"Single Addition" Substrates for the Synthesis of Specific Oligoribonucleotides with Polynucleotide Phosphorylase. Synthesis of 2′-*O*-(α-Methoxyethyl)nucleoside 5′-Diphosphates†

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ABSTRACT: A number of synthetic methods for the preparation of the 2′-*O*-(α-methoxyethyl) derivatives of the 5′-diphosphates of adenosine, cytidine, guanosine, and uridine have been studied in order to provide nucleotide substrates that can be applied to the synthesis of specific oligoribonucleotides using polynucleotide phosphorylase. The reaction of nucleoside 5′-diphosphates with methyl vinyl ether for a limited time produces low yields of the corresponding 2′-*O*-(α-methoxyethyl) derivatives because the rate of methoxyethylation of the 3′-hydroxyl groups is two to three times that of the 2′-hydroxyl groups. A study of the rates of acidic hydrolysis of α-methoxyethyl groups in the 2′ and 3′ positions of nucleosides and nucleotides has been made, and the results obtained form the basis of a more efficient method for the synthesis of the blocked nucleoside diphosphates. The method involves the reaction of nucleoside 5′-diphosphates with methyl vinyl ether to give the corresponding 2′,3′-di-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates, and exploits the fact that, in the acidic hydrolysis of these derivatives, the rate of removal of the 3′-methoxyethyl group is about twice that of the group in the 2′ position. Alternative syntheses were based on the phosphorylation of methoxyethylated nucleosides and nucleotides. The derivatives, 2′-*O*- and 2′,3′-di-*O*-(α-methoxyethyl)uridine, were prepared by the methoxyethylation of 3′,5′-di-*O*-acetyluridine and 5′-*O*-acetyluridine followed by removal of the acetyl groups. The corresponding guanosine derivatives were made by the synthetic routes: (i) guanosine → $O^{2'},O^{3'},O^{5'},N^2$-tetrabenzoylguanosine → 2-*N*-benzoylguanosine → $O^{3'}$-acetyl-$N^2,O^{5'}$-dibenzoylguanosine → 2′-*O*-(α-methoxyethyl)guanosine, and (ii) 2′,3′-*O*-isopropylideneguanosine → $N^2,O^{5'}$-diacetyl-2′,3′-*O*-isopropylideneguanosine → $N^2,O^{5'}$-diacetylguanosine → 2′,3′-di-*O*-(α-methoxyethyl)guanosine. These methoxyethylated nucleosides were converted to the corresponding 5′-phosphates by reaction with cyanoethyl phosphate and dicyclohexylcarbodiimide, and then to the corresponding 5′-diphosphates by subsequent reaction with 1,1′-carbonyldiimidazole and inorganic phosphate.

Recent studies in this laboratory have been concerned with a new approach to the synthesis of oligoribonucleotides of defined sequence (Mackey and Gilham, 1971; Bennett et al., 1973; Sninsky et al., 1974). The basis of the method involves the use of polynucleotide phosphorylase to catalyze the successive addition of nucleotide moieties to an acceptor oligonucleotide. The synthetic system employs monomers (2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates) containing a chemical blocking group that permits the enzymatic addition of single nucleotide units to the acceptor oligonucleotide and prevents the subsequent addition of further nucleotide residues. The blocking group can be removed from the terminus of the product to yield a new acceptor molecule that is then available for further single addition reactions. In the earlier studies, the syntheses of the monomer substrates were accomplished by the partial methoxyethylation of nucleoside 5′-diphosphates with methyl vinyl ether, followed by paper chromatographic separation of the products. Although this synthetic method requires relatively little effort, it is somewhat unsatisfactory in that the reaction produces low yields of the 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates because of the formation of large amounts of other products (the corresponding 3′ isomers and the 2′,3′-di-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates). The present work describes both the characterization of the four common 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates and a number of more efficient methods for their preparation.

Methoxyethylation of Nucleoside 5′-Diphosphates. The exposure of a nucleoside 5′-diphosphate to methyl vinyl ether in the presence of an acid catalyst produces a mixture of the two monomethoxyethyl derivatives as well as some of the dimethoxyethyl derivative (Mackey and Gilham, 1971). The yield of the 2′ isomer is quite low (5–15%) because the rate of its formation is about one-third that of the 3′ isomer. Since it has been subsequently shown that the 2′ isomer is the enzymatically active species in the synthesis of oligonucleotides (Bennett et al., 1973), it has become necessary to seek procedures by which the yield of this isomer can be increased. For reasons discussed below, it was considered that the partial hydrolysis of 2′,3′-di-*O*-(α-methoxyethyl)nucleoside diphosphates should produce a higher yield of the corresponding 2′ isomers. These disubstituted products were readily prepared by the reaction of the diphosphate with methyl vinyl ether for a longer time than was used in the partial methoxyethylation reactions, and the derivatives of the 5′-diphosphates of adenosine, cytidine, and uridine were isolated in 70–80% yield. Initially, some difficulty was experienced in the preparation of the corresponding guanosine derivative in that one of the major products of the reaction had λ_{max}, 270–275 nm, indicating that some modification of the guanine ring system had occurred. This product has

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an ultraviolet spectrum similar to that of 5-alkylformamido-2,4-diamino-6-hydroxypyrimidines that result from the treatment of 7-alkylguanosines with alkali (Lawley and Brookes, 1963). Thus, in the methoxyethylation reaction, the product may have arisen from alkylation at N-7 followed by opening of the imidazole ring at C-8. The structure of this modified guanosine diphosphate is probably analogous to that of a side product (λ_{max}, 275 nm) that was observed to result from the reaction of ethyl vinyl ether with guanosine 3′-phosphate (Brimacombe et al., 1968). In the current study of the methoxyethylation of nucleoside diphosphates, the acid-catalyzed reactions were normally terminated by the addition of aqueous ammonia, and it was subsequently found that, in the case of the guanosine derivative, the yield of the product with the modified base was substantially reduced when water was excluded from the reaction mixture and the termination was effected with triethylamine. Under these conditions, 2′,3′-di-*O*-(α-methoxyethyl)guanosine 5′-diphosphate (di-ME-GDP[1]) can be prepared in 50% yield.

