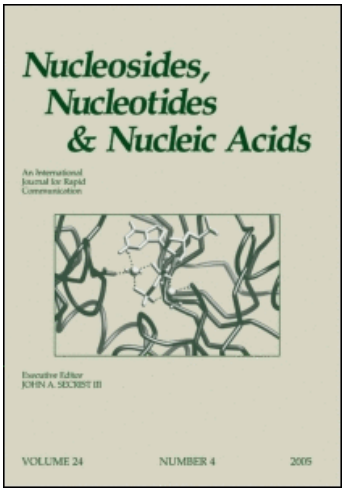

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Synthesis, Conformation and Hydrolytic Stability of p^1,p^3-Dinucleoside Triphosphates Related to mRNA 5'-cap, and Comparative Kinetic Studies on their Nucleoside and Nucleoside Monophosphate Analogs

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SYNTHESIS, CONFORMATION AND HYDROLYTIC STABILITY OF P^1,P^3-DINUCLEOSIDE TRIPHOSPHATES RELATED TO mRNA 5'- cap, AND COMPARATIVE KINETIC STUDIES ON THEIR NUCLEOSIDE AND NUCLEOSIDE MONOPHOSPHATE ANALOGS

Edward Darzynkiewicz,[a] Janusz Stepinski,[b] Stanley M. Tahara,[c] Ryszard Stolarski,[a] Irena Ekiel,[d] Dorota Haber,[a] Kari Neuvonen,[e] Pertti Lehikoinen,[e] Imre Labadi[f] and Harri Lönnberg*[e]

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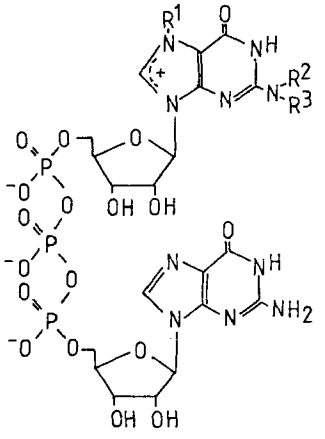
Abstract. P^1,P^3-Dinucleoside triphosphates, N(5')ppp(5')G, have been prepared in which N is 7-Me-, 7-Et-, 7-Bn, N^2,7-diMe- or N^2,N^2,7-triMe-guanosine. Conformations of the nucleoside moieties have been determined and compared with those of the corresponding nucleoside monophosphates. The hydrolytic stability of the 7-alkylguanine ring has been studied and the origin of the structural effects elucidated by comparative kinetic studies with monomeric nucleoside and nucleotide analogs. The mechanism of the alkaline decomposition has been established by following the cleavage of 7-methylguanosine by ^1H and ^{13}C NMR spectroscopy.

The 5'-terminus of eukaryotic mRNAs, called a cap, consists of 7-methylguanosine (m^7G) linked by a 5',5'-triphosphate bridge to the next nucleoside, which is often a 2'-O-methylated purine nucleoside.[1,2] Other naturally occurring 5'-end nucleosides of capped mRNAs include N^2,7-dimethyl- ($m_2^{2,7}G$) and N^2,N^2,7-trimethyl-guanosine ($m_3^{2,2,7}G$), found, besides 7-methylguanosine, in cells infected by

togaviruses.[3,4] Furthermore, snRNAs, participating in RNA splicing, are capped with $m_3^{2,2,7}G$.[5] Previous studies[6] have indicated that β-globin mRNAs capped with $m_2^{2,7}G$ were 1.5-fold more active, and those capped with $m_3^{2,2,7}G$ considerably less active, than m^7G capped mRNAs, when assayed in the reticulocyte lysate system. Furthermore, the unnatural 7-benzylguanosine cap has been shown to increase translation activity of mRNA relative to m^7G cap, whereas 7-ethylguanosine slightly reduces it.[7] In the present paper the syntheses of these extended cap structures have been described, and the effects of the structural modifications on their conformation and hydrolytic stability are considered. The factors affecting the rate of alkaline ring-opening of the 7-alkylguanosine moiety are further elucidated by comparative kinetic studies with a number of 7-alkylguanosines and 7-alkylguanosine 5'-monophosphates. The pathway for the subsequent breakdown of ring-opened intermediates has been established by using m^7G as a model compound.

