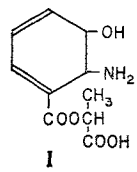
# THE STRUCTURES OF ORYZOXYMYCIN AND ITS DIMERIZATION PRODUCT

TOSHIHIRO HASHIMOTO, SHUJI TAKAHASHI, HIROSHI NAGANAWA, TOMOHISA TAKITA, KENJI MAEDA and HAMAO UMEZAWA

Institute of Microbial Chemistry,
Kamiosaki, Shinagawa-ku, Tokyo, Japan

(Received for publication April 25, 1972)

Oryzoxymycin (I), an antibiotic produced by *Streptomyces venezuelae* var. *oryzoxymyceticus*, is unstable and easily dimerizes to an inactive crystalline product. Structural study of this product led to the conclusion that the structure of oryzoxymycin is D-2-[(+)-6-amino-*trans*-5-hydroxy-1,3-cyclohexadiene-1-carbonyloxy]-propionic acid:




I

Oryzoxymycin (I)[1] is an antibiotic isolated from a cultured broth of *Streptomyces venezuelae* var. *oryzoxymyceticus*. It inhibits growth of *Xanthomonas oryzae* at 1.56 mcg/ml on glucose nutrient agar, but it does not show preventive or curative effect against infection of *X. oryzae* even at 1,000 mcg/ml in the pot test. One of the reasons of the ineffectiveness in the pot test seems to be due to the rapid dimerization of this antibiotic.

Oryzoxymycin (I) is obtained as amorphous powder, mp 155°C (dec.), $\lambda_{max}$ at 284 nm, $\nu_{c=0}$ at 1725 $cm^{-1}$ (ester), pKá 2.7 and 8.1. It is very unstable, losing the activity and yielding the crystalline dimerization product. Oryzoxymycin absorbs two moles of hydrogen on catalytic hydrogenation and gives tetrahydro-oryzoxymycin [colorless needles, mp 213~215°C (dec.), $\nu_{c=0}$ at 1724 $cm^{-1}$ (ester)] which has no uv maximum and has the molecular formula $C_{10}H_{17}NO_5$ determined by the elemental analysis and the mass spectrometry ($M^+$ 231). Therefore, the molecular formula of oryzoxymycin which was reported in a previous paper[1] should be revised to $C_{10}H_{13}NO_5$. At first, we studied the structure of the stable crystalline dimerization product and the result led to the structure of oryzoxymycin (I), D-2-[(+)-6-amino-*trans*-5-hydroxy-1,3-cyclohexadiene-1-carbonyloxy]-propionic acid. Hydrolysis of I with 2 N NaOH at 60°C for 5 hours gives D-lactic acid and (+)-6-amino-*trans*-5-hydroxy-1,3-cyclohexadiene-1-carboxylic acid which was isolated by MCCORMICK *et al.*[2] from *Streptomyces aureofaciens*.

Oryzoxymycin, kept at room temperature in a dry state, gradually loses its

activity and yields a crystalline substance (II), mp 185–193°C (dec.). The latter has no uv maximum, and ir spectrum of II indicates the presence of ester functions (1747 $cm^{-1}$). The molecular formula $C_{20}H_{26}N_2O_{10}$ is calculated from the results of the elemental analysis and the titration equivalent. Alkaline hydrolysis of II gives two crystalline products, (III) and (IV), and III still keeps an ester function (1742 $cm^{-1}$), but IV does not. Compound IV is also obtained by further alkaline hydrolysis of III. The molecular formulae $C_{17}H_{22}N_2O_8$ and $C_{14}H_{18}N_2O_6$ are obtained from their elemental analysis, respectively.

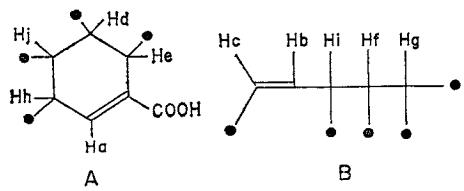
These observations indicate that III is II minus a $C_3$ fragment, and IV is III minus the same $C_3$ fragment. The $C_3$ fragment was isolated from effluent of cation-exchange resin chromatography of the alkaline hydrolyzate and identified to be D-lactic acid by comparison with an authentic sample.

