Preparation of *O'*-Alkyl Derivatives of Cytosine and Uracil Nucleosides†

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ABSTRACT: In strongly alkaline medium, 1-substituted cytosines are relatively resistant to ring N_3-alkylation. Dialkyl sulfate treatment of cytidine under these conditions leads predominantly to alkylation of the carbohydrate hydroxyls and, simultaneously, but to a lesser extent, of the exocyclic amino group. Procedures based on this principle are described for the preparation and isolation of some mono-, di-, and tri-*O'*-alkyl (-methyl or -ethyl) derivatives of cytidine. Subsequent bisulfite-catalyzed deamination of the products leads to the corresponding *O'*-alkyl derivatives of uridine. It is furthermore shown that this method is applicable to the preparation of all possible *O'*-methyl and/or *O'*-ethyl analogs of various cytosine and uracil nucleosides, including those not containing cis-vicinal hydroxyl systems, such as deoxynucleosides, arabinosyl nucleosides, etc. The overall findings are compared quantitatively with those based on the use of diazomethane, in relation to the mechanisms of action of the two types of alkylating agents and the participation of ionized sugar hydroxyls. The relatively broad versatility of these procedures and their possible extension to nucleotides, is outlined. Finally, the biological significance and applications of some of the products are pointed out.

The relative resistance to alkylation in strongly alkaline medium of the pyrimidine moiety of 1-substituted cytosines pointed to the possibility of alkylating under these conditions the sugar hydroxyls of cytosine nucleosides (and nucleotides) and, by subsequent deamination, to obtain the corresponding *O'*-alkyluracil nucleosides. The feasibility of this approach has been elsewhere demonstrated (Kuśmierek and Shugar, 1971) and the present communication presents details of the syntheses of some mono-, di-, and tri-*O'*-alkyl- (methyl- or ethyl-)cytosine and uracil ribonucleosides.

The 2'-*O*-methyl nucleosides are relatively widespread in Nature as components of tRNA (Hall, 1971) and, to a lesser extent, of rRNA (Tamaoki and Lane, 1968). The corresponding 2'-*O*-ethyl nucleosides are found in the tRNAs of L-ethionine-induced hepatic carcinoma (Farber, 1963; Ortwerth and Novelli, 1969). Synthetic polynucleotides containing 2'-*O*-methyl residues have proven quite useful in studies on the role of the 2'-hydroxyl in nucleic acid conformation (Bobst *et al.*, 1969; Żmudzka *et al.*, 1969, 1972; Żmudzka and Shugar, 1970, 1971) and in biological systems, *e.g.*, in studies on interferon induction (De Clercq, 1972; Shugar, 1972; De Clercq *et al.*, 1972), in *in vitro* transcription and translation (Gerard *et al.*, 1971; Dunlap 1971).

Potential applications of other *O'*-alkyl nucleosides have been discussed (Shugar, 1972), including studies of enzyme specificity. For example, 5'-*O*-alkyluridines have been employed to develop a ribonuclease substrate totally resistant to phosphodiesterase II (Kole *et al.*, 1971). The resistance to deaminases of *O'*-methylcytidines points to the possibility of development of improved antimetabolites (Darżynkiewicz *et al.*, 1972). The analogs described below are currently being employed in studies on the acidities of sugar hydroxyls and on the conformation of nucleosides. Some of them may prove useful as chain terminators in polymerase reactions.

The present widespread use of diazomethane for methylation of sugar hydroxyls (Broom and Robins, 1965; Khwaja and Robins, 1966; Martin *et al.*, 1968; Gin and Dekker, 1968; Robins and Naik, 1971a,b), a procedure which is extremely useful, is however, limited exclusively to the 2'(3')-*O*-monomethyl derivatives of *ribonucleosides*. The present use of dialkyl sulfate extends the foregoing to nucleosides which do not contain *cis*-hydroxyl groups (*e.g.*, Darżynkiewicz *et al.*, 1972), to nucleotides, and also makes accessible the *O'*-ethyl derivatives.

Results and Discussion

General Methods of Identification of Products. The derivatives described below were identified by various criteria. Ultraviolet (uv) spectra in the pH range 2–12 provided information with regard to the presence or absence of alkylation on the ring nitrogen, or on the exocyclic amino group of cytosine (Brookes and Lawley, 1962; Kulikowski *et al.*, 1969). The absence of any modification in spectrum over the pH range 12–14 provided evidence for alkylation of *all* the ribose hydroxyls (Fox and Shugar, 1952). Periodate oxidation and paper chromatography with a borate solvent differentiated 2'-O- and/or 3'-O-alkylated derivatives. Melting points confirmed the identity of those derivatives previously described in the literature. For several others, elementary analyses were obtained. Column chromatography on Dowex (OH^-) (Dekker, 1965; Gin and Dekker, 1968) provided additional identification. Finally, proton magnetic resonance spectra further confirmed the identity of all *O'*-methyl derivatives.

Mild Methylation of Cytidine. Following mild methylation of cytidine (**1**) with dimethyl sulfate (see Experimental Section), paper chromatography with solvent C (Table I) showed four spots identified as tri-*O'*-methylcytidine (4%), di-*O'*-methylcytidine (21%), mono-*O'*-methylcytidine (47%), and unreacted cytidine (28%). Column chromatography of the monomethylated fraction on Dowex OH^- led to separation of the three monomethylated derivatives. The monomethylated

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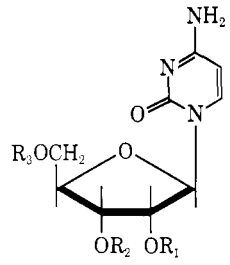
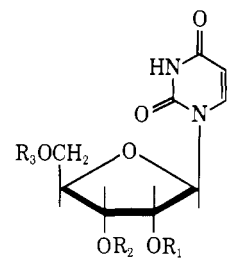