The four di-*O*-(α-methoxyethyl) derivatives were characterized by their R_f values (Table I), their ^1H nuclear magnetic resonance (NMR) spectra (Table II), and by a study of their conversion to the corresponding nucleoside 5′-diphosphates by mild acid hydrolysis. The assignment of the resonance positions of the protons in the α-methoxyethyl groups of the compounds, di-ME-adenosine, 2′-ME-, and 3′-ME-adenosine, has already been discussed (Bennett et al., 1973), and it was shown that the resonances of the OCH_3, CH, and CCH_3 protons in 2′-ME-adenosine are shifted upfield relative to those in the 3′ isomer, while the H-1′ resonance of the 2′ isomer is shifted downfield relative to that of the 3′ isomer. It will be noted that these effects are also seen in the di-ME derivatives of nucleoside diphosphates. For example, each of these derivatives displays a pair of resonances with lower δ values that correspond to the OCH_3 protons of the 2′-ME group and a resonance with a higher δ value corresponding to the same protons of the substituent group in the 3′ position. In addition, the differences between the chemical shifts of the 2′ and 3′ substituent groups for the purine derivatives are larger than those for the pyrimidine derivatives, a property that probably results from larger shielding effects of the ring currents in the purine compounds. In the earlier study on the methoxyethyladenosines, it was suggested that these differences in the δ values could be understood in terms of a model that permits only the methoxyethyl group at the 2′ position to be located within the positive shielding cone of the heterocyclic ring system. The dual resonances for the OCH_3 protons of the group in the 2′ position can be considered to be a consequence of differences in the interaction of the base, in each case, with the two diastereomeric configurations of the methoxyethyl group.

Partial Hydrolysis of Di-O-(α-methoxyethyl)nucleoside 5′-Diphosphates. The exposure of the di-ME-nucleoside diphosphates to ammonium formate at pH 3.5 results in partial hydrolysis to give, together with the unchanged starting material, the two monosubstituted derivatives and the parent nucleoside diphosphate. All four products can be readily separated by paper chromatography, and the results of a study of the time course of these hydrolyses are shown graphically in Figures 1 and 2. It will be noted that, at a time when only one-half of each disubstituted compound has undergone hydrolysis, there is about a 30% yield of the 2′-ME derivative and 10–15% yield of the corresponding 3′ isomer. Although 2′-ME-UDP and 3′-ME-UDP are not well separated by the analytical method used, it can be shown, by extended paper chromatography, that the ratio of the amount of the 2′ isomer to that of the 3′ isomer formed in this case is also equal to about 2. These studies, then, form the basis of a more efficient method for the production of the enzymatically active 2′ isomers. In addition, the efficiency of the synthetic method is enhanced by the fact that a substantial quantity of the unchanged di-ME derivative can be readily isolated from a patial hydrolysis in each case, and then subjected to a second partial hydrolysis.

Table I: R_f Values of Nucleotide Derivatives.[a]

Nucleotide	Solvent A[b]	Solvent B[c]	Solvent C[d]	Solvent D[e]
ADP	1.00	1.00	1.00	1.00
2′-ME-ADP	2.30	1.20	1.77	1.04
3′-ME-ADP	1.97	1.12	1.52	0.77
Di-ME-ADP	2.97	1.29	2.35	0.78
CDP	0.92	0.90	0.96	1.99
2′-ME-CDP	2.15	1.19	1.79	2.09
3′-ME-CDP	1.72	1.14	1.54	1.85
Di-ME-CDP	3.18	1.30	2.31	1.91
GDP	0.44	0.78	0.58	1.78
2′-ME-GDP	1.31	1.30	1.36	1.93
3′-ME-GDP	1.09	1.18	1.15	1.48
Di-ME-GDP	1.91	1.58	1.88	1.60
UDP	0.85	1.00	0.90	2.30
2′-ME-UDP	1.80	1.29	1.50	2.13
3′-ME-UDP	1.67	1.29	1.50	2.13
Di-ME-UDP	2.28	1.52	2.10	1.96
GMP	0.75	0.83	0.70	1.67
Di-ME-GMP	2.80	2.20	1.70	1.34
UMP	1.30	1.13	1.04	2.25
Di-ME-UMP	3.60	1.85	1.94	1.60

[a] The values given are relative to the R_f of ADP for each solvent system and were determined by descending paper chromatography on Whatman No. 1 paper (for the 5′-diphosphates) and on Whatman 3MM paper (for the 5′-phosphates). [b] Isopropyl alcohol–concentrated NH_4OH–water (7:1:2, v/v). [c] *n*-Propyl alcohol–concentrated NH_4OH–water (55:10:20, v/v). [d] Isopropyl alcohol–concentrated NH_4OH–water (6:1:3, v/v). [e] A mixture of water (100 ml) and $(NH_4)_2SO_4$ (40 g) adjusted to pH 8.5 with concentrated NH_4OH.

