MICROBIAL PRODUCTION OF VITAMIN B_{12} ANTIMETABOLITES. IV

ISOLATION AND IDENTIFICATION OF 4-KETO-5-AMINO-6-HYDROXYHEXANOIC ACID

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4-Keto-5-amino-6-hydroxyhexanoic acid was isolated from *Bacillus cereus* 102804 fermentations and found to inhibit the growth of Gram-positive and Gram-negative bacteria, when grown in a chemically defined medium. The mechanism appeared to be the inhibition of δ-aminolevulinic acid dehydratase. The Ki value of 4-keto-5-amino-6-hydroxyhexanoic acid in an enzyme preparation of *Propionibacterium shermanii* was 0.72 μM. Similar test conditions with 4-keto-5-aminohexanoic acid resulted in a Ki of 12.1 μM. In both cases competitive inhibition was found. The structure of 4-keto-5-amino-6-hydroxyhexanoic acid was determined.

Study of media fermented by *Bacillus cereus* 102804 showed that for a relatively short interval in the incubation period a vitamin B_{12} antagonist was formed[1]. This substance was isolated and found to inhibit the N^5-methyltetrahydrofolate-homocystein transmethylase[2]. The substance also caused growth inhibition in some Gram-positive and Gram-negative bacteria in chemically defined medium.

In this paper we will present the structure elucidation and chemical synthesis of the isolated fermentation product 4-keto-5-amino-6-hydroxyhexanoic acid. Physiological properties of this substance will be demonstrated, which explain the reason for growth inhibition in bacteria. Some other properties of 4-keto-5-amino-6-hydroxyhexanoic acid will be reported.

Materials and Methods

Isolation and identification of *B. cereus* 102804 was carried out as described before[1].

Bioassay of Vitamin B_{12} Antimetabolite Activity from *B. cereus* 102804

Samples for media fermented by *B. cereus* 102804 were assayed for the presence of vitamin B_{12} antagonist by an agar diffusion method. The agar diffusion assay involved placing 12.7 mm paper discs dipped in a test solution on the surface of agar plates seeded with *E. coli* (Davis 113–3) and noting the diameter of the inhibition zones after incubation of the agar plates at 37°C for 18 hours. (The plates were prepared as previously described[3] with the addition of 20 mcg of cyanocobalamin to 200 ml of the DAVIS-MINGIOLI medium). The activity which showed a 20 mm inhibition zone was defined as 1 unit. The slope of the dose response curve was usually about 3 mm. The ability of the amino acids and vitamin B_{12} to reverse the growth inhibitory effect of 4-keto-5-amino-6-hydroxyhexanoic acid (I) on *E. coli* (Davis 113–3) and other organisms was measured by a modification of the SMITH[4] agar diffusion method. The minimal inhibitory concentration (M.I.C.) of aminoglycoside antibiotics for a series of bacteria were determined by the gradient plate method with Difco antibiotic assay medium No. 1 (pH 6.6) using washed cells of 24 hours old cultures of *E. coli* (Davis 113–3), *B. subtilis* (ATCC 6633), *S. aureus* (FDA 209P), and *E. coli* B (ATCC 23226). The gradient agar for minimal inhibitory concentration determination in the presence of the reversant aminoglycoside antibiotic contained the reversant in both layers of the gradient.

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Fermentations by *B. cereus* 102804 were carried out as described before[1].

