ANTIMETABOLITES PRODUCED BY MICROORGANISMS. XIII[1] THE SYNTHESIS AND MICROBIOLOGICAL PRODUCTION OF A NOVEL AMINO ACID, L-2-AMINO-4-(2-AMINOETHOXY) BUTANOIC ACID

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A novel amino acid, L-2-amino-4-(2-aminoethoxy)-butanoic acid, was isolated from a fermentation broth of *Streptomyces* sp. X-11,085. It was shown to be identical with the chemical reduction product of an antimetabolite antibiotic, L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid, a co-product in the fermentation. Addition of the title compound to the fermentation led to an enhanced yield of the antimetabolite suggesting that the saturated amino acid serves as a precursor for the antimetabolite.

A *de novo* chemical synthesis and enzymatic resolution of the title compound are also reported.

We have previously[2] described the fermentation, isolation and chemical characterization of an antimetabolite antibiotic produced by an unclassified *Streptomyces* sp. X-11,085. Based primarily on spectral evidence, the antimetabolite was assigned[2] the structure, **1** (Fig. 1).

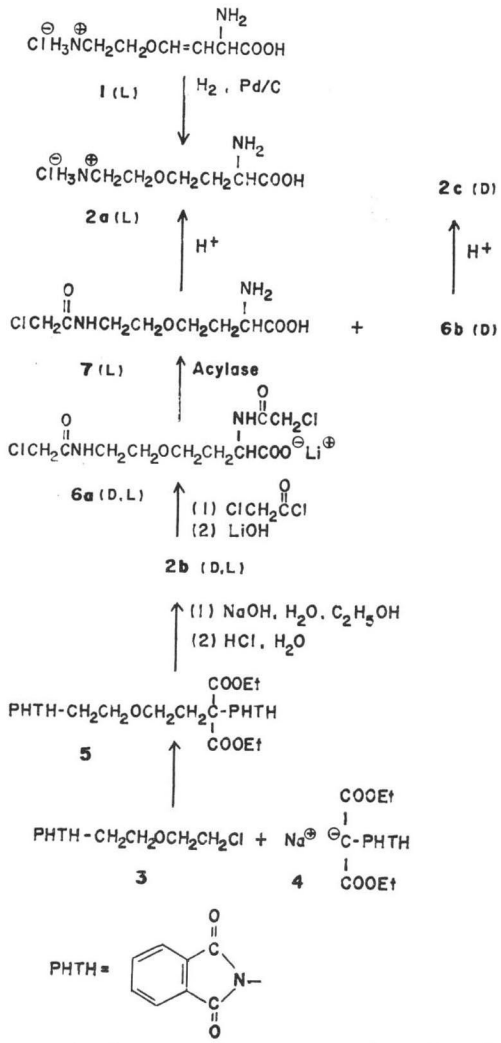
Amino acid analysis of the fermented broth revealed the presence of another basic amino acid which was isolated and shown to be identical with **2a**, a catalytic reduction product of **1**. The saturated compound, **2a**, was also synthesized by condensation of **3**[3] with **4**[4] followed by removal of protecting groups, decarboxylation, and enzymatic resolution of the chloroacetyl derivative, **6a**. Although **2a** did not show antibiotic or antimetabolite activity, it did stimulate the production of ethylene by plant tissues.

Experimental

General Methods and Materials

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 in D_2O using the Jeol C-60H instrument, and optical rotations were measured with a Perkin-Elmer 141 polarimeter. The purity of preparations was determined by thin-layer chromatography with Merck silica gel F-254 plates developed in ethanol - water - ammonia (49:49:2, v/v/v). Amino acid analysis of ultrafiltered broth samples was performed with the Jeol-6AH instrument using the 6-cm column at 45°C and a flow rate of 0.75 ml/min of 0.015 M sodium citrate, pH 5.5. The highly sensitive fluorescamine system[5] was originally used to detect trace amounts of **2** in the broth.