1, $R_1 = R_2 = R_3 = H$
2, $R_1 = CH_3$; $R_2 = R_3 = H$
3, $R_2 = CH_3$; $R_1 = R_3 = H$
4, $R_3 = CH_3$; $R_1 = R_2 = H$
5, $R_3 = C_2H_5$; $R_1 = R_2 = H$
6, $R_1 = R_2 = CH_3$; $R_3 = H$
7, $R_1 = R_2 = C_2H_5$; $R_3 = H$
8, $R_1 = R_2 = R_3 = CH_3$
9, $R_1 = R_2 = R_3 = C_2H_5$



10, $R_1 = R_2 = R_3 = H$
11, $R_1 = CH_3$; $R_2 = R_3 = H$
12, $R_2 = CH_3$; $R_1 = R_3 = H$
13, $R_3 = CH_3$; $R_1 = R_2 = H$
14, $R_3 = C_2H_5$; $R_1 = R_2 = H$
15, $R_1 = R_2 = CH_3$; $R_3 = H$
16, $R_1 = R_2 = C_2H_5$; $R_3 = H$
17, $R_1 = R_2 = R_3 = CH_3$

fraction contained one additional small peak, the uv spectrum of which showed it to be an N_3-methylated derivative, which was discarded.

It should be emphasized that the relative resistance of the ring N_3 to alkylation is not due to competition of the sugar hydroxyls for the alkylating reagent, since 1-methylcytosine is equally resistant to ring alkylation under these conditions.

2'-*O*-Methylcytidine (**2**) and 3'-*O*-methylcytidine (**3**) were isolated on a preparative scale directly from the initial reaction mixture by protracted chloroform extraction in a Soxlet apparatus, followed by fractionation on Dowex OH^-, with yields of 12 and 2.5%, respectively, relative to cytidine (**1**).

The cis-vicinal hydroxyls (2'-OH and 3'-OH) of ribonucleosides exhibit a p*K* value of about 12.5 (Levene and Simms, 1925; Fox and Shugar, 1952; Izatt *et al.*, 1965; Christensen *et al.*, 1970). The latter authors suggested that this relatively low value is due to stabilization of the anion by formation of an intramolecular hydrogen bond, as follows: 3'-OH···2'-$O^- \rightleftharpoons$ 2'-OH···3'-O^-. The p*K* of the 5'-OH was estimated by Gin and Dekker (1968) to be 2–3 units higher, *e.g.*, 15–15.5 for adenosine; but this value may be somewhat high, since spectral studies demonstrate appreciable dissociation of such hydroxyl groups in pyrimidine deoxynucleosides at pH values below 14 (Fox and Shugar, 1952).

Hence, under the conditions herein used for methylation of cytidine, *i.e.*, about 1 N NaOH, all the sugar hydroxyls are partially dissociated, thus allowing methylation to proceed. The observed ratio of 2'-*O*-methylcytidine to 3'-*O*-methylcytidine, about 4:1 (see above) is suggestive of preferential formation of the intramolecularly hydrogen bonded anion 3'-OH···2'-O^- as compared to 2'-OH···3'-O^-.

The results demonstrate that the hydroxyl group most reactive with dimethyl sulfate is the 2'-OH, as for the reaction of cytidine (Martin *et al.*, 1968; Robins and Naik, 1971a) and other ribonucleosides (Khwaja and Robins, 1966; Broom and Rogins, 1965; Robins and Naik, 1971b) with diazomethane. The reactivity of the various hydroxyls of adenosine toward diazomethane was found by Gin and Dekker (1968) to be 2'-OH > 3'-OH > 5'-OH; and this appeared to correlate with the degree of acidity of the three hydroxyls as estimated from the relative affinities towards a strongly basic ion exchange resin of the corresponding *O'*-methyl derivatives.

Exhaustive Alkylation of Cytidine. The principal product of extensive methylation of cytidine is 2',3',5'-tri-*O*-methylcytidine (**8**). Chloroform extracts of the reaction mixture in this instance were found to contain 80% of **8**, 15% of 2',3',5'-tri-*O*-N^4-methylcytidine and 5% of 2',3',5'-tri-*O*-methyluridine (**17**). The presence of the latter is due to the known deamination of cytidine in strongly alkaline medium, encountered during alkaline hydrolysis of RNA. Formation of the derivative with a methylated exocyclic amino group has been discussed elsewhere (Kuśmierek and Shugar, 1971), and shown to be due to direct methylation of the amino group, not to rearrangement (such as the Dimroth rearrangement) of the N_3-methyl derivative.

Several recrystallizations of the chloroform extracts from anhydrous ethanol yielded purified **8**. A completely analogous procedure, with diethyl sulfate as the alkylating agent, gave 2',3',5'-tri-*O*-ethylcytidine (**9**).

Alkylation of Cytidine with Protected Hydroxyls. Diazomethane treatment of ribonucleosides yields little, or only traces, of the 2',3'-di-*O*-methyl and 5'-*O*-methyl derivatives, due to the lower acidity of the 5'-OH, as well as of the 2'(3')-OH once the 3'(2')-OH is methylated. Khwaja and Robins (1966), for example, showed that 2'-deoxyadenosine is not methylated under conditions where adenosine readily methylates on the 2'-OH or 3'-OH. Robins and Naik (1971b) obtained low yields of 2',3'-di-*O*-methyl derivative on treatment with diazomethane of 1-(β-D-ribofuranosyl)-4-methoxypyrimidone-2.

