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56. Morio Ikehara: Studies on Coenzyme Analogs. I. Synthesis of 3-β-D-Ribofuranosyl-2-oxo-2,3-dihydropyrimidine (6-Deoxyuridine) and its 5'-Phosphate.

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In this decade, biochemical studies in the field of nucleotides have developed extensively and have supplied many interesting facts. Among these, mechanism of the action of a coenzyme was substantially clarified with the aid of biological and physical methods. Through the brilliant works of Todd and his colleagues, several important coenzymes, such as ATP,[*2,1] UDPG,[2] FAD,[3] and DPN[4] were chemically synthesized and tested as to their biological activities. Recently, the total synthesis of coenzyme-A was also achieved by Khorana.[5]

It is considered that it must be interesting to investigate the site of the action of naturally occurring coenzymes by accumulating the knowledge about chemically synthesized coenzyme analogs.

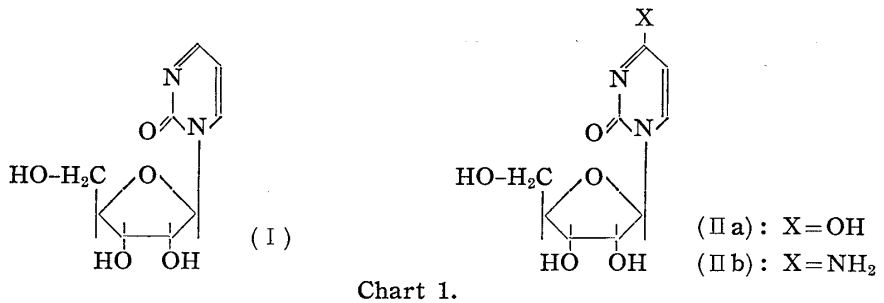

Chart 1.

In this paper, 3-β-D-ribofuranosyl-2-oxo-2,3-dihydropyrimidine[*3](6-deoxyuridine) (I) and its phosphates are described. Uridine (IIa) and cytidine (IIb) are two of the representative pyrimidine nucleosides which appear in the alkaline hydrolysate of ribonucleic acid. Both also occur naturally in the form of UDPG and CDP-choline as the coenzymes of carbohydrate and phosphatide metabolism. To examine the rôle of a substituent in the 6-position of the pyrimidine ring and to obtain a starting material to be led to a suitable substrate for biological studies, the synthesis of 6-deoxyuridine and its phosphates was first attempted.

The pyrimidine moiety of this nucleoside was synthesized according to Brown's method.[6] 2-Aminopyrimidine (III) was converted to 2-hydroxypyrimidine (IVa) by refluxing in 40% sodium hydroxide solution. (IVa) was then condensed with mercuric chloride to

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*2 Abbreviations used: ATP, adenosine triphosphate; UDPG, uridine diphosphate glucose; FAD, flavin adenine dinucleotide; DPN, diphosphopyridine nucleotide; UMP, uridine 5'-monophosphate; CMP, cytidine 5'-monophosphate.

*3 Numbering of the pyrimidine ring is according to C. A.

1) J. Baddiley, A. M. Michelson, A. R. Todd: J. Chem. Soc., **1949**, 582; A. M. Michelson, A. R. Todd: *Ibid.*, **1949**, 2487; V. M. Klark, G. W. Kirby, A. R. Todd: *Ibid.*, **1957**, 1497.
2) A. M. Michelson, A. R. Todd: *Ibid.*, **1956**, 3459; D. H. Hayes, A. M. Michelson, A. R. Todd: *Ibid.*, **1955**, 808.
3) M. H. Christie, G. W. Kenner, A. R. Todd: *Ibid.*, **1954**, 46.
4) I. J. Haynes, A. R. Todd: *Ibid.*, **1950**, 304; I. J. Haynes, N. A. Hughes, G. W. Kenner, A. R. Todd: *Ibid.*, **1957**, 3727.
5) J. G. Moffatt, H. G. Khorana: J. Am. Chem. Soc., **81**, 1265(1959).
6) D. J. Brown: Nature, **165**, 1010(1950).

afford the chloromercuric compound (IVb) as a white powder. The composition of (IVb) was calculated from its elementary analytical data $C_4H_3ON_2 \cdot HgCl$, which shows a rather unusual properties of this pyrimidine compared to other pyrimidine–mercury compounds.[7a,b]

