ANTIMETABOLITES PRODUCED BY MICROORGANISMS. V[1]

L-2-AMINO-4-METHOXY-*TRANS*-3-BUTENOIC ACID

JAMES P. SCANNELL, DAVID L. PRUESS, THOMAS C. DEMNY, LILIAN H. SELLO, THOMAS WILLIAMS and ARTHUR STEMPEL

Chemical Research Department, Hoffmann-La Roche Inc.
Nutley, New Jersey 07110, U.S.A.

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L-2-Amino-4-methoxy-*trans*-3-butenoic acid was isolated from a fermentation broth of *Pseudomonas aeruginosa* ATCC-7700. This substance inhibited the growth of *Bacillus* species 1283 B in a chemically defined medium. The growth inhibition was reversed by a variety of amino acids.

An amino acid antimetabolite which inhibited the growth of *Bacillus* sp. 1283 B was discovered in a fermentation broth of *Pseudomonas aeruginosa* ATCC-7700. The compound was isolated from the broth by adsorption onto an anion-exchange column followed by elution with trimethylammonium bicarbonate[2], a volatile buffer. The structure was determined to be L-2-amino-4-methoxy-*trans*-3-butenoic acid, I*, by physical chemical characterization and by reduction to L-2-amino-4-methoxybutanoic acid[3], **IIIa**, identical with a sample prepared by enzymatic resolution of the synthetic racemate[4], **IIIb**. An unexpected product, L-2-amino-butanoic acid, II, was isolated on reduction of I employing a platinum-charcoal catalyst. The title compound is the first amino acid reported to contain an enol ether group.

Experimental

General Methods

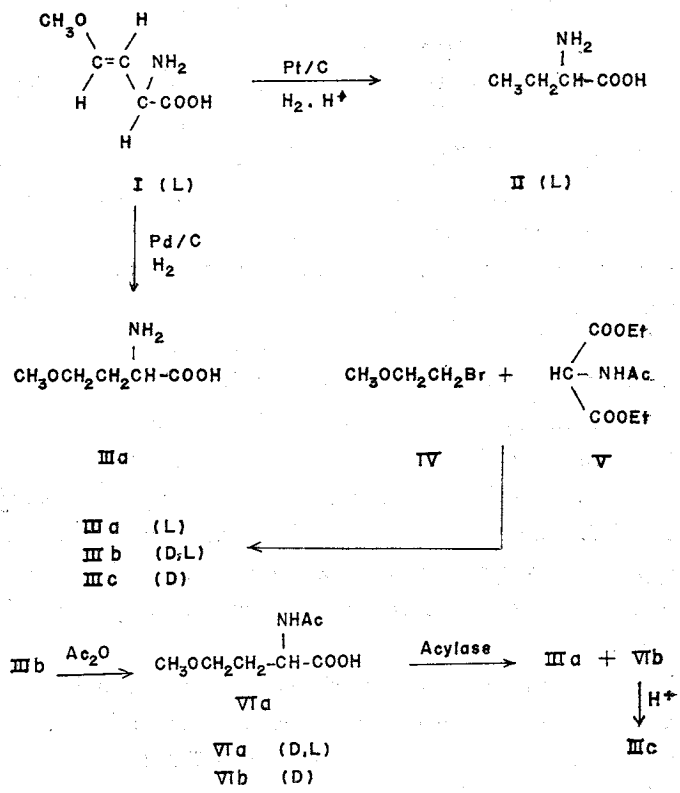
All melting points were taken with a Thomas Hoover capillary apparatus and are corrected. Spectral analyses were obtained as follows: ir spectra in KBr discs with either the Beckman IR-9 or the Perkin-Elmer Model 621 spectrophotometer, nmr spectra in D_2O using the Varian A-60 spectrometer, mass spectra with a CEC 21-110 mass spectrometer using a direct introduction probe, ord with a Durrum-Jasco recording spectropolarimeter and optical rotations with a Perkin-Elmer 141 Polarimeter. Amino acid analyses were performed with the Technicon Auto-Analyzer. Vpc analysed were carried out using the F & M 402 equipment. Brinkman cellulose F plates developed with ethanol/H_2O: 7/3 were used for thin-layer chromatography. Electrophoresis was done at 200 V using a Gelman apparatus.