Fig. 1. Synthesis of L-2-amino-4-(2-aminoethoxy)-butanoic acid



Fermentation

Inoculum for the seed-tank was prepared as described previously.[2] Four liters of inoculum was added to 230 liters of seed-tank medium containing (in g/liter): Bacto peptone (Difco), 17; soy peptone T1 (Sheffield), 3; NaCl, 5; K_2HPO_4, 2.5; and dextrose. 2.5. The culture was incubated at 28°C in a 380-liter fermentor, aerated at 113 liters per minute at 0.67 atm gauge pressure and agitated at 200 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 19 hours of incubation, 75 liters of the seed-tank culture was aseptically added to 2,650 liters of fermentation medium containing (in g/liter): Cerelose (Corn Products), 20; OM peptone (Oscar Mayer), 5; Ardamine Z (Yeast Products), 3: ferrous ammonium sulfate hexahydrate, 0.03. The pH had been adjusted to 7.0 before sterilization. The culture was incubated at 28°C in a 3,400-liter fermentor, aerated at 567 liters per minute at 0.34 atm gauge pressure and agitated at 70 rpm for the first six hours and 160 rpm for the remainder of the fermentation. At 48 hours additional Cerelose (20 g /liter) was added. During the fermentation silicone antifoam (Dow Corning AF) was added as needed to control frothing. At 71 hours the fermentation was terminated and filtered through infusorial earth with a rotary-drum vacuum filter and then through asbestos clarifying pads in a plate and frame filter. Amino acid analysis indicated that the broth contained 50 g of **1** and 4.1 g of **2**.

Isolation Procedure

The filtered broth (2,500 liters) was passed through a 12″-diameter column which contained 50 liters Dowex 50WX4 resin (50~100 mesh) in the pyridinium form. The resin was washed with 200 liters of 5 % aqueous pyridine solution followed by 200 liters of water. The diamino acid fraction was then eluted from the resin with 200 liters of 1 N NH_4OH solution. The eluate was evaporated under reduced pressure to 3.6 liters to which were then added 10.8 liters of denatured alcohol (95 % ethanol containing 0.5 % benzene) and 290 ml of concentrated ammonium hydroxide solution. Chromatography on silica gel followed by adsorption and elution from Bio-Rad AG50WX4 resin was carried out as previously described.[2] The eluate from the Bio-Rad cation exchange resin was then evaporated under reduced pressure and taken

up in 600 ml of denatured alcohol - H_2O - NH_4OH (75:25:2). This solution was then applied to a column (45×840 mm) of silica gel 60 (Merck, 70~230 mesh) which had been pre-conditioned with the same solvent. The column was developed with the same solvent and the fraction eluted between 1.4 and 4.8 liters was evaporated at reduced pressure to 200 ml, adjusted to pH 4.0 with 6 N HCl, treated with 40 g Darco G-60 charcoal, and filtered. The filtrate was further evaporated to a syrup which was taken up in methanol whereupon 22 g of **1** crystallized. The mother liquor was evaporated under reduced pressure; the residue, dissolved in 50 ml denatured alcohol - H_2O - NH_4OH (80:20:2), was resubmitted to silica gel chromatography on the same column pre-conditioned and developed with denatured alcohol - H_2O - NH_4OH (80:20:2). From the fraction eluted between 5.5 and 7.2 liters, **2a** (325 mg) was crystallized from methanol after pH adjustment and charcoal treatment as described above: ir spectrum identical with that of synthetic material; $[\alpha]_D^{25}$ +9.9° (*c* 1, 5 N HCl).

Anal. Calcd. for $C_6H_{14}N_2O_3 \cdot HCl$: C, 36.28; H, 7.61; N, 14.10.
Found: C, 36.36; H, 7.78; N, 13.92.

Catalytic Reduction of L-2-Amino-4-(2-aminoethoxy)-*trans*-3-butenoic Acid (1)

A solution of 7.85 g of **1** (40 mmoles) in 200 ml of H_2O was hydrogenated in a PARR apparatus at 16 lbs. pressure and 25°C for two hours in the presence of 1 g 5 % Pd on charcoal. The suspension was then filtered and the product adsorbed on 70 ml of AG50WX4 cation-exchange resin (100~200 mesh, H^+). The resin was washed with 10 % aqueous pyridine solution to remove L-2-aminobutanoic acid (see discussion). The resin was then eluted with 1 N NH_4OH, the eluate partially evaporated under reduced pressure, the concentrate adjusted to pH 5.0 with 18 ml of 2 N HCl and the remaining solvent evaporated under reduced pressure. The residue was taken up in hot methanol and **2a** crystallized after addition of ethanol; yield 7.26 g (37 mmoles) 92 %; mp, 208~211°C.

Anal. found: C, 36.01; H, 7.85; N, 13.99.

