MALIOXAMYCIN, A NEW ANTIBIOTIC WITH SPHEROPLAST-FORMING ACTIVITY

II. STRUCTURAL ELUCIDATION AND TOTAL SYNTHESIS

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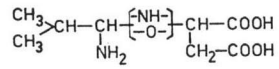
Malioxamycin (**1**) is a new antibiotic produced by *Streptomyces lydicus* No. 15748. The structure of malioxamycin has been determined by nmr and mass spectra to possess a hydroxamic acid bond between L-valine and D-malic acid. This structure was confirmed by total synthesis of the antibiotic with (*R*)-aminooxysuccinic acid and the active ester of L-valine.

In our preceding paper[1], we have reported the taxonomy of the producing organism and the fermentation, isolation and characterization of malioxamycin. The producing organism was identified as *Streptomyces lydicus* and the antibiotic was characterized as a water soluble, amphoteric substance with weak activity against limited genera of Gram-negative bacteria. In the preliminary study of its mechanism of action, malioxamycin inhibited peptidoglycan synthesis in the cell wall of bacteria. The cells of bacteria first swelled and finally formed spheroplasts. In the present paper the structural determination and a total synthesis of malioxamycin are described.

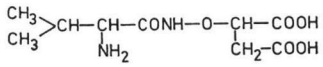
Structure of Malioxamycin

Malioxamycin (**1**) is a water-soluble crystalline substance melting at 158~160°C, molecular formula of $C_9H_{16}N_2O_6$, and no characteristic absorption in the UV region[1]. The molecular weight of **1** was not determined by mass spectroscopy. However, the molecular formula of the dimethyl ester (**2**) of **1** obtained by its reaction with diazomethane was assigned by high resolution mass spectrometry at *m/z* 275.1266 (calcd. for $C_{11}H_{19}N_2O_6$: 275.1243). The IR spectrum of **1** showed the presence of carboxylic acid at 3000 and 1680 cm^{-1} and amide bond at 1625, 1590, and 1565 cm^{-1}. On high voltage paper electrophoresis at 3,300 V for 20 minutes in formic acid - acetic acid - water (1: 3: 36), **1** moved to the cathode with an Rm (relative mobility to L-alanine) value of 0.73, and in 0.1 M tris-HCl buffer (pH 7.5), **1** moved to the anode with an Rm value of 1.10 (relative mobility to bromphenol blue). The data obtained were in good agreement with the presence of one amino group and two carboxyl groups in **1**. Determination of the N-terminal amino acid in **1** was performed to afford DNP-valine. Hydrolysis of **1** with 6 N HCl gave 1.0 mole of valine and 0.67 mole of ammonia as basic components by amino acid analysis. As acidic components of the hydrolysate, malic and fumaric acids were determined by gas chromatographic analysis of the esterified derivatives. The latter acid, however, was considered to be an artifact formed by dehydroxylation of the former acid under the conditions of this hydrolysis.

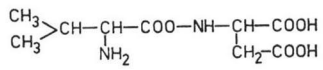
These results suggested that the amino group of valine was free and its carboxyl group formed a peptide bond with another amino group attached to the malic acid moiety as shown in the following partial structure.



The PMR spectrum of **1** displayed two doublet methyl protons at 0.92 and 0.89 each of which coupled (J=6.5 Hz) to methine proton at 1.94, and this methine proton also indicated vicinal coupling (J= 7.0 Hz) with a proton at 3.41 ppm. The signal of A_2X appeared as a double doublet (J_1=8.5, J_2= 5.0 Hz) at 2.62 ppm of the A_2 part and as a multiplet at 4.30 ppm of the X part. These assignments strongly supported the partial structure described above and the structure of **1** was then considered to be **(a)** or **(b)**.