Isolation of 4-Keto-5-amino-6-hydroxyhexanoic Acid from Fermented Media

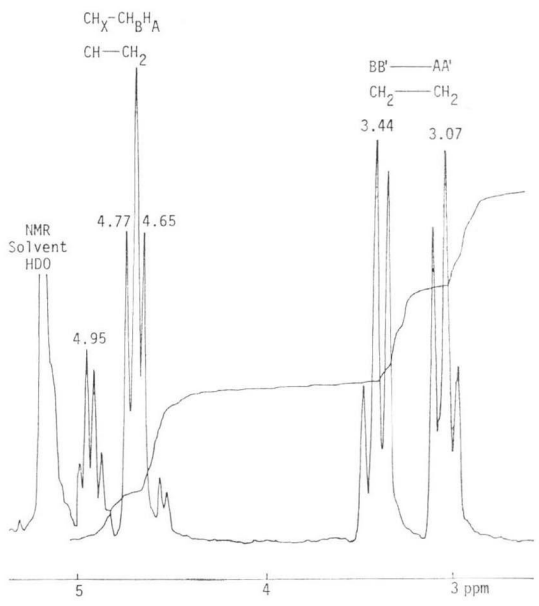
Four liters of fermented soybean meal-glucose medium with a potency of 25 units/ml were adjusted to pH 3 by addition of 6 N HCl and the mixture was then centrifuged. The supernatant solution was then passed through a column (5 cm × 60 cm) of Dowex 50W-×4 resin (100~200 mesh, H^+ cycle). The antimetabolite was eluted with 0.6 M aqueous pyridine. After removing the pyridine from the 'bioactive fractions' in vacuo, the solution was lyophilized. The 4 g of crude powder thus obtained had a potency of 22 units/mg. Three gram of this crude powder was dissolved in 10 ml of pyridine-acetic acid solution (pH 3.1)[5]. The pH adjusted to 2.7 with 6 N HCl and applied to a column (2×75 cm) of Dowex 50W-×4 resin (200~400 mesh) which has been equilibrated with pyridine-acetic acid solution (pH 3.1). The desired compound was separated by gradient elution (pH 3.1~pH 5.1) with 15 ml fractions being collected. A total of 90 fractions were collected. Each was concentrated in vacuo and analyzed for 4-keto-5-amino-6-hydroxyhexanoic acid (**I**) using the thin-layer chromatographic (TLC) system butanol - acetic acid - water (4: 1: 2). Fractions 59~66 contained **I**, giving a yellow color with ninhydrin reagent, similar to δ-aminolevulinic acid. They were pooled and evaporated and finally lyophilized to give 200mg of a powder of 160 units per mg. Further purification was obtained by dissolving this powder in water, acidification with 6 N HCl to pH 3 and passing through a column (2×30 cm) of Dowex 50W-×4 resin (100~200 mesh, H^+). The antimetabolite was eluted with 0.6 M pyridine and the 'bioactive fractions' pooled. Extremely hygroscopic powder (80~100 mg) was obtained which sometimes could be crystallized with difficulty from acetone-water mixtures. It gave a single spot on thin-layer chromatography (Rf 0.25) (silica gel) in the system butanol - acetic acid - water (4: 1: 2) and gave a yellow color with ninhydrin spray. It also gave a single spot in ionophoresis at pH 1.9 (acetic acid - formic acid buffer) with a mobility of 19 cm when the current was 85 volts per cm. The following data were obtained with the crystalline material: the potency of the pure material was 160 U/mg. The elemental analysis gave the molecular formula $C_6H_{11}NO_4$ (M.W. 161.16). The molecular weight by high resolution mass spectroscopy was misleading since it gave M.W. as 268.27 (This is also characteristic of δ-aminolevulinic acid.). U.V. and I.R. data, rotation and melting point were reported earlier[1].

Anal. Calcd. for $C_6H_{11}NO_4$: C 44.72; H 6.88; N 8.69
Found: C 44.59; H 6.94; N 8.49

Structure Elucidation

Structure elucidation was done by NMR (Fig. 1) and the conclusion reached that the B_{12} antimetabolite from *B. cereus* 102804 is 4-keto-5-amino-6-hydroxyhexanoic acid.

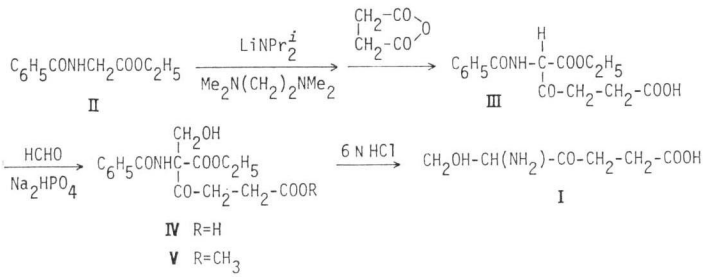
Fig. 1.