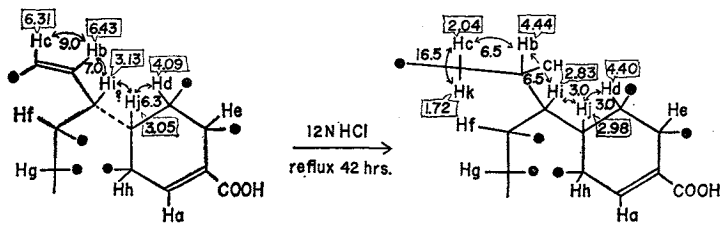
The nmr spectrum reveals that two hydroxyl groups of two lactic acids form esters in II, that is, protons on $\alpha$-carbons of two lactic acid moieties appear at 5.20 $\delta$ and 5.30 $\delta$, while the proton of free lactic acid is at 4.20 $\delta$.

Compound IV has two each of primary amino (VAN SLYKE method), carboxyl (formation of dimethyl ester), and hydroxyl groups (formation of tetraacetyl derivative). These functional groups account for all the nitrogen and oxygen present in IV. Compound IV absorbs two moles of hydrogen on catalytic hydrogenation.

Table 1. Chemical shifts and coupling constants of protons of IV (100 M Hz)

| Proton | Chemical shift | Coupling constant |
|---|---|---|
| Ha | 7.01 ($\delta$) | Jah=3.5 Jae=2.2 |
| Hb | 6.49 | Jbc=9.0 Jbi=7.0 |
| Hc | 6.31 | Jcb=9.0 Jcg=0.5 Jch=0.5 |
| Hd | 4.12 | Jde=9.5 Jdj=6.3 |
| He | 3.76 | Jed=9.5 Jeh=2.5 Jea=2.2 |
| Hf | 3.63 | Jfg=4.2 Jfi=3.0 |
| Hg | 3.51 | Jgf=4.2 Jgc=0.5 |
| Hh | 3.15 | Jhj=9.0 Jha=3.5 Jhe=2.5 Jhc=0.5 |
| Hi | 3.09 | Jib=7.0 Jif=3.0 |
| Hj | 3.05 | Jjh=9.0 Jjd=6.3 |



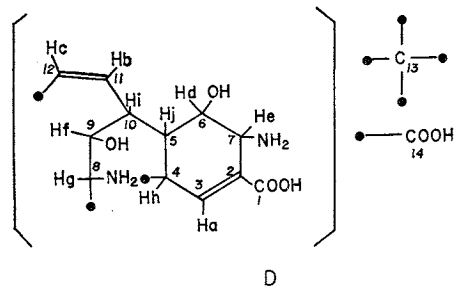

*Partial structure C of IV* *Partial structure of V*

Table 2. Chemical shifts and coupling constants of protons of V (100 M Hz)

| Proton | Chemical shift | Coupling constant |
|---|---|---|
| Ha | 7.18 ($\delta$) | Jah=4.0 |
| Hb | 4.44 | Jbi=6.0 Jbc=6.0 Jbk=<1 |
| Hc | 2.04 | Jcb=6.2 Jch=2.0 |
| Hd | 4.40 | Jdj=2.0 Jde=0 |
| He | 4.18 | Jed=0 |
| Hf | 3.71 | Jfi=2.8 Jfg=5.0 |
| Hg | 3.26 | Jgf=5.0 Jgk=1.5 |
| Hh | 2.99 | Jhc=2.0 Jha=4.0 Jhj=9.5 |
| Hi | 2.83 | Jib=6.0 Jif=2.8 Jij=3.0 |
| Hj | 2.98 | Jjd=2.0 Jjh=9.5 Jji=3.0 |
| Hk | 1.72 | Jkb=<1 Jkg=1.5 |

