Chemical Studies on Blasticidin S

Part II. The Structure of Cytosinine and Uracinine

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Acid hydrolysis of cytosinine gave each one mole of cytosine, levulinic acid, ammonia and carbon dioxide. Reduction of cytosinine with PtO_2 afforded a mixture of dihydro-cytosinine, 3-amino-tetrahydropyran-2-carboxylic acid and cytosine. Ozonolysis of N,N' -diacetylcytosinine methyl ester, followed by oxidation with hydrogen peroxide and acid hydrolysis gave erythro-D- β -hydroxyaspartic acid. These data permitted the assignment of structure (I) for cytosinine. Acid hydrolysis of uracinine gave uracil instead of cytosine, therefore, the structure (II) could be assigned to uracinine. Some stereochemical features and mechanism of levulinic acid formation were discussed.

The isolation of cytosinine and uracinine from the hydrolysate of blasticidin S¹⁾ and their characterization were described in the previous paper.²⁾ We have presented the structure of cytosinine and blasticidin S (III) in recent communications,^{3,4)} in which the nucleoside was demonstrated that it is consisted of cytosine and a peculiar sugar of 2,3-unsaturated 4-amino-4-deoxyuronic acid structure. Such a nucleoside is quite unique one which arises from natural source.

This paper concerns with the chemical evidences which permitted the assignment of structure I and II for cytosinine and uracinine respectively.

Cytosinine (I), $\text{C}_{10}\text{H}_{12}\text{O}_4\text{N}_4$, m.p. 244~245°C (dec.), $[\alpha]_D^{18} -20^\circ\text{C}$ (c, 1 in H_2O) was an amphoteric compound of pKa 2.4, 4.6 and 8.0. Van Slyke determination proved the presence of two free amino groups. By the action of methanol containing 3% hydrogen chloride,

I gave corresponding methyl ester dihydrochloride, $\text{C}_{10}\text{H}_{11}\text{O}_3\text{N}_4 \cdot (\text{OCH}_3) \cdot 2\text{HCl}$ (IV), m.p. 220~223°C (dec.), pKa 4.6 and 8.0, therefore, the presence of one free carboxylic function was demonstrated. UV spectra of I showed λ_{max} 274 $\text{m}\mu$ (ϵ 13,800) in 0.1N hydrochloric acid which shifted to λ_{max} 267 $\text{m}\mu$ (ϵ 7,500) in 0.1N sodium hydroxide. This data indicated probable presence of cytosine (V), on which a

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2) H. Yonehara, S. Takeuchi, N. Ōtake, T. Endō, Y. Sakagami and Y. Sumiki, *ibid.*, **16**, 195 (1963).

3) N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara, *Tetrahedron Letters*, **1965**, 1405.

4) N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara, *ibid.*, **1965**, 1411.

FIG. 1. NMR Spectrum of Sodium Cytosinate in D_2O .

substituent attached at N-I position.

The NMR spectra (in D_2O) supported this assumption as shown in Fig. 1, two protons appeared as doublet of AB type ($J=7.5$ c.p.s.) at 7.72 and 6.14 p.p.m. which could be assigned to the typical signals of protons at C_6 and C_5 of cytosine nucleus⁵⁾ respectively. Furthermore, the presence, of two ethylenic protons appeared at 6.45~6.00 p.p.m. as multiplet provided informative data for the elucidation of unknown portion.

Acid hydrolysis of I gave each one mole of V, levulinic acid, ammonia and carbon dioxide as characterizable products,²⁾ whereas further attempt to cleave the linkage of I into V and its substituent was unsuccessful without extensive decomposition. It was very reasonable to presume the presence of sugar in I by the levulinic acid formation.

In an effort to gain information regarding the sugar portion, reduction and oxidative cleavage of ethylenic linkage were next undertaken. On reduction with platinum oxide catalyst in acetic acid, I afforded a mixture of dihydrocytosinine (VI), $C_{10}H_{14}O_4N_4 \cdot HCl$, m.p. 230~233°C (dec.), a hydrogenolysis product (VII), $C_6H_{11}O_3N$, m.p. 275~278°C and V. The facile hydrogenolysis of I to VII and V indicated that an ethylenic function in C_6 moiety located at allylic position to the glycosidic C-N bond.