By contrast, alkylation with dimethyl (or diethyl) sulfate, in strongly alkaline medium, of the alkali-stable 2',3'-*O*-isopropylidenecytidine and 5'-*O*-tritylcytidine, provided, after removal of blocking groups, good yields of the 5'-*O*-methyl

TABLE I: Paper Chromatography Studies.[a]

Compound	R_F Value with Solvent				
	A	B	C	D	E
Cytidine	0.60	0.43	0.10	0.50	0.16
2'-*O*-Methyl-	0.70	0.61	0.27	0.58	0.70
3'-*O*-Methyl-	0.70	0.61	0.27	0.58	0.70
5'-*O*-Methyl-	0.70	0.61	0.27	0.58	0.29
5'-*O*-Ethyl-	0.78	0.78	0.43	0.70	0.40
2',3'-Di-*O*-methyl-	0.82	0.79	0.48	0.73	0.81
2',3'-Di-*O*-ethyl-	0.89	0.88	0.64	0.80	0.92
2',3',5'-Tri-*O*-methyl-	0.88	0.86	0.69	0.87	0.90
2',3',5'-Tri-*O*-ethyl-	0.93	0.94	0.85	0.92	0.97
Uridine	0.65	0.30	0.17	0.51	0.16
2'-*O*-Methyl	0.75	0.42	0.36	0.64	0.70
3'-*O*-Methyl-	0.75	0.42	0.36	0.64	0.70
5'-*O*-Methyl	0.75	0.42	0.36	0.64	0.28
5'-*O*-Ethyl	0.81	0.54	0.48	0.74	0.39
2',3'-Di-*O*-methyl	0.86	0.68	0.57	0.78	0.81
2',3'-Di-*O*-ethyl	0.92	0.81	0.77	0.90	0.92
2',3',5'-Tri-*O*-methyl	0.90	0.72	0.74	0.92	0.90

[a] Ascending, with Whatman paper No. 1 and the following solvent systems; all proportions v/v: (A) ethanol–0.5 M ammonium acetate, 5:2; (B) isopropyl alcohol–concentrated NH_4OH (d = 0.88)–water, 7:1:2; (C) 1-butanol–water, 84:16; (D) 1-butanol–acetic acid–water, 5:2:3; (E) isopropyl alcohol–concentrated NH_4 (d = 0.88)–1% H_3BO_3, 7:1:2, with paper previously impregnated with 1% ammonium borate.

TABLE II: Ultraviolet Absorption Spectral Data for Uridine, Cytidine, and Some of Their *O'*-Alkyl Derivatives at pH 2 (0.01 N HCl) and pH 12 (0.01 N NaOH).

Compound	pH	λ_{max} (nm)	ϵ_{max} ($\times 10^{-3}$)	λ_{min} (nm)	ϵ_{min} ($\times 10^{-3}$)
Cytidine	2	280	13.4	241	1.7
	12	271	9.2	249	5.8
2'-*O*-Methyl-	2	280	13.4	241	1.8
	12	271	9.4	250	6.4
3'-*O*-Methyl-	2	280	12.8	241	1.6
	12	271	8.9	250	6.3
5'-*O*-Methyl-	2	280	12.9	241	1.6
	12	271	8.8	249	6.3
5'-*O*-Ethyl-	2	280	12.9	241	1.8
	12	271	9.0	250	6.5
2',3'-Di-*O*-methyl-	2	280	12.9	241	1.3
	12	271	8.7	250	5.8
2',3'-Di-*O*-ethyl-	2	280	13.2	241	1.6
	12	271	9.0	249	6.3
2',3',5'-Tri-*O*-methyl-	2	280	13.2	241	1.6
	12	271	9.0	249	6.4
2',3',5'-Tri-*O*-ethyl-	2	280	13.3	241	1.5
	12	271	9.1	249	6.4
Uridine	2	262	10.0	230	2.1
	12	262	7.2	242	5.2
2'-*O*-Methyl-	2	262	10.2	230	2.3
	12	262	7.5	242	5.6
3'-*O*-Methyl-	2	262	9.7	231	2.1
	12	262	7.1	241	5.1
5'-*O*-Methyl-	2	262	9.9	230	2.0
	12	262	7.3	242	5.4
5'-*O*-Ethyl-	2	262	9.5	230	1.9
	12	262	6.9	242	5.0
2',3'-Di-*O*-methyl-	2	262	10.2	230	2.5
	12	262	7.9	242	5.8
2',3'-Di-*O*-ethyl-	2	262	10.2	230	2.0
	12	262	7.6	242	5.6
2',3',5'-Tri-*O*-methyl-	2	262	10.0	230	2.1
	12	262	7.4	242	5.4

TABLE III: Chemical Shifts (δ, in ppm with Respect to a Sodium 2,2-Dimethyl-2-silapentane-5-sulfonate Internal Standard) for the Methyl Protons in Various *O'*-Methyl Analogs of Cytidine (Deuteriochloride Salt) and Uridine, All in D_2O Solution.

Position of Methylation	Cytidine 2'	3'	5'	Uridine 2'	3'	5'
2'-*O*-Methyl	3.60			3.58		
3'-*O*-Methyl		3.50			3.52	
5'-*O*-Methyl			3.50			3.48
2',3'-Di-*O*-methyl	3.60	3.50		3.58	3.52	
2',3',5'-Tri-*O*-methyl	3.60	3.50	3.50	3.56	3.50	3.50

and 2',3'-di-*O*-methyl (and ethyl) derivatives (**4**, **5**, **6**, **7**). A smaller proportion of the N^4-alkyl analogs was not further studied at this time. Exhaustive methylation (and ethylation) of 5'-*O*-tritylcytidine required addition to the medium of 1,2-dimethoxyethane (and Me_2SO) to improve the solubility of the 5'-trityl derivative.