Table 3. Chemical shifts of Hd, He, Hf and Hg of IV and the derivative

| Proton | a) Hydrochloride ($D_2O$) | b) Free base ($D_2O$) | c) Tetraacetyldimethyl ester ($CDCl_3$) | (a—b) | (c—b) |
|---|---|---|---|---|---|
| Hc | 6.31 | 6.31 | 6.41 | 0 | 0.10 |
| Hd | 4.12 | 3.85 | 5.18 | 0.27 | 1.30 |
| He | 3.76 | 3.30 | 4.63 | 0.46 | 1.33 |
| Hf | 3.63 | 3.40 | 4.30 | 0.23 | 0.90 |
| Hg | 3.51 | 2.90 | 4.30 | 0.61 | 1.40 |
| Hh | 3.15 | 3.10 | ? | 0.05 | ? |



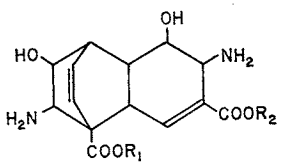
D

of double resonance technique. Then the results indicated the partial structures A and B.

Partial structures A and B account for all the protons presentin the nmr spectrum and twelve carbons of **IV**. One of the remaining two carbons should be a carboxyl carbon and the other could be considered to be a quaternary carbon. Concerning the linkage of partial structures A and B, the nmr spectrum of **IV** did not give definite information. The nature of the linkage (as shown by the partial structure C for **IV** and the partial structure **V**) was solved by nmr analysis (Table 2) of a hydrated product of **IV** (**V**), ($C_{14}H_{20}N_2O_7$, mp >260°C), which was obtained by refluxing **IV** in 12 N HCl for 42 hours.

Chemical shifts of Hc, Hd, He, Hf, Hg and Hh of **IV** and its tetraacetyl dimethyl ester are shown in Table 3. These data indicate that hydroxyl groups of **IV** are present at C-6 (Hd) and C-9 (Hf), and amino groups at C-7 (He) and C-8 (Hg), and lead to the partial structures D of **IV**.

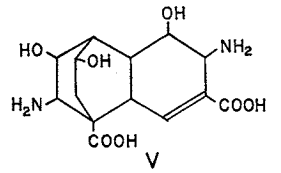
From the partial structure D, the structure **IV** can be directly constructed and structures **II**, **III** and **V** can be proposed as follows:




II $R_1 = R_2$ : $-CH(CH_3)-COOH$

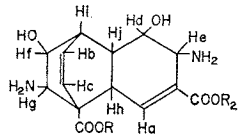
III $R_1$ : $-CH(CH_3)-COOH$, $R_2$: $-H$

IV $R_1 = R_2$ : $-H$



V

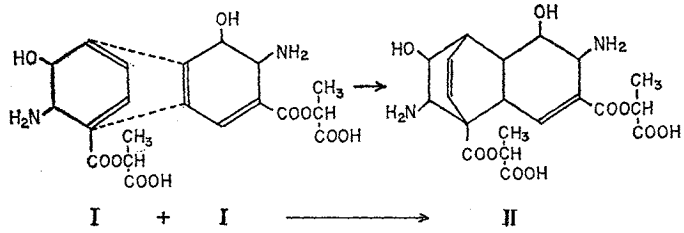
Table 4. Chemical shifts of Ha, He, Hh and Hg of compounds II, III and IV




| Compound | Chemical shift ($\delta$) | | | |
|---|---|---|---|---|
| | Ha | He | Hh | Hg |
| II | 7.15 | 3.90 | 3.65 | 3.70 |
| III | 7.05 | 3.75 | 3.65 | 3.72 |
| IV | 7.01 | 3.76 | 3.15 | 3.51 |

The position of the lactic ester group in **III** is shown by comparison of chemical shifts of neighboring protons of carbonyl functions of **II**, **III** and **IV** (Table 4).