Synthesis of 4-Keto-5-amino-6-hydroxyhexanoic Acid (**I**)

The following reaction sequence was used for the preparation of **I**: Ethyl hippurate (**II**) was treated through the lithio dianion with succinic anhydride according to the literature[6] to give the known **III**, a ketoaminoadipic acid derivative. Formylation of the methanol solution of **III** with 33% formaldehyde solution and Na_2HPO_4 gave in excellent yield the expected C-formyl derivative **IV**. This was purified for analytical purposes by treatment with diazomethane to form the methylester **V**. This after thick-layer chromatography on silica gel with the solvent system ethyl acetate - hexane (1: 1) gave pure **V** with correct elemental analysis and ^1H and ^{13}C NMR. 6 N HCl hydrolysis gave the expected **I** together

with a number of side products and serine. Separation was carried out through ion exchange chromatography on Dowex 50W ×4 resin with the gradient elution method as described for the isolation of the natural product. This gave a single ninhydrin positive spot on TLC with the characteristic yellow color, identical with the natural product. It had a bioactivity similar to the natural product and the NMR revealed the characteristic signals of the natural product.



Ethyl 2-Benzoylamino-3-ketoadipate (III)[6]

A solution of 4.2 ml of diisopropylamine (30 mmole) and 4.5 ml tetramethylethylenediamine (TMEDA) (30 mmole) in 50 ml tetrahydrofuran at −75°C was treated with 20 ml *n*-BuLi (1.5 mole in hexane) and stirred for 20 minutes. Ethyl hippurate (3.1 g, 15 mmole) dissolved in 25 ml anhydrous tetrahydrofuran was added dropwise at −75°C. The thick yellow suspension was stirred for an additional 1 hour at −75°C. Succinic anhydride (1.5 g, 15 mmole) dissolved in 30 ml anhydrous tetrahydrofuran was then added and stirred for 1 hour at −75°C. The mixture was allowed to warm to 0°C poured on ice and the layers separated. The water phase was acidified at 0°C with 6 N HCl and then extracted several times with ethyl acetate. The ethyl acetate was dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The residue crystallized. The crystals were washed with a small amount of cold ether and dried to give 2.5~2.8 g of white crystalline ethyl 2-benzoylamino-3-ketoadipate (**III**), m.p. 114~116°C[6].

4-Keto-5-carbethoxy-5-benzoylamino-6-hydroxyhexanoic Acid (IV) and the Methylester (V)

III (500 mg) was dissolved in 5 ml methanol and 5 ml 30% formaldehyde solution containing 0.2 g Na_2HPO_4 was added. The mixture was stirred for 16 hours at room temperature, the methanol evaporated under reduced pressure and to the residue 2~3 ml water added. The water phase was extracted several times with ethyl acetate, the ethyl acetate dried over anhydrous $MgSO_4$, filtered and evaporated. The residue was dissolved in ether and ethereal diazomethane solution added until the yellow color of the diazomethane stayed. The ether was then evaporated and the residue dissolved in a small amount of ether and applied to thick-layer silica gel plates (2 mm, Brinkmann). The plates were developed with ethyl acetate - hexane, 1: 1 solvent mixture. The fluorescent band contained the expected formyl compound which could be eluted with chloroform and methanol 95: 5 mixtures. The solvent was evaporated to give the pure formyl derivative as an oil.

Anal. Calcd. for $C_{17}H_{21}NO_7$ (MW 351.36): C 58.11; H 6.02; N 3.99
Found: C 57.89; H 6.14; N 4.07

PMR: ($CDCl_3$) 3.5 (s, 3H, OCH_3), 4.2 (q, 2 H, ethylester), 1.17 (t, 3H, ester), 4.38 (s, CH_2OH), 2.8 and 2.5 (2× m, 4H, succinyl CH_2-s), 7.3~7.5 (m, 5 aromatic).

^{13}CMR: ($CDCl_3$) 14.0 (methyl of ethylester), 27 and 32 (succinyl carbons or C_4 and C_5), 52 (methoxy carbon), 62 and 63 (hydroxymethyl carbon and ethylester O-CH_2), 73 (quaternary carbon), 127, 128, 142, 143 (phenyl carbons), 166, 167, 173, 190 (carbonyls).

