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161. Yoshiyasu Furukawa, Kunio Kobayashi, Yoshio Kanai, and Mikio Honjo: Synthesis of 2′-O-Methyluridine, 2′-O-Methylcytidine and Their Relating Compounds.

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In recent years several papers[1~6] reported the isolation from intact RNA[*2] of the substances which are presumed to be 2′-O-methylribonucleosides. Previously we have isolated the 2′-O-methyl derivatives of four naturally occurring 5′-ribonucleotides from enzymatically-digested[7] yeast RNA and obtained some evidence for the assignment of their structures.[8] These O-methylated nucleotides of RNA, however, were obtained in quantities insufficient for our research purposes and had to be separated by tedious procedures. Therefore we have attempted the chemical synthesis of 2′-O-methyl and 3′-O-methyl derivatives of uridine and cytidine.

Tritylation of uridine gives a mixture of 5′-O-trityluridine, 3′,5′-di-O-trityluridine (I) and 2′,5′-di-O-trityluridine (Ⅱ).[9] Recently Žemlička[10] has isolated I from the above mixture by silica gel chromatography. In our experiment we have methylated I with methyl iodide in presence of silver oxide and chromatographed the products on silica gel. Two fractions were obtained. The ultraviolet absorption maxima of the fractions were similar to those of N^3-methyluridine[11] and 1-(β-D-glucopyranosyl)-4-ethoxy-2(1*H*)-pyrimidinone,[11] and accordingly the fractions were assumed to be N^3,2′-O-dimethyl-3′,5′-di-O-trityluridine (Ⅲ) and 1-(2′-O-methyl-3′,5′-di-O-tritylribofuranosyl)-4-methoxy-2(1*H*)-pyrimidinone (Ⅳ), respectively. The substance from the former fraction (Ⅲ) was detritylated with methanolic hydrogen chloride and the product was examined by paper electrophoresis (pH 9.2, borate). A single ultraviolet absorbing spot was obtained which migrated only slightly to the (−) side from the point of application. Moreover, the ultraviolet absorption maximum and minimum of this substance

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[*2] RNA, ribonucleic acid.

1) J.D. Smith, D.B. Dunn: Biochim. Biophys. Acta, **31**, 573 (1959).
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did not change between pH values of 2~12 and were similar to those of N^3-methyluridine 5′-phosphate.[12] A similar experiment with the substance from the latter fraction (Ⅳ) gave a single ultraviolet absorbing spot (cm.=2.2), whereas uridine, in the same buffer (pH 9.2, borate) showed cm.=10. These results demonstrate that the compounds thus formed from Ⅲ and Ⅳ are N^3,2′-O-di-methyluridine (Ⅴ) and 2′-O-methyluridine (Ⅵ), respectively. A large scale chromatography of the mixture of Ⅲ and Ⅳ on silica gel was unsuccessful. Therefore the mixture was detritylated and then successfully separated with Dowex-1 (borate form) to give Ⅴ and Ⅵ.

After treatment of the mixture of Ⅲ and Ⅳ with ethanolic ammonia, which transformed the 4-methoxy-2(1*H*)-pyrimidinone moiety of Ⅳ into the cytosine analog, the reaction mixture was detritylated and separated with Dowex-50 (H$^+$) to give Ⅴ and 2′-O-methylcytidine (Ⅶ) respectively, the latter obtained as the hydrochloride. Ⅵ and Ⅶ thus obtained were found to be identical with specimens prepared from RNA by paper chromatography (Table I) and paper electrophoresis.

Table I. Paper Chromatography of 2′-O-Methyluridine, 2′-O-Methylcytidine and Their Relating Compounds

Compound		Asending paper chromatography (Rf) Solvent 1	Solvent 2
2′-O-Methyluridine	Natural	0.11	0.73
	Synthetic	0.11	0.73
2′-O-Methylcytidine	Natural	0.29	0.65
	Synthetic	0.29	0.65
3′-O-Methyluridine		0.10	0.72
3′-O-Methylcytidine		0.29	0.65
N^3,2′-O-Dimethyluridine		0.61	0.84
Uridine		0.05	0.59
Cytidine		0.11	0.48

Solvent 1: BuOH-H_2O-conc. NH_4OH (86:14:15)
Solvent 2: EtOH-PrOH-H_2O (4:1:2)

Methylation of Ⅱ under analogous conditions gave the 3′-O-methyl derivative only in ca. 50% yield. The low yield could be explained by the steric hindrance of 2′- and 5′-O-trityl groups (as suggested).[9] After repeated methylations, however, Ⅱ was methylated to give the 3′-O-methyl derivatives in ca. 80% yield, as determined by paper electrophoresis after detritylation. The reaction products were detritylated and chromatographed on Dowex-1 (borate form) to give pure samples of N^3,3′-O-dimethyluridine (Ⅷ) and 3′-O-methyluridine (Ⅸ).

