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The Mercuric Bromide Rearrangement and 1-β-D-Ribofuranosyl-4,6-pyrimidinedione, an Isomer of Uridine*

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ABSTRACT: The conformation of the pyrimidine ring in a uridine isomer, 1-β-D-ribofuranosyl-4,6-pyrimidinedione (isouridine, IV), is opposite to the pyrimidine conformation of 1-β-D-ribofuranosyluracil. Condensation of 2 molar equiv of 2,3,5-tri-*O*-benzoyl-D-ribosyl chloride with 4,6-pyrimidinedionemercury (I) led to the formation of 4,6-di-*O*-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranoside)-4,-6-dioxypyrimidine (II). Compound II was converted into 1,4-di-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranosyl)-4-oxy-6-pyrimidinone (III) by the mercuric bromide rearrangement; the mild alkaline degradation of III gave isouridine (IV). The position of the glycosyl bond in isouridine and the β-D-configurational assignment were confirmed by the conversion of isouridine into 2′,3′-*O*-isopropylidene-5′,6-anhydro-(cyclo)isouridine.

The anhydro derivative shows a specific 1-alkyl-4-pyrimidinone intense absorption band near 240 mμ which supports the structure assigned to isouridine. The new ribonucleoside behaves as a substrate in the formation of ribose 1-phosphate and 4,6-pyrimidinedione by uridine phosphorylase.

All of the 2-, 4-, and 6-oxygen-substituted derivatives of ribofuranosylpyrimidine are now known compounds. Ukita and his collaborators (1964a,b) have reported the preparation of 1-β-D-ribofuranosylbarbituric acid (Ukita *et al.*, 1964b), 1-β-D-ribofuranosyl-2-pyrimidinone, and 1-β-D-ribofuranosyl-6-pyrimidinone (Funakoshi *et al.*, 1961). The synthesis of 1-β-D-ribofuranosyl-4-pyrimidinone was performed in this laboratory (Lee and Wigler, 1968), and tribenzoylribofuranosyl chloride with 4-ethoxy-2-pyrimidinone gave 3-β-D-ribofuranosyluracil (Scannell and Allen, 1960). We wish to report the synthesis of the final member of this class of substances 1-β-D-ribofuranosyl-4,6-pyrimidinedione (isouridine).

Several derivatives of isouridine have been prepared from an appropriate halogenoribose and 4,6-pyrimidinedione blocked with 4-alkoxy or 6-alkoxy groups (Prystas and Sorm, 1968). The formation of the *N*-glycosyl bond was readily accomplished, but attempts to remove the alkyl substituents from the pyrimidine moiety led to destruction of the nucleoside (Prystas, 1967). In a recent report, however, Prystas (1968) described the preparation of the 5-methyl derivative of isouridine.

The synthesis of an *N*-glycosyl derivative from the unmodified 4,6-pyrimidinedione depends upon an oxygen to nitrogen transglycosylation, the mercuric bromide rearrangement of an oxygen–glycosidic bond to a nitrogen–glycosyl bond. Wagner and Pischel (1961) found that when 2-*O*-(2′,3′,4′,6′-tetra-*O*-acetyl glucopyranoside)-2-oxypyridine was treated with $HgBr_2$ at 160° for 3 hr the *N*-glucosyl-2-pyridone was produced. The rearrangement has been confirmed by Ukita *et al.* (1964a) and by Prystas and Sorm (1968).

The isomeric pair uridine and isouridine are opposite with respect to the structural conformation of the pyrimidine ring. This isomeric pair of potential substrates may be used to elucidate the conformational requirements of enzymatic reactions. For example, Ward and Reich (1968) suggested that the conformational selectivity of pancreatic ribonuclease A is an important factor in the specificity of the enzyme.

Results

Condensation of 4,6-pyrimidinedionemercury (I)

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with 2 moles of 2′,3′,5′-tri-*O*-benzoyl-1-D-ribofuranosyl chloride in dry acetonitrile gives a 4,6-di-*O*-(riboside)-4,6-dioxypyrimidine (II). If $HgBr_2$ is added to this mixture under anhydrous conditions, the di-*O*-glycosidic pyrimidine is converted into a 1,4-di-(ribosyl)-4-oxy-6-pyrimidinone (III). When the latter compound is treated with dilute sodium methoxide in dry methanol the *N*-glycosyl bond is preserved but the *O*-glycosidic bond at position 4 of the pyrimidine is cleaved. In addition, the benzoyl-blocking groups on the ribosyl moiety are removed by the mild alkaline degradation. The dipolar ionic structure of isouridine (IV), shown in Scheme I, is based on the report of Katritzky *et al.* (1966).