RESULTS AND DISCUSSION

Synthesis and characterization of cap analogs. P^1,P^3-Dinucleoside triphosphates, 1a-e, were obtained in 10 to 30 % yields by the method of Nagakawa et. al.,[8] and purified by column chromatography on DEAE-Sephadex A-25(HCO_3^-). The structures of the products were verified by ^1H and ^{31}P NMR spectroscopy. The ^1H resonance spectra exhibited two characteristic sets of signals, one similar to that of



1a : $R^1 = CH_3$, $R^2 = R^3 = H$

b : $R^1 = R^2 = CH_3$, $R^3 = H$

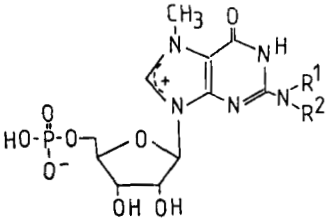
c : $R^1 = R^2 = R^3 = CH_3$

d : $R^1 = CH_2CH_3$, $R^2 = R^3 = H$

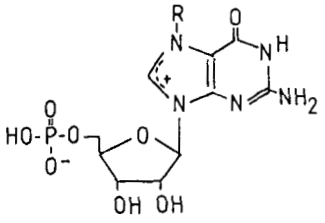
e : $R^1 = CH_2C_6H_5$, $R^2 = R^3 = H$

5'-GMP and the other to that of an appropriately substituted derivative of 5'-GMP (Table 1). Identity of the three phosphate groups was confirmed by ^{31}P NMR spectroscopy; two phosphorus atoms showed a doublet (protons decoupled) at -12.2 ppm and the third gave a triplet at -22.8 ppm (relative to external phosphoric acid); the $^{31}P,^{31}P$ coupling constants were 19.5 Hz.

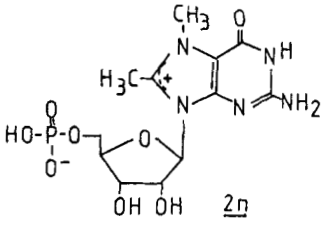
Conformational analysis. The values of the vicinal $^1H,^1H$ and $^1H,^{31}P$ coupling constants of the P^1,P^3-dinucleoside triphosphates, 1a-e, are listed in Table 2 together with the conformational parameters derived from them. These data strongly suggest that all the dinucleoside triphosphates studied are conformationally rather similar, and that the conformations of the guanosine and 7-alkylguanosine moieties closely resemble those of the corresponding nucleoside monophosphates, 2a-e. Firstly, analysis of the sugar ring-puckering by the two-state, N ⇌ S, model[11] showed that the N



2a: $R^1 = R^2 = H$
2b: $R^1 = CH_3$, $R^2 = H$
2c: $R^1 = R^2 = CH_3$



2d: $R = CH_2CH_3$
2e: $R = CH_2C_6H_5$
2f: $R = (CH_2)_2CH_3$
2g: $R = (CH_2)_3CH_3$
2h: $R = CH_2CH(CH_3)_2$
2i: $R = CH(CH_3)_2$
2j: $R = CH_2CH{=}CH_2$
2k: $R = CH(CH_3)C_6H_5$
2l: $R = (CH_2)_2C_6H_5$
2m: $R = (CH_2)_3C_6H_5$



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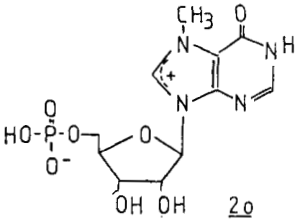

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Table 1: ^{1}H NMR chemical shifts for the P^1,P^3-dinucleoside triphosphates prepared.

