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The Chemistry of Pyridomycin

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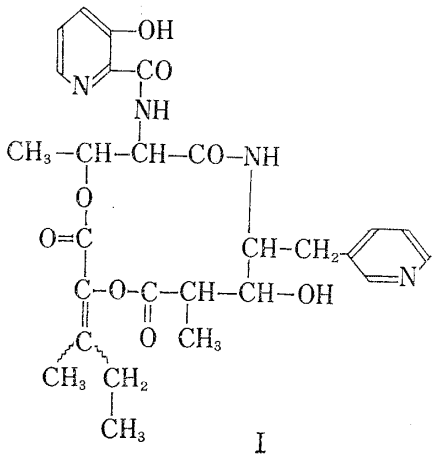
Structural studies on pyridomycin have been performed by chemical degradation such as ozonolysis and hydrolysis in alkaline and acidic media. New acids, α-oxo-β-methylvaleric acid, 4-amino-3-hydroxy-2-methyl-5-(3-pyridyl)pentanoic acid and its dehydration and deamination products are obtained as degradation products. The structure I, 10-hydroxy-6-(3-hydroxypicolinamido)-5,11-dimethyl-2-(1-methylpropylidene)-9-(3-pyridylmethyl)-8-aza-1,4-dioxa-cyclododecane-3,7,12-trione, has been proposed for pyridomycin. This structure including its stereochemistry was recently determined by X-ray analysis of the crystal of pyridomycin dihydrobromide ($C_{27}H_{32}O_8N_4 \cdot 2HBr \cdot H_2O \cdot CH_3CH_2OCOCH_3$).[2]

Pyridomycin (I) is an antimycobacterial antibiotic[3] produced by *Streptomyces pyridomyceticus*.[4] Elemental analysis of pyridomycin gives values consistent with a molecular formula $C_{27}H_{32}O_8N_4$. As reported in a previous paper,[5] 3-hydroxypicolinic acid, β-picoline, L-threonine, 2-ethylmalic acid, glycine and two unidentified products, $C_{11}H_{14}O_2N_2$ (mp 190°) and $C_{10}H_{10}O_4N_2$ (mp 184—185°) were obtained by acidic and alkaline degradations of I. A tentative structure proposed by Sugiyama, *et al.*[6] on the basis of infrared and ultraviolet absorption spectra of degradation products, can not reasonably explain the nuclear magnetic resonance spectrum (NMR) and mass spectrum of pyridomycin. In the present paper, we report the preparation of pyridomycin dihydrobromide (II), pyridomycin monoacetate (III), and results of degradation leading to the structure I.



I

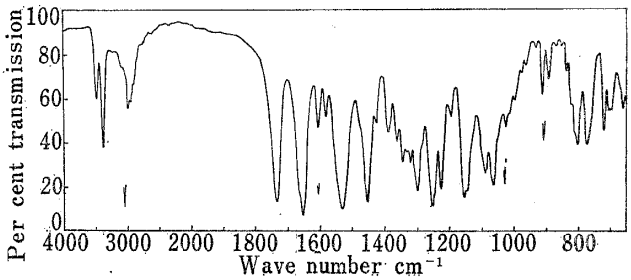


Fig. 1. Infrared Absorption Spectrum of I in Potassium Bromide Disc

1) Location: *a) 10-35, Kamiosaki 2-chome, Shinagawa-ku, Tokyo. b) 14-23, Kamiosaki 3-chome, Shinagawa-ku, Tokyo.*
2) G. Koyama, Y. Iitaka, K. Maeda, and H. Umezawa, *Tetrahedron Letters*, No. 37, 3587 (1967).
3) K. Maeda, H. Kosaka, Y. Okami, and H. Umezawa, *J. Antibiotics*, **6**, 140 (1953).
4) Y. Okami, K. Maeda, and H. Umezawa, *J. Antibiotics*, **7**, 55 (1954).
5) K. Maeda, *J. Antibiotics*, **10**, 94 (1957).
6) N. Sugiyama and K. Hori, The 5th Symposium on the Natural Organic Products (Japan), 1961.

Pyridomycin (I) is an optically active dibasic substance having phenolic group (positive ferric chloride reaction) and an ester band at 1735 cm^{-1} in the infrared absorption spectrum[7] (Fig. 1), and is converted by cold aqueous alkali into an inactive acid, named pyridomycin acid, which shows the same characteristic absorption of ultraviolet spectrum as the parent antibiotic but no ester band in the infrared absorption spectrum.

Acetylation of I with acetic anhydride in anhydrous methanol gives a monoacetate (III). The infrared spectrum shows absorption bands at 1768 and 1195 cm^{-1}, the NMR shows a signal at 7.62 τ (three protons, singlet)[8] and it is found to be negative for ferric chloride reaction. Therefore, these evidences indicate that phenolic hydroxyl group of I is acetylated.

The presence of methylpropylidene group in I was suggested at the early stage of the X-ray analysis. In order to confirm the suggestion, ozonolysis of I at the temperature of dry-ice/methanol has been carried out to give ethyl methyl ketone isolated as its 2,4-dinitrophenylhydrazone (IV). After removal of ethyl methyl ketone by distillation, oxalic acid (V) is obtained.