The characterization of the various 2′-ME and 3′-ME derivatives was accomplished by paper chromatography (Table I), ^1H NMR analysis (Table II), and by the comparison of their R_f values with derivatives obtained by different synthetic routes. In those chromatographic solvent systems in which the 2′-ME and the 3′-ME isomers can be separated (Table I), the 2′ isomer has the larger R_f value in each case. In addition, all of the 2′ isomers have been shown to be active in the single addition reaction catalyzed by polynucleotide phosphorylase. The identity of 2′-ME-ADP and 3′-ME-ADP has been previously established by dephosphorylation of the derivatives to the corresponding methoxyethyladenosines (Bennett et al., 1973), and the ^1H NMR data for the methoxyethyl groups in 2′-ME-ADP, 3′-ME-ADP, 2′-ME-CDP, and 3′-ME-CDP (Table II) follow the patterns discussed above for the corresponding di-ME derivatives. The structures of the monomethoxyethyl derivatives of GDP and UDP were assigned on the basis of a comparison of their properties with 2′-ME-GDP and 2′-ME-UDP that were synthesized by the phosphorylation of the appropriately substituted nucleosides as described below.

[1] Abbreviations used are: 2′-ME-, 2′-*O*-(α-methoxyethyl)-; 3′-ME-, 3′-*O*-(α-methoxyethyl)-; di-ME-, 2′,3′-di-*O*-(α-methoxyethyl)-.

Table II: ^{1}H NMR Spectra Data.

Compound	Chemical Shifts[a]				
	H-1′[b] (d)	H-6 (d)	H-8 (s)	CCH_3 (d)	OCH_3 or CH_3CO (s)
ADP	6.10 (4.3)		8.47		
2′-ME-ADP	6.20 (5.2)		8.50	1.25	3.02, 2.93
3′-ME-ADP	6.10 (5.1)		8.48	1.47	3.47
Di-ME-ADP	6.20 (6.3)		8.52	1.47, 1.27	3.48, 3.00, 2.80
CDP	5.98 (3.4)	7.97			
2′-ME-CDP[c]		7.97		1.35, 1.30	3.27, 3.25
3′-ME-CDP[c]		7.95		1.38	3.42
Di-ME-CDP[c]		7.95		1.35	3.42, 3.27, 3.20
GDP	5.92 (4.6)		8.10		
Di-ME-GDP	5.98 (6.3)		8.13	1.43, 1.28	3.47, 3.08, 2.95
UDP[c]		7.97			
Di-ME-UDP[c]		7.97		1.33	3.42, 3.33, 3.25
2′,3′-Di-*O*-(α-methoxyethyl)guanosine	5.88 (6.4)		8.02	1.32, 1.17	3.30, 2.95
2′-*O*-(α-Methoxyethyl)guanosine	5.86 (6.3)		7.98	1.22, 1.17	2.98
2′,3′-Di-*O*-(α-methoxyethyl)uridine[c]	5.67–5.97	7.94		1.17–1.30	3.13–3.32
2′-*O*-(α-Methoxyethyl)uridine[c]	5.66–5.97	7.97		1.26	3.18
2′,3′-*O*-Isopropylideneguanosine	5.99 (2.5)		7.97	1.56, 1.36 (s)	
N^2,$O^{5'}$-Diacetyl-2′,3′-*O*-isopropylideneguanosine	6.15 (1.7)		8.17	1.58, 1.38	2.25, 2.01
N^2,$O^{5'}$-Diacetylguanosine	5.88 (4.9)		8.19		2.23, 2.07

[a] Chemical shifts are given in ppm downfield from sodium 2,2-dimethylsilapentane-5-sulfonate as internal standard. The spectra were recorded on a Varian A-60A spectrometer with a sample concentration of 0.15 *M* in D_2O (pD = 7.4) for the nucleotide derivatives, and a concentration of 0.2 *M* in Me_2SO-d_6–D_2O (9:1, v/v) for the nucleoside derivatives. [b] The $J_{H\text{-}1'\text{-}H\text{-}2'}$ values in Hz are given in parentheses. [c] In these cases the H-1′ resonance is obscured by overlap with the H-5 resonance.

The rates of acidic hydrolysis of some methoxyethyl derivatives are listed in Table III. While the presence of the diphosphate group (or phosphodiester group in the case of the tetranucleotide) in the 5′ position decreases the stability of methoxyethyl groups, the group in each 3′ isomer is removed at a faster rate than the group in the corresponding 2′ isomer. In addition, it can be seen from Figures 1 and 2 that the 3′-ME group is also hydrolyzed at a faster rate in each di-ME-nucleoside diphosphate. Thus, in both the acid-catalyzed methoxyethylation and the acid-catalyzed demethoxyethylation of all of these compounds, it is the 3′ position that is the most reactive. This observation can be rationalized by considering that the 3′-hydroxyl group is more basic than the 2′-hydroxyl group, resulting in a more rapid attack at the 3′ position by the carbonium ion formed from the methyl vinyl ether during methoxyethylation. Similarly, the O-3′ in the 3′-ME derivatives must be more basic than the O-2′ in the corresponding 2′-ME isomers, resulting in more rapid protonation and subsequent demethoxyethylation of the 3′-ME isomers during the acid-catalyzed hydrolysis. These considerations are consistent with studies carried out by Gin and Dekker (1968) and Martin et al. (1968) who found that, in the reaction of adenosine with diazomethane, methylation occurs at the O-2′ position at three times the rate of methylation at the O-3′ position. This observed difference in the rate of methylation together with a study of the acidities of 2′-*O*-methyl- and 3′-*O*-methyladenosine led to the conclusion that, in adenosine, the 2′-hydroxyl group is slightly more acidic than the 3′-hydroxyl group (Gin and Dekker, 1968).