Deamination of O'-Alkylcytidine Derivatives. All the *O'*-alkylcytidines were converted to the corresponding *O'*-alkyluridines by deamination with sodium bisulfite according to Shapiro *et al.* (1970) and Hayatsu *et al.* (1970). The same procedure was applicable to deamination of N^4-alkyl derivatives, although under somewhat more drastic conditions, a result not altogether surprising in view of the bisulfite catalysis of transamination of cytosine derivatives (Shapiro and Weisgras, 1970; *cf.* Janion and Shugar, 1967). Details are given under Experimental Section.

Ultraviolet Spectra. Ultraviolet spectral data for the protonated and neutral forms of the cytidine derivatives, and for the neutral and monoanionic forms of the uridine analogs, are presented in Table II. Although λ_{max} is invariant for all compounds, it will be noted that the position of methylation in a number of instances leads to modifications of ϵ_{max} values. This testifies to the interaction between the heterocyclic and carbohydrate rings, an effect much more pronounced at pH values above 12, where ionization of the carbohydrate hydroxyl(s) is encountered. A more detailed study of the spectra in this region is under way.

Nuclear Magnetic Resonance (nmr) Spectroscopy. Table III presents data for the chemical shifts of the *O'*-methyl groups in some of the products. It will be noted that the 100-MHz spectrum did not resolve the signals for the 3'- and 5'-*O*-methyl groups in the tri-*O'*-methyl derivatives; the presence of two methyl groups in this case was indicated by the integral absorption of the signal at 3.50 ppm. We have since found that they are resolved in the 220-MHz spectrum, with a difference in chemical shifts of 0.01 ppm, but it has not yet been possible to unequivocally assign the two signals. As might be anticipated, the presence of the *O'*-methyl groups leads to shielding of the ribose nonexchangeable protons. The detailed nmr spectra of the foregoing compounds will be presented elsewhere in relation to the influence of O'-methylation on nucleoside conformation.

Experimental Section

Melting points, uncorrected, were determined on a Boetius microscope hot stage. Ultraviolet absorption spectra were run on a Zeiss (Jena) VSU-2P instrument, using 10-mm pathlength cuvets, and basic data are presented in Table II. Nmr spectra were recorded on a Jeol 100-MHz instrument (see Table III).

Thin-layer chromatography was ascending and, for analytical purposes, made use of HF_{254} silica gel on 7.5 × 2.5 cm plates. For preparative purposes PF_{254} gel was used, about 20 g on a 20 × 20 cm plate. The R_F values should be treated as relative.

Details regarding paper chromatography are given in Table I.

2'(3')-O-Methylcytidine (**2** *and* **3**) *by Mild Methylation of Cytidine.* To 4.86 g (20 mmol) of cytidine (**1**) dissolved in 100 ml of 1 N KOH and vigorously stirred, 5 ml (50 mmol) of dimethyl sufate was added during the course of 1 hr. Following several hours a sample was taken for paper chromatography which, with solvent C (see Table I), exhibited four spots characterized as follows: tri-*O'*-methylcytidine (4%), di-*O'*-methyl (21%), mono-*O'*-methyl (47%), and unreacted

material (28%). All three methylated fractions exhibited uv spectra with the same maxima and minima as cytidine. About 0.5 ml of the reaction mixture was then developed on Whatman No. 3MM paper with the same solvent, and the mono-O'-methylated fraction examined on a 33 × 1.8 cm Dowex 1-X2 (OH^-) column according to the procedure of Dekker (1965). Elution was initially with 30% aqueous methanol at a rate of 1 ml/min, 3-ml fractions being collected. Fractions 50–55 contained N_3-methyl derivative (7%), fractions 60–75 2'-*O*-methylcytidine (65%), and fractions 78–85 3'-*O*-methylcytidine (15%). Further elution was without effect and, at fraction 100, the eluent was changed to 50% methanol. This showed one peak between fractions 230 and 255, corresponding to 5'-*O*-methylcytidine (13%).

The entire reaction mixture was brought to neutrality with 10 N H_2SO_4, concentrated under reduced pressure to about 25 ml, and 150 ml of methanol added to give a precipitate of inorganic salt, which was removed and washed with 3 × 50 ml of methanol. The combined filtrates were mixed with 150 ml of cellulose powder, and the whole was brought to dryness and stored overnight over P_2O_5. The residue was extracted in a Soxlett for 2 days, with 350 ml of chloroform, from which precipitated the mono-*O'*-methyl fraction and traces of the di-*O'*-methyl; the tri-*O'*-, di-*O'*-, and traces of mono-*O'*-methyl fractions remained in the supernatant. Extraction with chloroform was continued for a further 6 days to obtain the monomethylated fraction, as shown by subsequent extraction with methanol, demonstrating the presence only of starting product and traces of the monomethyl derivative.

The combined chloroform extracts, containing the monomethylated fraction and traces of the dimethyl, were brought to dryness (1.47 g), the residue dissolved in 30 ml of 30% aqueous methanol, and deposited on a 55 × 6 cm column of Dowex 1-X2 (200–400, OH^-) previously equilibriated with 30% methanol. Fractions of 100 ml were collected at 10- to 15-min intervals, using 30% methanol as eluent. Fractions 36–42 contained the 2'-*O*-methylcytidine (**2**) and 43–49 the 3'-*O*-methylcytidine (**3**) as well-resolved peaks. Removal of solvent gave 0.63 g (12%) of **2** and 0.14 g (2.5%) of **3**. Crystallization of **2** from anhydrous ethanol gave 0.50 g in the form of colorless bipyramids, mp 251–253°; recrystallization from 85% aqueous ethanol yielded very large colorless bipyramids, mp 256–257°, lit. (Robins and Naik, 1971b) mp 255–258°. Two crystallizations of **3** from anhydrous ethanol resulted in 0.07 g of colorless needles, mp 209.5–210.5°, lit. mp (Robins and Naik, 1971b) 211–212°.