After treatment of the methylated product of Ⅱ with ethanolic ammonia, the reaction mixture was detritylated and chromatographed on Dowex-1 (borate form) to give a mixture of N^3,3′-O-dimethyluridine and 3′-O-methylcytidine. This mixture was further separated with Dowex-50 (ammonium form) into Ⅷ and 3′-O-methylcytidine (Ⅹ), the latter compound isolated as the hydrochloride. The detritylated mixture did not give 3′-O-methyluridine. However, the mixture contained comparable amounts of N^3,3′-O-dimethyluridine and 3′-O-methylcytidine or N^3-methyluridine and cytidine, respectively. These results indicate that the methylation of Ⅱ with methyl iodide and

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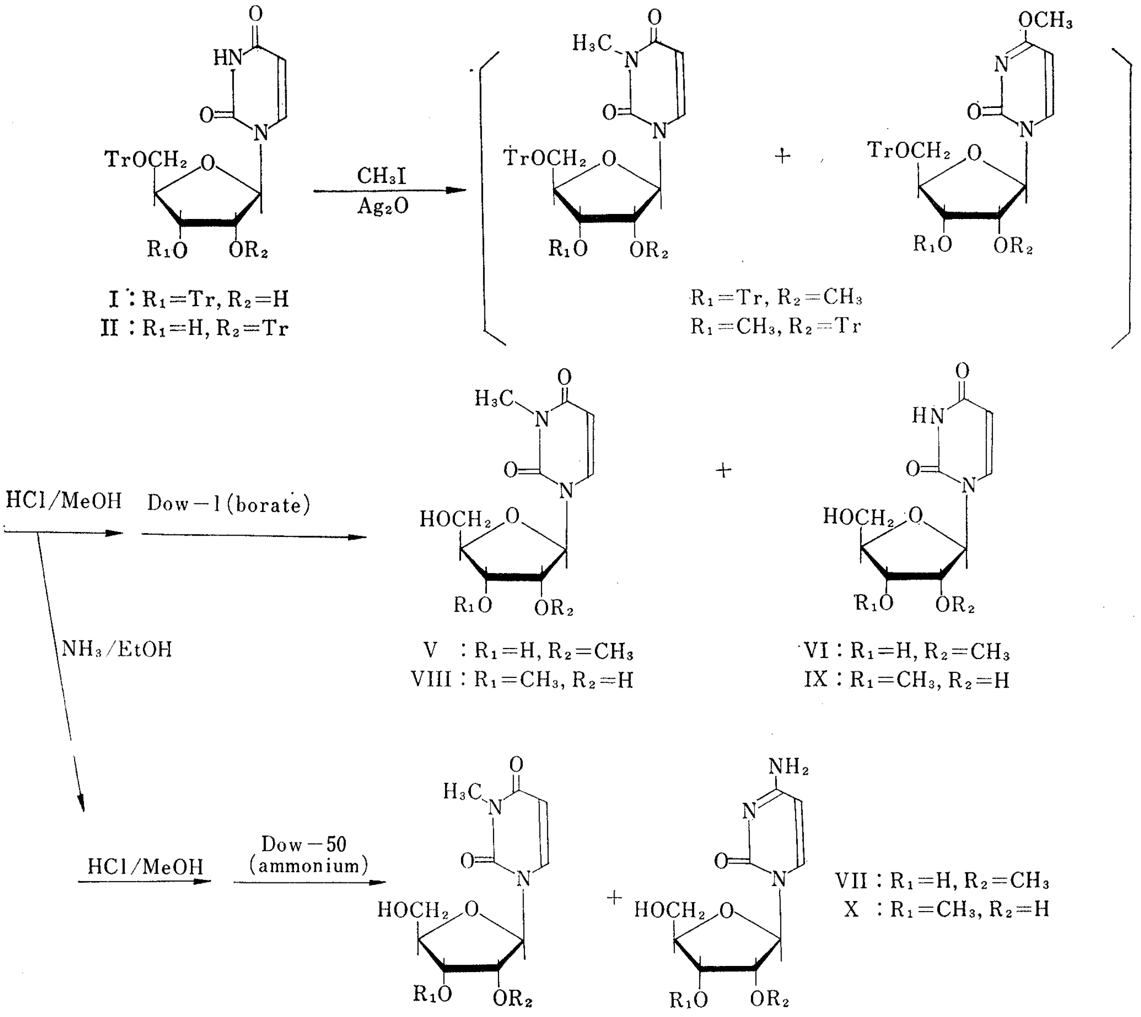
[12] N.K. Kochetkov, E.I. Budowsky, V.N. Shibaev: Biochim. Biophys. Acta, **53**, 415 (1961).