SCHEME I

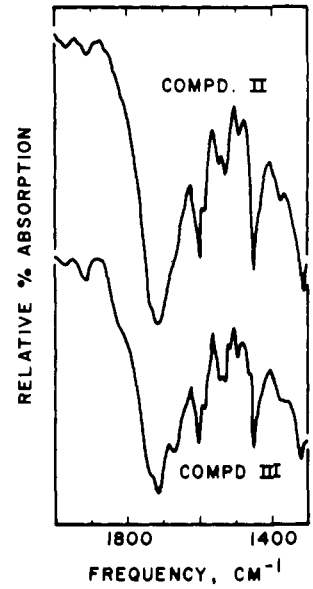

FIGURE 1: Infrared spectra (2% in KBr). Top curve: 4,6-di-*O*-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranosyl)-4,6-dioxypyrimidine (II); bottom curve: 1,4-di-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranosyl)-4-oxy-6-pyrimidinone (III). The same absorption scale was used in the measurement of the two spectra.

If $HgBr_2$ is not added to the reaction mixture *O*-glycosidic bonds are formed, but no *N*-glycosyl bond is observed. The 4,6-di-*O*-glycoside pyrimidine (II) may be isolated from the reaction mixture; the treatment of II with dilute alkali results in the destruction of both *O*-glycosidic bonds. The infrared absorption spectrum of the di-*O*-glycoside pyrimidine (II) is given in Figure 1; a strong band due to the six carbonyl groups of the benzoyl ester substituents is found with a maximum near 1710 cm^{-1}, but no pyrimidine carbonyl stretching band is observed. Compound II may be isolated, dissolved in dry acetonitrile, and converted into compound III by treatment with $HgBr_2$. The infrared spectrum of the latter compound contains a band with a maximum at 1665 cm^{-1} (see Figure 1) which corresponds to the stretching frequency of a pyrimidine carbonyl group.

The ultraviolet spectrum of isouridine (IV) in water, given in Figure 2, reveals an absorption band with a maximum at 256 mμ. When IV is hydrogenated in the presence of a palladium catalyst, the spectrum of the resultant 2,5-dihydro derivative does not show an absorption band at a wavelength longer than 230 mμ. The ultraviolet spectra of 4,6-pyrimidinedione and 1-methyl-4,6-pyrimidinedione in water are given in Figure 2 for a comparison with isouridine. The spectrum of the ribonucleoside was subtracted from the spectrum of the pyrimidine; the difference spectrum shows that cleavage of the N-1 glycosyl bond produces an absorbance increase of 2400 at 251 mμ. The compound, 1-methyl-4,6-pyrimidinedione, was prepared by a modification of the procedure of Kheifets and Khromov-Borisov (1965); the spectra of the *N*-methyl derivative and 4-methoxy-6-pyrimidinone in water (pH 6.8) are given in Figure 2. It is apparent that the spectrum of isouridine is different from the spectrum of the *O*-methylpyrimidine derivative. Furthermore, the pK_a of isouridine is 6.45, and the pK_a of

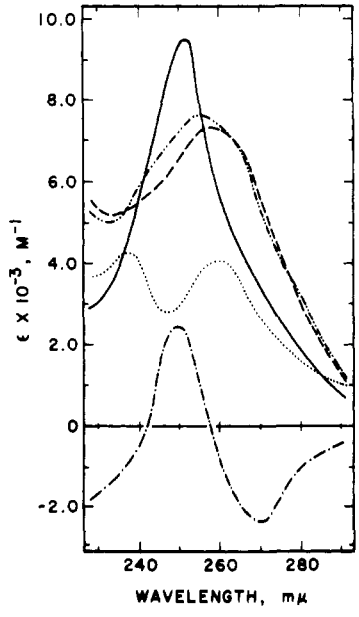

FIGURE 2: The ultraviolet absorption spectra of solutions in water at pH 6.8. (------) 1-β-D-Ribofuranosyl-4,6-pyrimidinedione, (·····) 1-methyl-4,6-pyrimidinedione, (——) 4,6-pyrimidinedione, (·····) 4-methoxy-6-pyrimidinone, and (-·-·-) a difference spectrum of 4,6-pyrimidinedione *minus* the ribosyl-4,6-pyrimidinedione.