4-Keto-5-amino-6-hydroxyhexanoic Acid (I)

Pure **V** (200 mg) was hydrolyzed with 5 ml 6 N HCl for 4 hours at reflux temperature. The hydrochloric acid was evaporated under reduced pressure, water added and again evaporated. This was repeated until most of the HCl was removed. The residue was dissolved in small amount of water, the benzoic acid removed by ether extraction and the water solution evaporated. The residue contained besides the expected **I** a number of other by-products, among them serine. Further purification was

achieved by gradient elution ion exchange chromatography as described for the isolation of the natural product. This resulted in one ninhydrin positive (yellow characteristic color) spot on TLC identical with the natural product. It also showed the same biological activity, *i.e.*, inhibition zone with *E. coli* (Davis 113–3) and gave the characteristic signals in NMR.

Enzyme Extraction and Assay

For the preparation of cell free δ-aminolevulinic acid dehydratase extract from *Propionibacterium shermanii* and for the enzyme assay the general procedure of NANDI, BAKER-COHEN and SHEMIN[7] was followed: *P. shermanii* was cultivated under vitamin B_{12} production conditions. The harvested cells were washed twice in 0.8% NaCl, lyophilized and stored at −20°C until used for extraction. The crude extract was used for the enzyme assay. No corrections for porphobilinogen condensing enzymes were necessary. The assay mixture was preincubated for 10 minutes at 37°C. Then the reaction was started by the addition of the substrate and the inhibitor (neutralized with concentrated tris-HCl to pH 7 and stopped after 30 minutes of incubation). The porphobilinogen content was determined with Ehrlich reagent according to the method of MAUZERALL and GRANIC[8].

Results and Discussion

In the agar diffusion test 4-keto-5-amino-6-hydroxyhexanoic acid was a potent inhibitor of vitamin B_{12} stimulated growth in *E. coli* (Davis 113–3). This effect could be reversed competitively by counter-diffusion of appropriate amounts of δ-aminolevulinic acid (ALA). ALA is an intermediate in the biosynthesis of porphobilinogen, which serves as a precursor for vitamin B_{12} and porphine biosynthesis.

ALA-dehydratase catalyzes an aldol condensation of two molecules of ALA to prophobilinogen. The enzyme is inhibited competitively by levulinic acid[9] in *Rhodopseudomonas spheroides*. LARTILLOT and BARON[10] also using an enzyme preparation of *R. spheroides* observed competitive inhibition with various synthetic levulinic acid analogs of which 4-keto-5-aminohexanoic acid was the most potent. In our studies we used an ALA-dehydratase preparation from *P. shermanii* for the enzyme assay with 4-keto-5-amino-6-hydroxyhexanoic acid as inhibitor. 4-Keto-5-amino-hexanoic acid, the synthetic substrate analog, served as a control for our assay system. The LINEWEAVER BURK plot (Fig. 2) demonstrates that both substances act as competitive inhibitors of the ALA-dehydratase. Comparison of the kinetic constants (Table 1) showed that 4-keto-5-amino-6-hydroxyhexanoic acid has a 17 times stronger inhibitory potency than the synthetic substrate analog, 4-keto-5-aminohexanoic acid. Other vitamin B_{12} antimetabolites inhibitory to *E. coli* (Davis 113–3) such as 2-amino-4-keto-3-methylpentanoic acid[11] or 2-amino-4-methyl-5-hexenoic acid do not inhibit the ALA-dehydratase from *P. shermanii*. Also the presence of 50 mmoles of vitamin B_{12} in the assay did not influence the enzyme activity.

In addition to the competitive inhibitory action on ALA-dehydratase, 4-keto-5-amino-6-hydroxyhexanoic acid reversed bacterial growth inhibition caused by the aminoglycoside antibiotic kanamycin, neomycin, dihydrostreptomycin and gentamicin. A similar phenomenon was observed with 4-keto-5-

Table 1. Studies with δ-aminolevulinic acid dehydratase from *Propionibacterium shermanii*.

MICHAELIS constant for δ-aminolevulinic acid	Km 0.36 μM
Inhibition constant for 4-keto-5-amino-6-hydroxyhexanoic acid from *B. cereus*	Ki 0.73 μM
Inhibition constant for synthetic 4-keto-5-aminohexanoic acid	Ki 12.1 μM
Effect of 2-amino-4-methyl-5-hexenoic acid	no inhibition
Effect of 2-amino-4-keto-3-methylpentanoic acid	no inhibition
Effect of vitamin B_{12}	no inhibition

Fig. 2. Inhibition of porphobilinogen formation in crude δ-aminolevulinic acid dehydratase extract by 4-keto-5-amino-6-hydroxyhexanoic acid from *B. cereus* 102804 and synthetic 4-keto-5-aminohexanoic acid.