Alkaline hydrolysis of I gives pyridomycin acid (VI) and a keto acid (VII). The latter is isolated as its 2,4-dinitrophenylhydrazone (VII-1, $C_{12}H_{14}O_6N_4$) and the NMR spectrum of VII-1 (Fig. 2) shows signals at 9.07 τ (three protons, triplet, J=7.0 cps) and 8.41 τ(two protons, multiplet, J=7.0 and 7.5 cps), suggesting the presence of ethyl group. Peaks at 8.80 τ (three protons, doublet, J=6.8 cps) and 7.06 τ (one proton, multiplet, J=6.8 and 7.5 cps) correspond to methyl and methine hydrogens, respectively. The doublet at 8.80 τ is decoupled to a sharp singlet by irradiation at low field (Fig. 2-1). A sharp singlet at −3.72 τ is coincident with the presence of one intramolecularly bonded hydrogen of carboxylic acid. On the basis of the NMR spectrum, infrared absorption spectrum (1623, 1597, 1340, 833 cm^{-1}, 2,4-dinitrophenylhydrazone, Fig. 3) and potentiometric titration (pK_a' 3.80, carboxyl), the

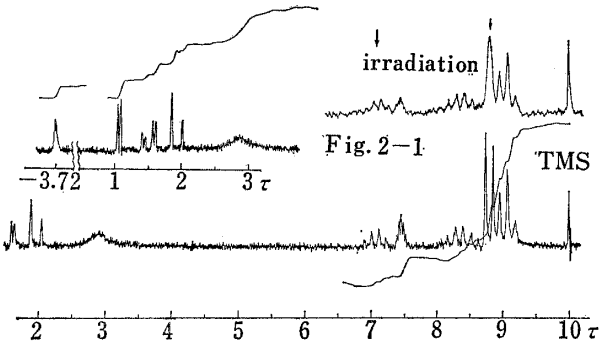


Fig. 2. Nuclear Magnetic Resonance Spectra of VII-1 in Deutero-dimethylsulfoxide Solution

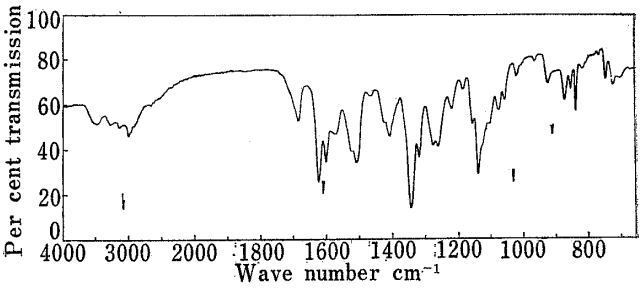
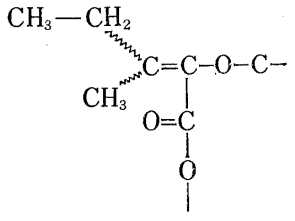


Fig. 3. Infrared Absorption Spectrum of VII-1 in Potassium Bromide Disc



derivative VII-1 was determined to be 2,4-dinitrophenylhydrazone of α-oxo-β-methylvaleric acid.

In consequence of the isolation of ethyl methyl ketone and oxalic acid by ozonolysis of I and of α-oxo-β-methylvaleric acid by alkaline hydrolysis of I, it is evident that I contains the following component in the molecule.

Hydrolysis of pyridomycin acid (VI) ($C_{21}H_{26}O_7N_4$·2HCl) with constant boiling hydrochloric acid (5.8 N) yields six components which are isolated by ion-exchange chromatography.

7) All infrared spectra were taken by Hitachi Model EPI-S-2 spectrophotometer in KBr disc.
8) NMR spectra were taken by Varian A-60 apparatus at 60 Mc in deuterochloroform, deuterodimethylsulfoxide using tetramethylsilane as an internal reference and in deuterowater taking the peak of hydrogen of water as 5.30 τ at room temperature and as 5.75 τ at 90°.

The first component of the eluate is ninhydrin positive and has been identified as L-threonine (VIII).

The next two components have strong ultraviolet absorption and are optically inactive and ninhydrin negative, but the existence of phenolic group is indicated by positive ferric chloride reaction. 3-Hydroxypicolinic acid (IX) was confirmed as one of them in a previous paper.[5] The other component is successfully isolated as a hydrochloride (X, $C_{10}H_{10}O_4N_2 \cdot HCl \cdot 2H_2O$) or a free base (X-1). Infrared absorption spectrum of X (Fig. 4), titration and the color reaction for amide bond (Rydon-Smith[9]) of X-1 indicate the presence of a carboxyl group (IR 1716 cm^{-1}, pK_a' 5.30) and an amide bond (IR 1676, 1507 cm^{-1}). Such appearance of the infrared absorption suggests that this amide group joins to aromatic nucleus or double bond. The ultraviolet absorption spectra of X-1 ($\lambda_{max}^{0.08N\ HCl}$ mμ: 217, 317. $\lambda_{max}^{0.08N\ NaOH}$ mμ: 234, 263, 363) indicate that it is a hydroxypicolinyl derivative. The peaks at 8.25 τ (methyl protons attached to double bond, doublet, J=6.8 cps) and 4.44 τ (*trans* one proton to carboxyl group, quartet) in the NMR spectrum of X (Fig. 5) and the result of their spin-decoupling indicates the presence of $\overset{CH_3}{H}\!\!>\!C\!=\!C\!<\!COOH$ group. These data and signals at aromatic region (1.85 τ, two protons; 1.35 τ, one proton) show that X has a chemical structure illustrated below. This compound is supposed to be identical with C_{10}-compound obtained from I by Sugiyama, *et al.*[6]

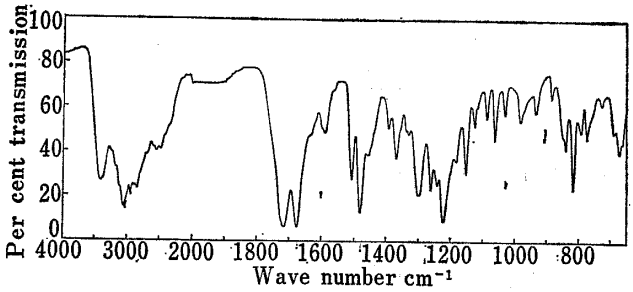


Fig. 4. Infrared Absorption Spectrum of X in Potassium Bromide Disc

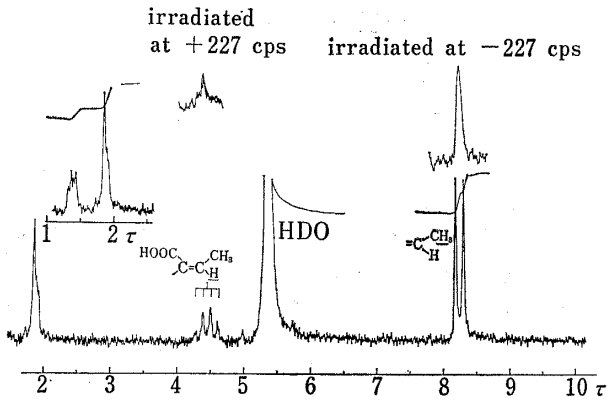
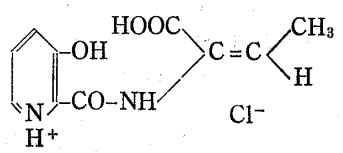