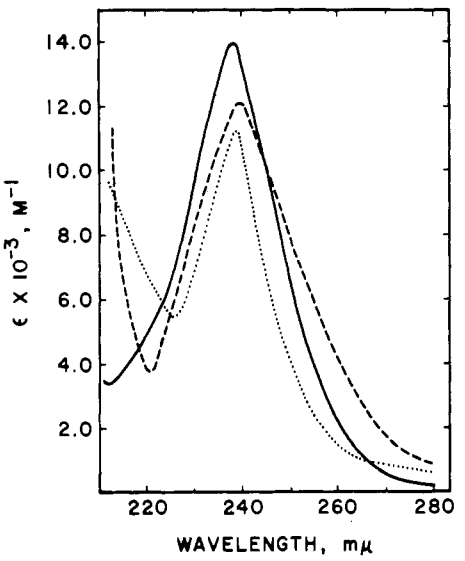

FIGURE 3: The ultraviolet absorption spectra of solutions in 95% ethanol. (——) 2′,3′-*O*-Isopropylidene-2,5′-anhydrouridine, (------) 2′,3′-*O*-isopropylidene-5′,6-anhydro-1-β-D-ribofuranosyl-6-oxy-4-pyrimidinone, and (·····) 1-β-D-ribofuranosyl-4-pyrimidinone.

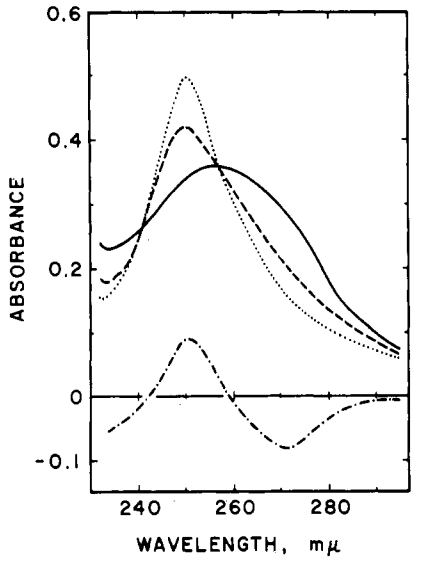

FIGURE 4: The effect of uridine phosphorylase on the ultraviolet absorption spectrum of 1-β-D-ribofuranosyl-4,6-pyrimidinedione at pH 7.4. (——) Zero-time sample of 50 μM substrate, 300 μM phosphate, and residual enzyme after denaturation; (------) 36-hr sample of the aforementioned solution; (·····) a solution of 50 μM 4,6-pyrimidinedione; (-·-·-) a difference spectrum of the 36-hr sample minus the zero-time sample.

4-methoxy-6-pyrimidinone is 8.47 (Brown and Teitei, 1963).

Isouridine was converted into a 2′,3′-isopropylidene derivative, and the 5′-hydroxyl group was replaced with a tosyl group. Treatment of this compound with NaI led to the formation of the 5′-iodo-5′-deoxy derivative; this substance was converted into the 5′,6-anhydro derivative by a routine procedure. The physical properties of the anhydro derivative provide an additional confirmation of the *N*-glycosyl structural assignment for isouridine. The conversion of an *O*-glycoside into the anhydro derivative would give 2′,3′-isopropylidene-5′,6-anhydro(cyclo)-4-β-D-ribofuranoside 4,6-dioxypyrimidine. Since two pyrimidine oxygen atoms would be bonded to the ribose group, the infrared spectrum of the latter compound would not show an absorption band for the pyrimidine carbonyl group. However, the infrared band observed at 1640 cm^{-1} suggests that the structure of the anhydro compound corresponds to that of an N-1 glycosyl of 6-oxy-4-pyrimidinone.

The 2′,3′-isopropylidene derivative of 2,5′-anhydrouridine was prepared by the method of Brown *et al.* (1957), and the 1-β-D-ribofuranosyl-4-pyrimidinone was prepared by a published method (Lee and Wigler, 1968). The ultraviolet spectra of these two compounds are compared with the spectrum of the anhydro derivative of 6-oxy-4-pyrimidinone in Figure 3. The absorption band with a maximum near 240 mμ is characteristic of an N-1 alkyl-4-pyrimidinone (Brown *et al.*, 1955). The formation of an anhydro compound also serves to confirm the β-anomeric configurational assignment for the new ribonucleoside.

The reaction of isouridine with inorganic phosphate, catalyzed by rabbit liver uridine phosphorylase, is shown in Scheme II. The substrates and products are represented in the predominant ionic forms for a pH of 7.4, based on the ionization constants and electrophoretic mobility of the compounds. The products of the enzymatic reaction were identified by paper chromatography.

SCHEME II

A spectrophotometric experiment was performed to demonstrate the chromogenic properties of the 4,6-pyrimidinedione-leaving group. The solution of 50 μM isouridine, 300 μM potassium phosphate, and 100 μM Tris-chloride was adjusted to pH 7.4 and treated with 3 drops of toluene/l. (to prevent bacterial contamination). The partially purified enzyme was added (to a final concentration of 1.3 mg of protein/ml), and an aliquot of the solution was removed rapidly and placed in a test tube in a boiling water bath for 2 min. This suspension was labeled the zero-time sample.

