the turbidimetric assay system. When a 3-disc arrangement was used in the agar diffusion assay with cyanocobalamin, N^5-hydroxy-L-arginine and L-lysine on the separate discs, the lysine reversed the growth inhibition of the N^5-hydroxy-L-arginine, and the lysine had some potentiating effect on the competition by N^5-hydroxy-L-arginine with cyanocobalamin.

Fig. 4. L-Lysine potention of N^5-hydroxy-L-arginine inhibition of cyancobalamin stimulated growth of *Escherichia coli* (Davis 113-3)



N^5-Hydroxy-L-arginine as a Vitamin B_{12} Antimetabolite in Bacterial Systems and KB Cells

Examination of the growth-inhibitory properties of N^5-hydroxy-L-arginine toward other microorganisms showed that it inhibited growth in a turbidimetric assay system when *Pseudomonas aeruginosa* (aminoglycoside antibiotic resistant), *E. coli* (ATCC 9732) and *Klebsiella pneumoniae* (ATCC 8724) were the test organisms. The inoculum for these experiments was grown

in nutrient broth and the cells were washed with sterile water prior to being used to inoculate DAVIS-MINGIOLI medium[5]. The growth inhibition was reduced if cyanocobalamin was added to the medium and potentiated if L-lysine was added.

N^5-Hydroxy-L-arginine·HCl was found to inhibit the growth of KB cells in the standardized tissue culture system[9]. Only slight inhibition was noted when the pure compund was used alone, *e.g.* ID_{50} was about 100 mcg/ml. However, when the N^5-hydroxy-L-arginine was mixed with L-lysine at a ratio of 1:50, marked inhibition was noted with an ID_{50} of the mixture of 1 mcg/ml.

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