$[\alpha]_D^{25}$ −1.4° (*c* 1, 0.1 M sodium phosphate buffer. pH 7.0), +12.3° (*c* 1, 5 N HCl); ir (KBr) 1120 cm^{-1} (C—O—C); nmr (D_2O, ext. TMS), δ 4.35 (t, 1, J=6Hz, $CH_2-\underset{\displaystyle NH_2}{\underset{|}{\underline{C}H}}-COOH$), 4.21 (t, 2, J=5Hz, $-C\underline{H}_2-O-CH_2-$), 4.18 (t, 2, J=6Hz, $-CH_2-O-C\underline{H}_2-$), 3.68 (t, 2, J=5Hz, $NC\underline{H}_2-CH_2-$), 2.65 (q, 2, J=6Hz, $OCH_2-C\underline{H}_2-CH$).

Synthesis of D, L-2-Amino-4-(2-aminoethoxy)-butanoic Acid (**2b**)

A solution of 2-chloroethyl-2′-phthalimidoethyl ether (**3**)[3] (9.4 g, 37.5 mmoles), sodium ethyl phthalimidomalonate (**4**)[4], (8.2 g, 25 mmoles) and potassium iodide (0.4 g, 2.5 mmoles) in 10 ml of N, N-dimethylformamide was maintained at 153°C for 4 hours, by which time titration of a small portion indicated that 99 % of **4** had reacted. Compound **5** crystallized with difficulty from ethanol, in 26 % yield, m.p. 96~98°C.

Anal. calcd. for $C_{27}H_{26}N_2O_9$: C, 62.06; H, 5.02; N, 5.36.
Found: C, 62.40; H, 5.13; N, 5.34.

It was more efficient, however, to proceed by precipitating crude **5** by addition of 4 volumes of water and triturating the precipitate 2 times with 40 ml water.

Crude **5** from 4/5 of the original reaction mixture was dissolved in 20 ml ethanol, 40 ml of an aqueous solution of 5 N NaOH was added and the solution was heated at reflux tempera-

ture for 1 hour. The ethanol was then allowed to boil off and the cooled solution was adjusted to pH 1 with 6 N HCl. The aqueous phase was decanted from the oil which formed, and the oil was heated at reflux temperature in 120 ml of 6 N HCl for 90 minutes. After cooling and filtering, the solvent was removed by evaporation under reduced pressure and the residue was dissolved in water and applied to 100 ml Bio-Rad AG50WX4 (50~100 mesh, H^+) cation-exchange resin. The resin was eluted first with 100 ml of 20 % aqueous pyridine solution and then with 200 ml of aqueous 1 N NH_4OH. The latter eluate was partially evaporated under reduced pressure and the pH was then adjusted to 3.5 with HCl. The water was removed by evaporation under reduced pressure, the residue dissolved in a small amount of methanol, and after addition of ethanol to a total volume of 50 ml, **2b** crystallized during storage at 0°C; yield 2.44 g, 62 % based on **4**: mp 175~177°C, ir (KBr) essentially identical to that of **2a**.

Anal. found: C, 36.12, H, 7.64, N, 13.88.

Resolution of 2-Amino-4-(2-aminoethoxy)-butanoic Acid (**2b**)

A solution of **2b**, 1 g (5 mmoles) in 5 ml 2 N NaOH, was treated with 1.25 g (11 mmoles) of chloroacetyl chloride and the pH was maintained at 10.2 by the addition of 2 N NaOH while the temperature was kept at 5°C. After 45 minutes, the pH was adjusted to 1.0 with 2 N HCl and the product was extracted with 5×10 ml ethyl ether. The combined extracts were back extracted with 10 ml H_2O and the organic phase evaporated at reduced pressure to a syrup (1.6 g). The syrup was dissolved in ethanol and a saturated solution of LiOH was added to an apparent pH of 7. The solvent was evaporated under reduced pressure and **6a** was crystallized from 5 ml of 50 % ether-ethanol; 951 mg, 63 %, mp 201~203°C.

Anal. calcd. for $C_{10}H_{15}Cl_2LiN_2O_5$: C, 37.41; H, 4.71, N, 8.72.
Found: C, 37.15; H, 4.63, N, 8.51.

A solution of 642 mg of **6a** in 20 ml of deionized water was treated with 30 mg hog renal acylase* at 37°C and pH 7.2 for 21 hours. The solution was then applied to a column containing 10 ml Bio-Rad AG50WX4 cation-exchange resin (50~100 mesh, H^+). The column effluent plus a 50-ml water wash was concentrated to 20 ml, the pH readjusted to 7.2 with LiOH solution, an additional 30 mg of acylase added and again incubated for 21 hours. The solution was then re-applied to the same column and the column effluent and water wash were again processed as above. From the final column effluent and wash, the lithium salt was made and **6b** crystallized from an aqueous ethanol suspension. After recrystallization 230 mg of **6b** was obtained; 72 %, mp 210°C.