The remainder of the enzyme–substrate mixture was incubated at 25°; aliquots were removed at 2 and 36 hr and placed in test tubes in a boiling-water bath for 2 min. Each of the suspensions was cooled, and the denatured protein was removed by centrifugation. The ultraviolet absorption spectrum of each supernatant

solution was determined from 230 to 300 mμ with a micro cell of 10-mm light path (see Figure 4).

Since the enzymatic reaction stops soon after 2 hr at 25°, the spectra of the solutions incubated for 2 and 36 hr were almost identical. The spectra of the solution incubated for 36 hr, the zero-time sample, and a solution of 50 μM 4,6-pyrimidinedione in water at pH 7.4 are reproduced in Figure 4. A difference spectrum of the 36-hr incubation solution *minus* the zero-time solution is also given in Figure 4.

Discussion

Rotation about the glycosyl bond of the natural pyrimidine nucleosides is restricted by steric hindrance of the oxygen atom at the 2 position of the pyrimidine moiety and the hydrogen atom at the 2′ position of the ribosyl group. Emerson *et al.* (1967) have suggested that the *anti* conformation is the preferred orientation for the N-1 pentofuranosyl derivatives of uracil, thymine, and cytosine in aqueous solution. In this conformation the carbonyl group at the 2 position is directed away from the furanose ring. In 1967, the 2,5′-anhydro derivatives of the pyrimidine nucleosides were the only known analogs with a ring conformation opposite to that of uridine (the pyrimidine ring has been rotated 180°). The 2,5′-anhydronucleosides are rigid molecules, however, with an abnormal ultraviolet absorption spectrum (see Figure 3).

An examination of the molecular models of 1-β-D-ribofuranosyl-5-methyl-4,6-pyrimidinedione (Prystas, 1968) and the corresponding 5-hydrogen compound (isouridine) reveals that the rotation of the glycosyl bond is hindered by the proximity of the C-6 oxygen atom and the C-2′ hydrogen atom. Thus, the conformation of the pyrimidine ring of this nonrigid molecule is rotated 180° in comparison with the preferred orientation of uridine. The chemical synthesis of isouridine was attempted to provide a compound with an ultraviolet spectrum similar to that of uridine, but a pyrimidine ring conformation opposite to that of uridine. A comparison of uridine with the new ribonucleoside may show the effect of molecular conformation on the physical (optical rotatory dispersion, for example), chemical, and biochemical properties of the ribofuranosylpyrimidinediones.

In a recent report on the mercuri procedure, satisfactory N-1 glycosyl formation was demonstrated with unblocked oxygen or sulfur substituents on the pyrimidine ring (Lee and Wigler, 1968). This result suggests that isouridine could be prepared by a condensation with unblocked 4,6-pyrimidinedione; the exposure of the latter compound to an equimolar amount of mercuric chloride gives a 95% yield of a 1:1 pyrimidine-mercury salt. The use of more than equimolar quantities of the $HgCl_2$ or the unblocked pyrimidine does not increase the quantity of 4,6-pyrimidinedione-mercury (I) that is obtained.

The O → N transribosylation of II with $HgBr_2$ in acetonitrile appears to be analagous to the experiment of Prystas and Sorm (1968). The formation of at least one pyrimidine carbonyl group in the rearrangement is consistent with the infrared spectra reported in Figure 1. The assignment of a structure to III is dependent upon the number of transribosylations that occur for each molecule. If one transfer occurs, the product would be a 1,4-disubstituted 4-oxy-6-pyrimidinone, but if two transribosylations occur the product would be 1,3-di-*N*-(β-D-ribofuranosyl)-4,6-pyrimidinedione. Thus, the structure of compound III cannot be assigned with any certainty.

In contrast with the nucleosides of uracil and thymine, the phosphorolytic cleavage of isouridine changes the ultraviolet wavelength maximum at neutral pH in water (see Figure 4). The change in absorbance at 251 mμ may provide the basis for a continuous rate determination of the uridine phosphorylase reaction. Furthermore, the results of Figure 4 suggest that the enzyme does not show an absolute specificity for a substrate with the same pyrimidine ring conformation as uridine. Since the pyrimidine ring of isouridine is rotated 180° opposite to the ring conformation of uridine, the new ribonucleoside is cleaved more slowly than uridine. Thus, the interaction of the active site with the hydrogen atom at position 3 and the oxygen atom at position 4 is not an absolute requirement for enzymatic activity. The oxo group at position 2 (or position 6) may be more important for phosphorolytic activity; however, 1-β-D-ribofuranosyl-4-pyrimidinone (Lee and Wigler, 1968) is inert as a substrate for uridine phosphorylase.