(a)

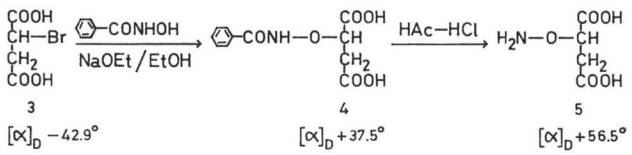


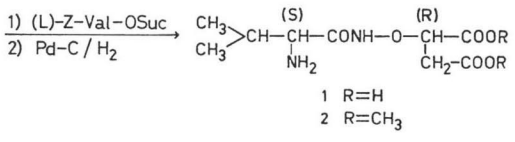
(b)

The structure **(b)** has an aspartic acid residue in its molecule. However, aspartic acid could not be obtained by hydrolysis.

In addition, the high resolution mass spectrum of **2**, contains a base peak at m/z 113.0715 originating from $C_5H_9N_2O$ (m/z 113.0714). This fragment ion in this mass spectrum supported the presence of an amide bond with a valine moiety in **1**. Thus the structure of **1** was established as **(a)**. The stereochemistry of valine and aminooxysuccinic acid in **1** was presumed on the basis of the enzyme reactions. In the reactions of valine with L- and D-amino acid oxidases, valine disappeared only from the former reaction mixture and not from the latter when examined by amino acid analysis and TLC. Similarly, malic acid, obtained by the hydrolysis of **1**, was found to be not susceptible to L-malate dehydrogenase by determination of absorbancy at 340 nm of the reaction mixture based on NADH. The valine and malic acid moieties in malioxamycin were therefore assigned to be L and D-forms, respectively. Furthermore, the structure and stereochemistry of **1** were confirmed by its stereospecific synthesis. Total synthesis of **1** bearing either *S* or *R* configuration was successfully achieved by the coupling of the active ester of (L)-valine and (*R*)-aminooxysuccinic acid as depicted in chart 1.

Chart 1.





This synthetic route involved a stereospecific sequence for aminooxysuccinic acid. The reaction of (*S*)-α-bromosuccinic acid (**3**)[2] with benzohydroxamic acid in the presence of sodium ethoxide in absolute

ethanol afforded N-benzoylaminooxysuccinic acid (**4**). The configuration was inverted in this reaction step. Hydrolysis of **4** with acetic acid - hydrochloric acid (1: 1) gave aminooxysuccinic acid (**5**), the key intermediate in the present synthetic procedure. The optical rotation of **5** was positive and equal to that of D-malic acid. This result suggested an *R*-configuration of **5**. Finally, malioxamycin was prepared by the reaction of **5** with the active ester of carbobenzyloxy-L-valine followed by hydrogenation of the resulting carbobenzyloxy group over 10% Pd-C. Synthetic malioxamycin was identified with natural malioxamycin by their spectra as well as their optical rotation and biological activity.

Experimental

The UV spectra were run on a Hitachi 124 recording spectrophotometer, and IR spectra on a JASCO IRA-2 spectrophotometer. Optical rotations were determined by Perkin-Elmer 241 polarimeter. The NMR spectra were measured with Varian HA-100 and Hitachi R-24 spectrometers using tetramethylsilane as an internal reference. The MS spectra were measured on a JEOL JMS-01SG spectrometer.

Dimethylester (2) of malioxamycin

A stirred solution of 10 mg of malioxamycin in 1 ml of methanol was treated with an excess amount of diazomethane in ether solution for 1 hour in an ice-water bath. After evaporation of the solvent, dimethylester (**2**) was obtained as a colorless powder. MS *m/z*: 275.1266 (M+1, Calcd. for $C_{11}H_{19}N_2O_6$: 275.1243), 113.0715 ($C_5H_9N_2O$: 113.0714). PMR (δ ppm, $CDCl_3$): 3.78 (3H, S, $-COOCH_3$), 3.72 (3H, S, $-COOCH_3$), 0.97 (6H, m, $-CH\langle^{CH_3}_{CH_3}$).

Hydrolysis of malioxamycin

A solution of 6 mg of malioxamycin in 0.5 ml of 6 N HCl was heated at 105°C for 8 hours in a sealed tube. After removal of the water under reduced pressure, the residue was dissolved in 1 ml of distilled water. A 0.1-ml aliquot of the hydrolysate gave valine and NH_3, 2.4180 μmole/mg and 1.6113 μmole/mg, respectively, by amino acid analysis. The remaining hydrolysate was applied on an SP-Sephadex column (H^+-form, 3 ml), and developed with distilled water. Acidic compounds in the eluate of fractions 1~3 were identified as malic and fumaric acids by nmr and after esterification by gas chromatography.