2'-O-Methyluridine (**11**). A solution of 0.35 g (1.36 mmol) 2'-*O*-methylcytidine (**2**) in 7 ml of 2.5 N $NaHSO_3$ was left for 16 hr at 37°. The mixture was diluted with several volumes of water and an excess of $Ba(OH)_2$ was added. The resulting precipitate of $BaSO_2$ was washed twice with water. The combined supernatants were treated with a suspension of Dowex (H^+) to give a strong acid reaction. The resin was removed by filtration and washed several times with water, and the combined filtrates were brought to dryness. The residue was dissolved in 15 ml of ethanol–ethyl acetate (1:1) and stored in a desiccator over P_2O_5 under slight vacuum. Slow evaporation led to crystallization. Filtration of the crystals gave 0.24 g (69%) of colorless needles of 2'-*O*-methyluridine (**11**), mp 158–160°. Recrystallization in the same manner gave a product with mp 160–161°, lit. (Robins and Naik, 1971b) mp 160–161°.

3'-O-Methyluridine (**12**). Deamination of 3'-*O*-methylcytidine (**3**) was carried out as for 2'-*O*-methylcytidine, but all attempts to crystallize **12** were unsuccessful. Thin-layer chromatography (tlc) of the product on HF_{254} silica gel with 85:15 chloroform–methanol showed, in addition to the main product with R_F 0.20, traces of some impurity at the start. The product was therefore separated on PF_{254} silica gel and eluted with methanol. Removal of solvent gave a colorless glass of **12**, which again could not be crystallized. It was dried overnight at 80° over P_2O_5 under vacuum and exhibited satisfactory spectral properties (uv and nmr) as shown in Tables II and III.

2',3',5'-Tri-O-methylcytidine (**8**), *by Exhaustive Methylation of Cytidine*. To 4.86 g (20 mmol) of cytidine (**1**) dissolved in 200 ml of 1.5 N KOH was added, with vigorous stirring, 5-ml portions of dimethyl sulfate at intervals of 15 min. Each portion of dimethyl sulfate was followed immediately by 10 ml of 10 N KOH. Following addition of a total of 50 ml of dimethyl sulfate (500 mmol) and 100 ml of 10 N KOH (during the course of 2.5 hr), the reaction mixture was left overnight at room temperature and extracted with ten 100-ml portions of chloroform. The combined chloroform extracts were washed with water to the point where the alkaline reaction of the aqueous phase had disappeared, dried over anhydrous $MgSO_4$, and brought to dryness (3.2 g) in the form of a glass.

Tlc on HF_{254} silica gel with chloroform–methanol (9:1, v/v) demonstrated the presence of three spots A, B, and C (in the proportion of 80:15:5) with R_F values of 0.4, 0.5, and 0.6, and the following spectral characteristics: for A; pH 2; λ_{max} 280 mm and λ_{min} 241 nm; pH 12; λ_{max} 271 nm and λ_{min} 249 nm; for B: pH 2, λ_{max} 281 nm and λ_{min} 242 nm; pH 12, λ_{max} 271 nm and λ_{min} 247 nm; for C: pH 2, λ_{max} 262 nm and λ_{min} 230 nm; pH 12 λ_{max} 262 nm and λ_{min} 243 nm. Products A and B, in 1 N KOH at 37° for several days, were slowly transformed to C. Product B was unaffected, whereas A was transformed to C on treatment with $NaNO_2$ in 25% acetic acid for 2 days at room temperature. The foregoing data show that C is a product of alkaline deamination (**17**), B is the N^4-methyl derivative, and A is the product methylated exclusively on the ribose, *i.e.*, 2',3',5'-tri-*O*-methylcytidine (**8**).

The glassy mixture, following three crystallizations from anhydrous ethanol, gave 0.40 g (9%) of chromatographically homogeneous A (**8**) in the form of colorless needles, mp 239–241°. *Anal.* Calcd for $C_{12}H_{19}N_3O_5$; C, 50.51; H, 6.71; N, 14.73. Found: C, 50.28; H, 6.60; N, 15.31.

2',3',5'-Tri-O-methyluridine (**17**). The glassy solid obtained from the chloroform extract following methylation of cytidine as above (3.0 g, about 10 mmol) was dissolved in 50 ml of 2.5 M $NaHSO_3$ and heated to 100° for 5 hr in a sealed tube. The solution was cooled to room temperature and added to 150 ml of 1 N NaOH (resultant reaction alkaline). To this solution, after 15 min, was added 150 ml of 1 M $BaCl_2$. The resulting precipitate was filtered and washed with a small quantity of water. The combined aqueous filtrates were acidified with concentrated HCl and then extracted with five portions of 200 ml of chloroform. The combined chloroform extracts were dried over anhydrous $MgSO_4$ and brought to dryness to give 2.49 g of a white glassy solid. This was dissolved in 10 ml of anhydrous ethanol and stored in a desiccator over P_2O_5 under slight vacuum. Following evaporation of most of the ethanol, an oil formed which crystallized slowly. A second crystallization carried out in the same manner gave, finally, 1.08 g (36%) of 2',3',5'-tri-*O*-methyluridine (**17**), in the form of colorless needles, mp 113.5–114.5°. *Anal.* Calcd for $C_{12}H_{18}N_2O_6$: C, 50.32; H, 6.34; N, 9.79. Found: C, 50.28; H, 6.40; N, 10.07.

2',3',5'-Tri-O-ethylcytidine (**9**). To 4.86 g (20 mmol) of

cytidine in 170 ml of 1.5 N KOH, with vigorous stirring was added 8 × 10 ml of diethyl sulfate (total 520 mmol) over a period of about 2 hr. Each portion of sulfate was followed by the addition of 13 ml of 10 N KOH. The reaction mixture was stored overnight at room temperature and then extracted with 10 × 100 ml of chloroform. The combined chloroform extracts were washed with water, dried over anhydrous $MgSO_4$, and brought to dryness. The resulting residue was crystallized twice from anhydrous ethanol to give 0.66 g (11%) of 2′,3′,5′-tri-*O*-ethylcytidine (**9**) in the form of colorless needles, mp 165.5–166°. *Anal.* Calcd for $C_{15}H_{25}N_3O_5$: C, 55.04; H, 7.70; N, 12.83. Found: C, 55.40; H, 7.81; N, 13.21.

