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The Action of Diazomethane on Ribonucleosides. Preparation of Ribonucleoside 2′- and 3′-Methyl Ethers*

D. M. G. Martin,† C. B. Reese,‡ and G. F. Stephenson†

ABSTRACT: Under the conditions described by Broom and Robins (Broom, A. D., and Robins, R. K. (1965), *J. Am. Chem. Soc. 87*, 1145) both adenosine and cytidine were reacted with diazomethane to give *ca.* three parts of their 2′- to one part of their 3′-*O*-methyl derivatives. 2′- and 3′-*O*-methyluridines have been prepared by deamination of the corresponding cytidine derivatives. The orientations of the 2′- and 3′-methyl ethers of all three nucleosides have been established by nuclear magnetic resonance spectroscopy; the orientations of the uridine derivatives have been confirmed by a chemical method. The stabilities of the glycosidic bonds of adenosine and its 2′- and 3′-methyl ethers are compared.

The study of the effect of methylating agents on nucleic acid components (Haines *et al.*, 1962, 1964; Brimacombe *et al.*, 1965; Griffin *et al.*, 1967) and the development of methods for the selective protection of the 2′- and 3′-hydroxyl functions of ribonucleosides (Reese and Trentham, 1965a; Griffin *et al.*, 1966, 1968; Fromageot *et al.*, 1967; Reese *et al.*, 1967) are two of the aspects of nucleic acid chemistry which have occupied our attention recently. The significance of chemical methylation studies has been considered previously (Haines *et al.*, 1962) and will not be discussed here. The development of methods whereby the secondary hydroxyl functions of a ribonucleoside *cis*-2′,3′-diol system may be differentiated is one of the fundamental problems of ribonucleoside chemistry and is of crucial importance in oligoribonucleotide synthesis (see Griffin *et al.*, 1968). This problem consists essentially of two parts: the first involves a search for reactions which occur specifically at the 2′- or 3′-hydroxyl group or, alternatively, satisfactory methods for the separation of mixtures of 2′- and 3′-isomers; the second part requires the development of a technique (or techniques) by which a pair of 2′- and 3′-isomers may be distinguished.

In connection with the second part of the problem, we have recently proposed a general method of orientation, based on nuclear magnetic resonance spectroscopy (Fromageot *et al.*, 1966), and have also devised a chemical procedure (Reese and Trentham, 1965a) for the orientation of uridine derivatives. However, it seems likely that it will, in general, be difficult to find reagents which attack ribonucleosides *exclusively* on either the 2′- or the 3′-hydroxyl functions, but that it will usually be possible to separate the mixtures of 2′- and 3′-isomers obtained. The electrophilic reagents, toluene-*p*-sulfonyl chloride (Brown *et al.*, 1958) and triphenylmethyl chloride (Reese and Trentham, 1965a), both appear to attack the 2′- approximately twice as fast as they attack the 3′-hydroxyl functions of ribonucleosides and thus, despite their presumably different steric requirements, exhibit similar and only marginal selectivity. No information relating to the behavior of acyl halides and anhydrides (derived from carboxylic acids) is available, as 2′- and 3′-*O*-acyl ribonucleoside derivatives readily isomerize (Reese and Trentham, 1965b) under the reaction conditions.

We were therefore interested in the recent reports by Robins and his coworkers (Broom and Robins, 1965;

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† D. M. G. M. and G. F. S. were holders of a Science Research Council research studentship and research fellowship, respectively.

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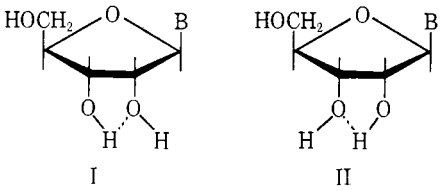
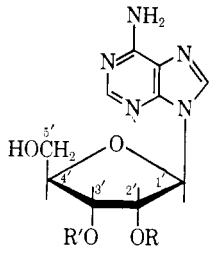
Khwaja and Robins, 1966) on the reaction between diazomethane, which acts as a nucleophilic reagent, and ribonucleosides in hot aqueous 1,2-dimethoxyethane solution. As well as developing a most convenient and valuable procedure for the preparation of the naturally occurring 2′-*O*-methyl ribonucleosides, these workers reported that diazomethane attacked adenosine and certain other ribonucleosides with marked selectivity at the 2′-hydroxyl functions; they suggested that, at most, only small proportions of the 3′-isomers might have been obtained.