Fig. 5. Nuclear Magnetic Resonance Spectra of X in D_2O Solution

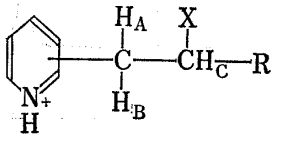
As these three components (VIII, IX and X) are found in the acid hydrolysate of VI, it is clear that 3-hydroxypicolinyl-L-threonine is present in VI, from which X is obtained as a result of dehydration during the hydrolysis.



X

Of the remaining three components in the hydrolysate, one is isolated as its hydrochloride, $C_{11}H_{14}O_2N_2 \cdot 2HCl$ (XI). The bands of ultraviolet absorption maxima ($\lambda_{max}^{0.08N\ NaOH}$ mμ: 256, 263, 270), infrared absorption spectrum (Fig. 6, 1705 cm^{-1}, carboxyl; 798 cm^{-1}, pyridine) and pK_a' 3.25 (carboxyl) indicate that XI is a monosubstituted pyridine derivative with carboxyl group. The NMR spectrum of XI (Fig. 7) shows the presence of a methyl group attached to double bond (8.35 τ, doublet, J=1.5 cps) and vinyl proton (3.25 τ, double quartet, J=1.5 and 10.5 cps). These results illustrate that XI contains the following function: $\overset{CH_3}{C}\!>\!C\!=\!CH\!-\!CH\!-$

Since signals at 6.4 τ (two protons, J_{AB}=14.5 cps) and 5.3 τ (one proton, J_{AC}=10.2, J_{BC}=9.2 cps) show ABC type multiplet, these three protons must possess the following relation:



9) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1952).

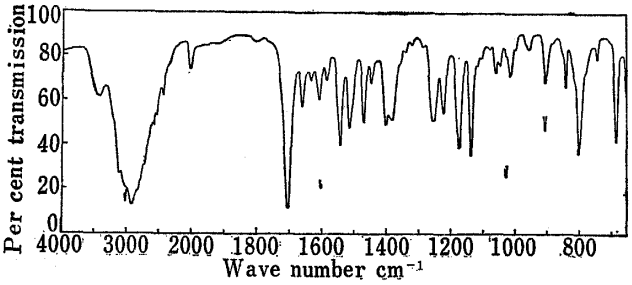


Fig. 6. Infrared Absorption Spectrum of XI in Potassium Bromide Disc

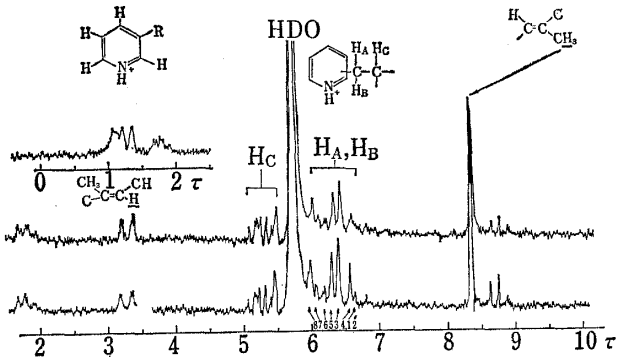
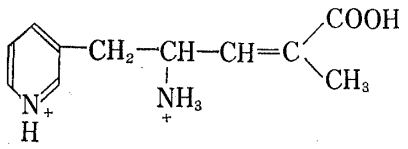
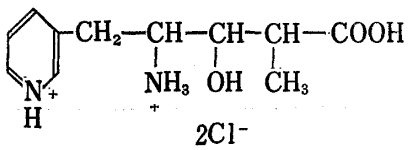


Fig. 7. Nuclear Magnetic Resonance Spectrum of XI in D_2O Solution at 90°

On the basis of these results and signals of aromatic region (1.71 τ, J=8.1 and 6.0 cps; 1.30 τ, J=8.1, 2.5 and 1.0 cps; 1.17 τ, J=6.0 and 1.0 cps; 1.10 τ, J=2.5 cps, suggesting a 3-substituted pyridine derivative), a structure shown below can reasonably be proposed for XI. It is further supported by the result of the potentiometric titration (pK_a' 3.25, carboxyl; 4.75, ring nitrogen; 8.80, primary amino group).



XI



XII

Another amino acid in the hydrolysate is isolated as its hydrochloride, $C_{11}H_{16}O_3N_2 \cdot 2HCl \cdot H_2O$ (XII). Similar pK_a' values and ultraviolet maxima of XI and XII and positive periodate reaction of XII, suggest the above structure for XII.

This structure can be proved by analysis of its NMR spectrum (Fig. 8). Peaks at 8.87 τ (three protons, doublet, J=7.0 cps) and peaks at 7.20 τ (one proton, quintet) correspond to methyl and methine protons, respectively (CH_3–$\underset{\underset{H}{|}}{C}$–). The doublet at 8.87 τ is decoupled to a sharp singlet by irradiation at low field. Peaks at 6.7 τ (two protons) and 6.2 τ(two protons) with fine structure splitting can be analysed as ABCD type coupling. Four proton signals at aromatic region (1.83 τ, J=8.5 and 5.8 cps; 1.30 τ, J=8.5, 2.0 and 0.7 cps; 1.17 τ, J=5.8 and 0.7 cps; 1.12 τ, J=2.0 cps) shows that XII must be a 3-substituted pyridinium compound. With the added knowledge that XII has a carboxyl (IR 1720 cm^{-1}, pK_a' 3.25) and a primary amino group (ninhydrin positive, pK_a' 8.80), the structure XII is completely expressed by the NMR spectrum.