Compd.	δ(H8)	δ(H1')	δ(H2')	δ(H3')	δ(H4')	δ(H5')	δ(H5'')	Others
1a m^7G^b	c	5.900	4.535	4.420	4.390	4.365	4.275	4.045[d]
G^b	8.005	5.800	4.670	4.475	4.340	4.260	4.240	
1b $m_2^{2,7}G^e$	c	5.935	4.550	4.415	4.370	4.400	4.280	4.045[d];2.930[f]
G	7.980	5.770	4.610	4.450	4.340	4.270	4.235	
1c $m_3^{2,2,7}G^g$	c	5.920	4.540	4.400	4.365	4.395	4.275	4.045[d];3.135[f]
G	7.945	5.740	4.550	4.425	4.330	4.270	4.230	
1d et^7G^h	c	5.885	4.530	4.425	4.345	4.375	4.285	4.430[i];1.505[j]
G	8.025	5.795	4.675	4.475	4.330	4.24[k]	4.24[k]	
1e bn^7G^h	c	5.920	4.620	4.475	4.37[k]	4.37[k]	4.260	5.610[l];7.35-7.40[m]
G	8.005	5.765	4.690	4.465	4.300	4.23[k]	4.23[k]	

[a]In ^2H_2O as ppm from internal TSP. [b]For the shifts of 7-methyl-5'-GMP, 2a, and 5'-GMP see Ref. 9. [c]Deuterated. [d]N7-C$\underline{H}_3$ [e]The shifts for N^2,7-dimethyl-5'-GMP, 2b, are 6.16, 4.69, 4.51, 4.38, 4.15, 4.02, 4.11 and 2.96, respectively. [f]N^2-C$\underline{H}_3$ [g]The shifts for N^2,N^2,7-trimethyl-5'-GMP, 2c, were 6.16, 4.69, 4.49, 4.36, 4.11, 4.00, 4.10 and 3.12, respectively. [h]For the shifts of 7-ethyl-, 2d, and 7-benzyl-5'-GMP, 2e, see Ref. 7. [i]N7-C$\underline{H}_2$CH$_3$ [j]N7-CH$_2$C$\underline{H}_3$ [k]Approximate values due to overlapping. [l]N7-C$\underline{H}_2$C$_6$H$_5$ [m]N7-CH$_2$C$_6$$\underline{H}_5$.

Table 2: Vicinal $^1H,^1H$ and $^1H,^{31}P$ coupling constants, and conformational parameters of the P^1,P^3-dinucleoside triphosphates prepared.

Comp.	J/Hz							Conformation		
	1',2'	2',3'	3',4'	4',5'	4',5''	5',P	5'',P	% N state	% gg	% g'g'
1a m^7G[a]	3.4	4.8	5.7	2.5	2.1	4.2	5.7	63	90	76
G[b]	6.3	5.1	3.3	3.6	4.5	5.5	7.0	34	55	63
1b $m_2^{2,7}G$[c]	3.3	4.8	5.8	2.6	2.4	4.0	5.8	64	86	77
G	6.1	5.2	3.5	3.7	4.3	5.6	7.0	36	56	63
1c $m_3^{2,2,7}G$[d]	3.4	4.8	5.8	2.5	2.3	4.0	5.8	63	88	77
G	5.9	5.1	3.7	4.1	3.9	5.8	7.0	39	57	62
1d et^7G[e]	3.6	4.9	5.4	2.7[f]	2.1[f]	4.3[f]	5.9[f]	60	89	75
G	6.3	5.2	3.3	3.9[f]	3.9[f]	6.1[f]	6.1[f]	34	59	65
1e bn^7G[g]	3.7	5.0	5.2	2.5[f]	3.4[f]	4.4[f]	5.7[f]	58	77	75
G	6.4	5.2	3.2	4.0[f]	4.0[f]	5.5[f]	5.5[f]	33	57	70