VII characterized as monoamino monocarboxylic compound of pKa 2.4 and 9.2, gave corresponding N-acetyl methyl ester, $C_6H_9O_2N \cdot (OCH_3) \cdot (CH_3CO)$, m.p. 130°C. The IR spectrum of VII and deuterated VII exhibiting characteristic bands of tetrahydropyran⁶⁾ at 1100 and 1060 cm^{-1} (C-O-C stretching vibration) indicated the presence of tetrahydropyran ring rather than tetrahydrofuran.⁷⁾ The NMR spectrum of VII in D_2O (p.p.m.) was most instructive, showing C_2 proton doublet at 3.80 ($J=10.5$ c.p.s.), C_3 proton in broad at 4.20, C_4 , C_5 methylene protons as multiplet at 1.70~2.20 and C_6 methylene protons as broad at 3.40 p.p.m. The NMR spectral evidence permitted the elucidation of VII as 3-aminotetrahydropyran-2-carboxylic acid.

The result of oxidative cleavage of ethylenic group in C_6 moiety provided unequivocal

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6) S. C. Barket and R. M. Badger, *J. Chem. Soc.*, **1950**, 4397.

7) S. A. Barker and R. Stephens. *ibid.*, **1954**, 4550.

FIG. 1.

FIG. 2. Infrared Spectrum of 3-Aminotetrahydropyran-2-Carboxylic Acid (VII).

— (VII)
 ---- deuterio (VII)

evidence for structure VII, and consequently for the complete structure of I. IV was acetylated with acetic acid and triethylamine in methanol to afford N,N'-diacetylcytosinine methyl ester (VIII), $C_{10}H_9O_3N_4 \cdot (OCH_3) \cdot (CH_3CO)_2$, m.p. $263 \sim 266^\circ C$ (dec.) as crystalline needles. Ozonolysis of VIII in acetic acid followed by oxidation with hydrogen peroxide and acid hydrolysis furnished β -hydroxyaspartic acid (IX), $C_4H_7O_5N$, m.p. $286 \sim 288^\circ C$ (dec.), $[\alpha]_D^{20} -47^\circ C$ (c, 1 in H_2O). Diazotization of IX gave *meso*-tartaric acid indicating

the erythro-D-configuration⁸⁾ of the amino acid. IX was a substantially important structural fragment of the sugar proton in I and was also obtained from the parent antibiotic itself by the ozonolysis.

The mechanism of levulinic acid formation is necessary to be considered. Transformation of hexoses through the intermediate (X) into levulinic acid by the action of acids was well established.⁹⁾ It seems probable that proto-

8) H. J. Salbach, *J. Biol. Chem.*, **229**, 437 (1957)

9) H. P. Teunissen, *Rec. Trav. Chem.*, **50**, 1 (1930).

tropic rearrangement of double bond coupled with the deamination and decarboxylation gave X, which was further degraded to levulinic acid.

Formation of formic acid in the hydrolysate of III by alkali was reported as structural fragment in the previous paper,²⁾ but according as the progress of our investigation, formic acid was demonstrated as an extensive decomposition product of I. Indeed, prolonged refluxing of I with 3N sodium hydroxide, gave one mole of formic acid almost quantitatively. One of the possible route of this reaction is presumed as follows; alkaline hydrolysis of I [give rise to a compound XI which affords an intermediate enal system XII, and the nucleo-

(S)-configuration at C'_4 and C'_5 respectively and, moreover, the NMR spectrum of VI which exhibited a distinct doublet of C'_5 proton at 3.65 p.p.m. ($J=10.5$ c.p.s.) demonstrated the C'_4 - C'_5 protons to be diaxial. Conclusively, the D-configuration of aminohexuronic acid moiety was established.¹⁰⁾ The configuration of glycosidic linkage was determined as β .¹¹⁾

Uracinine¹⁾ was obtained by the alkaline hydrolysis of I.²⁾ Acid hydrolysis of II gave uracil instead of cytosine, levulinic acid, ammonia and carbon dioxide.²⁾ Hydrogenation of II gave VII indicated that II differed from I at aglycone portion and the C_6 moiety remained intact, consequently structure II was assigned for uracinine.

phylic attack of OH^- to XII may release formic acid.

Finally, stereochemical feature of I was considered, e.g. the configuration of erythro-D- β -hydroxy aspartic acid obtained as structural fragment of I provided evidence for the

EXPERIMENTAL

Melting points were taken with a Kofler block and were not corrected. Infrared spectra were measured in

10) In the previous paper,⁸⁾ we have misassigned the configuration of erythro-D- β -hydroxyaspartic acid and led to the erroneous conclusion of (R)-configuration at C'_4 and C'_5 .