Phosphorylation of Methoxyethylated Nucleosides and Nucleotides. In order to provide procedures by which larger quantities of the methoxyethylnucleoside diphosphates might be prepared, it was considered desirable to explore alternative methods of synthesis employing somewhat less expensive starting materials such as nucleosides and nucleotides. These studies were particularly directed toward the synthesis of the uridine and guanosine derivatives because of the difficulty experienced in separating the 2′-ME-UDP and 3′-ME-UDP in the synthetic method described above and because of the lower yield that was obtained for the di-ME-GDP. Accordingly, the 2′-ME and di-ME derivatives of uridine and guanosine were synthesized. The synthesis of the 2′-ME, 3′-ME, and di-ME derivatives of adenosine has been described in the earlier study (Bennett et al., 1973).

5′-*O*-Acetyluridine and 3′,5′-di-*O*-acetyluridine (Fromageot et al., 1967) were each treated with methyl vinyl ether and the products were exposed to ammonia to remove the acetyl groups, giving essentially quantitative yields of di-ME- and 2′-ME-uridine, respectively. The starting materials used for the synthesis of the corresponding guanosine derivatives were $O^{3'}$-acetyl-N^2,$O^{5'}$-dibenzoylguanosine and N^2,$O^{5'}$-diacetyl-2′,3′-*O*-isopropylideneguanosine. The acetyldibenzoyl derivative was prepared by the route: guanosine → tetrabenzoylguanosine (Reese and Saffhill, 1972) → 2-*N*-benzoylguanosine (Chladek and Smrt, 1964) → $O^{3'}$-acetyl-N^2,$O^{5'}$-dibenzoylguanosine (Neilson et al., 1973). This product was treated with methyl vinyl ether and then with methanolic ammonia to remove the acyl groups. The resulting 2′-*O*-methoxyethylguanosine required chromatographic purification to remove from the product some 3′ isomer that had arisen from the methoxyethylation of $O^{2'}$-acetyl-N^2,$O^{5'}$-dibenzoylguanosine, a contaminant that was present in the starting material. For the preparation of the di-ME derivative, isopropylideneguanosine was converted to N^2,$O^{5'}$-diacetyl-2′,3′-*O*-isopropylideneguanosine (Jahn, 1965), and this compound was treated with aqueous formic acid to give N^2,$O^{5'}$-diacetylguanosine in 86% yield. The product was methoxyethylated and then treated with methanolic ammonia to give the di-ME guanosine in 87% yield. This compound could also be converted into a mixture of 2′-ME- and 3′-ME-guanosine by partial hydrolysis at pH 3.5.

The ^{1}H NMR spectral constants for these derivatives and some of the intermediates used in their synthesis are listed in Table II. The 2′-ME derivatives of uridine and guanosine display resonances corresponding to the CCH_3 and OCH_3 groups while the di-ME deriatives show resonances corre-

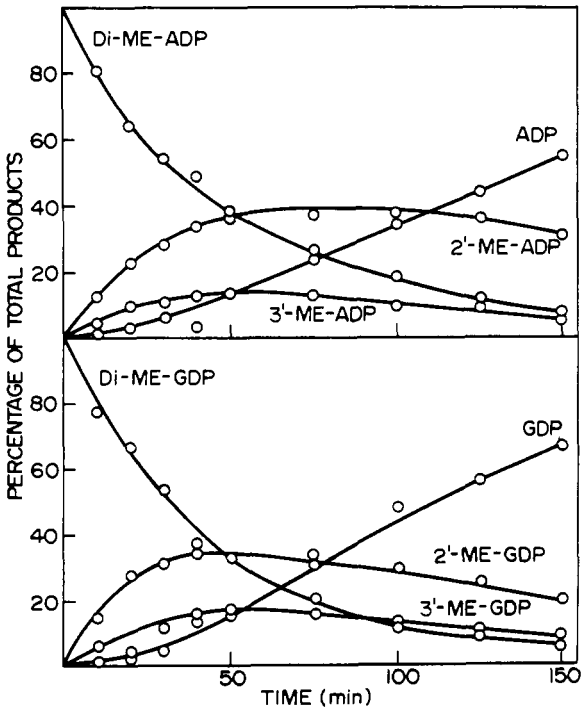

FIGURE 1: Graphs derived from a study, with respect to time, of the products arising from the hydrolysis of the 2′,3′-di-*O*-(α-methoxyethyl) derivatives of ADP and GDP. The hydrolyses were carried out in 0.2 *M* ammonium formate (pH 3.5) at 37°, and the amounts of each product were determined spectrophotometrically from samples that were withdrawn at various times and subjected to paper chromatographic separation.