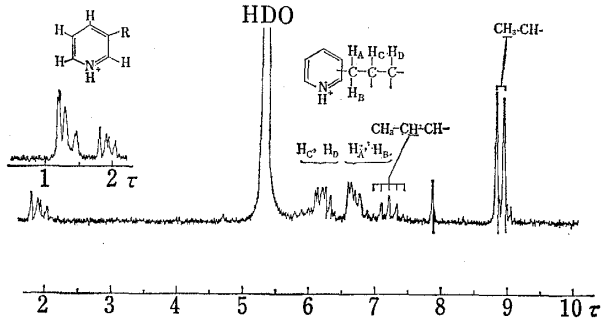


Fig. 8. Nuclear Magnetic Resonance Spectrum of XII in D_2O Solution

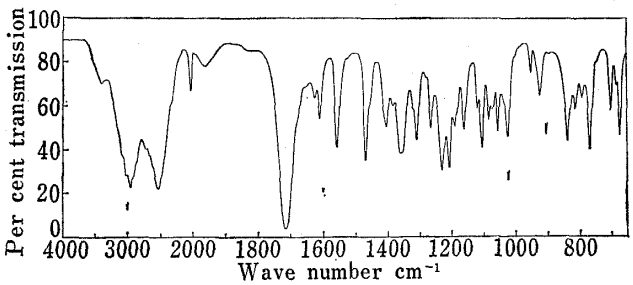
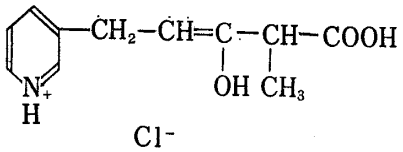


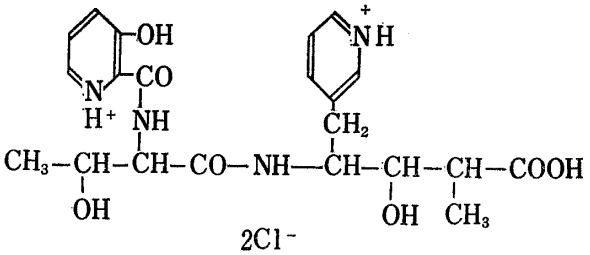
Fig. 9. Infrared Absorption Spectrum of XIII in Potassium Bromide Disc

As stated above, VI is constituted of 3–hydroxypicolinyl–L–threonine and C_{11} component (XI or XII). The molecular formula of VI ($C_{21}H_{26}O_7N_4 \cdot 2HCl$) indicates that the hydrated compound XII is preferable to XI. This fact can be verified by the isolation of deamination product of XII.

The deamination product is obtained as its hydrochloride, $C_{11}H_{13}O_3N \cdot HCl$ (XIII), which has a carboxyl group (IR Fig. 9, 1716 cm^{-1}, pK_a' 4.11) and similar ultraviolet absorption spectrum as XI and XII, but no primary amino group (titration and negative ninhydrin reaction). From these results, XIII has the following structure.



XIII



VI

Pyridomycin acid (VI) has a carboxyl (IR 1727 cm^{-1}, pK_a' 4.10), an amide bond (IR 1670, 1530, 1250 cm^{-1}) but no primary amino group (negative ninhydrin). From the foregoing results and its NMR spectrum described below, the structure of VI can be shown as VI.

The NMR spectrum of VI (Fig. 10) shows the presence of two methyl groups which must be adjacent to methine group (8.87 τ and 8.81 τ, each three protons, doublet). In addition there are one–proton multiplet (–CH–CH_3), two–proton multiplet (–CH_2–) between 7.0 τ and 6.5 τ, and multiplets of four hydrogens (–CH–) between 6.1 τ and 5.4 τ. In the aromatic region, peaks of seven hydrogens are observed. Thus, the NMR spectrum can be satisfied by the structure VI which accounts satisfactorily for all facts so far presented. In this molecule the pK_a' values show 4.10, 5.43 and 7.80 which can be assigned to carboxyl, pyridinium nitrogen and phenolic hydroxyl groups, respectively.

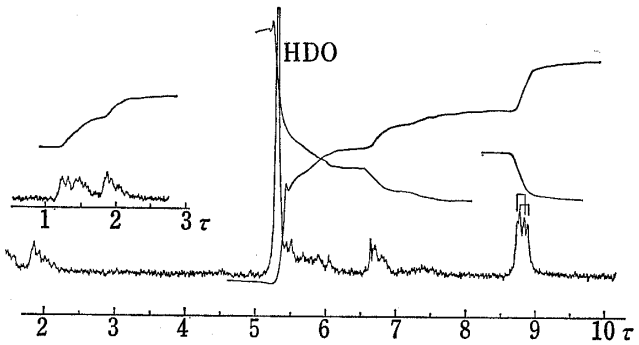


Fig. 10. Nuclear Magentic Resonance Spectrum of VI in D_2O Solution

Evidences that I has a macrocyclic lactone are as follows. I does not contain carboxyl and primary amino groups, but contains phenolic hydroxyl (pK_a' 9.17, positive ferric chloride reaction) and two tertiary nitrogens of 3–hydroxypicolinic acid (pK_a' below 2.0) and XII (pK_a' 4.17). The infrared spectrum of I possesses a peak at 1735 cm^{-1} characteristic of an ester function. Treatment with dilute sodium hydroxide at room temperature produces two products, such as α–oxo–β–methylvaleric acid (VII) and pyridomycin acid (VI). The latter has a peak at 1727 cm^{-1} attributable to the carboxyl group. Since I has no keto function (negative 2,4–DNP reaction[10]), a hydroxyl group in the enol form of VII has to link with carboxyl group in VI. It is supported by the isolation of ethyl methyl ketone and oxalic acid and alkaline titration, that is, I consumes two moles of sodium hydroxide during

10) 2,4–dinitrophenylhydrazine was abbreviated to 2,4–DNP in this paper.

the titiration at pH 11.0. Thus, the other lactone link involves the carboxyl group of VII with the hydroxyl group of either threonine or XII moiety.