11) The details will be published.

nujol mull with Koken DS 301 spectrophotometer and ultraviolet spectra with Cary 11 spectrophotometer. NMR spectra of 60 megacycle were measured with Varian A-60 or JNM-C-60 spectrometers.

Dissociation constant

Potentiometric titration was carried out by the procedure described by Parke and Davis.¹²⁾ About 10 mg of the sample was dissolved in 2 ml of 0.1 N hydrochloric acid and titrated with 1 N sodium hydroxide using microsyringe with the calibration of 0.002 ml. During the titration the mixture was constantly stirred by microstirrer and the surface of the solution was covered with gentle current of nitrogen. pH was measured using pH meter model HM-5 (Toa Dempa Kogyo Co. Ltd.)

Cytosinine (I)

The sample of I was prepared by the procedures in the previous paper.²⁾ After recrystallization from hot water I gave fine needles of m.p. 244~245°C (dec.), $[\alpha]_D^{18} - 20^\circ\text{C}$ (c, 1 in H_2O), λ_{max} 274 m μ (ϵ 13,800) in 0.1 N hydrochloric acid, λ_{max} 267 m μ (ϵ 7,500) in 0.1 N sodium hydroxide. *Anal.* Found: C, 47.51; H, 4.92; N, 21.63. Calcd. for $\text{C}_{10}\text{H}_{12}\text{O}_4\text{N}_4$: C, 47.62; H, 4.80; N, 22.22.

Cytosinine methyl ester dihydrochloride (IV)

I (3 g) was suspended in dry methanol (15 ml) containing 3% hydrogen chloride and the mixture was refluxed gently for one hour. After cooling a crystalline precipitate deposited (2.8 g). Recrystallization from hot methanol gave fine needles of m.p. 220~223°C (dec.). Potentiometric titration showed two titrable functions as base of pKa 4.6 and 8.0. The NMR spectrum exhibited characteristic singlet of $-\text{COOCH}_3$ at 3.57 p.p.m. *Anal.* Found: C, 39.08; H, 4.50; N, 16.38; Cl, 20.83. Calcd. for $\text{C}_{11}\text{H}_{16}\text{O}_4\text{N}_4\text{Cl}_2$: C, 38.95; H, 4.75; N, 16.52; Cl, 20.91.

Hydrogenation of I

I (2.2 g) was hydrogenated over platinum oxide catalyst (500 mg) in acetic acid until hydrogen uptake had ceased. About 1.5 mole of hydrogen was absorbed. After filtration of the catalyst, the filtrate was evaporated in vacuo with addition of water. The residue was separated by partition chromatography on cellulose powder using solvent system of *n*-butanol-acetic acid-water (2:1:1) to afford three compounds in pure form.

The first compound has been eluted at earlier fractions, m.p. over 300°C, which was identified with

¹²⁾ T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).

cytosine by the comparison of infrared spectrum.

Next was a hydrogenolysis product (VII), being distributed in the fractions of no UV absorption but ninhydrin positive, obtained as crystalline precipitate (800 mg). Recrystallization from aqueous ethanol gave needles, m.p. 275~278°C, pKa 2.4 and 9.2, ν_{max} 3200 (shoulder), 2750, 2640, 1610 (COO^-), 1105 and 1048 (C-O-C) cm^{-1} . *Anal.* Found: C, 49.50; H, 7.25; N, 9.56. Calcd. for $\text{C}_6\text{H}_{11}\text{O}_3\text{N}$: C, 49.64; H, 7.64; N, 9.65.

The third compound was distributed in the last fractions showing UV absorption and ninhydrin reaction. After removal of the solvent, resulting syrup showed no tendency to crystallize. Trituration of the syrup with ethanol (20 ml) containing concentrated hydrochloric acid (2 ml) afforded a crystalline needles (980 mg). Recrystallization from 80% ethanol gave fine needles of m.p. 230~233°C (dec.) of VI λ_{max} 274 m μ (ϵ 12,000) in 0.1 N HCl, λ_{max} 267 m μ (ϵ 7,000) in 0.1 N NaOH. *Anal.* Found: C, 41.14; H, 5.51; N, 19.15; Cl, 12.37. Calcd. for $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_4 \cdot \text{HCl}$: C, 41.31; H, 5.20; N, 19.27; Cl, 12.15.