On the other hand, yeast ribonucleic acid, prepared by a slightly modified procedure of Mizuno's method,[8] was subjected to pyridine–water hydrolysis to obtain nucleosides. Guanosine thus obtained was protected by benzoylation of its ribose–OH residue, and acetolyzed by acetic acid–acetic anhydride–sulfuric acid mixture.[9] Resulting 1–O–acetyl–2,3,5–tri–O–benzoyl–D–ribofuranoside (V) was converted to 1–chloro compound (VI) by reaction with dry hydrochloric acid in anhydrous ether. Well–dried (IVb) and (VI) were condensed in boiling xylene under vigorous stirring for one hour. The benzoylated nucleoside (VII) thus obtained formed pale yellow needles, m.p. 132~133°(from ethanol), $[\alpha]_D^{13}$ −1.12°(c=1.49, $CHCl_3$). These properties and the 1,2-*trans* rule of Baker[10] confirm the configuration of the nucleoside linkage of this compound to be β.

Compound (VII) was then debenzoylated by treating with anhydrous methanol saturated with ammonia. The nucleoside (I) thus obtained formed colorless micro–needles, m.p.

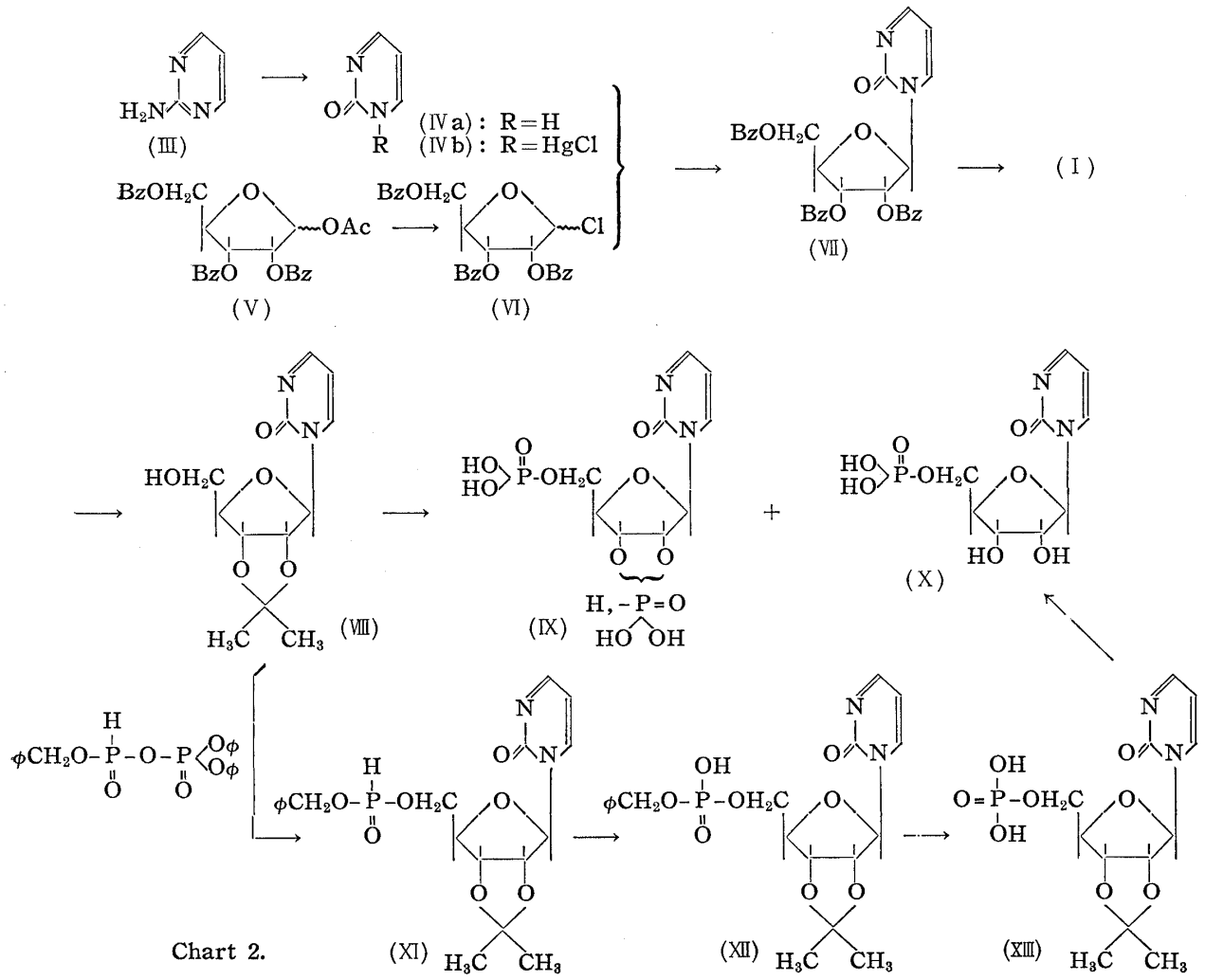

Chart 2.

7) *a*) J. J. Fox : J. Am. Chem. Soc., **78**, 2117(1956). *b*) J. J. Fox, N. Yung, I. Wempen, I. L. Doerr: J. Am. Chem. Soc., **79**, 5060(1957).
8) Y. Mizuno, K. Nakamura, T. Ueda : Yakugaku Zasshi, **77**, 683(1957).
9) Y. Mizuno, M. Ikehara, T. Ueda, A. Nomura, F. Ishikawa : This Bulletin, to be published.
10) B. R. Baker : J. Org. Chem., **19**, 1786(1954); CIBA Symposium, **1957**, 120.

183° (ethanol–water), and showed absorption maximum at 298 mμ in ethanol, closely resembling the ultraviolet absorption spectra of 1-ethyl-2-oxo-1,2-dihydropyrimidine. A strong absorption band at 1650 cm^{-1} in its infrared spectrum agreed with the above fact and showed that (I) was actually N-glucoside and not O-glucoside. The hydrolytic behavior in various concentrations of hydrochloric acid showed the nucleoside linkage of (I) to be rather weak in comparison with natural pyrimidine nucleoside and similar to that of purine nucleoside. This may be explained by the larger proton-accepting tendency of N(1)-atom in the case of acid hydrolysis.