Treatment of VI with chromic acid in acetic acid–pyridine by the method described by Sheehan, *et al.*[11] degradates both threonine and XII. Under the same condition XII in pyridomycin is destroyed, but threonine is unaffected. Consequently, a lactone linkage is between the carboxyl group of VII and the hydroxyl group of threonine, and another lactone linkage is between the terminal carboxyl group of pyridomycin acid and the hydroxyl group in the enol form of VII. Therefore, the whole structure of pyridomycin can be deduced as formula I. The NMR spectrum of I supports the structure, and the assignment of signals was described in Fig. 11 and Table I.

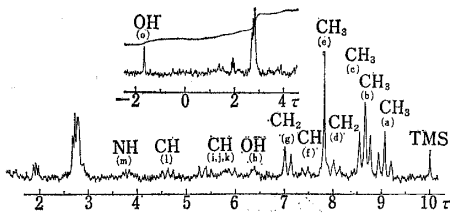
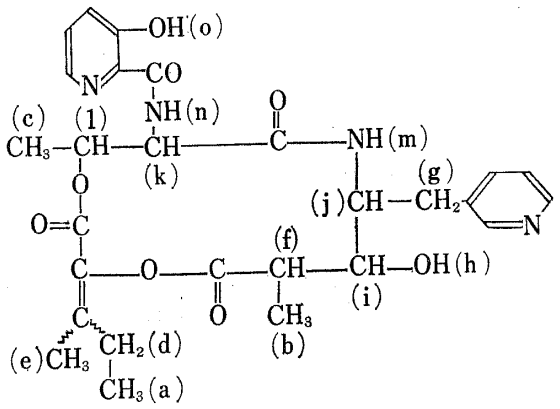


Fig. 11. Nuclar Magnetic Resonance Spectrum of I in $CDCl_3$ Solution

TABLE I. Nuclear Magnetic Resonance Spectral Analysis of Pyridomycin



Chemical shifts (τ values)	Coupling constants (cps)	Ratio of intensity of signals	Assignment
9.07	7.7 (triplet)	3	CH_3 (a)
8.70	7.0 (doublet)	3	CH_3 (b)
8.60	7.8 (doublet)	3	CH_3 (c)
7.95	7.7 (quartet)	2	CH_2 (d)
7.82	(singlet)	3	CH_3 (e)
7.50	(multiplet)	1	CH (f)
7.07	(doublet–like)	2	CH_2 (g)
6.37	(singlet)	1	OH (h)
6.1—5.1	(multiplet)	3	CH (i, j, k)
4.63	(multiplet)	1	CH (l)
3.80	(singlet–like)	1	NH (m)
3.0—1.5	(multiplet)	8	ring H and NH (n)
−1.82	(singlet)	1	OH (o)[a]

a) This signal disappeared in NMR spectrum of III.

The pyridomycin structure containing the moiety of 3–hydroxypicolinyl–L–threonine is closely related to those of etamycin[12] and mikamycin.[13] However, it contains α–oxo–β–methylvaleric acid and 4–amino–3–hydroxy–2–methyl–5–(3–pyridyl)pentanoic acid (XII), neither

11) J.C. Sheehan, H.G. Zachau, and W.B. Lawson, *J. Am. Chem. Soc.*, **80**, 3349 (1958).
12) J.C. Sheehan, H.G. Zachau, and W.B. Lawson, *J. Am. Chem. Soc.*, **79**, 3933 (1957).
13) K. Watanabe, *J. Antibiotics*, **14**, 293 (1960).

of which has been encountered previously in natural products, and pyridomycin possesses one 12-membered ring containing two ester functions.

Experimental[14]

Pyridomycin (I)——The crude pyridomycin, as supplied by Toyo Jozo Company, Ltd., was recrystallized three times from EtOH to yield colorless needles, mp 231—233° (decomp.). $[\alpha]_D^{21}$ −62.0° (c=1.0, dioxane–H_2O=2:1). pK_a' 4.17, 9.17 (titration equivalent 540, EtOH–H_2O=1:1). It gave positive tests with $FeCl_3$ and Biuret reagents, but negative with ninhydrin, Fehling and 2,4-DNP reagents. UV λ_{max}^{EtOH} mμ ($E_{1cm}^{1\%}$): 264 (92), 270 (80), 305 (177). UV $\lambda_{max}^{0.1N\ HCl}$ mμ ($E_{1cm}^{1\%}$): 228 (446), 265sh (170), 304 (197). UV $\lambda_{max}^{0.1N\ NaOH}$ mμ ($E_{1cm}^{1\%}$): 333 (168). *Anal.* Calcd. for $C_{27}H_{32}O_8N_4$: C, 59.99; H, 5.97; O, 23.67; N, 10.37; M.W., 540.56. Found: C, 59.55; H, 6.14; O, 23.50; N, 10.13; M.W. (mass spectrum), 540.

Pyridomycin Dihydrobromide (II)——To a solution of I (332.10 mg) in EtOH (10 ml), 0.6 ml of ethanolic HBr (2.2 N) was added and precipitated crystals were recrystallized from H_2O–EtOH to give 105.70 mg of colorless needles, mp 218—220° (decomp.). pK_a' 4.50, 8.90 (titration equivalent 650, EtOH–H_2O=1:1). IR cm^{-1}: 3450, 3050—2600, 1730, 1670, 1650, 1525, 1465, 1327, 1250, 800. *Anal.* Calcd. for $C_{27}H_{32}O_8N_4 \cdot 2HBr$: C, 46.20; H, 4.88; O, 18.22; N, 7.98; Br, 22.75; M.W., 702.43. Found: C, 46.07; H, 5.29; O, 17.90; N, 8.33; Br, 22.17.