2′,3′-O-Isopropylidenecytidine was prepared as described by Fromageot *et al.* (1967), with the exception that the final extraction with chloroform was carried out in a Soxhlet apparatus, as for extraction of the products of mild methylation of cytidine (see above). The final product, obtained in 86% yield as a pale yellow glass, was more than 95% pure by thin-layer chromatography, and was used as such in the alkylation reactions described below.

Methylation of 2′,3′-O-Isopropylidenecytidine. To a stirred solution of 3.6 g (12.7 mmol) of 2′,3′-*O*-isopropylidenecytidine in 150 ml of 1 N KOH was added dimethyl sulfate, in 5 ml portions, followed by 10 ml of 10 N KOH, until tlc with HF_{254} silica gel and chloroform–methanol (9:1) demonstrated disappearance of the starting product. This point was reached following addition of a total of 40 ml (400 mmol) of dimethyl sulfate and 70 ml of 10 N KOH over a period of 6 hr. The mixture was stored overnight and then extracted with 10 × 100 ml of chloroform. The combined extracts were washed with water, dried over $MgSO_4$, and concentrated to dryness under reduced pressure, to give 3.25 g of a yellow oil which, on HF_{254} silica gel with 9:1 chloroform–methanol, showed traces of 2′,3′-*O*-isopropylidenecytidine (R_F 0.2), two intense spots with R_F values of 0.30 and 0.35, and several extremely faint spots with higher R_F values, not identified. The oil was dissolved in 50 ml of chloroform and deposited on HF_{254} silica gel thin-layer plates (340 g of gel), which were developed twice in the same direction with 9:1 chloroform–methanol. The two most intense bands were eluted with methanol to yield 2.14 g (57%) of 2′,3′-isopropylidene-5′-*O*-methylcytidine (R_F 0.30) and 0.67 g (18%) of 2′,3′-*O*-isopropylidene-N^4,5′-*O*-dimethylcytidine (R_F 0.35), both identified by means of their uv absorption spectra.

5′-O-Methylcytidine (**4**). A solution of 1.5 g (5 mmol) of 2′,3′-*O*-isopropylidene-5′-*O*-methylcytidine in 80 ml of 15% aqueous acetic acid was heated on a water bath for 2 hr. The solution was then brought to dryness under reduced pressure, and the residue was dried several times from water and, finally, from several milliliters of concentrated HCl. Two crystallizations from methanol yielded 0.64 g (43%) of the HCl salt of 5′-*O*-methylcytidine (**4**) in the form of colorless needles, mp 190–193°. *Anal.* Calcd for $C_{10}H_{15}N_3O_5 \cdot HCl$: C, 40.90; H, 5.49; N, 14.38. Found: C, 40.89; H, 5.47; N, 14.13.

5′-O-Methyluridine (**13**). A solution of 2.14 g (7.2 mmol) of 2′,3′-*O*-isopropylidene-5′-*O*-methylcytidine in 50 ml of 2.5 M $NaHSO_3$ was stored for 24 hr at 37°. Alkali (1 N KOH) was added to give an alkaline reaction, followed by an excess of 1 M $BaCl_2$, and the precipitated $BaSO_3$ was filtered off and washed with water. The combined filtrates were carefully brought to approximately pH 5 with 1 N HCl and extracted with seven portions of 100 ml of chloroform. The chloroform extract was washed with water, dried with anhydrous $MgSO_4$, and evaporated to yield 1.8 g of 2′,'3-*O*-isopropylidene-5′-*O*-methyluridine in the form of a foamy glass. This residue was dissolved in 50 ml of 10% aqueous acetic acid, and the solution was heated on a water bath for 1.5 hr and then brought to dryness under reduced pressure. The residue was brought to dryness several times from toluene, and then ethanol, following which it was dissolved in 30 ml of anhydrous ethanol and stored in a desiccator over P_2O_5 under slightly reduced pressure. Slow evaporation led to crystallization. A second crystallization in the same manner gave 1.25 g (58%) of 5′-*O*-methyluridine (**13**) in the form of long, colorless, needles, mp 134–135°.

Ethylation of 2′,3′-O-Isopropylidenecytidine. 2′,3′-*O*-Isopropylidenecytidine (3.1 g, 11 mmol) was dissolved in 110 ml of 1 N KOH and, as above, treated with seven portions of 9 ml of dimethyl sulfate (total 420 mmol and 7 × 10 ml of 10 N KOH over a period of 13 hr, the reaction being followed by tlc after each addition. The reaction mixture was left overnight, extracted with 10 × 100 ml of chloroform; and the extracts, following washing with water and drying over $MgSO_4$, were concentrated to give an oil. On HF_{254} silica gel with chloroform–methanol (9:1), the oil exhibited traces of isopropylidenecytidene (R_F 0.2), two intense spots with R_F values of 0.35 and 0.40, and several additional faint spots. The oil was dissolved in 50 ml of chloroform and chromatographed on PF_{254} silica gel plates (360 g of gel) with 9:1 chloroform–methanol. The two most intense bands were eluted with methanol to yield 1.82 g (53%) of 5′-*O*-ethyl-2′,3′-*O*-isopropylidenecytidine (R_F 0.35) and 0.38 g (11%) of N^4-ethyl-5′-*O*-ethyl-2′,3′-*O*-isopropylidenecytidine (R_F 0.40), both of which were identified on the basis of their uv spectra at different pH values.