In order to obtain 5′-phosphate of 6-deoxyuridine (I), 2′,3′-isopropylidene derivative (X) was synthesized by toluenesulfonic acid method.[11] Although this compound was obtained as a vitreous substance, elementary analytical data and negative periodate test on paper chromatogram confirmed (Ⅷ) as isopropylidene-6-deoxyuridine. According to Michelson's procedure[12] for synthesis of UMP and CMP, (Ⅷ) was dissolved in phosphoric acid–phosphorus pentoxide mixture and heated at 60° for 2 hours. Barium hydroxide precipitation of phosphates resulted in two compounds (Ⅸ and X). (Ⅸ) was diphosphate, calculated from its analytical data, and showed negative periodate test. Furthermore it had no labile phosphorus when tested in hydrochloric acid hydrolysis. Accordingly, (Ⅸ) was proved to be 2′(or 3′),5′-diphosphate of (I). Rf values in various solvent systems (see Experimental) also agreed well with above facts. This diphosphate may be the result of partial hydrolysis of protective isopropylidene group during phosphorylation reaction.

The second phosphate (X) had the composition corresponding to a monophosphate and showed a higher Rf value in various solvent systems relative to (Ⅸ) (see Experimental). From positive periodate test, it was considered to be in free state. The yield of each phosphate (Ⅸ and X) was 17.0% and 8.2%, respectively, calculated from their ultraviolet absorption. In order to establish the structure of 5′-monophosphate (X), rather mild conditions of phosphorylation was then adopted. Todd's O-benzylphosphorus O,O-diphenylphosphoric anhydride[13] was employed as the phosphorylating agent. Treatment of isopropylidene-6-deoxyuridine (Ⅷ) with the fresh preparation of above reagent afforded 5′-benzylphosphite (Ⅺ), which was converted to 5′-phosphate (X) via 5′-benzylphosphate (Ⅻ) through several steps. Hydrolysis of isopropylidene group of (Ⅻ) by hydrochloric acid in methanol yielded 6-deoxyuridine 5′-monophosphate (X) in a poor yield. Comparison of two specimens of (X) by paper chromatography in various solvent systems showed good agreement. (X) was further analyzed on a column of Amberlite

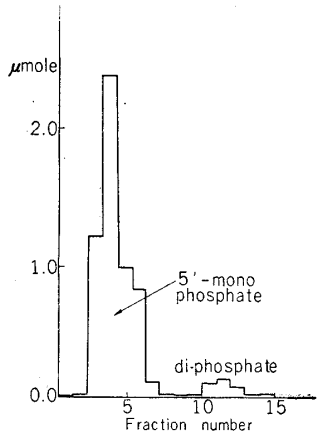

Fig. 1.