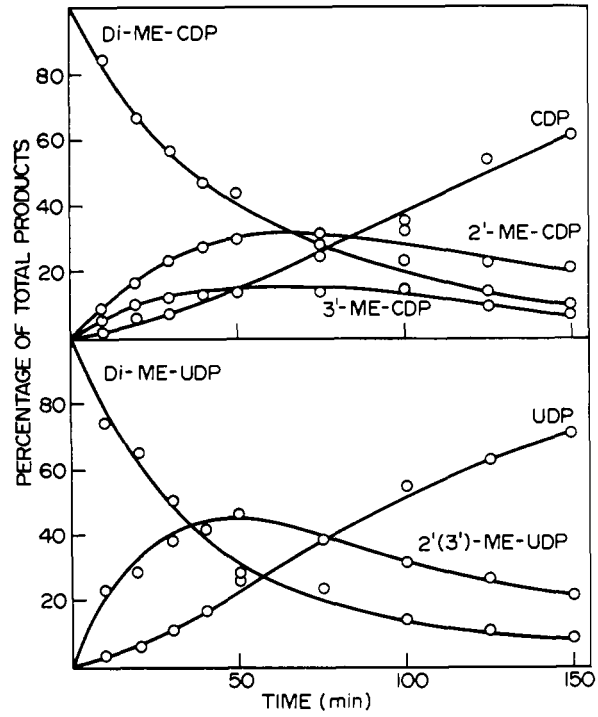

FIGURE 2: Graphs derived from a study, with respect to time, of the products arising from the hydrolysis of the 2′,3′-di-*O*-(α-methoxyethyl) derivatives of CPD and UDP. The hydrolyses were carried out in 0.2 *M* ammonium formate (pH 3.5) at 37°, and the amounts of each product were determined spectrophotometrically from samples that were withdrawn at various times and subjected to paper chromatographic separation.

sponding to two CCH_3 and two OCH_3 groups in each case. The δ values obtained are consistent with those discussed above for the nucleoside diphosphate derivatives, and with the published values for 2′-ME-, 3′-ME-, and di-ME-adenosine (Bennett et al., 1973). The ^1H NMR spectra of the isopropylideneguanosine and its diacetyl derivative show the presence of the two CCH_3 groups together with small coupling constants for the H-1′ protons. The small $J_{H\text{-}1'\text{-}H\text{-}2'}$ values are undoubtedly a consequence of the restricted conformation of the ribose rings arising from the fused five-membered ring systems in these derivatives. The diacetylguanosine exhibits resonances corresponding to the two acetyl groups and a much larger H-1′-H-2′ coupling constant.

The phosphorylation of the di-ME-nucleosides was carried out by the method of Tener (1961). The nucleoside was treated with an excess of β-cyanoethyl phosphate and dicyclohexylcarbodiimide, and, after the removal of the cyanoethyl groups with base, the 5′-phosphates of di-ME-uridine and di-ME-guanosine were obtained in yields of 81 and 61%, respectively. For the conversion of 2′-ME-nucleosides to their 5′-phosphates, the phosphorylation procedure was modified in order to exploit the greater reactivity of the 5′-hydroxyl group, and to prevent the phosphorylation of the unprotected 3′-hydroxyl group. Thus, in the case of 2′-ME-guanosine, the reaction of the nucleoside with 1 equiv of cyanoethyl phosphate in the presence of dicyclohexylcarbodiimide gave, after removal of the cyanoethyl groups, a 53% yield of 2′-ME-guanosine 5′-phosphate. This product was shown to be free of detectable quantities of the isomeric 3′-phosphate.

The conversion of these substituted nucleoside 5′-phosphates to the corresponding 5′-diphosphates was achieved by a modification of the procedure used by Hoard and Ott (1965) who employed the reaction of 1,1′-carbonyldiimidazole and inorganic pyrophosphate with nucleoside 5′-phosphates to prepare nucleoside 5′-triphosphates. The procedure is based on the work of Cramer et al. (1961), Schaller et al. (1961), and Cramer and Neunhoeffer (1962) who showed that phosphorimidazolidates, formed from the reaction of phosphomonoesters with the carbonyldiimidazole, are useful intermediates in the synthesis of pyrophosphate bonds. In the present work, the substituted nucleoside 5′-phosphates were converted to the corresponding 5′-phosphorimidazolidates, and these products were then treated with inorganic phosphate. The 5′-diphosphates of di-ME-uridine, di-ME-guanosine, and 2′-ME-guanosine were obtained in 75–80% yields.

Table III: Rates of Hydrolysis of α-Methoxyethyl Derivatives.

Derivative	$t_{1/2}$ (min)[a]
Di-ME-ADP	35
2′-ME-ADP	90
3′-ME-ADP	40
2′-ME-adenosine	150
3′-ME-adenosine	70
2′-ME-pA-A_2-G	90

[a] Approximate time of the half-life of the derivative when heated at 37° in the 0.2 *M* ammonium formate (pH 3.5).

Experimental Section

Materials. Methyl vinyl ether was purchased from K and K Laboratories, Plainview, N.Y., and the gas was condensed in a test tube kept at 0°, just prior to use. Nucleoside 5′-diphosphates were obtained from P-L Biochemicals,

Table IV: R_f Values of Nucleoside Derivatives.

Nucleoside	Solvent D[a]	Solvent E[b]	Solvent F[c]
Guanosine	2.4		0.03
2',3'-*O*-Isopropylidene-guanosine	1.0		0.43
N^2,$O^{5'}$-Diacetyl-2',3'-*O*-isopropylideneguanosine			0.60
N^2,$O^{5'}$-Diacetylguanosine			0.30
2',3'-Di-*O*-(α-methoxyethyl)guanosine	1.6		0.50
2'-*O*-(α-Methoxyethyl)-guanosine	2.6		0.33, 0.38
3'-*O*-(α-Methoxyethyl)-guanosine	1.6		0.25
2',3'-*O*-Isopropylidene-uridine		0.37	
5'-*O*-Acetyluridine		0.13	
3',5'-Di-*O*-acetyluridine		0.25	
2'-*O*-(α-Methoxyethyl)-uridine		0.20	
2',3'-Di-*O*-(α-methoxyethyl)uridine		0.35	

[a] Descending chromatography on Whatman No. 1 paper with a mixture of water (100 ml) and $(NH_4)_2SO_4$ (40 g) adjusted to pH 8.5 with concentrated NH_4OH. Values listed are relative to the R_f value of 2',3'-*O*-isopropylideneguanosine. [b] Tlc R_f values determined on Eastman 6060 silica gel sheets with $CHCl_3$–CH_3OH (96:4, v/v). [c] Tlc R_f values determined on Eastman 6060 silica gel sheets with $CHCl_3$–CH_3OH (9:1, v/v).