5′-O-Ethylcytidine (**5**). A solution of 1 g (3.2 mmol) of 5′-*O*-ethyl-2′,3′-*O*-isopropylidenecytidine in 40 ml of 10% acetic acid was heated on a water bath for 3 hr, brought to dryness, and the residue was dried several times from toluene, ethanol, and finally from 10 ml of concentrated HCl. The resulting oil was crystallized from anhydrous ethanol on addition of ethyl acetate. Recrystallization gave 0.43 g (44%) of colorless hygroscopic crystals of the HCl salt of 5′-*O*-ethylcytidine (**5**), mp 110–112°. *Anal.* Calcd for $C_{11}H_{17}N_3O_5 \cdot HCl$: C, 42.93; H, 5.90; N, 13.66. Found: C, 42.58; H, 5.71; N, 13.57.

5′-O-Ethyluridine (**14**). A solution of 1.2 g (3.9 mmol) of 5′-*O*-ethyl-2′,3′-*O*-isopropylidenecytidine in 30 ml of 2.5 M $NaHSO_3$ was maintained for 24 hr at 37°, and then brought to alkalinity with an excess of $Ba(OH)_2$. The precipitated $BaSO_3$ was filtered off and washed with water, and the combined filtrates were brought to about pH 5 with 1 N HCl and extracted with 7 × 100 ml of chloroform. The chloroform extract was washed with water, dried with $MgSO_4$, and concentrated to give 1 g of 5′-*O*-ethyl-2′,3′-*O*-isopropylidenecytidine as an oil, which was dissolved in 30 ml of 10% acetic acid, heated for 2 hr on a water bath, and brought to dryness. The residue was dried twice from water and several times from toluene and anhydrous ethanol, then treated with Norit in boiling ethanol, and finally crystallized twice from anhydrous ethanol to yield 0.50 g (48%) of long, colorless needles of 5′-*O*-ethyluridine (**14**), mp 135–136.5°.

5′-O-Tritylcytidine. This was prepared essentially as described by Andersen *et al.* (1959) for 2′-deoxyadenosine. The final product, crystallized from ethanol–ether, mp 162–165°, in 75% yield, was chromatographically homogeneous.

Methylation of 5′-O-Tritylcytidine. To 5 g (10.3 mmol) of 5′-*O*-tritylcytidine, dissolved in 100 ml of 1,2-dimethoxyethane and 100 ml of 2 N KOH, and vigorously stirred, was added, portionwise, dimethyl sulfate and 10 N KOH. Fol-

lowing each addition, the course of the reaction was checked on HF_{254} silica gel with chloroform–methanol (85:15). The reaction was interrupted when, in addition to the main product (R_F 0.5), traces of the mono-O'-methylated product (R_F 0.4) were visible. This occurred following addition of a total of 20 ml (200 mmol) of dimethyl sulfate and 10 ml of 10 N KOH over a period of 3 hr. At this point, starting product (R_F 0.3) had disappeared, but the mixture contained about 20% of a product with R_F 0.6, which was probably N^4-methylated. The mixture was left several hours at room temperature, concentrated to one-half volume, and extracted with 5 × 100 ml of chloroform. The combined chloroform extracts were washed with 100 ml of water, dried with anhydrous $MgSO_4$ and brought to dryness, to yield 4.8 g (90%) of crude 2',3'-di-*O*-methyl-5'-*O*-tritylcytidine.

2',3'-Di-O-Methylcytidine (**6**). Crude 2',3'-di-*O*-methyl-5'-*O*-tritylcytidine (4.7 g; about 7 mmol of pure compound) was dissolved in 100 ml of 80% acetic acid and heated on a water bath for 2 hr. The solution was brought to dryness, the residue was taken up in water and again brought to dryness, and the latter was repeated several times. The final residue was suspended in 50 ml of water, to which was added several milliliters of concentrated HCl, and the resulting precipitate of tritanol was filtered off and washed with water. The combined filtrates were brought to dryness, the residue was taken up in anhydrous ethanol and brought to dryness, and the latter procedure was repeated several times. The final residue was crystallized (twice) from anhydrous ethanol to give 0.76 g (35%) of the HCl salt of **6** in the form of colorless needles, mp 195–197°. *Anal.* Calcd for $C_{11}H_{17}N_3O_5 \cdot HCl$: C, 42.93; H, 5.90; N, 13.66. Found: C, 42.73; H, 6.12; N, 13.87.

2',3'-Di-O-Methyluridine (**15**). The filtrates left from the crystallization of 2',3'-di-*O*-methylcytidine, above, were brought to dryness, dissolved in 40 ml of 2.5 M $NaHSO_3$, and kept at 100° for 16 hr. The solution was brought to alkalinity with 1 N NaOH and, after 30 min, a slight excess of 1 M $BaCl_2$ was added. The $BaSO_3$ precipitate was washed with water and the combined filtrates were acidified to pH 5 with 1 N HCl and extracted with 20 × 50 ml of chloroform. The chloroform extract was treated as above, following which tlc with HF_{254} silica gel and 9:1 chloroform–methanol demonstrated the presence of about 50% impurities. The extract was therefore concentrated to small volume and chromatographed on PF_{254} plates with 120 g of silica gel, using the 9:1 chloroform–methanol solvent system. The most intense band (R_F 0.3) was eluted with methanol, the latter was removed under reduced pressure and the resulting residue was crystallized from anhydrous ethanol with addition of ethyl acetate to yield 0.05 g of thick colorless needles of 2',3'-di-*O*-methyluridine (**15**), mp 175–176°, lit. (Robins and Naik, 1971b) mp 176–177°.