Microbiological Assay

A paper disc agar-diffusion microbiological assay was used to detect and quantitatively measure I. Inoculum was prepared from *Bacillus* sp. 1283 B grown overnight at 35 °C on a rotary shaker in 500-ml Erlenmeyer flasks containing 100 ml of medium

* The Roman numerals refer to structures in Fig. 1.

composed of (in g/liter): Bacto-peptone (Difco), 6.0; N-Z Amine type A (Sheffield), 4.0; yeast autolyzate (National Yeast Products), 3.0; beef extract (Wilson), 1.5; and glucose, 1.0. The cells were washed three times with water to remove excess nutrients and adjusted to an optical density of 1.2 (wave length, 500 nm; path length, 15.5 mm); 30 ml of washed cell suspension was added to one liter of liquefied minimal-agar of DAVIS and MINGIOLI[5] just prior to distribution. Five ml portions of this inoculated medium were pipetted into Petri dishes (100×15 mm). After the agar solidified, the dishes were stored at 4 °C and used within a week. Samples to be assayed were applied to paper discs which were then placed on the agar. After incubation overnight at 35 °C the diameters of the zones of inhibition were measured; the zone diameters were found to be proportional to the log of the concentrations between 0.05 and 10 mcg/ml of I. A two-fold increase in the concentration of I increased the zone diameter by 4 mm.



The effect of known metabolites on the antibacterial activity of I was measured on the inoculated agar plates described above. A paper disc containing I was placed on an agar plate one inch (2.54 cm) from a paper disc containing 10 mcg of a known metabolite(s). If the zone of inhibition around the disc containing I was distorted (*i.e.*, crescent shaped, semicircular), it was assumed that the metabolite was reversing the antibacterial activity of I and this effect was characterized as a "positive reversal". The common amino acids, various nucleosides and the water-soluble vitamins were all tested as possible reversing agents.

Antimetabolite Production

Cells of *Pseudomonas aeruginosa* ATCC-7700 were inoculated into 6-liter Erlenmeyer flasks containing 2 liters of the inoculum medium described above. The flasks were incubated at 28 °C for 72 hours on a rotary shaker (240 rpm with a 2-inch (5.1-cm) stroke). Four liters of inoculum was then added to 1,320 liters of fermentation medium containing (in g/liter): Cerelose (Corn Products), 11.0; Bacto-yeast extract (Difco), 2.0; asparagine, 0.5; and K_2HPO_4, 0.5. The culture was incubated at 30°C in

a 1,900-liter fermentor, aerated at 565 lpm and agitated at 150 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 42 hours the fermentation broth was clarified by Sharples centrifugation.

Isolation Procedure

The clarified broth containing 8 g/liter solids and 12 mg/liter antimetabolite was treated at pH 7.0 with 20 g/liter of Darco G-60. After filtration through Celite, the filtrate was concentrated under reduced pressure to one-tenth of its original volume. Five volumes of acetone was added to the concentrate. After standing for 24 hours at 4°C, the suspension was filtered with the aid of Celite and the filtrate concentrated and lyophilized. The recovery, based on bioactivity, was 85 %; the purification, based on specific activity, was 10 fold.

Preliminary experiments had shown the activity to be stable at 50 °C for 1 hour at pH values of 7 and 12 but unstable at pH 2 at this temperature. The antimetabolite moved as an anion and a cation respectively when subjected to electrophoresis at pH 12 and 2. Since the acid instability contraindicated use of cation-exchange chromatography, the major emphasis was on anion-exchange chromatography. To avoid salt removal problems the volatile buffer trimethylammonium bicarbonate[2] was used as eluent.