The conformational substrate requirements of RNase, based on the enzymatic degradation of a 3′,5′-phosphodiester-bonded polymer of formycin in the *syn* conformation, have recently been discussed (Ward and Reich, 1968). These authors utilized a structural comparison of the polynucleotide of formycin with polyuridylic acid, to suggest that RNase activity depends upon bonding of the active site with the 3 and 4 positions of uracil in the *anti* conformation. The RNase mechanism of Gassen and Witzel (1967), on the other hand, is based on a requirement for a 2-oxopyrimidine or 6-oxopyrimidine group on the substrate. It was of interest, therefore, to prepare isouridine 2′:3′-cyclic phosphate as a potential substrate for RNase.

Isouridine was blocked with a trityl group at the 5′ position and the 5′-trityl compound was converted to a mixture of 2′-(3′)-cyanoethyl phosphate derivatives with dicyclohexylcarbodiimide. Hydrolysis of these compounds under mild conditions, with acid and alkali, gave the mixed nucleotides; the action of dicyclohexylcarbodiimide in dry methanol provided isouridine 2′:3′-cyclic phosphate. Since the cyclic nucleotide proved to be completely resistant to hydrolysis at neutral pH in the presence of RNase, the conformational orientation of isouridine may prevent pyrimidine binding to the active site. The specificity of RNase for isouridine is consistent with the conformational model of Ward and Reich (1968).

It is suggested that the derivatives of isouridine may provide further information on the conformational specificity requirements of enzymes. The binding of the hydrogen at N-3 to the active site may be required

for RNase activity, but not for uridine phosphorylase activity. The 3′,5′-linked polyribonucleotide of isouridine would also be of interest as a potential substrate for enzymatic reactions.

Experimental Section

Synthesis of 1-β-D-*Ribofuranosyl*-4,6,(1*H*,5*H*)-*pyrimidinedione.* A solution of 5.45 g (20 mmoles) of $HgCl_2$ in 100 ml of 60% aqueous ethanol was slowly added to 2.25 g (20 mmoles) of 4,6-pyrimidinedione (Sigma Chemical Co.) in 50 ml of 0.4 M sodium methoxide in methanol. The mixture was stirred gently to suspend the white precipitate of pyrimidinemercury salt, and the suspension was heated at 50° for 15 min. The suspension was stored at 25° for 15 hr, the filtrate was discarded, and the precipitate was purified with hot water and ethanol. The 4,6-pyrimidinedionemercury (yield 6.2 g) was dried at high vacuum over P_2O_5 at 45° for 2 days.

A sample of 1-acetyl-2,3,5-tri-*O*-benzoyl-D-ribose (9.8 g, 20 mmoles) was converted into the corresponding ribosyl chloride by the method of Fox *et al.* (1956). The 4,6-pyrimidinedionemercury (I, 3.5 g, 11 mmoles) was suspended in 250 ml of dry acetonitrile, 3 g of Celite filter aid was added, and the suspension was stirred. The syrup of a blocked ribosyl chloride (20 mmoles in 20 ml of dry benzene) was added, and the suspension was heated under reflux for 15 min. The suspension was cooled to room temperature and 9.0 g (25 mmoles) of $HgBr_2$ was added; the suspension was stirred and heated under reflux for 3 hr. The suspension was cooled and filtered, the solvent was removed from the filtrate with a rotary evaporator, and the residue was suspended in 5 ml of ethyl acetate. This mixture was applied to an anhydrous 1.5 × 30 cm column of 80–200 mesh neutral alumina (Fisher Scientific), the column was washed with 200 ml of benzene–ethyl acetate (10:1, v/v), the eluate was collected, and the solvent was removed with a rotary evaporator. The residue was dissolved in $CHCl_3$, the solution was shaken with 30% KI in water, and the $CHCl_3$ solution was dried over Na_2SO_4. The $CHCl_3$ solution was treated with Norit A, filtered, and the solvent was removed under vacuum. The residue was dissolved in dry ethyl acetate, treated with petroleum ether (bp 30–60°) for precipitation, and the precipitate was recrystallized from ethanol to give 3.4 g (30%) of a compound tentatively identified as 1,4-di-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranosyl)-4-oxy-6-pyrimidinone (III): mp 116–118°, infrared spectrum (KBr) 1665 cm^{-1} (pyrimidine C=O).

The blocked diglycosyl-4-oxy-6-pyrimidinone (3.35 g, 3.4 mmoles) was dissolved in 70 ml of 0.1 M sodium methoxide in dry methanol (prepared from sodium hydride), and the solution was stirred gently at 25° for 48 hr and cooled. Cold water (25 ml) was added, the solution was neutralized with Dowex 50W-X8 (H^+ form) and filtered, and the solvent was removed under vacuum. The residue was dissolved in 30 ml of water, shaken with two portions of cold ethyl ether, and the ether fraction was discarded. Water was removed from the aqueous fraction with a rotary evaporator to give a pale yellow residue which was triturated with ethanol; recrystallization from ethanol gave 740 mg (90%) of 1-β-D-ribofuranosyl-4,6-pyrimidinedione: mp 138–140°; $[\alpha]_D^{20}$ + 16.5° (*c* 0.38, H_2O); pK_a = pH 6.45, determined at an ionic strength of 0.1 by potentiometric titration with tetraethylammonium hydroxide; ultraviolet spectrum λ_{max} (H_2O, pH 6.8) 256 mμ (ε 7300); infrared spectrum (KBr) 1650 cm^{-1}.