Anal. found: C, 37.45, H, 4.56, N, 8.61.

The above-described resin was then eluted with 100 ml 10 % aqueous pyridine solution. The eluate was concentrated under reduced pressure and **7** crystallized from aqueous ethanol in two crops, 190 mg, 80 %, mp after recrystallization 139~141°C, $[\alpha]_D^{25}$ −16.0° (*c* 1, H_2O).

Anal. calcd. for $C_8H_{15}ClN_2O_4$: C, 40.26; H, 6.34; N, 11.74.
Found: C, 40.03; H, 6.46; N, 11.58.

The chloroacetyl groups were removed from **6b** and **7** by heating 0.8 mmoles of each for 2 hours in 10 ml 2 N HCl at reflux temperature. After evaporation at reduced pressure each preparation was taken up in water and applied separately to 5-ml columns of Bio-Rad

* Purchased from Mann Research Laboratory, New York City, New York, U.S.A.

AG50WX4 (50~100 mesh, H^+) cation-exchange resin. After washing the columns with aqueous pyridine solution, the products were eluted with 50 ml 1 N NH_4OH and after evaporation and pH adjustment, the products crystallized from ethanol-water. Thus, from **6b**, 126 mg **2c** was obtained; 79 %, mp 206°C, $[\alpha]_D^{25}$ −11.5° (*c* 1, 5 N HCl), ir identical with that of **2a** prepared by reduction of **1**.

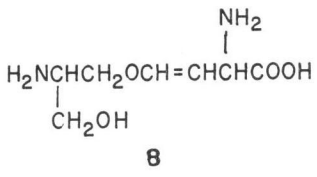
Anal. found: C, 36.41; H, 7.64; N, 13.83.

From **7**, 103 mg of **2a** was obtained, 65 %; mp 204~206°C, $[\alpha]_D^{25}$ +11.4° (*c* 1, 5 N HCl), ir identical with that of **2a** prepared by reduction of **1**.

Anal. found: C, 36.12; H, 7.64; N, 13.88.

Results and Discussion

The synthetic method and the physical properties prove the structure of **2a** to be L-2-amino-4-(2-aminoethoxy)-butanoic acid. Since **2a** is the major reduction product of **1**, the structure previously assigned[2] to **1** is confirmed. The formation of a small amount of L-2-aminobutanoic acid during the reduction of **1** is not surprising since catalytic hydrogenation of the related compound, L-2-amino-4-methoxy-*trans*-3-butenoic acid, also resulted in partial hydrogenolysis to L-2-aminobutanoic acid[6]. When reduction of **1** was carried out with a 10 % Pt on charcoal catalyst, a 25 % yield of L-2-aminobutanoic acid was obtained crystalline and proved to be identical with an authentic sample.



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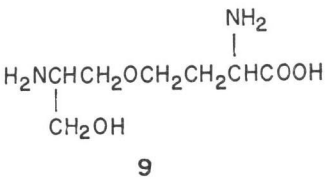

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When **2b** (D, L) at 480 mg/liter was added to shaken flask fermentations at 24 hours, the amount of **1** present at 72 hours was 119 mg/liter compared to 37 mg/liter for an untreated control fermentation. The fermentation conditions (media, temperature, *etc.*) have been described previously.[2] Amino acid analyses were performed as described in the experimental part. The amount of **2** remaining at 72 hours was 236 mg/liter compared to less that 1 mg/liter in the control. After isolation of **1** and **2** from the treated broth by an appropriately scaled down version of the method described above, it was found that **1** was 100 % L-isomer while **2** was 62 % D-isomer. These facts seem to indicate that **2a** is a precursor of **1**, and that synthetically derived **2a** or **2b** may be used to increase the yield of **1**. A material balance calculation shows that not all of the D-isomer, **2c**, is accounted for, thus indicating that it may be racemized or otherwise metabolized.

Unlike **1**, no antimicrobial activity was observed for **2a** nor did **2a** reverse the antimicrobial activity of **1** in agar diffusion bioassays carried out in a chemically defined minimal medium.[7] However, **2a** has been found[8] to increase the production of ethylene by plant tissues while, by contrast, **1** causes a decrease in ethylene production by plants.[9] The structurally related compounds, rhizobitoxine, **8**[10,11,12] and dihydrorhizobitoxine, **9**[13], which were isolated from a fermentation of *Rhizobium japonicum*, have been found to have analogous biological activities in plants.[9,14]

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