silver oxide affected the N^3 and $O=C^4$ positions of the uracil ring first (at almost comparable rates) and then attacked the hydroxyl group at the 3′-position of the ribose moiety.



I : $R_1=Tr, R_2=H$
II : $R_1=H, R_2=Tr$

V : $R_1=H, R_2=CH_3$
VIII : $R_1=CH_3, R_2=H$

VI : $R_1=H, R_2=CH_3$
IX : $R_1=CH_3, R_2=H$

VII : $R_1=H, R_2=CH_3$
X : $R_1=CH_3, R_2=H$

Chart 1.

Acid hydrolysis of 2′-O-methyluridine (Ⅵ) and 3′-O-methyluridine (Ⅸ) with 6*N* hydrochloric acid at 100° for 90 minutes (in a sealed tube) resulted in the decomposition of 11% and 24% of the substances, respectively. Under similar conditions uridine decomposed to the extent of 40%. Paper electrophoresis (pH 9.2, borate) of the hydrolysates gave two spots corresponding to those of uracil and the starting materials in the above three cases. Good first-order kinetics were obtained for the three nucleosides (Fig. 1). These results show that the introduction of one methoxyl group in the sugar moiety considerably enhanced the stability of the glycosylic linkage towards acid hydrolysis.

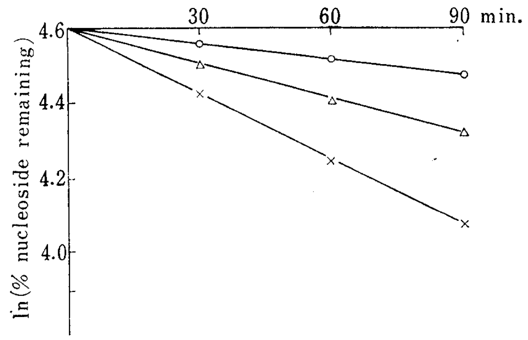


Fig. 1. Kinetics of the Hydrolysis of 2′-O-Methyluridine (—○—), 3′-O-Methyluridine (—△—), and uridine (—×—) in 6*N* Hydrochloric Acid at 100°

2′-O-Methyluridine obtained from RNA was resistant to pyrimidine riboside hydrolase, uridine phosphorylase and pyrimidine deoxyriboside phosphorylase.[8] Synthetic 3′-O-methyluridine is also resistant to uridine phosphorylase from *Escherichia coli* or *Aerobacter aerogenes*.[13]

Experimental

Paper Electrophoresis (PEP) and Nuclear Magnetic Resonance (NMR)——PEP was carried out on Whatman No. 1 filter paper at 22 v./cm. for 60 min. using 0.05*M* borate buffer (pH 9.2), unless otherwise stated. NMR was measured in D_2O using $(CH_3)_4Si$ as an external reference.

Methylation of 3′,5′-Di-O-trityluridine (I)——To a solution of I[10] (3.6 g.) in acetone (68 ml.) were added MeOH (6.8 ml.), CH_3I (70 ml.) and Ag_2O (2.8 g.), and the stirred mixture was refluxed. Ag_2O (1.6 g.) was added further 5 times after every 30 min. CH_3I (20 ml.) was also added at the third time interval. The reaction mixture was filtered, the AgI was washed well with acetone, and the combined filtrate and washings were evaporated to dryness *in vacuo* to give a syrupy residue.