Anal. Calcd for $C_9H_{12}N_2O_6$: C, 44.26; H, 4.95; N, 11.47. Found: C, 44.40; H, 4.92; N, 11.55.

Preparation of 4,6-Di-O-(2′,3′,5′-tri-O-benzoyl D-*Ribofuranoside) 4,6-Dioxypyrimidine (II).* A mixture of 3.5 g of 4,6-pyrimidinedionemercury, 250 ml of dry acetonitrile, 3 g of Celite, and 20 mmoles of the blocked 1-chlororibose was prepared. The suspension was heated under reflux for 30 min, the hot suspension was filtered, and the filtrate was cooled to 25°. The filtrate was concentrated under vacuum to a volume of approximately 25 ml, and excess petroleum ether was added to give a white precipitate. The precipitate was dissolved in $CHCl_3$ and shaken with 30% aqueous KI solution. The solvent was removed from the $CHCl_3$ fraction, and the residue was dissolved in ethyl acetate and applied to a 2 × 30 cm column of silica gel (J. T. Baker Chemical Co.). The column was washed with 150 ml of benzene–ethyl acetate (10:1, v/v), the eluate was collected, and the solvent was removed under vacuum. The residue was recrystallized from ethanol to give 3.6 g (32%) of II, mp 76–80°. The infrared spectrum (KBr) of this compound does not show an absorption band for the pyrimidine carbonyl group. A solution of 112 mg of II in 0.13 M NaOH in acetonitrile was stored at 25° for 30 min; the only ultraviolet-absorbing product found was 4,6-pyrimidinedione, mp 228–230° dec. The di-*O*-glycosidic compound was completely hydrolyzed in the same alkaline acetonitrile solution in 3 hr at 5°.

Anal. Calcd for $C_{56}H_{44}N_2O_{16}$: C, 67.19; H, 4.43; N, 2.80. Found: C, 66.91; H, 4.36; N, 2.93.

The Mercuric Bromide Rearrangement. Mercuric bromide (9.0 g, 25 mmoles) and 5.6 g (5.6 mmoles) of the blocked 4,6-di-(ribofuranoside) dioxypyrimidine (II) were mixed with 150 ml of dry acetonitrile, and the solution was heated under reflux for 2 hr. The solvent was removed under vacuum, the residue was suspended in $CHCl_3$, and the suspension was filtered. The $CHCl_3$ fraction was treated with 35 g of activated alumina (with agitation), shaken with a 30% aqueous KI solution, and dried with Na_2SO_4. The $CHCl_3$ was removed under vacuum, the residue was dissolved in ethyl acetate, and petroleum ether was added to give a precipitate. The suspension was cooled, and the precipitate was recrystallized from ethanol to give 2.8 g (50%) of III.

*Preparation of 2,5-Dihydro-*1-β-D-*ribofuranosyl-4,6-pyrimidinedione for Comparison with the Compound of Prystas (1967).* A 1.0-g sample of isouridine (IV) was dissolved in 50 ml of dry methanol; the solution was added to 0.6 g of 10% palladium-on-charcoal catalyst. The suspension was shaken under H_2 gas for 3 hr at 25°. The suspension was filtered, the filtrate was col-

lected, and the solvent was removed under vacuum. The residue was recrystallized from 95% ethanol to give 420 mg (40%) of a hydrogenated compound (which may contain a small amount of ribose): mp 130–132°, $[\alpha]_D^{20}$ +31.2° (*c* 0.32, H_2O); the ultraviolet spectrum of a water solution showed no absorption maximum (lit. (Prystas, 1967) mp 136–137°, $[\alpha]_D^{25}$ +43.5°; *c* 0.51, H_2O).