A column (6 cm diameter) was filled to a height of 60 cm with 2 liters AG 1×8 (200~400 mesh anion-exchange resin, chloride form). The resin was converted to the bicarbonate form by passing 6 liters of 3.7 M $KHCO_3$ through the column. A charge of 25 g of solids dissolved in 200 ml distilled water and adjusted to pH 10.0 with 10 % sodium hydroxide solution was then applied to the resin and the resin was eluted with 0.05 M trimethylammonium bicarbonate solution, pH 9.5, prepared by sparging CO_2 into 25 % aqueous trimethylamine solution followed by appropriate dilution. Fractions containing antimetabolite activity greater than 20 mcg/ml, occurring at an elution volume of 4~9 liters, were evaporated at reduced pressure to an oil (667 mg; recovery 80 %; purification 30 fold). The oil was taken up in 7 ml hot methanol and after storage for 48 hours at 0°C crystals of I were removed by filtration. After recrystallization from 80 % aqueous ethanol, 133 mg I was obtained (recovery 70 %; purification 3.5 fold; overall recovery 47 %; overall purification 1,050 fold); m.p. 240~245 °C (dec.); ir 1620 (carboxylate C=O), 1665 and 1220 cm^{-1} (O-C=C); nmr (15 mg ext TMS), δ 7.25 (d, 1, J=13 Hz, O-C<u>H</u>=C), 5.38 (d of d, 1, J=10 and 13 Hz, -CH=C<u>H</u>-C), 4.70 (d, 1, J=10 Hz, CH-C<u>H</u>-NH_2), 4.24 (s, 3, C<u>H_3</u>-O); mass spectrum m/e 86 (only major peak); pKa_1, 2.47, pKa_2, 9.55; $[\alpha]_D^{25}$ +115° (*c* 1, H_2O); ord (*c* 0.13, 1 N HCl) $[\phi]_{700}$ = +148, $[\phi]_{589}$ = +192, $[\phi]_{233}$ = +16,800 (peak), $[\phi]_{244}$ = 0, $[\phi]_{215}$ = −30,300.

Anal. calcd for $C_5H_9NO_3$: C 45.71, H 6.92, N 10.68, OCH_3 23.66.
Found: C 45.96, H 6.94, N 10.58, OCH_3 22.66.

Large Scale Isolation

A six-inch (15.24 cm) diameter column was filled to a height of 90 cm with 25 liters Dowex 1×4 resin, (50~100 mesh, Cl^- form) and 100 gal (378 liters) of 5 % $NaHCO_3$ solution was passed through the column to convert the resin to the bicarbonate form. After washing with 100 gal. of 0.1 % aqueous ammonia solution and backwashing with

distilled water, the resin was charged with 500 g solids obtained from the acetone supernatant dissolved in 6 liters distilled water adjusted to pH 9.5 with 25 % aqueous trimethylamine solution. The resin was then eluted with 0.05 M trimethylammonium bicarbonate solution (prepared as above). Fractions containing greater than 15 mcg/ml activity were combined in early and late pools of elution volumes, 100~150 liters and 150~200 liters respectively. The antimetabolite in the latter, about 30 % pure on a non-volatile solids basis, could be crystallized from methanol in up to 60 % yield but the mother liquors from this and the crude concentrate from the early pool required further purification on a small anion-exchange column as described above.