Pyridomycin Monoacetate (III)——To a suspension of I (116.20 mg) in anhydrous MeOH (8 ml), acetic anhydride (0.2 ml) was added, and the mixture was allowed to stand at room temperature for 7 hr, and clarified during the reaction. To the clear solution, 8 ml of H_2O was added. After 17 hr, the mixture was extracted with $CHCl_3$ (8 ml×5). The $CHCl_3$ extracts were combined, dried with Na_2SO_4 and concentrated to dryness under reduced pressure. The residue (145.50 mg) was recrystallized from acetone–petr. ether to give 92.50 mg of white needles, mp 235—237° (decomp.), which gave a negative $FeCl_3$. IR cm^{-1}: 1768, 1195. NMR ($CDCl_3$) τ: 7.62 (3H, singlet). *Anal.* Calcd. for $C_{27}H_{31}O_8N_4 \cdot COCH_3$: C, 59.78; H, 5.88; O, 24.72; N, 9.62; CH_3CO, 7.39. Found: C, 60.16; H, 6.06; O, 24.47; N, 9.36; CH_3CO, 9.26.

Isolation of Ethyl Methyl Ketone as Its 2,4-Dinitrophenylhydrazone (IV)——A stream of ozone was introduced into a solution of I (102.20 mg) in CH_2Cl_2 (15 ml) at the temperature of dry-ice/methanol for 30 min and the solution changed pale yellowish violet. To the solution, 15 ml of AcOH, 1 g of zinc dust and 1 ml of H_2O was added and refluxed for 30 min. At the end of this period, the mixture showed negative KI–starch reaction. After addition of H_2O (10 ml), the solution was steam-distilled to obtain 40 ml of distillate. To the distillate, 2 ml of 2,4-DNP solution (2,4-DNP 182 mg, conc. H_2SO_4 1 ml and EtOH 5 ml) was added. After evaporation, the mixture gave precipitate (63.50 mg) by the evaporation of ethanol and the precipitate was dissolved in $CHCl_3$, mixed with small amount of silicic caid, dried *in vacuo*, and put on a column of silicic acid (1×30 cm, 15 g). The column was eluted with benzene–petr. ether (2:1), and the eluate cut into 7 ml fractions. Fractions 15 to 29 gave 9.90 mg of crude crystals (yield 20.7%), which were recrystallized from EtOH–H_2O yielding 2.45 mg of yellow needles, mp 114°. It was identical with ethyl methyl ketone 2,4-dinitrophenylhydrazone in mixed melting point determination, IR spectra and chromatographic behavior.

Isolation of Barium Oxalate and Oxalic Acid (V)——A solution of I (300.62 mg) in CH_2Cl_2 (50 ml) was treated with ozone at the temperature of dry-ice/methanol for 15 min and steam-distilled. From distillate, ethyl methyl ketone 2,4-dinitrophenylhydrazone was obtained as described above. The undistilled solution (100 ml) was concentrated to dryness under reduced pressure, and the residue was redissolved in H_2O (10 ml) containing barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$ 260 mg) and kept at room temperature for 18 hr yielding 74.30 mg of crystalline powder (yield 79.1%). Identity with barium oxalate was confirmed by IR spectrum and chromatographic behavior. A solution of this powder (70.30 mg) in 1 N HCl (5 ml) was lyophilized, dissolved in EtOH (5 ml), and concentrated to dryness under reduced pressure to obtain 35.10 mg of crystals. Recrystallization from ether–petr. ether gave 11.90 mg of colorless needles, mp 187°, mixed mp 187°. *Anal.* Calcd. for $C_2H_2O_4$: C, 26.68; H, 2.24; M.W., 90.04. Found: C, 26.76; H, 2.61. It was identical with oxalic acid in IR spectrum.

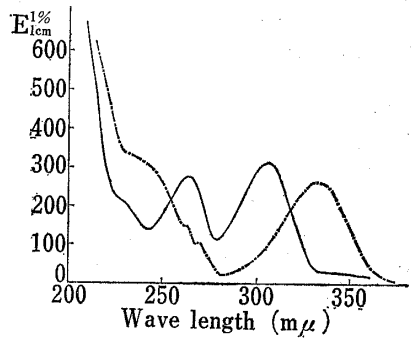


Fig. 12. Ultraviolet Absorption Spectra of VI

——— 0.08 N HCl
— - - - — 0.08 N NaOH

Isolation of Pyridomycin Acid (VI)——A mixed solution of I (544.40 mg) in dioxane–H_2O (1:1, 20 ml) and 1 N NaOH (4 ml) was kept at room temperature for 17 hr and lyophilized. The residual solid was dissolved

14) All melting points were uncorrected. Ultraviolet spectra were measured Hitachi Model EP-2 recording spectrophotometer, and pK_a'- values were measured with Radiometer Titrator Type TTT 1c.

in H_2O (20 ml), acidified with 1 N HCl and extracted with ether (30 ml × 5). The water layer was lyophilized, and the solid was dissolved in MeOH (10 ml) and precipitated by the addition of ether (100 ml). Five repetition of the procedure with MeOH–ether afforded 461.90 mg of white powder, mp 187—188° (decomp), which gave negative ninhydrin. pK_a' 4.10, 5.43, 7.80 (titration equivalent 500). UV (Fig. 12). IR cm^{-1}: 1727, 1670, 1530, 1250, 800. *Anal.* Calcd. for $C_{21}H_{26}O_7N_4 \cdot 2HCl$: C, 48.56; H, 5.43; O, 21.56; N, 10.79; Cl, 13.66; M.W., 519.40. Found: C, 48.55; H, 6.07; O, 22.06; N, 10.42; Cl, 12.78.