It is well known that alcoholic hydroxyl functions are not readily attacked by diazomethane under normal conditions (Schmidt and Zeiser, 1934). However, adenosine has a comparatively acidic function ($pK_a \simeq 12.5$) (Levene *et al.*, 1926), which has been identified (Izatt *et al.*, 1965) as its 2′,3′-*cis*-diol system. It would seem reasonable that the acidity of either the 2′- or the 3′-hydroxyl group of a ribonucleoside could be increased by hydrogen bonding (as in I and II, respectively), but that neither I nor II would be energetically much more favored than the other. As methylation by diazomethane is supposed to result in the replacement of an acidic proton by a methyl group (Eistert, 1941), the reaction between this reagent and adenosine (IIIa) would be expected to lead to a mixture of the isomeric ethers, IIIb and c.



I II



IIIa, R = R′ = H
b, R = Me; R′ = H
c, R = H; R′ = Me

We therefore repeated the methylation of adenosine (IIIa) under the conditions described by Broom and Robins (1965). Examination of the products by thin-layer chromatography revealed approximately equal quantities of starting material and a more mobile component, and a small amount of a third component with an even higher mobility. The products were then allowed to react with an excess of 2,2-dimethoxypropane in the presence of toluene-*p*-sulfonic acid (Hampton, 1961; Fromageot *et al.*, 1967). In this way, the unchanged adenosine was converted into its 2′,3′-*O*-isopropylidene derivative, and the mixture of products was thereby made sufficiently soluble in organic solvents to enable it to be fractionated by chromatography on silicic acid.

TABLE I: Nuclear Magnetic Resonance Spectra[a] of Ribonucleoside 2′- and 3′-Methyl Ethers.

Compound	H(1′) ($J_{1',2'}$)	OCH_3 Protons	Δ[b]
2′-*O*-Methyladenosine (IIIb)	3.93 (6)	6.54	
3′-*O*-Methyladenosine (IIIc)	4.03 (6)	6.43	0.10
2′-*O*-Methylcytidine[c] (IVb)	4.06 (2.8)	6.45	
3′-*O*-Methylcytidine[c] (IVc)	4.11 (3.3)	6.52	0.05
2′-*O*-Methyluridine (Vb)	4.04 (3.9)	6.48	
3′-*O*-Methyluridine (Vc)	4.12 (4.6)	6.52	0.08[d]

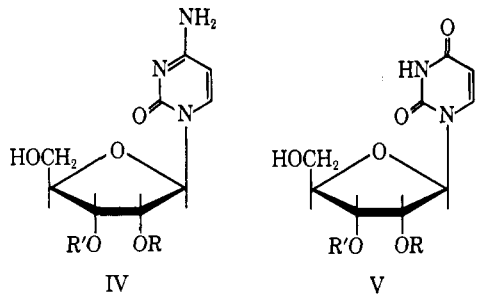
[a] Spectra of 0.15–0.2 M solutions in D_2O were run at 100 Mcps. Chemical shifts are given in parts per million on a τ scale; coupling constants (J) are expressed in cycles per second. [b] Δ = difference in chemical shifts between H(1′) resonances of 2′- and 3′-isomers. [c] The spectra of the cytidine derivatives were taken in acidified (0.1 N with respect to HCl) D_2O to avoid the overlap of the H(1′) and H(5) signals. [d] This value of Δ was not obtained from the spectrum of a mixture of 2′- and 3′-*O*-methyluridines, and may consequently be less accurate.

Following this procedure, the major product was isolated as a thin-layer chromatographically homogeneous glass; when this material was dissolved in ethanol it deposited colorless crystals with properties corresponding closely to those of 2′-*O*-methyladenosine (IIIb), described by Broom and Robins (1965). The nuclear magnetic resonance spectrum of this material (see Table I) in deuterium oxide solution included a doublet at τ 3.93, assignable to the resonance of H(1′) and a singlet at τ 6.54, assignable to the resonance of the methoxyl protons. However, the nuclear magnetic resonance spectrum of the thin-layer chromatographically homogeneous glass included additional signals at τ 4.02 (doublet) and 6.43 (singlet) which were approximately one-third as intense as the signals at τ 3.93 and 6.54, respectively. The immediate conclusion was that the glass was composed of 75% of 2′-*O*-methyladenosine (IIIb) and *25% of 3′-O-methyladenosine (IIIc)*. We recently observed (Fromageot *et al.*, 1966) that the H(1′) resonance of a 2′-ribonucleoside derivative generally occurs at slightly lower field (0.05–0.25 ppm) than that of the corresponding 3′-isomer. This generalization provides additional support for the above conclusion, and for the orientation of the crystalline compound obtained.