[a]For 7-methyl-5'-GMP, 2a, %N 54, %gg 87, %g'g' 83.[10] [b]For 5'-GMP %N 36, %gg 58, %g'g' 81.[10] [c]For N^2,7-dimethyl-5'-GMP, 2b, the values of the coupling constants were 4.2, 4.8, 5.3, 2.7, 2.3, 4.0 and 4.8, respectively. %N 56, %gg 86, %g'g' 82. [d]For N^2,N^2,7-trimethyl-5'-GMP, 2c, the values of the coupling constants were 4.0, 4.9, 4.9, 2.8, 2.6, 4.2 and 4.8, respectively. %N 55, %gg 81, %g'g' 81. [e]For 7-ethyl-5'-GMP, 2d, %N 56, %gg 85, %g'g' 81.[7] [f]Approximate values due to signal overlap. [g]For 7-benzyl-5'-GMP, 2e, %N 54, %gg 82, %g'g' 80.[7]

form population of the 7-alkylguanosine residue ranged from 58 to 64 % with 1a-e and from 54 to 58 % with 2a-e. The unsubstituted guanosine moiety, in turn, prefers S type puckering (%N 34 to 39), analogous to 5'-GMP (%N 37). Secondly, the 4'-hydroxymethyl group of the 7-alkylguanosine moiety adopts a gg conformation, i.e. a gauche orientation of O5' relative to O4' and C3'. The gg populations, determined on the basis of the coupling constants of the conventional gauche-gauche, gauche-trans and trans-gauche forms,[12] varied from 77 to 90 % with 1a-e and from 82 to 92 % with 2a-e. The predominance of this conformation is less marked in the unsubstituted guanosine moiety (%gg 55 to 57) and 5'-GMP (%gg 58). Thirdly, the P^1 and P^3 atoms prefer the g'g' conformation with a transoidal orientation of the P and C4' atoms,[13] the population of this form being slightly lower than with nucleoside monophosphates. In particular, the g'g' population of the unsubstituted guanosine moiety is about 15 % smaller than that of 5'-GMP. Finally, comparison of the sugar proton shifts of 1a-e with those of 2a-e suggests that the conformation about the N-glycosidic bond is mainly anti, as with the latter compounds.

Hydrolytic stability of P^1,P^3-dinucleoside triphosphates. It has been shown previously that 7-alkylguanosines and their derivatives undergo opening of the imidazole ring when treated with aqueous alkali.[14-18] Table 3 records the second-order rate constants for this reaction of P^1,P^3-dinucleoside triphosphates, 1a-e, and their monomeric counterparts, 2a-e. As seen, the hydrolysis rates of the former compounds are almost invariably 60 to 70 % of those of the corresponding nucleoside monophosphates. The fact that the structural effects are almost identical within both series of compounds, is consistent with the preceding assumption on the conformational similarity of compounds 1a-e. It has been shown[18] that a 5'-phosphate group electrostatically retards the nucleophilic attack of hydroxide ion on C8 of 7-methylguanosine by one order of magnitude. Accordingly, marked changes in the conformation of 1a-e

Table 3: Hydrolytic stability of the 7-alkylguanine ring of P^1,P^3-dinucleoside triphosphates (1a-e) and nucleoside monophosphates (2a-e). Second-order rate constants for the opening of the imidazole ring in aqueous sodium hydroxide at 298.2 K.[a]

Compd.	$k/dm^3\ mol^{-1}\ s^{-1}$	Compd.	$k/dm^3\ mol^{-1}\ s^{-1}$	b
1a	0.0905(8)	2a	0.129(1)	0.70
1b	0.0490(5)	2b	0.0755(8)	0.65
1c	0.0315(6)	2c	0.0481(20)	0.65
1d	0.0237(3)	2d	0.0387(4)	0.61
1e	0.139(2)	2e	0.203(3)	0.68

[a]Ionic strength adjusted to 0.1 mol dm^{-3} with sodium chloride. [b]The ratio of the rate constants obtained with 1a-e and 2a-e.

could be expected to influence their hydrolytic stability. The lower reactivity of these compounds compared to their nucleoside monophosphate counterparts, 2a-e, may tentatively be attributed to intramolecular base-stacking. Previous studies[19] with 9-(1-ethoxyethyl)purine have indicated that stacking of this substance with other heteroaromatic nitrogen bases retards the nucleophilic attack of hydroxide ion on the C8 atom by 30 to 40 %. In contrast, replacement of a 5'-monophosphate group with a triphosphate group is probably of minor importance. For comparison, the rates for the alkaline ring opening of 7-methylguanosine mono- and tri-phosphates are almost equal.[18]