Ethylation of 5'-O-Tritylcytidine. To a solution of 7.1 g (14.5 mmol) of 5'-*O*-tritylcytidine in 160 ml of Me_2SO and 1,-2-dimethoxyethane (1:1) was added 150 ml of 2 N KOH. To this, with vigorous stirring over a period of 7 hr, was added 27 ml of diethyl sulfate (180 mmol) and 24 ml of 10 N KOH. The reaction mixture was left overnight and extracted with 10 × 100 ml of chloroform. The combined extracts were washed with 5 × 100 ml of water to remove Me_2SO, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to give 7.2 g (~90%) of a glassy material. Tlc demonstrated, apart from the main product, 20% material as the N^4-ethyl derivative.

2',3'-Di-O-Ethylcytidine (**7**). The product of ethylation of 5'-*O*-tritylcytidine, above (7.2 g, about 10 mmol of 2',3'-di-*O*-ethyl-5'-*O*-tritylcytidine), was dissolved in 150 ml of 80% acetic acid and heated 1.5 hr on a water bath, then brought to dryness. The residue was dissolved in the minimal volume of 50% aqueous methanol, which was then concentrated to about one-third volume under reduced pressure. The tritanol was filtered off, the filtrate was strongly acidified with concentrated HCl, and then extracted with 2 × 100 ml of benzene to remove traces of tritanol. The aqueous phase was brought to dryness and the residue was crystallized several times from anhydrous ethanol to yield 0.39 g (10%) of colorless needles of the HCl salt of 2',3'-di-*O*-ethylcytidine (**7**), mp 166–168°. *Anal.* Calcd for $C_{13}H_{21}N_3O_5 \cdot HCl$: C, 46.50; H, 6.31; N, 12.51. Found: C, 46.49; H, 6.27; N, 12.59.

2',3'-Di-O-Ethyluridine (**16**). The filtrates left from the crystallization of 2',3'-di-*O*-ethylcytidine, foregoing section, were brought to dryness, dissolved in 50 ml of 2.5 M NaHSO and heated at 100° for 14 hr. The solution was made alkaline with 1 N NaOH, left for 20 min, and an excess of 1 M $BaCl_2$ was added. The $BaSO_3$ precipitate was washed with water, and the combined filtrates were acidified by addition of concentrated HCl and extracted with 20 × 50 ml of chloroform. The chloroform extract was concentrated to small volume and deposited on PF_{254} silica gel plates (120 g of gel). Following development with chloroform–methanol, the main band (R_F 0.4) was eluted with methanol, the eluate brought to dryness, the residue was dissolved in 15 ml of anhydrous ethanol, which was filtered to remove traces of gel, and subjected to slow evaporation in a desiccator under slightly reduced pressure over P_2O_5. Crystallization occurred readily to yield 0.19 g of 2',3'-di-*O*-ethyluridine (**16**) in the form of colorless needles, mp 140–142°.

Concluding Remarks

It is now pertinent to inquire into the relative merits of the foregoing procedures, both with respect to others described in the literature, and as regards their possible extension.

As concerns the preparation of the 2'-*O*- and 3'-*O*-monomethyl derivatives of cytidine and adenosine, diazomethane treatment in the presence of catalytic amounts of $SnCl_2$ (Robins and Naik, 1971a) is probably the method of choice, and we have verified the high yields obtained.

By contrast, dialkyl sulfate treatment in alkaline medium, while presently limited to cytosine nucleosides (and uridine nucleosides by subsequent deamination), is presently the only suitable procedure for the preparation of (a) 5'-*O*-methyl nucleosides, (b) the di-*O'*-methyl nucleosides, (c) the tri-*O'*-methyl nucleosides, (d) *O'*-methyldeoxy (which we have verified experimentally with deoxycytidine), and other, nucleosides (*e.g.*, Darżynkiewicz *et al.*, 1972), (e) the corresponding *O'*-ethyl derivatives of all the foregoing, as well as combinations of methyl and ethyl.

The dialkyl sulfate method has also proven useful in the direct preparation, in high yield, of the 2'(3')-*O*-methyl (and 2'(3')-*O*-ethyl) derivatives of 5'-CMP; but attempts to fractionate the 2' and 3' isomers at the nucleotide level have hitherto been unsuccessful. Somewhat to our surprise, the mixture of 2'(3')-*O*-methylcytidine 5'-phosphates, following conversion by standard procedures to the pyrophosphates, proved to be a suitable substrate for polynucleotide phosphorylase, yielding poly(2'-*O*-methylcytidylic acid) in good yield, with sedimentation constants higher than 7 (Kuśmierek and Shugar, 1971). The apparent absence of chain termination by the 3'-*O*-methyl isomer is of interest in relation to the recent use of chain terminators with more bulky

substituents in the 3′ (and 2′) positions (Kaufman *et al.*, 1971). This is the object of a separate investigation.

While application of the dialkyl sulfate procedure to alkylation exclusively of the sugar hydroxyls of purine nucleosides will probably prove more complex, it should be possible to circumvent the difficulties by the use of suitable protected aglycone moieties.

Finally, mention should be made of the fact that all three mono-*O*′-methylcytidine derivatives were found to be resistant to enzymatic deamination, using a highly active mouse kidney cytidine deaminase preparation, with cytidine as a control. This fact may be of some significance in relation to 2′-*O*-methylcytidine, which is found in tRNA. Possible antimetabolic activities of all the methylated cytidines and uridines are presently being investigated.

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Added in Proof

In a systematic study on the susceptibility to enzymatic deamination of various *O*′-alkylcytidines, it has been found by Dr. Estera Krajewska in this laboratory that 2′-*O*-methylcytidine is deaminated, at about half the rate for cytidine, by the cytidine deaminase of an extract of *Salmonella typhimurium* strain LT-2. 3′-*O*-Methylcytidine and 5′-*O*-methylcytidine were fully resistant under the same conditions.

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