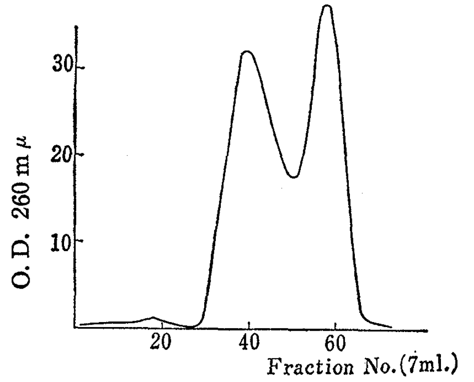


Fig. 2. Silica Gel Chromatography of the Methylated Products of 3′,5′-Di-O-trityluridine

N³-2′-O-Dimethyl-3′,5′-di-O-trityluridine (III)——A solution in $CHCl_3$ (1 ml.) of 1/14 weight of the above methylated I was subjected to column chromatography on silica gel (E. Merck, fine powder for chromatography, 25 g.) containing 5% H_2O and eluted with $CHCl_3$ to yield two fractions (Fig. 2). The first fraction (70 ml., TOD*[3] 1600) was concentrated to a white powder, crystallization of which with various solvents was unsuccessful. UV λ_{max}^{MeOH} mμ: 263; λ_{min}^{MeOH} mμ: 246.

1-(2′-O-Methyl-3′,5′-di-O-trityl-β-D-ribofuranosyl)-4-methoxy-2(1*H*)-pyrimidinone (IV)——The second fraction (70 ml., TOD 1100) in the above chromatography was concentrated to a white powder, UV λ_{max}^{MeOH} mμ: 270; λ_{min}^{MeOH} mμ: 248.

N³,2′-O-Dimethyluridine (V)——a) A mixture of a solution of 1/3 weight of Ⅲ in MeOH (4 ml.) containing HCl gas (20 mg.) was refluxed for 10 min. and, after standing overnight, was evaporated to dryness *in vacuo*. The residue was dissolved in H_2O (5 ml.), washed twice with $CHCl_3$ (5 ml. each) and concentrated. UV $\lambda_{max}^{pH\,4\text{ or }12}$ mμ: 261; $\lambda_{min}^{pH\,4\text{ or }12}$ mμ: 234.

b) One-fourth weight of the methylated I was detritylated as in a) and the aqueous solution of the product passed through a column (8×1.8 cm. in diam.) of Dowex-1 (borate form, 100~200 mesh). The column was washed with H_2O and the washing (130 ml., TOD 4100) was adsorbed on a column of ated charcoal (3 g.) and eluted with 2% NH_4OH-50% EtOH-5% BuOH (200 ml.) (This procedure is called the desalting process by activated charcoal). The eluate was evaporated to dryness and the residue was recrystallized from AcOEt to give colorless needles, m.p. 140~141° (100 mg., 32% based on I). On PEP, the product gave only one and the same UV-spot as in a) and its UV spectrum was the same as in a). *Anal.* Calcd. for $C_{11}H_{16}O_6N_2$: C, 48.55; H, 5.93; N, 10.28; OCH_3, 11.38. Found: C, 48.78; H, 5.72; N, 10.59; OCH_3, 11.86. $[\alpha]_D^{21}$ +50 (c=1.1, H_2O). IR ν_{max}^{KBr} cm^{-1}: 1700, 1665, 1625. NMR (τ): 6.52 (OCH_3), 6.79 (NCH_3).

2′-O-Methyluridine (VI)——a) One half the weight of Ⅳ was detritylated by the process used to obtain V and the product investigated by PEP, giving a UV-spot, whose mobility ratio to uridine was 0.22. UV $\lambda_{max}^{pH\,4}$ mμ: 261; $\lambda_{min}^{pH\,4}$ mμ: 231; $\lambda_{max}^{pH\,12}$ mμ: 262; $\lambda_{min}^{pH\,12}$ mμ: 244.

b) The column of Dowex-1 used in process b) for V was then eluted with 0.025*M* borate, the fraction (400 ml., TOD 7500) was desalted with activated charcoal and evaporated to dryness. The residue was recrystallized from AcOEt giving colorless needles, m.p. 159° (150 mg., 50% based on I). The product gave only one UV-spot in PEP, showed no coloration with $NaIO_4$-benzidine reagent spray,[14] and its UV spectrum was the same as in a). *Anal.* Calcd. for $C_{10}H_{14}O_6N_2$: C, 46.50; H, 5.47; N, 10.83; OCH_3, 12.0. Found: C, 46.72; H, 5.40; N, 11.10; OCH_3, 11.91. $[\alpha]_D^{20}$ +41 (c=1.6, H_2O). NMR (τ): 6.40 (OCH_3).

2′-O-Methylcytidine Hydrochloride (VII)——One-fourth weight of the methylated I was dissolved in hot EtOH (20 ml.) and the solution, after being saturated with NH_3 at 0°, was heated in a sealed tube at

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*[3] TOD=Optical density at 260 mμ×ml., 1 cm. light path.