It was clearly necessary to isolate and characterize the 3′-*O*-methyladenosine (IIIc). It was hoped that fractionation of the mixture of isomers might be possible by silicic acid chromatography, and it was indeed estab-

lished by nuclear magnetic resonance spectroscopy that the later fractions were richer in the assumed 3′-isomer (IIIc) than the earlier fractions. However, it did not prove possible to achieve a complete separation by this technique. We then learned (C. A. Dekker, private communication) that Gin and Dekker (1968) had come to the same conclusion as us regarding the action of diazomethane on adenosine. The latter workers chromatographed the mixture of products on Dowex 1-X2 (hydroxide form) anion-exchange resin (Dekker, 1965) and isolated both isomers (IIIb and c) in a pure crystalline state. We have also used this procedure to separate the synthetic mixture of isomers, and have thus confirmed our nuclear magnetic resonance estimate that it contained about one part of 3′- to three parts of 2′-*O*-methyladenosine. The 3′-isomer (IIIc) crystallized readily, and had properties corresponding to those described by Tong *et al.* (1967), who had synthesized it by a different route.

Unlike Gin and Dekker (1968), we have not isolated the other methylation products of adenosine. However we have examined the reaction between diazomethane and cytidine under Broom and Robins' (1965) conditions. As cytidine (IVa) is also not readily attacked by diazomethane on its base residue (Haines *et al.*, 1964), we hoped that this would lead to a convenient source of 2′- and 3′-*O*-methylcytidines (IVb and c, respectively), and hence of the corresponding uridine derivatives (Vb and c). Since we commenced this work, synthetic procedures for all four of these compounds have been described (Furukawa *et al.*, 1965), but by a rather less direct route.



IV V

a, R = R′ = H
b, R = Me; R′ = H
c, R = H; R′ = Me

It was found that, for methylation to proceed to a reasonable extent, cytidine required a larger excess of diazomethane than adenosine. The methylated products were again allowed to react with 2,2-dimethoxypropane in the presence of toluene-*p*-sulfonic acid, but it was necessary to use a modified procedure to solubilize the products for silicic acid chromatography (see Experimental Section). In this way a 30% yield of a paper chromatographically homogeneous glass was obtained. Examination of this material by nuclear magnetic resonance spectroscopy suggested that it contained about three parts of 2′- to one part of 3′-*O*-methylcytidine. A solution of this glass in ethanol deposited colorless crystals of the major component, which has been orientated as 2′-*O*-methylcytidine (IVb) on the basis of the chemical shift and splitting of its H(1′) proton (Fromageot *et al.*, 1966) (see Table I). The synthetic mixture of 2′- and 3′-*O*-methylcytidines was not easily susceptible to fractionation by anion-exchange chromatography (Dekker, 1965). However the later fractions, which were found to be enriched in 3′-isomer, were rechromatographed twice to give a small amount of IVc in a pure state. This material readily crystallized from ethanol.

As expected, when 2′-*O*-methylcytidine (IVb) was treated with nitrous acid, it underwent quantitative deamination to give 2′-*O*-methyluridine (Vb). It was found that, although 3′-*O*-methylcytidine (IVc) reacted more slowly with nitrous acid, it could be quantitatively converted into 3′-*O*-methyluridine (Vc). However, the latter compound is not easily accessible by the present approach as its cytidine precursor (IVc) is difficult to obtain in a pure state.

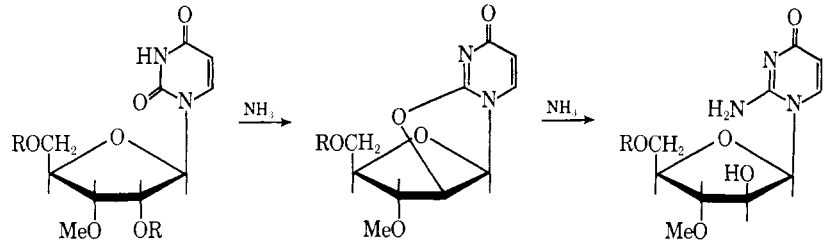
Although both 2′- and 3′-*O*-methylcytidines and the corresponding uridine derivatives were orientated by nuclear magnetic resonance spectroscopy (Fromageot *et al.*, 1966) (see Table I), it was decided to confirm the structural assignments by a chemical method (Reese and Trentham, 1965a). Both the uridine ethers Vb and c were converted into their di-*O*-methanesulfonyl derivatives, and the latter was treated with methanolic ammonia. Whereas the dimesyl derivative of 2′-*O*-methyluridine was unchanged by this ammoniacal treatment, the corresponding 3′-*O*-methyluridine derivative (VI) was converted into a mixture of two more polar products, which had higher electrophoretic mobilities (toward the cathode) in sodium formate buffer at pH 3.5. The rationalization of these observations is indicated in Scheme I. An anhydrouridine derivative (VII) (which then undergoes ammonolysis to give the isocytosine arabinoside (VIII)) can be formed from a 2′- but not from a 3′-methanesulfonate, under the reaction conditions (Reese and Trentham, 1965a). Thus the orientations of 2′- and 3′-*O*-methyluridines (Vb and c, respectively), and hence those of 2′- and 3′-*O*-methylcytidines (IVb and c, respectively) are confirmed.