The proposed structure of **II** indicates the following dimerization process from **I**:




## Experimental

The melting points were determined in capillary tubes and are uncorrected. The ultraviolet spectra were recorded on a Hitachi EPS-2U spectrophotometer. The infrared spectra were recorded on a Hitachi EPI-S2 spectrometer. The nuclear magnetic resonance spectra were measured on Varian A-60D and HA-100 instruments, and the internal standards used in deutrium oxide and in organic solvents were sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and tetramethylsilane (TMS), respectively. The mass spectra were measured on a JEOL JMS-01SG spectrometer by direct inlet method. Rotations were measured on a Carl Zeiss LEP-A2 photoelectric polarimeter. The pKá values were determined on a Metrohm Herisau E-436 potentiograph, and samples were dissolved in 0.1 N HCl and titrated with 1 N NaOH. Silica gel "G" (Merck) was used in thin-layer chromatography and spots were detected by spraying with one of the following reagents; 0.5% $KMnO_4$ aqueous solution, 10% $H_2SO_4$ solution, and 0.2% ninhydrin in acetone solution containing 5% pyridine. For the latter two reagents, the plate was heated at 105°C after spraying.

Oryzoxymycin: D-2-[(+)-6-amino-*trans*-5-hydroxy-1,3-cyclohexadiene-1-carbonyloxy]-propionic acid (I)

Compound I was obtained as colorless amorphous powder from a cultured broth of *Streptomyces venezuelae* var. *oryzoxymyceticus* as previously described[1]: mp 155°C (dec.); $[\alpha]_D^{22}$ +349° (*c* 1, water); $\lambda_{max}$ (ε), 284 nm (6470) in 0.1 N HCl[1]; $\lambda_{max}$ (KBr disk), 1725 $cm^{-1}$ (ester). Anal. calcd. for $C_{10}H_{13}NO_5$: C 52.86, H 5.77, N 6.17, O 35.21, (M.W. 227.21). Found: C 53.02, H 5.82, N 6.30, O 35.21, M.W. 224 (titration).

Oryzoxymycin dimer (II)

Three grams of I was kept at room temperature for 3 days and dissolved in 10 ml of water. By addition of 10 ml of ethanol, 2.1 g of crystalline substance was obtained. Recrystallization from ethanol-water gave 1.75 g (58% yield) of colorless needles; mp 185~193 °C (dec.), $[\alpha]_D^{22}$ +30° (*c* 1, water), pKá <2, 2.5, 7.2, 8.6; uv, end absorption; $\lambda_{max}$ (KBr disk), 1747 $cm^{-1}$ (ester). Anal. calcd. for $C_{20}H_{26}N_2O_{10}\cdot H_2O$: C 50.84, H 5.97, N 5.93, O 37.25, (M.W. 472.46). Found: C 50.95, H 6.10, N 5.78, O 37.00, M.W. 470 (titration).

Monodeslactic oryzoxymycin dimer (III) and dideslactic oryzoxymycin dimer (IV)

A 10 g of II was dissolved in 150 ml of 2 N NaOH, and was hydrolyzed at 60°C for 5 hours. The reaction mixture was added to a column of Dowex 50W-X8 (H type, $\phi$=4 cm, 500 ml). The column was washed with water, and eluted with 0.5 N ammonia. The fractions which showed positive by ninhydrin reaction were combined and concentrated under reduced pressure to dryness. The powder was dissolved in 0.2 M pyridine-acetate buffer (pH 3.75) and chromatographed on a colmun of Dowex 50W-X8 ($\phi$=6 cm, 2200 ml) which had been previously treated with the same buffer. The eluate was collected in 20-ml fractions. The fractions from 47 to 77 contained 580.5 mg of II, and the fractions from 95 to 209 contained 6.50 g of III (65% yield). A yield of 1.85 g of IV (18.5%) was obtained from 267 to 291 using the buffer of pH 4.5.