Isolation of α-Oxo-β-methylvaleric Acid as 2,4-Dinitrophenylhydrazone (VII-1)——The ether extracts (150 ml) described in the isolation of VI were dried with Na_2SO_4 and concentrated under reduced pressure. The residual solid was dissolved in H_2O (20 ml) and treated with 2,4–DNP solution (2,4–DNP 220.20 mg, conc. H_2SO_4 1.2 ml and H_2O 2.0 ml). Twice recrystallization of the precipitate (290.20 mg, yield 93.6%) from MeOH–H_2O yielded 224.10 mg of yellow needles, mp 167.5—168° (identical with the data described in *J. Biol. Chem.*[15]). pK_a' 3.80 (titration equivalent 317, EtOH–H_2O 1:1). UV λ_{max}^{EtOH} $m\mu$ ($E_{1cm}^{1\%}$): 372 (795). *Anal.* Calcd. for $C_{12}H_{14}O_6N_4$: C, 46.45; H, 4.55; O, 30.94; N, 18.06; M.W., 310.26. Found: C, 46.71; H, 4.55; O, 30.48; N, 17.38.

Hydrolysis of Pyridomycin Acid——Pyridomycin acid obtained from 2.35170 g of pyridomycin was hydrolyzed with constant boiling HCl (20 ml) at 105° for 11 hr in a sealed tube. On thin-layer and paper chromatograms the hydrolysate showed three ninhydrin positive spots and three ninhydrin negative spots with marked ultraviolet absorption. The hydrolysate was adsorbed on a column of Dowex 50W–X8 (3.5 × 42 cm, H^+ form) and the column was eluted initially with 1 N HCl (1 liter), linear gradient (4 N HCl to 1 N HCl, each 2100 ml) and finally 4 N HCl. The eluate was cut in 10 ml fractions and detected by absorption at 253.7 $m\mu$ (Uvicord) and reactions with $FeCl_3$ and ninhydrin on paper. L–Threonine (VIII) (fractions 89—102) was eluted first, followed by 3–hydroxypicolinic acid (IX) (fractions 197—265), compound X (fractions 270—297), compound XIII (fractions 351—430), compound XII (fractions 435—482) and compound XI (fractions 496—570).

L-Threonine (VIII)——The appropriate fractions (89—102) of Dowex 50 column eluate were passed through Dowex 1−X2 (acetate form) column and the effluent gave the free amino acid (238.00 mg, yield 46.0%). It crystallized from aqueous EtOH–acetone in colorless plates, mp 245° (decomp.), mixed mp 245° (decomp.). $[\alpha]_D^{22}$ −30.0° (c=0.5, H_2O). pK_a' 2.05, 9.15 (titration equivalent 116). *Anal.* Calcd. for $C_4H_9O_3N$: C, 40.33; H, 7.62; O, 40.29; N, 11.76; M.W., 119.12. Found: C, 40.27; H, 7.65; O, 40.51; N, 11.48. The IR spectrum and chromatographic behavior were indistinguishable from those of L–threonine.

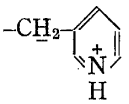
3-Hydroxypicolinic Acid (IX)——Appropriate Dowex 50 column fractions (197—265), treated with Dowex 1–X2 (acetate form), gave an amino acid (293.50 mg, yield 48.5%) crystallizing from MeOH in neeldes, mp 215—216°, mixed mp 215—216°. pK_a' 5.20, 11.3 (titration equivalent 148). Test with $FeCl_3$ was positive (brown). UV $\lambda_{max}^{H_2O}$ $m\mu$ ($E_{1cm}^{1\%}$): 304 (642). *Anal.* Calcd. for $C_6H_5O_3N$: C, 51.80; H, 3.62; O, 34.51; N, 10.07; M.W., 139.11. Found: C, 51.10; H, 3.67; O, 34.16; N, 9.61. It was identical with 3–hydroxypicolinic acid in IR, UV spectra and chromatographic behavior.

Compound X——The fractions (270—297) were concentrated to dryness under reduced pressure, compound X was obtained as its crystalline hydrochloride (249.80 mg, yield 22.2%), recrystallizing three times from EtOH in pale yellow needles, mp 211—212° (decomp.). *Anal.* Calcd. for $C_{10}H_{10}O_4N_2 \cdot HCl \cdot 2H_2O$: C, 40.76; H, 5.13; O, 32.57; N, 9.51; Cl, 12.03; H_2O, 11.52; M.W., 294.71 Found: C, 41.61; H, 4.87; O, 31.98; N, 9.66; Cl, 12.69; H_2O, 9.84 (180°,2 hr). Free base was obtained by passage through Dowex 1–X2 column (acetate form), mp 196—198° (decomp.). pK_a' 5.30 (titration equivalent 200). Tests with $FeCl_3$ and Rydon-Smith reagents[6] were positive. UV $\lambda_{max}^{H_2O}$ $m\mu$($E_{1cm}^{1\%}$): 216 (787), 318 (310). UV $\lambda_{max}^{0.08N\ HCl}$ $m\mu$($E_{1cm}^{1\%}$): 217(845), 317 (342). UV $\lambda_{max}^{0.08N\ NaOH}$ $m\mu$($E_{1cm}^{1\%}$): 234 (733), 263 (305), 363 (350). *Anal.* Calcd. for $C_{10}H_{10}O_4N_2$: C, 54.05; H, 4.54, N, 12.61; M. W., 222.20. Found : C, 53.35; H, 4.78; N, 12.27.