L-2-Aminobutanoic Acid, II, by Reduction of I

Reduction of 164 mg (1.25 mmoles) of I was carried out in a Brown hydrogenator* using 250 mg 4 % platinum on charcoal as catalyst with *in situ* generation of hydrogen from sodium borohydride in 0.67 N HCl. The uptake of almost 2.5 mmoles hydrogen was complete in less than 5 minutes. The reaction mixture was filtered and the filtrate passed through 20 ml AG 50WX4 (200~400 mesh cation-exchange resin in the hydrogen ion form). After washing the resin with 100 ml distilled water, the reduction product was eluted from the resin with 10 % aqueous pyridine solution. After concentration to 132 mg dry powder, crystallization from 80 % aqueous ethanol gave 99 mg **II**, m.p. 295°C (dec) [lit[7]. 292 °C], $[\alpha]_D^{25}$+8.0 (*c* 1, H_2O); [lit.+8.0 for L isomer][7]. Ir, nmr and mass spectra of **II** were essentially identical to those of authentic D,L-2-amino butanoic acid. The mother liquors from the above crystallization contained predominantly **IIIa**.

L-2-Amino-4-methoxybutanoic Acid, **IIIa**, by Reduction of I

Reduction of 140 mg (1.07 mmoles) of **I**, using 300 mg 5 % palladium on charcoal in 100 ml 90 % aqueous methanol at 25°C and 1 atmosphere resulted in an uptake of almost one mmole hydrogen in one minute. After filtration and evaporation to 144 mg dry powder, crystallization from aqueous ethanol gave **IIIa** (90 mg). Recrystallization gave a product with m.p. 230~239°C (char); ir (KBr), 1610 (carboxylate C=O), 1120 cm^{-1} (C-O-C); nmr (20 mg, int. TMS), δ=3.92 (t, 1, C<u>H</u>-NH_2), 3.72 (t, 2, -C<u>H_2</u>-O), 3.44 (s, 3, C<u>H_3</u>-O), 2.22 (m, 2, C-C<u>H_2</u>-C); mass spectrum m/e 45 (base peak), 88 (major peak), $[\alpha]_D^{25}$−8.0° (*c* 0.5, H_2O).

Anal. calcd. for $C_5H_{11}NO_3$: C 45.10, H 8.33, N 10.52.
Found: C 44.95, H 8.49, N 10.37.

The powder obtained after hydrogenation contained less than 0.5 % **II**.

D,L-2-Acetylamino-4-methoxybutanoic acid, **VIa** from **IIIb**

A solution of 250 mg **IIIb**[4] in 30 ml acetic acid and 2 ml of acetic anhydride was heated to reflux for 10 minutes. After concentration at reduced pressure, 3×20 ml water was added and evaporated, and the residue was crystallized from a mixture of ethanol and ethyl ether to yield **VIa** (226 mg, 69 %, m.p. 155°C). Ir (KBr), 3375 (amide NH), 1730 (carboxylic C=O), 1120 cm^{-1} (C-O-C).

Anal. calcd for $C_7H_{13}NO_4$: C 47.99, H 7.48, N 8.00.
Found: C 48.05, H 7.51, N 8.01.

* Purchased from Delmar Scientific Laboratories, Maywood, Ill., U.S.A.

Enzymatic Resolution of VIa

A solution of 100 mg of **VIa** in 4 ml of deionized water was adjusted to pH 7.6 with 2N LiOH. Hog kidney acylase* (100 mcg, 5.5 units) was added and the solution was incubated at 38°C for 16 hours. The enzymatic digest was poured directly onto 3.5 ml AG 50WX8 (200~400 mesh cation-exchange resin in the hydrogen ion form). The effluent plus 30 ml distilled water wash of the column were combined, evaporated to dryness, and the residue crystallized from a mixture of ethanol and ethyl ether to yield 41.8 mg of **VIb** (84 %, m.p. 174~175°C). Ir (KBr) identical to that of **VIa**. $[\alpha]_D^{25}$ +27.3° (*c* 1, H_2O).

Anal. calcd for $C_7H_{13}NO_4$: C 47.99, H 7.48, N 8.00.
Found: C 47.90, H 7.51, N 7.91.

A 10 % aqueous pyridine solution (30 ml) was then passed through the ion-exchange column and the eluate evaporated to dryness. The residue crystallized from aqueous ethanol to yield 24 mg of **IIIa** (67 %, m.p. 234~237°C [char]). Ir (KBr) identical to that of **IIIa** prepared by reduction of **I** using Pd/C catalyst. $[\alpha]_D^{25}$ −9.6° (*c* 0.5, H_2O).