Preparation of 1-Methyl-4,6-pyrimidinedione. Methyl iodide was reacted with 4,6-dimethoxypyrimidine (Brown and Harper, 1961) by the method of Brown and Teitei (1964) to give 1-methyl-4-methoxy-6-pyrimidinone. The latter compound (2.2 g, 50 mmoles) was dissolved in 30 ml of HBr-saturated glacial acetic acid, the suspension was shaken at 20° for 24 hr, and the filtrate was discarded (Kheifets and Khromov-Borisov, 1965). The precipitate was washed with cold acetic acid and ethyl ether and dissolved in water. The solution was applied to a 2 × 10 cm column of Dowex 1-X8 (100–200 mesh, acetate form). The column was washed with 190 ml of water, and this portion of the eluent was discarded. The product was then eluted in 60 ml of water, the water was removed with a rotary evaporator, and the residue was suspended in benzene–ethanol (1:1, v/v). The solvent was removed under vacuum, and the residue was resuspended twice in benzene–ethanol and dried twice. The product was recrystallized from ethyl acetate to give 0.9 g of 1-methyl-4,6-pyrimidinedione: mp 151–152°; ultraviolet spectrum λ_{max} (H_2O, pH 6.8) 254 mμ (ε 7400); infrared spectrum (KBr) 1655 cm^{-1}; pK_a at 0.1 ionic strength is pH 5.98 (lit. (Kheifets and Khromov-Borisov, 1965) mp 155–156°, pK_a = 5.75).

Preparation of 2′,3′-O-Isopropylidene-5′,6-anhydro-(cyclo)-1-β-D-ribofaranosyl-6-oxy-4-pyrimidinone. A sample of isouridine (IV) was converted into the isopropylidene derivative by the procedure of Hampton (1961). 2,2-Dimethoxypropane (8 ml) and di-*p*-nitrophenyl hydrogen phosphate (180 mg) were dissolved in 50 ml of dry acetone, and 1.2 g (5 mmoles) of the glycosylpyrimidinedione was added. The suspension was stirred for 2 hr at 25°, stored for 3 hr at the same temperature, and cooled to 3°. The mixture was added to a stirred solution of 500 ml of 0.25 M ammonium bicarbonate in methanol–water (1:1, v/v). The mixture was applied to a 1.5 × 30 cm column of Dowex 1-X8 (200–400 mesh, bicarbonate form), and the column was developed with 500 ml of 0.25 M ammonium bicarbonate in methanol–water. The eluent was pooled, the solvent was removed under high vacuum, and the residue was dried to give 900 mg (65%) 2′3′-*O*-isopropylideneisouridine, mp 128–130°.

p-Toluenesulfonyl chloride (400 mg) and the isopropylidine compound (570 mg) were dissolved in 25 ml of dry pyridene, and the solution was stored for 18 hr at 3°. The solution was treated with 5 ml of water, stored for 3 hr at 25°, and the solvent was removed under vacuum. The residue was suspended in $CHCl_3$, and the $CHCl_3$ fraction was filtered and shaken with water. The $CHCl_3$ fraction was treated with anhydrous Na_2SO_4, and the solvent was removed under vacuum. The residue was recrystallized from acetone and petroleum ether to give 700 mg (80%) of a fully blocked ribonucleoside.

The tosyl compound (660 mg), 830 mg of sodium iodide, and 20 ml of dry acetone were placed in an aerosol pressure tube (Fischer and Porter No. 110–023). The tube was evacuated, sealed, and placed in an oil bath at 105° for 1 hr. The reaction mixture was cooled, filtered, and the filtrate was concentrated under vacuum. The residue was suspended in water, the water fraction was discarded, and the residue was recrystallized from methanol and water to give 270 mg (50% yield) of 2′,3′-*O*-isopropylidene-5′-iodo-5′-deoxyisouridine, mp 121–122°. Chromatography on Whatman No. 3MM paper (descending) with a 1-butanol–glacial acetic acid–water solvent (5:2:3, v/v) gave one zone with an R_F of 0.38. To detect the iodo derivative the chromatograms were dried and sprayed with a mixture of 0.1 N ceric sulfate solution (in 1.0 N H_2SO_4, G. F. Smith Chemical Co.) and 5% sodium arsenite (in water) (2:3) (Bowden *et al.*, 1955). The chromatograms were dried and examined with an ultraviolet lamp; the 5′-iodo compound gives a bright blue fluorescent spot on a dark background.

The 5′-iodo derivative (270 mg), silver acetate (320 mg), triethylamine (2 ml), and dry methanol (120 ml) were mixed and heated under reflux for 90 min. The hot mixture was applied to a 2 × 3 cm column of Hyflo Super-Cel (Fisher Scientific Co.); the eluent was collected, cooled, and treated with H_2S. The precipitate of Ag_2S was discarded, and the supernatant was concentrated to a volume of 10 ml under vacuum. The product was recrystallized from methanol and benzene to give 40 mg of 2′,3′-*O*-isopropylidene-5′,6-anhydro(cyclo)isouridine: mp 116–118°, ultraviolet spectrum λ_{max} (95% ethanol) 240 mμ (ε 12,100), infrared spectrum (KBr) 1640 cm^{-1} (C=O). The test for the 5′-iodo group, performed as described before, was negative; treatment of the anhydro derivative with 0.5 N HCl at 40° for 1 hr gave a spot on paper chromatograms that corresponds to 2′,3′-*O*-isopropylideneisouridine.