13) A. Imada: Personal communication.

14) M. Viscontini, D. Hoch, P. Karrer: Helv. Chim. Acta, 38, 642 (1955).

80° for 4 days. The reaction mixture was evaporated to dryness *in vacuo*, the residue was detritylated as in the process a) for V, and the resulting solution was passed through a column (5 × 1.0 cm. in diam.) of Dowex-50 (H^+). The column was washed with H_2O (100 ml., TOD 3100) and eluted with $2N$ HCl (150 ml., TOD 4300). The eluate was desalted with activated charcoal and evaporated to dryness *in vacuo*. To a solution of the residue in $1N$ HCl (1 ml.) were added MeOH (1.5 ml.), acetone (5 ml.) and ether (50 ml.) after which crystals separated. The crystals were dissolved in 50% MeOH (1.5 ml.) and acetone (5 ml.) and ether (30 ml.) were added to the solution to yield colorless needles, m.p. 211° (decomp.) (100 mg., 32% based on I). The product gave only one UV-spot as the same position as cytidine in PEP (citrate, pH 3.7) and showed no coloration with $NaIO_4$-benzidine reagent. UV $\lambda_{\max}^{\text{pH 2}}$ mμ: 280; $\lambda_{\min}^{\text{pH 2}}$ mμ: 241; $\lambda_{\max}^{\text{pH 13}}$ mμ: 273; $\lambda_{\min}^{\text{pH 13}}$ mμ: 250. *Anal.* Calcd. for $C_{10}H_{15}O_5N_3 \cdot HCl$: C, 40.85; H, 5.49; N, 14.29; OCH_3, 10.55. Found: C, 40.89; H, 5.49; N, 14.54; OCH_3, 10.58. $[\alpha]_D^{20}$ +64° (c=1.2, H_2O). NMR (τ): 6.45 (OCH_3).

Methylation of 2′,5′-Di-O-trityluridine (II)——II (1.0 g.) was methylated as I and a part of the reaction mixture, after detritylation, was investigated by PEP, giving four UV-spots, whose mobility ratio to uridine was 1.00 (probably uridine), 0.80 (probably N^3-methyluridine), 0.22 (probably 3′-O-methyluridine). The rest which migrated a little to the (−) side is probably N^3,3′-O-dimethyluridine. The AgI was filtered from the above reaction mixture and the filtrate was evaporated to dryness. The same methylation was repeated 3 times on this residue after which methylation of 3′-OH proceeded to ca. 80%. The AgI was filtered and the filtrate was evaporated *in vacuo* to give a syrupy residue.

N^3,3′-O-Dimethyluridine (VIII)——The methylated II was detritylated as in the process a) for V. The resulting aqueous solution was adjusted to pH 8 with NH_4OH and passed through a column (13 × 1.8 cm. in diam.) of Dowex-1 (borate form, 100∼200 mesh). The column was washed with H_2O, the resulting fraction (340 ml., TOD 5400) was desalted with activated charcoal and evaporated to dryness. The residue was recrystallized from AcOEt to yield colorless needles, m.p. 125∼127° (120 mg., 33% based on II). On PEP the product gave only one UV-spot migrating slightly to the (−) side (identical to V). UV $\lambda_{\max}^{\text{pH 2 or 12}}$ mμ: 261; $\lambda_{\min}^{\text{pH 2 or 12}}$ mμ: 234. *Anal.* Calcd. for $C_{11}H_{16}O_6N_2$: C, 48.55; H, 5.93; N, 10.28; OCH_3, 11.38. Found: C, 48.63; H, 6.00; N, 10.42; OCH_3, 11.29. $[\alpha]_D^{21}$ +38° (c=1.0, H_2O). IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 1725, 1670.

3′-O-Methyluridine (IX)——The column of Dowex-1 (borate) used in the preparation of VIII was washed with H_2O and then eluted as follows.