Double reciprocal plot of velocity and δ-aminolevulinic acid concentration. 58 U enzyme per test were used; 1 U forms 13 nmole porphobilinogen under test conditions.

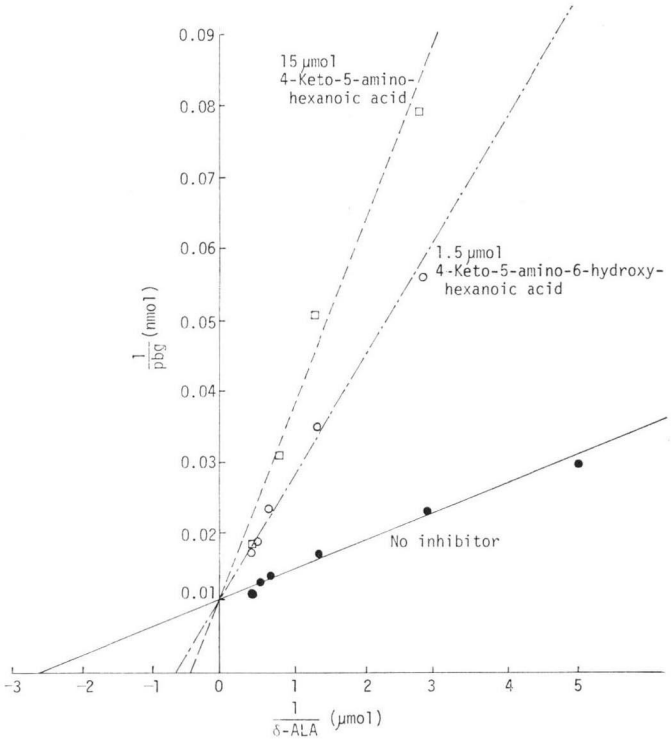


Table 2. Reversal by 90 U/ml 4-keto-5-amino-6-hydroxyhexanoic acid of bacterial growth inhibition by aminoglycoside antibiotics.

A 20 mm inhibition zone in *E. coli* (Davis 113-3) agar diffusion test was considered as 1 unit of activity. The antibiotics were applied as sulfate salts. (+; with reversant)

Test strain	Minimal inhibitory concentration μg/ml							
	Neomycin		Kanamycin		Gentamicin		Dihydrostreptomycin	
	Control	+	Control	+	Control	+	Control	+
E. coli B	11	50	3	7	2	6	4	4
E. coli (B_{12}^-)	39	50	4	7	4	7	5	10
B. subtilis	7	12	2	7	3	3	14	20
S. aureus	3	6	1	2	1	4	3	3

aminohexanoic acid. In both cases counterdiffusion test with *E. coli* (Davis 113-3) demonstrated a non-competitive inhibition mechanism. Determination of M.I.C. values for the aminoglycosides in the presence of both hexanoic acids in Difco antibiotic assay medium No. 1 (pH 6.6) clearly showed that the tolerance towards the aminoglycoside antibiotics was in most cases remarkably higher than in the presence of the pure antibiotics (Tables 2,3). This phenomenon seems to be unique for 4-keto-5-aminohexanoic acid and its 6-hydroxyalcohol. It still has to be shown whether this effect is due to a physiolo-

Table 3. Reversal by 4-keto-5-aminohexanoic acid (3 μmole/ml) of bacterial growth inhibition caused by aminoglycoside antibiotics.

Antibiotics were applied as sulfate salts. (+; with reversant)

Test strain	Minimal inhibitory concentration μg/ml							
	Neomycin		Kanamycin		Gentamicin		Dihydrostrepto-mycin	
	Control	+	Control	+	Control	+	Control	+
E. coli B	11	38	3	3.5	2	4	4	8
E. coli (B_{12}^-)	39	44	4	6	4	7	5	7
B. subtilis	7	24	2	4	3	3	14	30
S. aureus	3	30	1	4	1	4	3	12

gical mechanism or a chemical inactivation of the aminoglycoside antibiotics by these hexanoic acid derivatives.

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