N-Acetyl methyl ester of VII

VII (500 mg) was acetylated with acetic anhydride and triethylamine in methanol and the mixture was stood over night at room temperature. After removal of the solvent in vacuo, a residual syrup was dissolved in 15 ml of methanol and slight excess of diazomethane saturated in ether solution (10 ml) was added. After three hours, the solvent was removed in vacuo, and the residue crystallized as needles. Recrystallization from ethyl acetate-ligroin gave fine needles of m.p. 130~130.5°C. ν_{max} 3390, 1740, 1670, 1230, 1130 and 1047 cm^{-1} . *Anal.* Found: C, 53.65; H, 7.30; N, 6.82. Calcd. for $\text{C}_9\text{H}_{15}\text{O}_4\text{N}$: C, 53.72; H, 7.51; N, 6.96.

N,N'-diacetyl cytosinine methyl ester (VIII)

To a mixture of IV (2.5 g) in dry methanol (30 ml) cooled in ice water, triethylamine (3 ml) and acetic anhydride (5 ml) was added dropwise and the solution stood overnight. The mixture was carefully evaporated in vacuo with addition of water twice, a crystalline solid deposited. Recrystallization from hot water gave VIII as needles, m.p. 263~266°C (dec.). ν_{max} 1740, 1650, 1600, 1100 and 1050 cm^{-1} . *Anal.* Found: C, 51.38; H, 5.31; N, 15.89. Calcd. for $\text{C}_{15}\text{H}_{18}\text{O}_5\text{N}_4$: C, 51.42; H, 5.18; N, 15.99.

Erythro-D- β -hydroxyaspartic acid (IX)

A mixture of VIII (2.5 g) in acetic acid (100 ml) was ozonolysed for three hours at room temperature.

Acetic acid was carefully evaporated in vacuo and resulted ozonide was treated with hydrogen peroxide (30%, 5 ml) at room temperature overnight. Subsequently 3 N hydrochloric acid (15 ml) was added to the reaction mixture and the solution was refluxed three hours under the current of nitrogen. The hydrolysate was continuously extracted with ether, the ether extracts gave a yellow oil. Digestion of this with chloroform afforded oxalic acid (520 mg). The aqueous layer was dried up in vacuo and the residual solids were chromatographed on cellulose powder using *n*-butanol-acetic acid-water (5:1:4). The marked ninhydrin positive fractions were collected and evaporated in vacuo to afford almost pure crystals (350 mg). Recrystallization from 50% aqueous ethanol gave crystalline rods of m.p. 287~288°C (dec.), $[\alpha]_D^{20} - 47^\circ$ (c, 1 in H₂O). The infrared spectrum of this compound coincided completely with that of authentic β -hydroxyaspartic acid.

From the cellulose column, it has been eluted some unreacted cytosine, ammonium chloride and an intractable oil in the preceding fractions of IX.

meso-Tartaric acid from IX

To the aqueous solution of IX (60 mg in 5 ml H₂O), acetic acid (3 ml) and sodium nitrate (30 mg) were added, the mixture was kept 5°C with stirring for two hours. The solution was evaporated in vacuo at 40°C with addition of water to remove excess of acetic acid and finally the residue was made up to 10 ml. Calcium chloride (40 mg) was added to this solution and insoluble crystals of calcium *meso*-tartarate (40 mg) deposited immediately. The identity was confirmed by comparison of infrared spectrum.

Uracinine (II)

II was obtained by the procedures described pre-

viously²⁾ and was recrystallized from hot water. m.p. 286~289°C (dec.) $[\alpha]_D^{13} - 28.87^\circ$ (c, 2 in 0.1 N HCl). pKa 2.4, 8.0 and 9.8. *Anal.* Found: C, 47.25; H, 4.31; N, 16.61. Calcd. for C₁₀H₁₁O₅N₃: C, 47.43; H, 4.38; N, 16.60.

Hydrogenation of uracinine (II)

II (1.0 g) in acetic acid (20 ml) was hydrogenated over PtO₂ until the hydrogen up take had ceased, the filtrate was evaporated in vacuo and residual syrup was chromatographed on cellulose powder using *n*-butanol-acetic acid-water (2:1:1). The fractions showed ninhydrin positive and lack of UV absorption were collected and evaporated in vacuo to afford crystalline needles (250 mg), which was identified with VII by infrared spectrum. The fractions has been eluted afterward of VII showed ultraviolet absorption peak at 267 m μ and also ninhydrin positive, presumably corresponded to dihydro-uracinine, were not further characterized.

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