Finally, the effect of O methylation on the acid stability of the glycosidic linkage of adenosine was examined. The rates of hydrolysis of adenosine and its 2′- and 3′-*O*-methyl derivatives were determined in 1 N hydrochloric acid at 41°. In all cases, first-order kinetics were observed: the half-times of hydrolysis of IIIa, b, and c, were 7.9, 19.6, and 12.8 hr, respectively. Thus while both methyl ethers (IIIb and c) are more resistant to acidic hydrolysis than adenosine (IIIa), methylation on *O*(2′) has a greater stabilizing effect than methylation on *O*(3′). These results correspond to those observed by Furukawa *et al.* (1965) for uridine (Va) and its 2′- and 3′-methyl ethers (Vb and c, respectively), despite the considerably greater acid stability of the latter compounds.

Experimental Section

Nuclear magnetic resonance spectra were run at 100 Mcps on a Varian HA 100 spectrometer. Solutions (0.15–0.2 M) of substrates in D_2O were used, with *t*-bu-

SCHEME I



VI VII, R = $MeSO_2$ VIII

tyl alcohol as internal standard. Chemical shifts were expressed in parts per million on a τ scale; coupling constants, J, are given in cycles per second. (Singlets and doublets are represented by (s) and (d), respectively.) The spectrophotometric estimations, in connection with the hydrolysis experiments, were made with the aid of a Zeiss Model PMQ II ultraviolet spectrometer. Ultraviolet spectra were obtained with a Cary recording spectrometer, Model 14M-50.

Ascending paper chromatograms on Whatman No. 1 paper (No. 42 paper for quantitative purposes) were run in the following solvent systems: (A) butan-1-ol–acetic acid–water (5:2:3), (B) propan-2-ol–ammonia (*d*, 0.88)–water (7:1:2), and (C) butan-1-ol–water (86:14). Paper electrophoresis on Whatman No. 4 paper was conducted in a CCl_4-cooled apparatus in 0.028 M–sodium formate buffer (pH 3.5). Glass plates (20 × 10 cm), coated with Merck Kieselgel GF_{254}, were used for thin-layer chromatography. The chromatograms were developed with methanol–chloroform solutions. Mallinckrodt analytical grade silicic acid (100 mesh) was used for adsorption chromatography.

Action of Diazomethane on Adenosine in Aqueous 1,2-Dimethoxyethane. *N*-Nitrosomethylurea (100 g, 0.97 mole) was added portionwise over a period of 1 hr to a stirred solution of 40% aqueous KOH (600 ml) and 1,2-dimethoxyethane (400 ml), maintained at 0°. The reaction mixture was stirred for a further 20 min and the supernatant yellow solution was poured by decantation onto KOH pellets. The diazomethane solution was filtered and then added in one portion to a solution of adenosine (10 g, 0.038 mole) in water (100 ml), maintained at 80°. A vigorous reaction ensued and the yellow coloration was rapidly discharged. The products were concentrated under reduced pressure, redissolved in water (100 ml), and treated again at 80° with an equal quantity of diazomethane in aqueous 1,2-dimethoxyethane solution. After this process had been repeated once more, thin-layer chromatography ($CHCl_3$–MeOH (3:1, v/v) of the products revealed adenosine, an approximately equal amount of a more mobile component, and a small amount (*ca.* 10%) of a component of even higher R_F.

The products were concentrated under reduced pressure to give an oil which was dissolved in ethanol (100 ml) and the solution was reconcentrated. This reevaporation process was repeated three more times. The final residue was dissolved in anhydrous dimethylformamide (50 ml) and 2,2-dimethoxypropane (20 ml), and the solution was treated with toluene-*p*-sulfonic acid monohydrate (9.4 g, 0.046 mole). The reaction solution was allowed to stand at 20° for 24 hr, neutralized with 2 M methanolic sodium methoxide, and then concentrated under reduced pressure. The residue was dissolved in water (10 ml), and the solution was continuously extracted with chloroform for 16 hr. The dried chloroform extract was applied to a silicic acid column (120 g, 19 cm × 7 cm^2), which was eluted first with 2% MeOH–$CHCl_3$ to give 2′,3′-*O*-isopropylideneadenosine (4.3 g of glass, 37%; mp 214–219° (lit. (Fromageot *et al.*, 1967) mp 220°) after crystallization from ethanol). Subsequent elution with 4% MeOH–$CHCl_3$ gave a mixture of 2′- and 3′-*O*-methyladenosines (3.5 g of glass, 37%) which was paper chromatographically (Table II) homogeneous (R_F 0.78 and 0.67 for systems A and B, respectively). Nuclear magnetic resonance spectrum (D_2O) of the glass included the following signals assignable: (a) to the proton resonances of 2′-*O*-methyladenosine: τ 1.70 (s), 1.88 (s), 3.93 (d, J = 6 cps), and 6.54 (s); (b) to the proton resonances of 3′-*O*-methyladenosine: τ 1.73 (s), 1.92 (s), 4.02 (d, J = 6 cps), and 6.43 (s). All the signals assigned to the 2′-isomer were about three times as intense as the corresponding signals assigned to the 3′-isomer.