11) M. P. Gordon, D. J. Magrath, G. B. Brown : J. Am. Chem. Soc., **79**, 3256(1958).
12) A. M. Michelson : J. Chem. Soc., **1958**, 1959.
13) A. R. Todd, W. S. Corby, G. W. Kenner : *Ibid.*, **1952**, 3669.

IRA-400 (Cl′) by gradient elution technique[14] (Fig. 1). From the number of fractions containing ultraviolet absorbing eluents, (X) was confirmed to be a monophosphate.

Experimental*4

2-Hydroxypyrimidin-1-ylmercury Chloride (IVb)—To a solution of 2.0 g. (0.02 mole) of 2-hydroxypyrimidine dissolved in 200 cc. of water containing 0.8 g. of NaOH, 50 cc. of EtOH containing 5.4 g. of $HgCl_2$ was added at room temperature under vigorous stirring. Resulting white heavy precipitate was collected on a filter, washed with small portions of EtOH-H_2O (1:4), and dried at 60° for 5 hr. over P_2O_5 at 2 mm. Hg. Yield, 6.3 g. (90.1%). *Anal.* Calcd. for $C_4H_3ON_2 \cdot HgCl$: C, 14.49; H, 0.96; N, 8.46. Found : C, 14.39; H, 1.26; N, 8.81.

1-Chloro-2′, 3′, 5′-tri-O-benzoylribofuranose (VI)—A solution of 7.2 g. (0.014 mole) 1-O-acetyl-2,3,5-tri-O-benzoylribofuranose (V) (m.p. 126°, dried over P_2O_5 for 8 hr. at 60°, 2 mm. Hg) dissolved in 300 cc. of dry Et_2O, previously saturated with dry HCl, was set at 0° to 5° in an ice box for 8 days under exclusion of moisture. Solvent was removed under reduced pressure below 30°, dry benzene was added, and evaporated several times to remove HCl entirely.

3-(2′, 3′, 5′-Tri-O-benzoylribofuranosyl)-2-oxo-2, 3-dihydropyrimidine (VII)—4.7 g. (0.014 mole) of (IVb) was suspended in 300 cc. of dry xylene and the moisture was removed azeotropically. Into this, 20 cc. of benzene solution of (VI) (from 7.2 g. of (V)) was added dropwise under vigorous stirring. After 5 min. of refluxing, the solution became clear and then turbidity increased again. To avoid further yellow coloring (probably caused by polymerization of pyrimidine), the reaction was stopped after 1 hr. The solvent was removed under a reduced pressure and the residual yellow syrup was taken up in $CHCl_3$. The extract was filtered, washed with saturated NaI solution and water, and dried over Na_2SO_4. Evaporation of the solvent left yellow vitreous substance (9.8 g.), which was recrystallized from EtOH to small needles, m.p. 132~133° (5.2 g, 67.7%).

A small portion of this material was purified by Al_2O_3 chromatography with $CHCl_3$ and gave a sample of 134~136°. Rf 0.87 (BuOH-H_2O=86:14). UV λ_{max}^{EtOH} (pH 7.0) mμ (ε) : 229 (34380), 273 (3300), 280 (2780), 298 (1380). IR : 1670 cm^{-1} (no 3400 cm^{-1}), $[\alpha]_D^{13}$ −1.12° (c=1.49, $CHCl_3$). *Anal.* Calcd. for $C_{30}H_{24}O_8N_2$: C, 66.60; H, 4.45; N, 5.18. Found : C, 65.62, H, 4.60; N, 4.87.