Fraction No.	Solvent	Volume (ml.)	TOD	Substance
1	$0.002M$ $Na_2B_4O_7$ + $0.04M$ H_3BO_3	600	5700	IX
2	$0.004M$ $Na_2B_4O_7$ + $0.08M$ H_3BO_3	1100	1900	N^3-methyluridine and uridine

The first fraction was desalted with activated charcoal and evaporated to dryness, and the residue was extracted with hot AcOEt (20 ml.). When the extract was concentrated and scratched against the wall of the vessel, an amorphous powder, m.p. 142∼145°, precipitated (140 mg., 40% based on II). The product gave only one UV-spot at the same position as VI on PEP. UV $\lambda_{\max}^{\text{pH 2}}$ mμ: 261; $\lambda_{\min}^{\text{pH 2}}$ mμ: 230; $\lambda_{\max}^{\text{pH 12}}$ mμ: 262; $\lambda_{\min}^{\text{pH 12}}$ mμ: 243. *Anal.* Calcd. for $C_{10}H_{14}O_6N_2$: C, 46.50; H, 5.47; N, 10.83; OCH_3, 12.00. Found: C, 46.84; H, 5.28; N, 11.01; OCH_3, 12.06. $[\alpha]_D^{21}$ +26° (c=1.1, H_2O). NMR (τ): 6.52 (OCH_3).

3′-O-Methylcytidine Hydrochloride (X)——II (1.4 g.) was methylated as in the methylation of I (3′-OH was methylated to ca. 50%) and the syrupy residue was aminated and then detritylated (as in the preparation of VII). The resulting aqueous solution was passed through a column of Dowex-1 (15 ml., borate form, 100∼200 mesh) and eluted.

Fraction No.	Solvent	Volume (ml.)	TOD	Substance
1	H_2O	250	8400	VIII and X
2	$0.05M$ $Na_2B_4O_7$	200	6900	N^3-methyluridine and cytidine

The first fraction was adjusted to pH 2 with HCl, passed through a column of Dowex-50 (2 ml., NH_4^+ form, 50∼100 mesh), and the column was washed with H_2O (300 ml., TOD 3800) and eluted with 5% NH_4OH (100 ml., TOD 3200).

The second fraction was evaporated to dryness *in vacuo*, the residue was dissolved in $1N$ HCl (1 ml.), and to the solution were added MeOH (1 ml.), acetone (10 ml.) and ether to yield a precipitate, which was dissolved in 50% MeOH (2 ml.) and mixed with acetone (10 ml.) and ether. Colorless needles separated, m.p. 220～222° (decomp.) (140 mg., 25% based on II). The product gave only one UV-spot on PEP (citrate, pH 3.7) at the same position as cytidine and was negative to $NaIO_4$-benzidine reagent. UV $\lambda_{max}^{pH\ 2}$ mμ: 280; $\lambda_{min}^{pH\ 2}$ mμ: 240; $\lambda_{max}^{pH\ 13}$ mμ: 272; $\lambda_{min}^{pH\ 13}$ mμ: 250. *Anal.* Calcd. for $C_{10}H_{15}O_5N_3 \cdot HCl$: C, 40.85; H, 5.49; N, 14.29; OCH_3, 10.55. Found: C, 41.05; H, 5.45; N, 14.22; OCH_3, 10.53. $[\alpha]_D^{21}$ $+54°(c=1.1, H_2O)$. NMR (τ): 6.52 (OCH_3).

Kinetics of Acid Hydrolysis of Uracil Nucleosides——One milligram each of 2′-O-methyluridine, 3′-O-methyluridine and uridine was dissolved in 6*N* HCl (0.25 ml.) and a part (0.05 ml.) of the solution was heated in a sealed tube at 100° (boiling water bath). After a definite time, 0.2*N* NaOH (ca. 5 ml.) was added to the contents (0.02 ml.) and the extent of the hydrolysis was calculated from the optical densities at 263 mμ and 295 mμ, using molar absorptivities in 0.2*N* NaOH (Table II). The results are shown in Fig. 1. All three nucleosides obeyed first-order kinetics.

Table II. Molar Absorptivity of Uracil Nucleosides in 0.2*N* Sodium Hydroxide

Compound	263 ($\times 10^{-3}$)	295 ($\times 10^{-3}$)
Uracil	4.8	3.3
Uridine 2′-O-Methyluridine 3′-O-Methyluridine	7.6	0.1

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Summary

Methylation of 3′,5′-di-O-trityluridine (I) gave a mixture of two compounds, which was detritylated and then separated to give N^3,2′-O-dimethyluridine (V) and 2′-O-methyluridine (VI). After treatment of the above mixture with ethanolic ammonia, the reaction products were detritylated and separated to give V and 2′-O-methylcytidine (VII). By a similar method 3′-O-methyl nucleosides were obtained from 2′,5′-di-O-trityluridine (II). The relative rates of acidic hydrolysis of uridine, VI, and 3′-O-methyluridine have been determined and were shown to obey first order kinetics. Introduction of a methoxyl function at the 2′- or 3′-position of uridine enhances the stability of the glycosyl linkage to acid hydrolysis.

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