Compound XI——This was isolated from fractions (496—570) as its hydrochloride (218.50 mg, yield 18.0%), crystallizing from MeOH–EtOH in needles, decomposing at 267—268°. pK_a' 3.25, 4.75, 8.80 (titration equivalent 290). It gave yellowish violet ninhydrin. UV $\lambda_{max}^{H_2O}$ $m\mu$($E_{1cm}^{1\%}$): 262 (167). UV $\lambda_{max}^{0.08N\ HCl}$ $m\mu$($E_{1cm}^{1\%}$): 262 (186). UV $\lambda_{max}^{0.08N\ NaOH}$ $m\mu$($E_{1cm}^{1\%}$): 256 (110), 263 (118), 270 (85). *Anal.* Calcd. for $C_{11}H_{14}O_2N_2 \cdot 2HCl$: C, 47.33; H, 5.78; O, 11.46; N, 10.04; Cl, 25.40; M.W., 279.18. Found: C, 47.11; H, 5.42; O, 12.01; N, 9.83; Cl, 25.10.

Compound XII——From fractions (435—482), 389.80 mg of prism crystals (yield 28.5%) were obtained. The crystals were dissolved in MeOH (10 ml) and treated with charcoal (100 mg). To the methanol solution, acetone (100ml) was added yielding colorless powder. Recrystallization of the power from MeOH–EtOH gave colorless prisms, mp 177—180° (decomp.). pK_a' 3.25, 4.75, 8.80 (titration equivalent 300). It gave positive ninhydrin and periodate reactions. UV $\lambda_{max}^{H_2O}$ $m\mu$($E_{1cm}^{1\%}$): 262 (154). UV $\lambda_{max}^{0.08N\ HCl}$ $m\mu$($E_{1cm}^{1\%}$): 262 (180). UV $\lambda_{max}^{0.08N\ NaOH}$ $m\mu$ ($E_{1cm}^{1\%}$): 258 (91), 263 (108), 270 (78). IR cm^{-1}: 1720, 1555, 1472, 790, 678. *Anal.* Calcd. for $C_{11}H_{16}O_3N_2 \cdot 2HCl \cdot H_2O$: C, 41.90; H, 6.39; N, 8.88, Cl, 22.51; H_2O, 5.71; M.W., 315.21. Found: C, 42.52; H, 7.10; N, 8.43; Cl, 20.97; H_2O, 6.18 (150°, 2 hr).

15) A. Meister, *J. Biol. Chem.*, **190**, 269 (1951).

Compound XIII——The fractions (351—430) gave 620.90 mg (yield 58.4%) of crude crystals. Recrystallization from MeOH–EtOH yielded colorless needles, mp 160—161°. pK_a' 4.11, 5.40 (titration equivalent 250). It gave negative ninhydrin reaction. UV $\lambda_{max}^{H_2O}$ mμ ($E_{1cm}^{1\%}$): 262 (127). UV $\lambda_{max}^{0.08N\ HCl}$ mμ ($E_{1cm}^{1\%}$): 262 (141). UV $\lambda_{max}^{0.08N\ NaOH}$ mμ ($E_{1cm}^{1\%}$): 256sh (67), 262 (75), 270sh (40). IR cm^{-1}: 3400, 3050, 1716, 1557, 1468, 835, 700, 672. NMR (D_2O) τ: 8.79 (3H, doublet, CH_3–CH–) and 7.00 (2H, doublet, )

Anal. Calcd. for $C_{11}H_{13}O_3N \cdot HCl$: C, 54.20; H, 5.77; O, 19.72; N, 5.75; Cl, 14.56; M.W., 243.70. Found: C, 53.75; H, 5.74; O, 19.62; N, 5.85; Cl, 15.02.

Chromic Acid Oxidation of Pyridomycin and Pyridomycin Acid——A solution of I (9.3 mg) in 0.15 ml of chromium trioxide solution (CrO_3 100 mg, AcOH 3 ml, pyridine 0.1 ml) was allowed to stand at room temperature for 16 hr as described by Sheehan, Zachau and Lawson.[11] The reaction mixture, diluted with 5 ml of H_2O, was extracted with $CHCl_3$ (5 ml × 5), and the extract was dried with Na_2SO_4, and evaporated to dryness. The residue was hydrolyzed in 3 ml of constant boiling HCl at 105° for 16 hr, and the hydrolysate was evaporated, and dissolved in 0.50 ml of H_2O. This solution was subjected to paper (BuOH–AcOH–H_2O, 4:1:5) and thin-layer (BuOH–AcOH–H_2O, 3:1:1) chromatography. When the chromatograms from oxidized I was developed with ninhydrin, $FeCl_3$ and detected with UV light, three spots corresponding to hydroxypicolinic acid, threonine and compound X appeared and spots to XI, XII and XIII disappeared. The acid hydrolysate of I gave all six spots on chromatograms. When VI (15 mg) was subjected to the same reaction (CrO_3 solution, 0.2 ml), only hydroxypicolinic acid was detected on chromatograms. *Rf* values of pyridomycin and its degradation products were shown in Table II.

Table II. *Rf* Values of Pyridomycin and Its Degradation Products

	System 1	System 2	System 3	System 4	System 5
I	0.25				
VI	0.52				
VII	0.76				
VIII	0.30	0.21	0.68	0.64	0.11
IX	0.57	0.51	0.61	0.43	0.42
X	0.10	0.10			
XI	0.50	0.34			
XII	0.33	0.25			
XIII	0.07	0.08			

System 1. Thin-layer chromatography on silicic acid plates.[a]
n-Butanol–AcOH–H_2O (3:1:1)
System 2. Paper chromatography.
n-Butanol–AcOH–H_2O (4:1:5)
System 3. Amberlite ion exchanger SA-2.
Pyridine–AcOH–H_2O (1:10:89)
System 4. Amberlite ion exchanger SA-2.
Formic acid (2M)–NH_3 (1M)–H_2O (200:200:600) pH 3.7
System 5. Amberlite ion exchanger SA-2.
Formic acid (2M)–NH_3 (1M)–H_2O (450:50:500) pH 2.5
a) Thin-layer chromatography was performed using the Eastman Chromagram Sheet Type K301R2.

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