3-β-D-Ribofuranosyl-2-oxo-2,3-dihydropyrimidine (6-Deoxyuridine) (I)—A solution of 10 g. of (VII) dissolved in 350 cc. of anhyd. MeOH, previously saturated with NH_3 at 0°, was set aside for 50 hr. at 5~15°. The extent of the reaction was tested by paper chromatography (BuOH-H_2O=86:14). After 14 hr. of reaction, three spots appeared at Rf 0.15, 0.68, and 0.84. After 50 hr. the first spot increased and the third almost disappeared. The whole solution was evaporated *in vacuo* and the vitreous residue was separated from oily benzamide by decantation. The residue was taken up in 50 cc. of water and extracted several times with $CHCl_3$. Aqueous layer was evaporated to dryness *in vacuo* below 30° and the residue was triturated with EtOH and Me_2CO. The amorphous substance thus obtained was removed by filtration, and evaporation of the solvent gave 3.5 g. (46.5%) of a vitreous mass. Recrystallization from EtOH-H_2O gave colorless needles, m.p. 183°. Rf 0.15 (BuOH-H_2O=86:14). UV λ_{max}^{EtOH} mμ (ε) : 298 (2365); λ_{min}^{EtOH} 264 (1745). IR cm^{-1} : 3400 (OH), 1650 (C=O). *Anal.* Calcd. for $C_9H_{12}O_5N_2 \cdot H_2O$: C, 43.92; H, 5.69; N, 11.87. Found : C, 43.93; H, 5.92; N, 11.87.

Hydrolysis of 6-Deoxyuridine—i) A solution of 10 mg. of (I) dissolved in 2*N* HCl was heated at 100° for 30 min. The reaction mixture was tested by paper chromatography (BuOH-H_2O=86:14), and showed a spot corresponding to ribose (Rf 0.29) and 2-hydroxypyrimidine (Rf 0.48).

ii) A solution of 10 mg. of (I) dissolved in 2*N* NaOH was heated at 100° for 2 hr. When tested on paper chromatography, there was no evidence of decomposition.

2′,3′-Isopropylidene-6-deoxyuridine (VIII)—2.0 g. of (I) and 12.5 g. of *p*-toluenesulfonic acid (hydrate) were added to 250 cc. of Me_2CO, and the turbid solution was stirred vigorously at room temperature until all the precipitate disappeared (about 1 hr.). The whole solution was added to saturated $NaHCO_3$ solution and extracted with 250 cc. of $CHCl_3$, which was dried over Na_2SO_4. Evaporation of the solvent in vacuum afforded pale yellow vitreous residue (2.0 g.). In spite of efforts to recrystallize it, all attempts failed. Rf 0.71 (BuOH-H_2O=86:14). UV λ_{max}^{EtOH} 300 mμ : λ_{min}^{EtOH} 287. *Anal.* Calcd. for $C_{12}H_{16}O_5N_2$: C, 53.75; H, 5.97; N, 10.45. Found : C, 53.30; H, 6.08; N, 10.21.

Phosphorylation of 6-Deoxyuridine with P_2O_5-H_3PO_4—700 mg. of (VIII) (dried at 50~60° for 3 hr. at 2 mm. Hg) was well mixed with H_3PO_4-P_2O_5 mixture (made from 3.5 g. of 85% H_3PO_4 and 2.5 g. of P_2O_5), the vessel was tightly stoppered and heated in an oil bath at 60° for 2 hr. Color of the mixture gradually darkened. 25 cc. of water was added to this solution and heated at 100° for a further

*4 All m.p.s are uncorrected. Ultraviolet absorption spectra were taken by Beckman DK-II and infrared spectra by Koken DS-301 spectrophotometer.

14) M. Smith, H. G. Khorana : J. Am. Chem. Soc., **80**, 1141 (1958).