Structural effects. Since the substituent effects were observed to be identical in the alkaline cleavage of 1a-e and 2a-e, the origin of these effects were further elucidated by kinetic studies on a more extensive series of 7-alkylguanosine 5'-monophosphates ,2a-n, and their nucleoside analogs. The second-order rate constants obtained are listed in Table 4 together with the pK_a values of 2a-n. Muller and Eisenbrand[20] have suggested that the rate of alkaline ring-opening of 7-alkylguanosines is linearly related to the polar substituent constant, σ^*, of the alkyl

Table 4: Second-order rate constants for the opening of the imidazole ring of 7-alkylguanosine 5'-monophosphates (2a-o) in aqueous alkali at 298.2 K, the pK_a values of their base moieties, and the ratio of the rate constants obtained with 7-alkylguanosine 5'-monophosphates and the corresponding nucleosides.[a]

Compd.	$k/dm^3\ mol^{-1}\ s^{-1}$	pK_a	k(NMP)/k(N)[b]
2a	0.110(1)	7.20	0.10
2b	0.0644(8)	-	0.13
2c	0.0410(18)	7.27	0.13
2d	0.0330(4)	7.26	0.13
2e	0.173(3)	7.18	0.17
2f	0.0234(5)	7.26	
2g	0.0225(4)	7.29	0.19
2h	0.0095(2)	7.24	0.22
2i	0.0051(1)	7.37	
2j	0.160(10)	-	0.16
2k	0.0380(16)	7.02	0.34
2l	0.0403(5)	7.33	0.24
2m	0.0136(1)	-	0.12
2n	0.0080(1)	7.52	0.27
2o	1.94(3)	6.28	

[a]Ionic strength adjusted to 0.10 mol dm^{-3} with sodium chloride. [b]The ratio of the rate constants obtained with 7-alkylguanosine 5'-monophosphates (NMP) and their nucleoside analogs.

group, the reaction constant being 2.0. The results of the present work, however, reveal that this is the case only when the 7-substituents are structurally closely related (Fig. 1). In the correlation analysis of Muller and Eisenbrand all the substituents were 2-substituted ethyl groups. When the size and chemical nature of the substituents are varied, the structural effects are not adequately described by a one parameter equation. As seen form Fig. 1, saturated and unsaturated substituents appear to fall on different correlation lines. Moreover, branched-chain substituents deviate markedly from the line referring to unbranched alkyl groups. Inclusion of a steric substituent constant, E_s,[21] as an additional parameter into