In a separate experiment, in which the conditions de-

TABLE II: Paper Chromatography[a] of Ribonucleoside 2′- and 3′-Methyl Ethers.

Compound	R_F Values		
	A	B	C
2′-*O*-Methyladenosine (IIIb)	0.78	0.67	0.47
3′-*O*-Methyladenosine (IIIc)	0.78	0.67	0.47
2′-*O*-Methylcytidine (IVb)	0.72	0.49	0.26
3′-*O*-Methylcytidine (IVc)	0.72	0.49	0.26
2′-*O*-Methyluridine (Vb)	0.69	0.62	0.41
3′-*O*-Methyluridine (Vc)	0.69	0.62	0.41

[a] R_F values are given for ascending chromatography on Whatman No. 1 paper in systems A–C.

scribed by Broom and Robins (1965) for the methylation reaction were followed precisely, the mixture of 2′- and 3′-*O*-methyladenosines was eluted with 6% MeOH–$CHCl_3$. Examination of each fraction by nuclear magnetic resonance spectroscopy revealed that the later fractions were much richer in the 3′-isomer than the earlier fractions. The first fraction was composed of *ca.* 85% of the 2′- and 15% of the 3′-isomer, whereas the last fraction was composed of 60% of the 2′- and 40% of the 3′-isomer.

2′-O-Methyladenosine. A solution of the above mixture of 2′- and 3′-*O*-methyladenosines (3.5 g) in ethanol (20 ml) deposited 2′-*O*-methyladenosine (*Anal.* Calcd for $C_{11}H_{15}N_5O_4$: C, 47.0; H, 5.3; N, 24.9. Found: C, 46.7; H, 5.65; N, 24.7.) as colorless prisms (1.8 g, 17% based on adenosine), mp 199–201°, lit. (Broom and Robins, 1965) mp 200–202°. The ultraviolet absorption in 95% ethanol showed λ_{max} 260 mμ (ε 15,200), λ_{min} 228 mμ (ε 2360). The nuclear magnetic resonance spectrum (D_2O) includes the following signals: τ 1.70 (s) and 1.87 (s), each of weight 1, assigned to H(2) and H(8); τ 3.93 (d, J = 6 cps), weight 1, assigned to H(1′); τ 6.54 (s), weight 3, assigned to methoxyl protons; R_F 0.78 (system A) and 0.67 (system B); $[\alpha]_D^{22}$ −53° (*c* 1, H_2O). This compound gives a negative periodate–Schiff spray test.

Separation of 2′- and 3′-O-Methyladenosines by Anion-Exchange Chromatography. A solution of a mixture of 2′- and 3′-*O*-methyladenosines (3.5 g, prepared as above from 10 g of adenosine) in ethanol–water (5 ml; 2:1, v/v) was applied to a column (50 cm × 3 cm^2) of Dowex 1-X2 (HO^- form) anion-exchange resin. The column was eluted with ethanol–water (2:1, v/v), and 20-ml fractions were collected. Fractions 6–18 were combined and concentrated to give a glass (1.7 g), which was found by nuclear magnetic resonance spectroscopy to be free from 3′-*O*-methyladenosine. A solution of this material in ethanol (20 ml) deposited crystals (1.1 g) of 2′-*O*-methyladenosine, mp 199–200°.

Fractions 29–43 were combined and concentrated to give a glass (0.53 g) which was found by nuclear magnetic resonance spectroscopy to be free from 2′-*O*-methyladenosine. A solution of this material in ethanol (7 ml) deposited 3′-*O*-methyladenosine (*Anal.* Calcd for $C_{11}H_{15}N_5O_4$: C, 47.0; H, 5.3; N, 24.9. Found: C, 46.9; H, 5.5; N, 24.6.) as colorless crystals (0.32 g; 3% based on adenosine), mp 177–180°, lit. (Tong *et al.*, 1967) mp 177–178°. The ultraviolet absorption in 95% ethanol showed λ_{max} 260 mμ (ε 14,600), λ_{min} 228 mμ (ε 2050). The nuclear magnetic resonance spectrum (D_2O) includes the following signals: τ 1.74 (s) and 1.94 (s), each of weight 1, assigned to H(2) and H(8); τ 4.03 (d, J = 6 cps), weight 1, assigned to H(1′); τ 6.43 (s), weight 3, assigned to methoxyl protons; R_F 0.78 (system A) and 0.67 (system B); $[\alpha]_D^{22}$ −56° (*c* 1, H_2O). This compound gives a negative periodate–Schiff spray test.