0.5 hr. After cool, saturated $Ba(OH)_2$ solution was added until pH became 6.4. Precipitated $BaSO_4$ was filtered off, the supernatant was concentrated to a small volume (ca. 20 cc.), and adjusted to pH 7.3 by the addition of $Ba(OH)_2$. When 2 volumes of EtOH was added and set aside overnight in a refrigerator, white precipitate (IX) appeared, which was collected by centrifugation and dried. Further addition of EtOH to this supernatant gave a second precipitation (X). *Anal.* Calcd. for $C_9H_{10}O_{11}N_2Ba_2P_2 \cdot 7H_2O$ (IX): C, 13.78; H, 3.03; N, 3.57; P, 7.90. Found: C, 13.82; H, 2.98; N, 3.08; P, 9.06. Labile P (1*N* HCl, 100°, 7 min.), no inorganic P was detected. Yield, 350 mg. (17.0%), Purity, 92.6% (calculated from UV absorption). *Anal.* Calcd. for $C_9H_{10}O_8N_2BaP \cdot 2H_2O$ (X): C, 23.08; H, 2.99; N, 5.9; P, 6.63. Found: C, 23.38; H, 3.24; N, 5.28; P, 6.75. Labile P (1*N* HCl, 100°, 7 min.); no inorganic P was detected. Yield; 100 mg. (8.2%), Purity: 95.0%. Paper chromatographical data are listed in Table I. Another experiment from 1.8 g. of (VIII) gave 550 mg. of (X) (17.5%).

Table I. Rf Values

Subst.	(IX)	(X)	(X′)	UMP	inorg. P
Solvent A	0.09	0.24	0.25	0.32	0.22
B	0.11	0.86		0.80	
C	0.45				0.48
D		0.10	0.13	0.10	0.13

Solvent A iso-PrOH-1% $(NH_4)_2SO_4$=2:1 (v/v) ↓
B iso-AmOH-5% KH_2PO_4=0.5 cc.:1 cc. ↑
C EtOH-1*N* AcOH=75:30 (pH 7.5 w. NH_3) ↑
D BuOH-AcOH-H_2O=40:10:50 ↑
Detection: Base by UV absorption
Sugar by IO_4^-+benzidine
P by Hanes-Isherwood method

Phosphorylation of 6-Deoxyuridine with O-Benzylphosphorus O,O-Diphenylphosphoric Anhydride—i) Isopropylidene 6-deoxyuridine 5′-benzylphosphite (XI): To a solution of 700 mg. of (IX) dissolved in 1.3 cc. of acetonitrile, 0.6 cc. of 2,4,6-collidine was added, followed by addition of O-benzylphosphorus O,O-diphenylphosphoric anhydride (prepared from 0.97 g. of sodium benzylphosphite, 1.04 cc. of diphenyl phosphorochloridate, and 0.7 cc. of triethylamine) in 15 cc. of benzene, and the mixture was set aside for 30 min. The solution was evaporated, the residual, viscous syrup was dissolved in $CHCl_3$ (50 cc.), and $CHCl_3$ solution was washed with water, saturated $NaHCO_3$ solution, saturated $KHSO_4$ solution, and finally with water. Dried (Na_2SO_4) solution was evaporated *in vacuo* to afford 2′,3′-isopropylidene-6-deoxyuridine 5′-benzylphosphite (XI) as a pale yellow syrup (0.8 g.). Rf 0.84 (BuOH-H_2O=86:14).

ii) Isopropylidene-6-deoxyuridine 5′-benzylphosphate (XII): 0.8 g. of crude (XI) was dissolved in 20 cc. of acetonitrile, 0.3 g. of N-chlorosuccinimide was added, and set aside for 2 hr. at room temperature. Then 20 cc. of $NaHCO_3$ (saturated) solution was added and the mixture was stirred mechanically for 6 hr. at room temperature. After standing overnight acetonitrile was removed by vacuum distillation, acidified with HCl (Congo Red), and extracted thoroughly with $CHCl_3$. Evaporation of $CHCl_3$ gave a hygroscopic solid (250 mg.). Rf 0.78 (BuOH-H_2O=86:14), 0.87 (BuOH-AcOH-H_2O=4:1:5).

iii) Isopropylidene 5′-phosphate (XIII): 250 mg. of (XII) was dissolved in 50 cc. of H_2O, acidified with HCl (giving a final concentration of *N*/200), and hydrogenated overnight at atmospheric pressure over a mixture of PdO_2 (0.1 g.) and Pd-C (0.1 g., 10%). The catalyst was removed by filtration, washed with water, filtrate and washings were combined, and evaporated under a reduced pressure. 175 mg. of (XIII) was obtained. Rf 0.19 (BuOH-H_2O=86:14), 0.54 (iso-PrOH-1% $(NH_4)_2SO_4$=2:1).

iv) 6-Deoxyuridine 5′-phosphate (X): 100 mg. of (XIII) was allowed to stand for 15 hr. in a 5% MeOH solution of HCl, 2 cc. of water was added, and the solvent was concentrated to one-half the volume by vacuum distillation. The solution was adjusted to pH 7.3 with $Ba(OH)_2$ and on addition of 2 volumes of EtOH, white precipitate appeared, which was collected and dried (60 mg.). Rf values are listed in Table I as (X′). This substance is quite identical with the sample obtained as above.