III: mp 205~210°C (dec.); $[\alpha]_D^{22}$ −30° (*c* 1, 0.1 N HCl); pKá <2, 2.9, 7.5, 9.3; uv, end absorption; ir (KBr disk); 1742 $cm^{-1}$ (ester). Anal. calcd. for $C_{17}H_{22}N_2O_8\cdot H_2O$: C

C 50.99, H 6.04, N 7.00, O 35.97, (M.W. 400.38). Found: C 50.78, H 6.04, N 7.17, O 35.36, M.W. 404 (titration).

**IV**: mp 230~237°C (dec.); $[\alpha]_D^{22}$ −50° (*c* 1, 0.1 N HCl); pKá <2, 3.4, 7.7, 9.6; uv, end absorption; nmr, Table 1. Anal. calcd. for $C_{14}H_{18}N_2O_6 \cdot H_2O$: C 51.21, H 6.14, N 8.53, O 34.11, (M.W. 328.32). Found: C 50.27, H 6.16, N 8.46, O 34.02, M.W. 323 (titration).

Tetrahydro-IV (IX)

A sample (176 mg) of **IV** was dissolved in 30 ml of 1 N HCl solution, and was hydrogenated over 50 mg of ADAMS platinum oxide under atmospheric pressure at room temperature for about 5 hours. Two moles of hydrogen were absorbed. After removal of the catalyst, the solution was concentrated under reduced pressure to yield 192 mg of hydrochloride of **IX**. The hydrochloride was dissolved in 3 ml of water, and subjected to a column of Dowex 50W-X8 (H type, $\phi$=1.0 cm, 6 ml). After washing with water, **IX** was eluted with 0.5 N ammonia and was dried under reduced pressure. Crystallization with water-ethanol yielded 164 mg of colorless needles (95% yield); mp 260°C (dec.). Anal. calcd. for $C_{14}H_{22}N_2O_6 \cdot H_2O$: C 50.59, H 7.28, N 8.43, O 33.70, (M.W. 332.35). Found: C 50.08, H 7.48, N 8.40, O 33.40.

D-Lactic acid ammonium salt

A 3 g quantity of **III** was dissolved in 100 ml of 2 N NaOH, and was hydrolyzed at 60°C for 18 hours. The reaction mixture was passed through a column of Dowex 50W-X8 (H type, $\phi$=2.5 cm, 150 ml). The effluent was neutralized with 0.5 N ammonia and evaporated under reduced pressure to dryness. The residue was identified with ammonium lactate by comparison with an authentic sample; 722 mg (yield 94.5%), $[\alpha]_D^{22}$ −2.8° (*c* 5, water), ir (liquid film), 1725 (sh.), 1670, 1580, 1450, 1415, 1318, 1123, 1083, 1040, 922, 851, 775 $cm^{-1}$.

Tetraacetyl-dimethyl ester of IV (VIII)

A 300 mg of **IV** was dissolved in 10 ml of methanol containing 10% HCl, and was refluxed for 3 days. The reaction mixture was evaporated under reduced pressure to yield 210 mg of a pale yellow syrup. A 130 mg quantity of the syrup was dissolved in 5 ml of pyridine, and 2.5 ml acetic anhydride was added dropwise. The reaction mixture was kept overnight at room temperature and was evaporated under reduced pressure to dryness. The residue was dissolved in 5 ml of chloroform and the solution was passed through a column of silica gel (Mallinckrodt, $\phi$=1.0 cm, 5 g). After washing with chloroform (*ca.* 50 ml), the column was developed with chloroform-methanol (100:40 in volume). The fractions which gave one spot on thin-layer chromatography were collected, and concentrated under reduced pressure to yield 30 mg of a pale yellow powder; mp 156°C (dec.), nmr ($\delta$); 1.90, 1.98, 2.05, 2.12 (3H singlets×4, tetraacetyl), 3.71, 3.80 (3H singlets×2, dimethyl ester). MS; $M^+$ *m/e* 506.