The amino acid in fractions 18~23 was identified as valine by amino acid analysis.

Configuration of valine in malioxamycin

The valine from the hydrolysate of malioxamycin and the authentic D- and L-valines were treated with L-amino acid oxidase (L-AAO) and D-amino acid oxidase (D-AAO) according to the procedure described by Boehringer Catalogue No. 15050 and No. 15051. When examined by TLC and amino acid analysis, L-valine and the valine from the hydrolysate disappeared from the reaction mixture with L-AAO but D-valine did not. On the other hand, only D-valine disappeared from the reaction mixture with D-AAO.

Configuration of malic acid in malioxamycin

The malic acid from the hydrolysate of malioxamycin and the authentic D- and L-malic acids were treated with malate dehydroxidase (MDO) under the condition described by Boehringer Catalogue No. 15048. Only L-malic acid showed absorption at 340 nm based on NADH in the UV spectra of the reaction mixture.

Synthesis of compound 4

To a stirred solution of 40 ml of ethanol containing 1 g of sodium ethoxide, a solution of 1.37 g of benzohydroxamic acid in 20 ml of ethanol was added dropwise at room temperature. After stirring for 1 hour, a solution of 1.97 g of S-bromosuccinic acid in 10 ml of ethanol was added to this reaction mixture, which was allowed to stand at room temperature overnight and then refluxed for 7 hours. After addition of water and neutralization with HCl, the reaction mixture was concentrated to a small volume.

The residue was acidified with HCl and was extracted with ethyl acetate. The solvent layer was dried with Na_2SO_4, and evaporated to dryness. The residue was charged on a silica gel column (40 g) and developed with chloroform - ethyl acetate (7: 3), yielding 1.04 g of crude compound. By recrystallization of the crude compound from ether 540-mg aliquot of pure compound **4** was obtained: m.p. 145~147°C. $[\alpha]_D^{25}$ +37.5° (*c* 2.07, acetone). IR (ν_{max}^{KBr} cm^{-1}): 3450, 3250, 2650, 2500, 1740, 1720, 1620, 1580, 1280, 690. PMR (δ ppm, $(CD_3)_2CO$): 7.5~8.0 (5H, m), 4.95 (1H, q), 3.0 (2H, d-d).

Anal. Calcd. for $C_{11}H_{11}NO_6$: C, 52.17; H, 4.83; N, 5.53.
Found: C, 51.46; H, 4.28; N, 5.73.

Hydrolysis of **4**

A solution of 750 mg of **4** in 11 ml of 12 N HCl - AcOH (1 : 1) was allowed to stand for 2 days at room temperature. After removal of the solvent under reduced pressure, crystalline benzoic acid obtained in the residue was removed with ethyl acetate leaving 470 mg of compound **5**. $[\alpha]_D^{21}$ +56.5° (*c* 1.98, H_2O). PMR (δ ppm, D_2O=4.8 ppm), 5.0 (1H, t), 3.0 (2H, d).

Condensation of **5** with L-valine

To a stirred solution of 50 mg of **5** in 4 ml of DMF, 0.2 ml of triethylamine and 100 mg of L-(*Z*)-Val-OSuc was added under N_2 gas. After stirring at room temperature overnight, 3-dimethylaminopropylamine was added to this reaction mixture to destroy an excess of the active ester. It was acidified with HCl and extracted with ethyl acetate to yield a condensation product. After removal of the solvent, the residue was dissolved in 10 ml of methanol - water (7: 3) and hydrogenated over 120 mg of 10% Pd-C under H_2 gas for 1 hour. The solution was filtered and concentrated under reduced pressure. The residue was chromatographed on SP-Sephadex (2 ml) and developed with water to give 6 mg of the desired malioxamycin: $[\alpha]_D^{25}$ +106° (*c* 0.66, water). The NMR and IR spectra of synthetic malioxamycin were identical with those of the natural antibiotics.

References

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