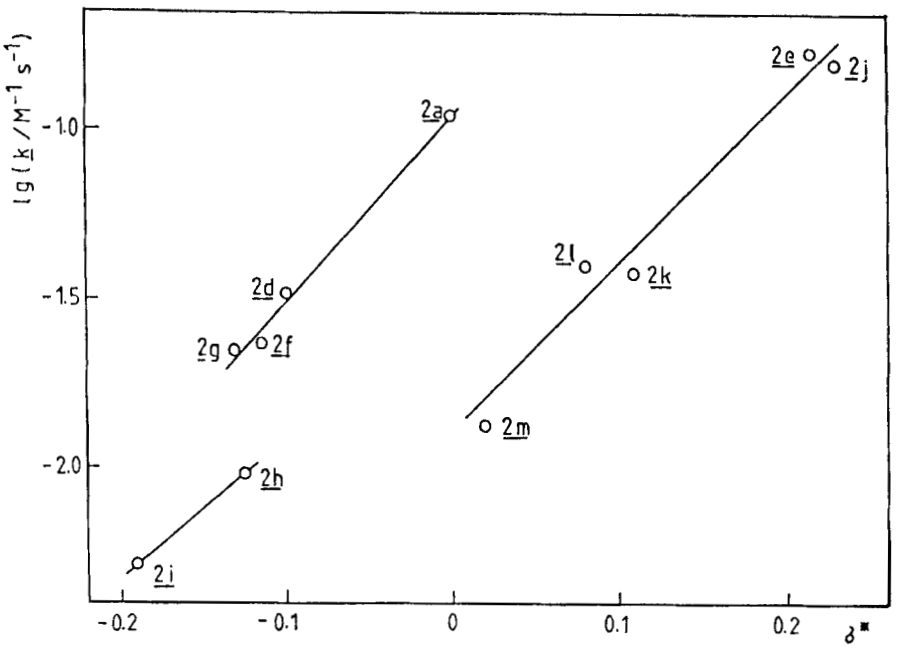


Fig. 1: Logarithmic rate constants for the imidazole ring-opening of 7-alkylguanosine 5'-monophosphates in aqueous alkali plotted against the polar substituent constant, σ^*, of the 7-alkyl group.

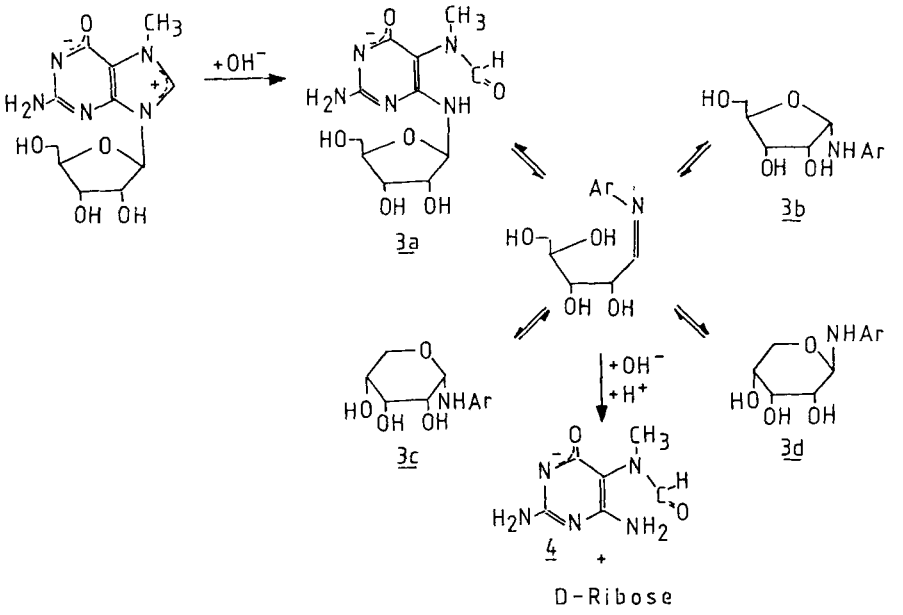
the analysis of structural does not lead to a satisfactory correlation. The observed substituent effects are obviously combinations of several different factors, the importance of which cannot be estimated quantitatively on the basis of the data available. One factor that has been demonstrated to affect the hydrolytic stability of 7-alkylguanosine 5'-monophosphates, is the electrostatic interaction between the positively charged imidazole ring and the negatively charged phosphate moiety.[15,18] Due to this interaction, the nucleophilic attack of hydroxide ion on the C8 atom of 7-methylguanine is retarded by one order of magnitude. As seen from Table 4, the reactivity difference between a given 7-alkylguanosine and its 5'-monophosphate is usually de-

creased when the size of the 7-alkyl group is increased. Possibly the presence of a bulky group at N7 weaken the intramolecular electrostatic interaction between the imidazole ring and the phosphate group, and hence affect indirectly the reactivity. It should be noted, however, that this is not a sufficient explanation for the deviations observed. The reactivities of 7-alkylnucleosides do not obey a simple two parameter equation, having σ^* and E_s as variables, although the reaction rate cannot be influenced by intramolecular electrostatic interactions.

The effects of the 7-alkyl substituents on the acidity of the base moiety is expectedly small; electronegative substituents, as expected, slightly decrease the pK_a value whereas electropositive groups increase it.