Hydrated product of IV (V)

A 3 g of **III** was dissolved in 100 ml of 12 N HCl and was refluxed for 18 hours. The reaction mixture was evaporated under reduced pressure to dryness. The material was dissolved in 0.2 M pyridine-acetat buffer (pH 3.75), and chromatographed on a column of Dowex 50W-X8 which was previously treated with the same buffer ($\phi$=4.0 cm, 500 ml). The column was eluted with the same buffer. The eluate was collected in 20-ml fractions. The fractions from 33 to 99 contained 900 mg of **III**. The pH of the buffer was raised to 4.5 from fraction No. 151. The eluate from 167 to 291 contained 1.89 g of the mixture of the hydrated products of **III** and **IV**. Finally 432 mg of **V** (14.4% yield) was obtained from the eluate from 333 to 368 by using 0.4 M buffer of pH 4.75. mp >260°C, uv; end absorption, nmr; Table 2. Anal. calcd. for $C_{14}H_{20}N_2O_7 \cdot H_2O$: C 48.55, H 6.40, N 8.09, O 36.96, (M.W. 346.33). Found: C 48.23, H 6.26, N 8.00, O 36.45.

Tetrahydro I (VII)

A 1.00 g of **I** was dissolved in 200 ml of water and hydrogenated over 100 mg of ADAMS platinum oxide under atmospheric pressure at room temperature for about 3 hours. Two moles of hydrogen was absorbed. After removal of the catalyst, the solution was

evaporated under reduced pressure to dryness. Crystallization from water - ethanol yielded 835 mg of colorless needles (83.5 % yield). mp 213~215°C (dec.), ir (KBr disk); 1724 $cm^{-1}$ (ester). Anal. calcd. for $C_{10}H_{17}NO_5$: C 51.94, H 7.41, N 6.06, O 34.60, (M.W. 231.24). Found: C 51.86, H 7.43, N 5.99, O 34.72, M.W., 231 (MS $M^+$ *m/e*).

(+)-6-Amino-*trans*-5-hydroxy-1,3-cyclohexadiene-1-carboxylic acid (**VI**)

A 1.00 g quantity of **I** was dissolved in 200 ml of 2 N NaOH solution, and hydrolyzed at 60°C for 5 hours. After cooling, the reaction mixture was added to a column of Dowex 50W-X8 (H type, $\phi$=2.5 cm, 50 ml). After washing with water, the column was eluted with 0.5 N ammonia. The fractions containing **IV** and **VI** were combined and evaporated under reduced pressure to dryness. The material was chromatographed on a cellulose column (Whatmann CF 11, $\phi$=2.5 cm, 50 g) with *n*-butanol - acetic acid - water (3 : 1 : 1 in volume). The eluate was collected in 15-ml fractions. The fractions from 86 to 156 contained 680 mg of **VI** (68.0 % yield) and the fractions from 186 to 235 contained 130 mg of **IV** (13.0 % yield). mp 192°C, $[\alpha]_D^{22}$ +446° (*c* 1, water), $\lambda_{max}$ ($\varepsilon$); 272 nm (5425). Anal. calcd. for $C_7H_9NO_3$: C 54.19, H 5.85, N 9.03, O 30.94, (M.W. 151.15). Found: C 54.23, H 5.85, N 9.00, O 30.88.

## References

1) Hashimoto, T.; S. Kondo, T. Takita, M. Hamada, T. Takeuchi, Y. Okami & H. Umezawa: Oryzoxymycin, a new antibiotic. J. Antibiotics 21 : 653~658, 1968
2) McCormick, J.R.D.; J. Reichenthal, U. Hirsh & N. O. Sjolander: Biosynthesis of the tetracyclines. III. A new amino acid from *S. aureofaciens*; (+)-*trans*-2,3-dihydro-3-hydroxyanthranilic acid. J. Amer. Chem. Soc. 84 : 3711~3714, 1962