Ion Exchange Chromatography of (X)—A solution of 3 mg. of (X) dissolved in 25 cc. of water was applied on top of the column of Amberlite IRA-400 (Cl^--form, 100~200 mesh, 0.7×7 cm.). Washing with water was continued until optical density of the fraction (20 cc.) at 260 mμ diminished to 0.025, followed by elution with 0.003*N* HCl and 0.003*N* HCl containing 0.1 mole of LiCl by gradient elution technique. Results are shown in Fig. 1. It was found that (X) was slightly contaminated with the diphosphate.

The author wishes to express his deep gratitude to Daiichi Seiyaku Co. for the gift of 2-aminopyrimidine. This study was partly supported by the Grant-in-Aid for Scientific Experimental Research from the Ministry of Education, which is gratefully acknowledged.

Summary

A new nucleoside, 3-(β-D-ribofuranosyl)-2-oxo-2,3-dihydropyrimidine (6-deoxyuridine), was synthesized from 2-hydroxypyrimidin-1-ylmercury chloride and 2,3,5-tri-O-benzoylribofuranosyl chloride, followed by removal of protecting groups. 6-Deoxyuridine 5′-monophosphate and 2′(or 3′), 5′-diphosphate were obtained by phosphorylation of 6-deoxyuridine.

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57. Hiroshi Mitsuhashi and Yuzuru Shimizu: Studies on the Constituents of Asclepiadaceae Plants. I.[1] On the Components of *Cynanchum caudatum* MAX.

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Cynanchum caudatum MAX. (Japanese name, Ikema. Asclepiadacea family) is a plant widely distributed in Japan especially in the Hokkaido and its root has been used as a crude drug among the people of the Ainu race, and the natives of Hokkaido and Sakhalin have employed it for all kinds of diseases as a home remedy[2] (Ainu name: Ikema or Penup). This plant, however, is very toxic and sometimes causes very serious poisoning with vomiting, diarrhea, convulsion, arhythmea, etc.[3]

Concerning the components of the root of this plant, Kunitomo[4] reported the isolation of sucrose and an alkaloidal substance, about which not much detail was given. While Iwakawa gave a name cynanchotoxin[*2] to the ether-soluble and petroleum ether-insoluble fraction of the ethanol extract, and reported that it had picrotoxin-like action and LSD 0.002 g./20 g. frog,[5] it now seems that the substance was a crude mixture. Further investigation was attempted in order to determine the components of the root of this plant.

Percolation of the powdered root with chloroform afforded a powdery extract, which showed strong Keller-Kiliani reaction (blue), suggesting the presence of a glycoside containing 2-deoxy-suger component. Active methylene reaction was negative in the extract. Therefore, it is reasonable to assume that the glycoside is not a cardiac glycoside. The extract was precipitated several times with petroleum ether and the crude glycoside

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*1 Kita-12-jo, Nishi-5-chome, Sapporo, Hokkaido (三橋 博, 清水 譲).

*2 N. Nagai reported the isolation of phytolaccotoxin from the root of *Phytolacca esculenta* VAN HOUTTE (Yakugaku Zasshi, **10**, 214(1890)), but according to Iwakawa, phytolaccotoxin should be cynanchotoxin itself, and this may have been caused by confusion of the starting material, owing to the resemblance of the appearance of these roots.

1) Part of this work was reported at the 3rd Hokkaido Local Meeting of the Pharmaceutical Society of Japan, July 27, 1959.
2) M. Chiri: "The Dictionary of Ainu Language," Vol. I (1953). Oka Shoin.
3) Y. Narumi: Tohoku J. Med., **19**, 439(1936).
4) Y. Kunitomo: Yakugaku Zasshi, **18**, 653(1898).
5) K. Iwakawa: Tokyo J. Med., **26**, 359(1912); Arch. exptl. Path. Pharmakol., **67**, 118(1912).