Decomposition of 7-methylguanosine. The structures of the ring-opened intermediates and their subsequent reactions were elucidated by following the decomposition of m^7G in aqueous alkali. ^1H and ^{13}C NMR spectroscopic analyses of the aliquots withdrawn at appropriate intervals revealed that the disappearance of the starting material was accompanied by formation of four relatively stable intermediates (3a-3d in Scheme 1), the time-dependent distribution of which is depicted in Fig. 2.

Prolonged treatment at 363 K (6h, 0.1 mol dm^{-3} NaOH) converted the mixture of these intermediates to a single UV-absorbing product. This final product was crystallized from aqueous solution and identified as N^5-formyl-N^5-methyl-2,5,6-triaminopyrimidin-4-one (4 in Scheme 1) by ^1H and ^{13}C NMR and UV spectroscopy (Tables 5 and 6). It is worth noting that compound 4 gives, like other formamidopyrimidines,[22] two sets of NMR signals due to a hindered rotation of the formyl group. Formation of the corresponding N^6-formyl derivative may be excluded; the ^1H NMR spectrum of this compound would include only one NH_2 signal and three NH signals, while the N^5-isomer exhibits two NH_2 resonances and one NH resonance. The presence of the



Scheme 1

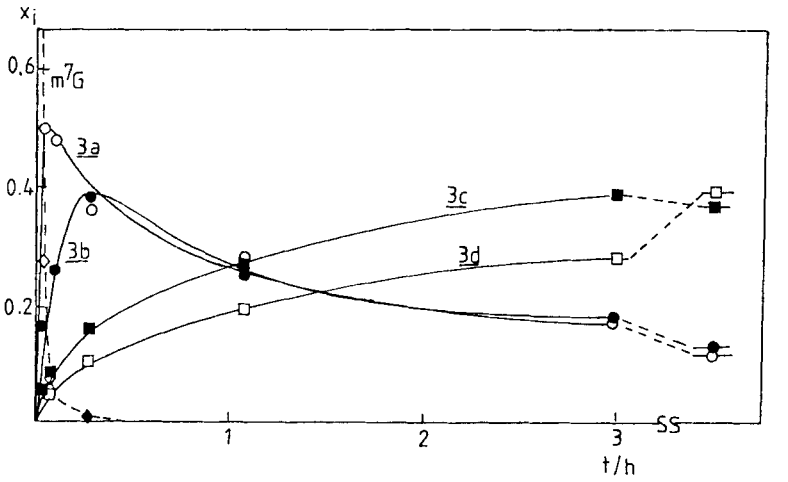


Fig. 2: Interconversion of the ring-opened intermediates (3a -d) during the decomposition of 7-methylguanosine (m^7G) in 0.010 mol dm^{-3} aqueous sodium hydroxide at 298.2 K. The mole fractions are based on ^1H NMR data.

Table 5: ^{13}C NMR chemical shifts for the intermediates and products of the alkaline cleavage of 7-methylguanosine.[a]

Compd.	Sugar moiety	Base moiety
3a	61.9, 70.2, 73.2, 83.2, 84.9[b]	31.2, 95.4, 154.9, 159.8, 161.6, 165.7
3b	61.8, 69.9, 71.4, 80.4, 82.7[c]	31.1, 95.3, 155.3, 159.5, 161.6, 165.8
3c	59.5, 66.6, 66.7, 71.5, 77.1[d]	31.1, 95.4, 155.4, 160.0, 161.3, 165.7
3d	64.0, 67.2, 69.1, 71.1, 77.7[e]	31.3, 95.5, 155.0, 160.3, 161.4, 166.1
4		32.4, 95.3, 154.8, 161.9, 162.6, 167.3

[a] In DMSO-d_6 as ppm from TMS. [b] For N-phenyl-β-D-ribofuranosylamine 62.1, 70.0, 73.9, 83.1, 87.9.[23] [c] For N-phenyl-α-D-ribofuranosylamine 61.3, not det., 71.4, 81.5, 83.7.[23] [d] For N-phenyl-α-D-ribopyranosylamine 62.1, 67.8, 69.9, 69.9, 81.3.[23] [e] For N-phenyl-β-D-ribopyranosylamine 63.1, 67.4, 70.1, 70.4, 81.6.[23]