Preparation of 1-O-Acetyl-2,3,5-tri-O-benzoyl-D-ribose from Guanosine.[1] The procedure of Weygand and Sigmund (1953) was modified and simplified. The 2′,3′,5′-tri-*O*-benzoylguanosine was prepared by the treatment of guanosine with benzoyl chloride in dry pyridine. The blocked ribonucleoside (29 g) was dissolved in 150 ml of dry pyridine–acetic anhydride (1:3, v/v), and the solution was heated under reflux for 1 hr. The mixture was cooled, the precipitate was discarded, and the filtrate was concentrated to a volume of 50 ml under vacuum. The solution was mixed with 300 ml of water and 120 ml of $CHCl_3$, the two fractions were separated, and the aqueous fraction was discarded. The $CHCl_3$ fraction was treated with water, three times with 30-ml portions of saturated sodium bicarbonate in water, and dried with Na_2SO_4. The

[1] The preparation of 1-acetyl-2,3,5-tribenzoyl-β-D-ribose by the treatment of guanosine with acetic anhydride has been described in a patent by Zellstoff-fabrik Waldhof, Britain 1,106,588, March 20, 1968.

mixture was filtered, and the solvent was removed from the $CHCl_3$ fraction under vacuum. The residue was dissolved in ethanol, treated with activated charcoal, and recrystallized from ethanol to give 24 g (90%) of 1-*O*-*acetyl*-2,3,5-tri-*O*-benzoyl-D-ribose: mp 130–131°, $[\alpha]_D^{25}$ +43.8° (*c* 1.32, $CHCl_3$) (lit. (Ness *et al.*, 1954) mp 130–131°, $[\alpha]_D^{20}$ +44.2° (*c* 1.32, $CHCl_3$)).

Enzymatic Formation of 4,6-Pyrimidinedione and Ribose 1-Phosphate from Isouridine and P_i. A fractionation of rabbit liver acetone powder (General Biochemicals) was performed by salt precipitation with ammonium sulfate (Krenitsky *et al.*, 1964) followed by adsorption to a calcium phosphate gel (Yamada, 1961). The partially purified pyrimidine nucleoside phosphorylase was found to utilize the substrates, uridine, cytidine, thymidine, ribose 1-phosphate, and uracil.

A solution of 5 mM isouridine, 30 mM potassium phosphate, and partially purified enzyme (at 0.16 mg of protein ml^{-1} based on the method of Lowry *et al.*, 1951) was adjusted to a pH of 7.2 and incubated at 25° for 90 min. The reaction (final volume 2.5 ml) was terminated by the immersion of the incubation tube into a boiling-water bath for 2 min. The suspension was cooled, and the denatured protein was removed by centrifugation.

A sample (0.5 ml) of the supernatant was applied to Whatman No. 3MM paper, and the chromatogram was developed in descent with a solvent of 1-butanol–glacial acetic acid–water (5:2:3, v/v). The unreacted isouridine was observed with an ultraviolet lamp at an R_F value of 0.52 and 4,6-pyrimidinedione was detected at an R_F value of 0.42. The presence of the pyrimidine base was dependent upon active enzyme; the chromatographic migration of this product was identical with an authentic sample of 4,6-pyrimidinedione. The fluorescent zones were removed from the chromatogram, and the fractions eluted with water. The absorbance at 252 mμ (ϵ 9450) was used to determine the amount of 4,6-pyrimidinedione formed in the reaction; under these conditions the cleavage is approximately 60% completed.

A portion of the protein-free supernatant (2.0 ml) was mixed with 1.0 ml of 4.0 M LiOH, and the precipitate of Li_3PO_4 was removed by centrifugation. The supernatant was applied to filter paper, and the chromatogram was developed with 2-propanol–concentrated ammonia–water (7:1:2, v/v) solvent in descent. The chromatogram was then treated with a spray to detect phosphate compounds (Bandurski and Axelrod, 1951). Ribose 1-phosphate was located at an R_F of 0.16, but no inorganic phosphate was found.

Analytical Methods. The spectra were determined with a Gilford Model 240 ultraviolet spectrophotometer and a Perkin-Elmer Model 700 infrared spectrophotometer. The ionization constant of 1-β-D-ribofuranosyl-4,6-pyrimidinedione was determined from titration curves prepared with a Radiometer Model TTT1 recording titrator. A solution of 2.88×10^{-3} M ribonucleoside in 0.1 M tetraethylammonium chloride was acidified to pH 2 with HCl and titrated to pH 11 with 0.023 M tetraethylammonium hydroxide. The titrations were performed in a closed vessel under moist N_2 gas at 20°. The pK_a value of 6.45 is based on the average of four determinations of the pH observed at the addition of 0.5 equiv of titrant.

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