Milwaukee, Wis. Adsorption column chromatography was carried out with silicic acid powder, 100 mesh (Mallinckrodt). Dowex 50W-X8 ion-exchange resin was purchased as AG 50W-X8 (100–200 mesh) from Bio-Rad Laboratories, Richmond, Calif. The tetranucleotide 2'-ME-pA-A_2-G, was prepared by the reaction of pA-A-A with 2'-ME-guanosine 5'-diphosphate in the presence of polynucleotide phosphorylase using the procedure of Sninsky et al. (1974). The nucleoside derivatives, 2'-ME-adenosine, 3'-ME-adenosine, and di-ME-adenosine, were prepared as described previously (Bennett et al., 1973). Paper chromatography was carried out on Whatman No. 1 and Whatman 3MM paper by the descending technique, and thin-layer chromatography was performed by the ascending method on Eastman 6060 silica gel sheets. The solvent systems used are listed in the footnotes to Tables I and IV.

Methoxyethylation of Nucleoside 5'-Diphosphates. The sodium salt (200 mg) of the 5'-diphosphate of adenosine, cytidine, or uridine and *p*-toluenesulfonic acid monohydrate (400 mg) were dissolved in dry dimethyl sulfoxide (3 ml). The solution was then frozen in an ice bath and methyl vinyl ether (6 ml) at 0° was added. The mixture formed a clear solution with shaking and it was kept at 0° for 30 min. Triethylamine (1 ml) was then added and the mixture was applied to Whatman 3MM chromatographic paper (150 cm) in a hood. Purification was effected by descending chromatography with solvent A (Table I), and the resulting major band was subsequently eluted with water containing a few drops of ammonia. Spectrophotometric analysis showed that the yield of the di(α-methoxyethyl) derivative in each case was 70–80%. For the preparation of the corresponding guanosine derivative, the sodium salt of the diphosphate (200 mg) and *p*-toluenesulfonic acid monohydrate (400 mg) were dissolved in dry dimethyl sulfoxide (10 ml). The mixture was cooled to 0° and treated with methyl vinyl ether (10 ml) at 0°. The mixture formed a homogeneous solution within a few minutes and it was kept at 0° for 50 min. Triethylamine (2 ml) was added and the excess methyl vinyl ether was removed on a rotary evaporator. The product was purified as described above except that solvent B (Table I) was used for the chromatography, and the di(α-methoxyethyl) derivative was obtained in 50% yield. A portion of each derivative was dried in vacuo for ^1H NMR analysis and the remainder was converted to the corresponding mono(methoxyethyl) derivatives by acid hydrolysis.

Rates of Hydrolysis of 2',3'-Di-O-(α-methoxyethyl)nucleoside 5'-Diphosphates. A solution of the di(α-methoxyethyl)nucleoside diphosphate (75 A_{260nm} units) in dilute ammonia was concentrated to dryness in vacuo. The residue was dissolved in 0.5 ml of 0.2 *M* ammonium formate that had been brought to pH 3.5 with concentrated HCOOH. The solution was kept at 37° and, at various times, a 25-μl sample was withdrawn and, after being treated with 15 μl of concentrated NH_4OH, it was applied to Whatman 3MM paper. After chromatography ith solvent A, the spots on the paper corresponding to the nucleoside diphosphate and its mono- and di(methoxyethyl) derivatives were cut out, together with appropriate paper blanks, and each was eluted with 5 ml of 0.1 *M* sodium phosphate (pH 7). The A_{260nm} of each extract was determined and the yield of each product was calculated as a percentage of the total A_{260nm} units contained in each sample. The graphs of the variation of these yields with time are shown in Figures 1 and 2. In contrast to the other methoxyethylated compounds, the 2'- and 3'-(methoxyethyl) derivatives of uridine 5'-diphosphate do not separate well in this chromatographic system, and their combined yields are shown. However, the ratio of the yields of the 2' and 3' derivatives in this case was obtained in a separate analysis in which the paper chromatography was extended over a period of days. The rates of hydrolysis of the 2'- and 3'-(methoxyethyl) derivatives of adenosine and of adenosine 5'-diphosphate, and the 2'-(methoxyethyl) derivative of pA-A_2-G were determined in a similar way except that solvent B was used for the chromatographic separation. The R_f values of these adenosine compounds have been reported previously (Bennett et al., 1973), and the ratio of the R_f of 2'-ME-pA-A_2-G to that of the underivatized tetranucleotide in solvent B is 1.7. The rates of hydrolysis of these compounds are indicated in Table III.

Preparation of 2'-O-(α-Methoxyethyl)nucleoside Diphosphates. A solution of the ammonium salt of the di(α-methoxyethyl)nucleoside diphosphate (1 mmol) in about 10 ml of water was adjusted to pH 3.5 with concentrated HCOOh and kept at 37° for 30 min. Concentrated NH_4OH was then added to raise the pH above 8, and the mixture was applied to Whatman 3 MM chromatographic paper (150 cm). Separation was effected with solvent A, and the resulting bands of unchanged material and the two monosubstituted products were cut out and eluted with water containing a few drops of NH_4OH. The yield of the 2'-(methoxyethyl) derivative was determined spectrophotometrically, and the product was converted to the trisodium salt by adding 3 molar equiv of NaOH to the aqueous extract and then concentrating the solution to a small volume in vacuo. In the case of uridine diphosphate the monosubstituted product was isolated as a mixture of the 2' and 3' isomers.