Anal. calcd for $C_5H_{11}NO_3$: C 45.10, H 8.33, N 10.52.
Found: C 45.32, H 8.61, N 10.61.

D-2-Amino-4-Methoxybutanoic Acid, IIIc, by Hydrolysis of VIb

A solution of 78 mg of **VIb** in 3 ml 2N HCl was refluxed for 2 hours. After evaporation to dryness at reduced pressure, the residue was dissolved in 3 ml H_2O and adsorbed onto 3.5 ml AG 50 WX 8 (200~400 mesh cation-exchange resin in the hydrogen ion form). After washing the column with water, a 5 % aqueous pyridine solution was passed through the column. The pyridine eluate was evaporated to a dry powder under reduced pressure and the product was crystallized from aqueous ethanol to yield 24 mg of **IIIc** (41 %, m.p. 233~236°C [char]). Ir (KBr) identical to that of **IIIa**; $[\alpha]_D^{25}$ +10.4° (*c* 0.5, H_2O).

Anal. found: C 44.87, H 8.30, N 10.43.

Discussion

The method of purification, the pKa values and the infrared spectrum indicated that **I** was an α-amino acid. It gave a yellow reaction product with ninhydrin which differed from that formed by imino acids in that no intermediate purple product extractable into benzene was formed[8]. Unsaturated amino acids also produce a yellow color with nihydrin[9]. Elemental analysis** and the strong M-45 peak shown by mass spectrometry (due to loss of COOH) indicated an empirical formula, $C_5H_9NO_3$. The acid lability of **I**[10], the rapid iodine uptake[11], the ir spectrum[12] and the presence of a methoxy group (analysis and nmr) pointed to the presence of an enol ether. The allylic grouping with electron withdrawing substituents was consistent with the three downfield protons in the nmr spectrum and the coupling constant, J=13 Hz, indicated a *trans* configuration[13]. The compound had a high rotation consistent with polarizable substituents on the asymmetric carbon atom, and the L configuration was likely because of the greater positive value in acidic solution (CLOUGH-LUTZ-JIRGENSON rule[14]), and the increasing positive rotation at lower wavelengths[15]. Conclusive evidence of structure **I** was obtained by hydrogenation of the antimetabolite. Under acidic conditions, using platinum on charcoal as the catalyst, an

* Purchased from Mann Research Laboratories, New York, N. Y.

** Since the analytical sample contained about 4 % of other amino acids which were not substantially removed by repeated recrystallization, the correct analysis could have been fortuitous.

unexpected product, L-2-amino-butanoic acid, II, was obtained. This, however, confirmed the L-configuration of the original antimetabolite. Under neutral or acidic conditions, using palladium on charcoal as the catalyst, reduction gave the expected product, L-2-amino-4-methoxybutanoic acid. **IIIa** whose physicochemical data were consistent with the structure assigned. Moreover, the ir and nmr spectra were essentially identical to racemic material, **IIIb**, synthesized[4] from **IV** and **V**. The optical rotation of **IIIa** corresponded to that of the L-isomer obtained by enzymatic resolution of **VIa**. Recently[3] **IIIa** was shown to be produced by *Corynebacterium* sp. E 17 grown in a medium which contained methanol and L-homoserine. The D-isomer, **IIIc**, has not been reported previously.

Antimetabolite Activity of I

A variety of metabolites were tested for reversal of antibacterial activity as described under Microbiological Assay. Positive reversals were observed only with amino acids; water-soluble vitamins and nucleosides were inactive in this respect. Of the common amino acids, D- and L-alanine, D- and L-2-aminobutanoic acid, D-glutamic acid, and D-aspartic acid reversed the antibacterial activity of **I**. The basis for this interesting reversal pattern is currently under investigation in our laboratory.

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