Determination of the Rates of Acid-Catalyzed Hydrolysis of 2′- and 3′-O-Methyladenosines. A solution of the substrate (0.005 g) in 1 N hydrochloric acid (1 ml), contained in a stoppered vessel, was maintained at 41° by immersion in a Grant constant-temperature water bath. After suitable intervals of time, aliquots (20 μl) of the reaction solution were removed, treated with methanolic ammonia (0.1 ml), and applied to a strip of Whatman No. 42 paper. The chromatogram was developed in system B (ascending) for 12 hr and then dried. The spots corresponding to the starting material and adenine were cut out from the chromatogram, as was an equivalent area of paper required as a blank. Each of the pieces of paper was cut into strips and allowed to stand, with occasional shaking, in a stoppered tube with 0.1 N hydrochloric acid (6 ml) at 20° for 24 hr. The optical densities of the solutions containing starting material and adenine were then measured against the blank at λ_{max}. The mole fractions of both components could then be calculated. Adenosine, 2′-, and 3′-*O*-methyladenosines were found to display simple first-order kinetics in their conversion into adenine; their respective half-times of hydrolysis were 7.9, 19.6, and 12.8 hr.

Action of Diazomethane on Cytidine in Aqueous 1,2-Dimethoxyethane. *N*-Nitrosomethylurea (200 g, 1.94 moles) was added portionwise to a stirred mixture of 40% aqueous KOH (600 ml) and 1,2-dimethoxyethane (750 ml), maintained at 5°. The diazomethane solution was poured, by decantation, in one portion into a stirred solution of cytidine (5 g, 0.019 mole) in water (200 ml) at 80°. A vigorous reaction ensued. The stirred products were allowed to cool and remain at room temperature for 16 hr before being concentrated under reduced pressure. The residue was dissolved in aqueous ethanol, and the solution was reconcentrated.

This procedure was repeated three times with absolute ethanol, and the final residue was shaken with toluene-*p*-sulfonic acid monohydrate (5.72 g, 0.03 mole) at 20° for 15 min. After the reaction mixture had been allowed to remain at 20° for a further period of 1 hr, the required amount of 2 M methanolic sodium methoxide was added to neutralize the acid. The products were concentrated to dryness, dissolved in pyridine (30 ml), and treated with acetic anhydride (29 ml). The reactants were shaken mechanically for 12 hr and then methanol (25 ml) was added. After 30 min the products were concentrated under reduced pressure, the residue was partitioned between chloroform and water, and the aqueous layer was then extracted twice with chloroform. The combined chloroform layers were dried, concentrated, and applied to a silicic acid column (40 g, 8 cm × 6 cm^2) which was first eluted with chloroform. The eluate was concentrated to a glass (5.1 g) which was redissolved in 4.5 M methanolic dimethylamine (200 ml).

The latter solution was allowed to stand at 20° for 9 hr, then concentrated under reduced pressure, and the residue was redissolved in the minimum volume of methanol. Chloroform was added and the solution was concentrated at atmospheric pressure (to remove most of the methanol) before it was applied to a silicic acid column (60 g, 25 cm × 2.5 cm^2). After the 2′,3′-*O*-isopropylidenecytidine had been eluted with 5% MeOH–$CHCl_3$, the desired mixture of 2′- and 3′-*O*-methylcytidines (1.56 g of glass, 30%) was eluted with 10% MeOH–$CHCl_3$. The latter material was paper chroma-

tographically homogeneous in systems A and B (R_F 0.72 and 0.49, respectively). The nuclear magnetic resonance spectrum (D_2O–HCl) of the glass included the following signals assignable: (a) to the proton resonances of 2′-*O*-methylcytidine: τ 1.80 (d, $J \simeq 8$ cps), 3.76 (d, $J \simeq 8$ cps), 4.04 (d, $J \simeq 3$ cps), and 6.46 (s); (b) to the proton resonances of 3′-*O*-methylcytidine: τ 1.82 (d, $J \simeq 8$ cps), 4.13 (d, $J \simeq 3.5$ cps), and 6.54 (s). All the signals assigned to the 2′-isomer were about three times as intense as the corresponding signals assigned to the 3′-isomer.