2'-O-(α-Methoxyethyl)uridine and 2',3'-Di-O-(α-methoxyethyl)uridine. A mixture of 2 g of 3',5'-di-*O*-acetyluridine (Fromageot et al., 1967) and *p*-toluenesulfonic

acid monohydrate (88 mg) dissolved in dioxane (17 ml) was cooled in an ice bath. Methyl vinyl ether (6 ml) at 0° was added and the mixture was kept at 15° for 30 min. Ammonium hydroxide (7 *M*, 25 ml) was added, and the mixture was stirred overnight and then concentrated in vacuo. The residue was dissolved in CH_3OH and the solution was evaporatd onto silicic acid (5 g) which was then placed on top of a column (54 × 2.4 cm) of silicic acid. The column was eluted with $CHCl_3$–CH_3OH (85:15, v/v) and the product, which accounted for 98% of the original A_{260nm} units of starting material, was obtained in fractions collected between elution volumes of 200 and 800 ml. These fractions were combined and evaporated to dryness, and the residue was dissolved in warm ethanol (100 ml). The solution, on being concentrated and cooled, yielded a crystalline product (0.7 g, mp 170–172°) that corresponded to the diastereomer possessing the higher R_f value on extended chromatography in solvent E (Table IV). *Anal.* Calcd for $C_{12}H_{18}N_2O_7$: C, 47.68; H, 6.00; N, 9.27. Found: C, 47.78; H, 5.93; N, 9.41.

A solution of 1.43 g of 5′-*O*-acetyluridine (Fromageot et al., 1967) and *p*-toluenesulfonic acid monohydrate (80 mg) in dioxane (15 ml) was treated with methyl vinyl ether (10 ml) for 20 min at 15°. Triethylamine (2 ml) was then added and the solution was concentrated in vacuo. Methanolic ammonia (saturated at 0°, 15 ml) was added to the residue, and the resulting solution was kept at room temperature for 24 hr. Chromatographic analysis showed that there was an essentially quantitative yield of the di(methoxyethyl) derivative and the product was purified by paper chromatography with solvent A (Table I).

2′-O-(α-Methoxyethyl)guanosine. $O^{3'}$-Acetyl-N^2,$O^{5'}$-dibenzoylguanosine, prepared by the method of Neilson et al. (1973), was found by ^1H NMR analysis to contain about 20% of the isomeric $O^{2'}$ acetate. The derivative (4 mmol) and *p*-toluenesulfonic acid monohydrate (210 mg) were dissolved in cold dioxane (20 ml) and treated with methyl vinyl ether (8 ml) at 0°. The solution was kept at room temperature for 5 hr and was then treated with concentrated NH_4OH (100 μl). The mixture was concentrated in vacuo to an oil, which was dissolved in 50 ml of CH_3OH that had been saturated with NH_3 at 0°. The solution was kept at room temperature for 4 days, and was then evaporated to dryness. Column chromatography of the residue on 50 g of silicic acid, using $CHCl_3$–CH_3OH (9:1, v/v) as solvent, yielded 3.2 mmol of 2′(3′)-*O*-(α-methoxyethyl)guanosine. This product was purified by the chromatographic technique that was used by Christensen and Broom (1972) for the separation of 2′- and 3′-*O*-benzyl derivatives of nucleosides. After removal of the solvent, the product was dissolved in 1.5 *M* NH_4OH and applied to a column (80 × 2.7 cm) of DEAE-cellulose (Whatman DE 52), and elution with 1.5 *M* NH_4OH gave the 2′ isomer (1.8 mmol) first, followed by the 3′ isomer (0.68 mmol). The products were concentrated and dried in vacuo. Anal (2′ isomer). Calcd for $C_{13}H_{19}N_5O_6$: C, 45.74; H, 5.61; N, 20.52. Found: C, 45.73; H, 5.68; N, 20.39.

2′,3′-Di-O-(α-methoxyethyl)guanosine. N^2,$O^{5'}$-Diacetyl-2′,3′-*O*-isopropylideneguanosine (12 g), prepared by the method of Jahn (1965), was dissolved in 88% formic acid (720 ml), and the solution was kept at room temperature for 9 hr. The solution was evaporated to dryness and the residue was recrystallized from CH_3OH to yield N^2,$O^{5'}$-diacetylguanosine (9.3 g, 86%), mp 210–211°, λ_{max} 258 nm in water at pH 7. The diacetate (736 mg, 2 mmol) and *p*-toluenesulfonic acid monohydrate (80 mg) were dissolved in dioxane (40 ml) and treated with methyl vinyl ether (16 ml) at 0°. The mixture was kept at room temperature for 24 hr. Triethylamine (0.6 ml) was then added and the mixture was evaporated to dryness in vacuo. The product was dissolved in CH_3OH (15 ml) that had been saturated with NH_3 at 0°, and the solution was kept at room temperature 24 hr. The solution was concentrated in vacuo and a portion of the product that was subjected to paper chromatography with solvent A gave the di(α-methoxyethyl) derivative in 87% yield.

For the conversion of this product to the 2′- and 3′-ME derivatives, 1.5 mmol of the compound was dissolved in 15 ml of 0.2 *M* ammonium formate (pH 3.5), and the solution was kept at 37° for 65 min. Concentrated NH_4OH (1 ml) was added and the solution was evaporated to dryness in vacuo. The product was applied to a column (110 × 2.8 cm) of silicic acid, which was then washed with 1.3 l. of $CHCl_3$–CH_3OH (96:4, v/v). The mixture of 2′- and 3′-ME derivatives (0.56 mmol) was subsequently eluted with $CHCl_3$–CH_3OH (92:8, v/v) and the two isomers were then separated by DEAE-cellulose chromatography as described above.

Preparation of 2′,3′-Di-O-(α-methoxyethyl)guanosine 5′-Phosphate and 2′,3′-Di-O-(α-methoxyethyl)uridine 5′-Phosphate. The di(α-methoxyethyl)nucleoside (0.5 mmol) was rendered anhydrous by repeated evaporation in vacuo of its pyridine solution, and it was finally dissolved in dry pyridine (5 ml) containing 2.5 mmol of the pyridinium salt of cyanoethyl phosphate. Dicyclohexylcarbodiimide (1 g) was added and the mixture was shaken for 24 hr. Water (1 ml) was added and the mixture was allowed to stand for 1 hr, and was then concentrated in vacuo to a small volume. The product was extracted with water (2 × 10 ml) and the extracts were filtered and combined. The pH of the extract was adjusted to about 8 by the addition of 0.5 *M* $Ba(OH)_2$. Ethanol (120 ml) was added and the solution was filtered. The filtrate was concentrated in vacuo to a small volume and mixed with 0.4 *M* LiOH (100 ml), and then heated under reflux for 1 hr. The mixture was cooled and filtered, and the pH of the filtrate was adjusted to 7.5 with Dowex 50W-X8 (H^+) ion-exchange resin. The resin was removed and the solution was applied to a column (50 × 2.5 cm) of DEAE-cellulose (Whatman DE 23). Elution was effected with 6 l. of water containing a linear gradient of 0–0.3 *M* $Et_3NH^+HCO_3^-$ (pH 7.5), at a flow rate of 80 ml/hr. The fractions containing the major uv-absorbing peak were pooled and the product was rendered free of the volatile salt by the repeated evaporation to dryness of its aqueous solution. The yields of the 5′-phosphates of di(α-methoxyethyl)guanosine and di(α-methoxyethyl)uridine were 61 and 81%, respectively. These products were also readily prepared by the direct methoxyethylation of the corresponding 5′-nucleotides using the method described above for the methoxyethylation of nucleoside 5′-diphosphates.

Phosphorylation of 2′,3′-Di-O-(α-methoxyethyl)guanosine 5′-Phosphate and 2′,3′-Di-O-(α-methoxyethyl)uridine 5′-Phosphate. The triethylammonium salt of the di(α-methoxyethyl)nucleoside 5′-phosphate (0.2 mmol) was converted to the pyridinium salt by passing a solution of the material in dilute pyridine through a column of Dowex 50W-X8 (pyridinium form) ion-exchange resin. The solution was then concentrated to a small volume and was treated with a solution of tri(*n*-butyl)amine (50 μl) in pyridine (5 ml). The mixture was rendered anhydrous by the repeat-

ed evaporation in vacuo of its pyridine solution. The product was finally dissolved in dry dimethylformamide (2 ml) and added to a solution of 1,1′-carbonyldiimidazole (160 mg) in dry dimethylformamide (2 ml). The solution was allowed to stand overnight and was then treated with CH_3OH (70 μl). After 1 hr, a solution of mono[tri(*n*-butyl)ammonium] phosphate (1 mmol) in dimethylformamide (10 ml) was added, and the mixture was allowed to stand for 4 days. Methanol (20 ml) was added and the mixture was concentrated in vacuo to about 10 ml. Water (100 ml) was added and the pH of the solution was adjusted to 7.5 with NH_4OH. The solution was applied to a column (50 × 2.5 cm) of DEAE-cellulose (Whatman DE 23), and elution was effected with 6 l. of water containing a linear gradient of 0–0.3 M $Et_3NH^+HCO_3^-$ (pH 7.5) at a flow rate of 80 ml/hr. The diphosphates corresponding to the guanosine and uridine derivatives were obtained between elution volumes of 3.0–3.5 l. and 2.6–3.2 l. with yields of 75 and 78% respectively. The products were desalted by repeatedly evaporating, in vacuo, their aqueous solutions to remove the volatile buffer.

Phosphorylation of 2′-O-(α-Methoxyethyl)guanosine. 2′-*O*-(α-Methoxyethyl)guanosine (0.05 mmol) and pyridinium cyanoethyl phosphate (0.05 mmol) dissolved in dry pyridine (0.5 ml) were shaken with dicyclohexylcarbodiimide (0.1 g) for 2 days. Water (0.5 ml) was added and, after 4 hr, the mixture was cooled to 0° and treated with 2 *M* NaOH (5 ml) at 0°. The mixture was kept at 0° for 40 min and the pH of the solution was adjusted to 8 by adding Dowex 50W-X8 (pyridinium form) ion-exchange resin. The mixture was filered, and the filtrate was treated with NH_4OH (0.5 ml). After the solution was concentrated in vacuo it was applied to Whatman 3MM paper (80 cm), and separation was effected with solvent B. Elution of the band with R_f = 1.36R_f of GMP gave the 5′-phosphate in 53% yield. A portion of this derivative was hydrolyzed (pH 3.5, 37°, 11 hr), and the resulting product was shown, by paper chromatography with 2-propanol–3 *M* NH_4OH–0.1 *M* boric acid (7:2:1, v/v) as solvent, to consist of 5′-GMP, with no contaminating 3′-GMP. In this system the R_f of 5′-GMP = 0.2R_f of 3′-GMP. The 2′-*O*-(α-methoxyethyl)guanosine 5′-phosphate was converted to the corresponding 5′-diphosphate by the method described above for the phosphorylation of the 2′,3′-di-*O*-(α-methoxyethyl)nucleoside 5′-phosphates.

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