2′-O-Methylcytidine. A solution of the above mixture of 2′- and 3′-*O*-methylcytidines (1.56 g) was crystallized from ethanol to give *2′-O-methylcytidine* (*Anal.* Calcd for $C_{10}H_{15}N_3O_5$: C, 46.7; H, 5.8; N, 16.3. Found: C, 46.7; H, 5.4; N, 16.6.) as colorless rhomboids (0.713 g; 13.5% based on cytidine), mp 252–253°. The ultraviolet absorption in 95% ethanol showed λ_{max} 273 mμ (ϵ 8190), λ_{infl} 231 mμ (ϵ 7200), and λ_{min} 252 mμ (ϵ 6250); in 0.1 N hydrochloric acid: λ_{max} 278 (ϵ 13,700), λ_{min} 240 mμ (ϵ 1870). The nuclear magnetic resonance spectrum (D_2O, 0.1 N with respect to HCl[1]) includes the following signals: τ 1.71 (d, $J \simeq 8$ cps), weight 1, assigned to H(6); τ 3.74 (d, $J \simeq 8$ cps), weight 1, assigned to H(5); τ 4.06 (d, $J = 2.8$ cps), weight 1, assigned to H(1′); and τ 6.45 (s), weight 3, assigned to methoxyl protons. This compound gives a negative periodate–Schiff spray test, R_F 0.72 (system A) and 0.49 (system B).

Separation of 2′- and 3′-O-Methylcytidines by Anion-Exchange Chromatography. The ethanol mother liquors, obtained after some 2′-isomer had crystallized from a mixture of 2′- and 3′-*O*-methylcytidines, were concentrated to a glass (0.49 g) and dissolved in water (5 ml), and the solution was applied to a column (150 cm × 6 cm²) of Dowex 1-X2 (HO^- form) anion-exchange resin. The column was eluted with ethanol–water (2:1, v/v) at a flow rate of *ca.* 50 ml/hr. Fractions (20 ml) were collected. Fractions 24–30 were combined and concentrated under reduced pressure to give a glass (0.11 g), which was found by nuclear magnetic resonance spectroscopy to be pure 2′-*O*-methylcytidine. Fractions 31–45 were similarly concentrated to a glass (0.28 g), which was found by nuclear magnetic resonance spectroscopy to contain about one part of 2′-isomer to two parts of 3′-isomer.

The latter mixture was rechromatographed on the same column. Fractions 29–33 gave a glass (0.023 g) composed solely of 2′-isomer, and fractions 34–44 gave a glass (0.22 g) further enriched in 3′-isomer. This material was rechromatographed again on the same column. Fractions 37–44 were combined and found to contain solely 3′-isomer (0.11 g). The latter material crystallized from ethanol to give 3′-*O*-methylcytidine (*Anal.* Calcd for $C_{10}H_{15}N_3O_5$: C, 46.7; H, 5.8; N, 16.3. Found: C, 46.4; H, 5.6; N, 16.6.) as colorless needles, mp 211–212°. The ultraviolet absorption in 95% ethanol showed λ_{max} 273 mμ (ϵ 8450), λ_{infl} 232 mμ (ϵ 7250), and λ_{min} 253 mμ (ϵ 6480); in 0.1 N hydrochloric acid: λ_{max} 279 mμ (ϵ 13,400), λ_{min} 240 mμ (ϵ 1670). The nuclear magnetic resonance spectrum (D_2O, 0.1 N with respect to HCl) includes the following signals: τ 1.78 (d, $J = 8$ cps), weight 1, assigned to H(6); τ 3.73 (d, $J = 8$ cps), weight 1, assigned to H(5); τ 4.11 (d, $J = 3.3$ cps), weight 1, assigned to H(1′); and τ 6.52 (s), weight 3, assigned to methoxyl protons. This compound gives a negative periodate–Schiff spray test, R_F 0.72 (system A) and 0.49 (system B).

2′-O-Methyluridine. Potassium nitrite (0.80 g) was added to a solution of 2′-*O*-methylcytidine (0.20 g) in 25% aqueous acetic acid (2 ml), maintained at 20°. After 5 hr, thin-layer chromatography ($CHCl_3$–MeOH (3:1, v/v) revealed a single product and the absence of starting material. The reaction solution was then neutralized with solid sodium bicarbonate, and continuously extracted with chloroform for 18 hr. When the oil, obtained by evaporation of the dried ($MgSO_4$) chloroform extract, was dissolved in ethyl acetate, colorless crystals of *2′-O-methyluridine* (0.13 g, 65%) (*Anal.* Calcd for $C_{10}H_{14}N_2O_6$: C, 46.5; H, 5.5; N, 10.8. Found: C, 46.6; H, 5.7; N, 11.2.) were obtained, mp 157–158.5°, lit. (Furukawa *et al.*, 1965) mp 159°. The ultraviolet absorption in 95% ethanol showed: λ_{max} 263 mμ (ϵ 10,100), λ_{min} 232 mμ (ϵ 2020). The nuclear magnetic resonance spectrum (D_2O) includes the following signals: τ 2.06 (d, $J = 7.8$ cps), weight 1, assigned to H(6); τ 4.04 (d, $J = 3.9$ cps), weight 1, assigned to H(1′); τ 4.11 (d, $J = 8.0$ cps), weight 1, assigned to H(5); and τ 6.48 (s), weight 3, assigned to methoxyl protons. This compound gives a negative periodate–Schiff spray test, R_F 0.69 (system A) and 0.62 (system B).

3′-O-Methyluridine. Potassium nitrite (0.10 g) was added to a solution of 3′-*O*-methylcytidine (0.10 g) in 25% aqueous acetic acid (1 ml), maintained at 20°. Two additional portions of potassium nitrite (each of 0.02 g) were added after 6 and 9 hr, and more 25% aqueous acetic acid (1 ml) was added after 12 hr. After a further period of 24 hr, thin-layer chromatography ($CHCl_3$–MeOH (3:1, v/v)) indicated that no starting material remained. The products were neutralized and extracted with chloroform as above. The dried (Na_2SO_4) chloroform extract was evaporated to an oil (0.09 g) which was first crystallized from ethanol–ethyl acetate and then recrystallized from methanol to give *3′-O-methyluridine* (*Anal.* Calcd for $C_{10}H_{14}N_2O_6$: C, 46.5; H, 5.5; N, 10.8. Found: C, 46.25; H, 5.5; N, 10.7.), mp 143–144.5°, lit. (Furukawa *et al.*, 1965) mp 142–145°. The ultraviolet absorption in 95% ethanol showed: λ_{max} 263 mμ (ϵ 9380), λ_{min} 230 mμ (ϵ 1760). The nuclear magnetic resonance spectrum (D_2O) includes the following signals: τ 2.13 (d, $J = 7.9$ cps), weight 1, assigned to H(6); τ 4.12 (d, $J = 8$ cps), weight 1, assigned to H(5); τ 4.12 (d, $J = 4.6$ cps), weight 1, assigned to H(1′); and τ 6.52 (s), weight 3, assigned to methoxyl protons. This compound gives a

[1] The chemical shifts of H(5) and H(6) in cytidine derivatives are very sensitive to acid (Fromageot *et al.*, 1966). Previously, the nuclear magnetic resonance spectrum (at 60 Mcps) of 2′-*O*-methylcytidine had been measured (a) in D_2O and (b) after the original solution had been made *ca.* 0.1 N with respect to HCl by the addition of 10 N hydrochloric acid. The principal signals in the two spectra were as follows: (a) τ 2.16 (d, $J = 7.8$ cps), 3.99 (d, $J \simeq 8$ cps), 4.08 (d, $J \simeq 3$ cps), and 6.48 (s); (b) τ 1.85 (d, $J \simeq 8$ cps), 3.76 (d, $J \simeq 8$ cps), 4.05 (d, $J \simeq 3$ cps), and 6.48 (s).

negative periodate–Schiff spray test, R_F 0.69 (system A) and 0.62 (system B).

Action of Methanolic Ammonia on the Dimethanesulfonates of 2'- and 3'-O-Methyluridines. Solutions of (a) 2'-*O*-methyluridine (0.005 g) and (b) 3'-*O*-methyluridine (0.005 g) in anhydrous pyridine (1 ml) were prepared. Methanesulfonyl chloride (0.02 ml, 10 mole equiv) was added to each solution, and the reaction flasks were sealed and allowed to stand at 0°.

The following work-up procedure was adopted for both reactions. After 12 hr, water (0.2 ml) was added and, after a further period of 1 hr, the products were concentrated under reduced pressure, dissolved in water (1 ml), and the solution was reevaporated. This process was repeated, the residue was dissolved in water (1 ml), and the solution was extracted with dichloromethane (three 1-ml portions). In both cases, thin-layer chromatography ($CHCl_3$–MeOH (85:15, v/v)) of the dried (Na_2SO_4) organic extract revealed a single component (R_F 0.73) and no starting material (R_F 0.44).

The dichloromethane solutions were evaporated to dryness and dissolved in saturated methanolic ammonia at 0°. After 36 hr, thin-layer chromatography ($CHCl_3$–MeOH (85:15, v/v)) of the products derived from 2'-*O*-methyluridine revealed one component (R_F 0.73), whereas thin-layer chromatography of the products derived from 3'-*O*-methyluridine revealed two components (R_F 0.19 and 0.26). The products from both reactions were submitted to paper electrophoresis (sodium formate buffer, pH 3.5; 60 V/cm) on Whatman No. 4 paper for 2 hr. The material derived from 2'-*O*-methyluridine migrated as a single component, with a mobility of 5.5 cm toward the cathode (mobility of 2'-*O*-methyluridine is also 5.5 cm); the material derived from 3'-*O*-methyluridine migrated as a single component, with a mobility of 9.0 cm toward the cathode.

Acknowledgment

We thank Dr. C. A. Dekker for communicating his results to us, prior to publication.

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