Table 6: ^{1}H NMR spectroscopic data for the intermediates and products of the alkaline cleavage of 7-methylguanosine.[a]

Compd.	$\delta(CH_3)$	δ(H1')	J(1',2')	$\delta(NH_2)$	δ(CHO)	δ(NH)
3a	2.67 (2.82)[b]	5.35-5.43[c]	6 Hz		7.58 (7.88)[b]	
3b	2.70 (2.85)	5.59-5.63	4 Hz		7.63 (7.93)	
3c	2.73 (2.87)	5.16-5.20	3 Hz		7.64 (7.94)	
3d	2.68 (2.81)	5.03-5.13	9 Hz		7.59 (7.89)	
4	2.78 (2.91)[d]			6.21 (5.97) 6.45 (6.32)	7.71(7.99)	10.32

[a] In ^2H_2O as ppm from external TMS, if not otherwise stated. [b] The values in parentheses refer to the less stable rotamer. Both signals show splitting of 0.01-0.02 ppm. [c] 4 doublets. [d] In DMSO-d_6.

C8 atom of the starting material in the final product was additionally verified by ^{14}C isotopic labeling.

Each of the intermediates, 3a-3d, showed four sets of ^{1}H NMR signals, most probably due to hindered rotations around the N^5-CHO and C6-N bonds (Table 6). The UV spectra (λ_{max} = 264 nm and λ_{min} = 240 nm) of all four intermediates were similar to that of 4, and the ^{1}H and ^{13}C chemical shifts of the base moiety signals closely resembled those of the final product (Table 5). Accordingly, it appears clear that the intermediates all are N^6-ribosyl derivatives of 4. The ^{13}C chemical shift patterns of the sugar moieties are rather similar to those reported for anomeric N-phenyl-D-ribosylamines.[23] The pathway for the alkaline cleavage of m^7G may thus be depicted by Scheme 1. The first step most probably proceeds by a nucleophilic attack of hydroxide ion on the C8 atom of m^7G with a concomitant formation of 7,8-dihydro-8-hydroxy-7-methylguanosine as a transient intermediate. The resulting ring-opened intermediate, 3a, then undergoes anomerization to α-furanoid and α- and β-pyranoid structures via an acyclic Schiff base. m^7G does not undergo a similar anomerization, since the N9 atom is in this molecule part of an aromatic system, and hence the mesomeric electron release from this nitrogen atom, which would stabilize the acyclic Schiff base, is impeded. The reaction is completed by a nucleophilic attack of hydroxide ion on the anomeric carbon atom and rapid subsequent release of the free pyrimidine base. The mechanism of alkaline decomposition of m^7G thus differs from that described for 9-(β-D-ribofuranosyl)purine[24] and its 6-substituted derivatives[25] only in the respect that deribosylation takes place prior to deformylation. Evidently the N^5-methyl group retards the nucleophilic attack of hydroxide ion on the carbonyl carbon. The consecutive steps are kinetically exceptionally well separated, owing to the facile attack of hydroxide ion on the positively charged imidazole ring of m^7G. While the half-time for the imidazole ring opening is 63 s in 0.010 mol dm^{-3} aqueous sodium

hydroxide at 298.2 K (I = 0.10 mol dm^{-3}),[18] the isomerization of the sugar moiety of 3a is completed in 10 to 20 h, and the half-time for the rupture of the N-glycosidic bond is of the order of 1 h in 0.10 mol dm^{-3} aqueous sodium hydroxide at 363 K. The opening of the imidazole ring is practically irreversible, since no sign of 7-methylguanine nucleosides was detected during the conversion of the mixture of 3a-d to 4. It is also noteworthy that no radioactivity was released, when 7-methyl-[8-^{14}C]guanosine was converted to 4 in aqueous sodium hydroxide containing unlabelled formate ion (0.1 mol dm^{-3}). In other words, the N^5-formyl group does not equilibrate with free formate ion during the course of alkaline decomposition.

Chetsanga et al. have previously described a slightly different mechanism for the reaction of m^7G with aqueous alkali.[16,17] According to these authors, the first detectably stable intermediate is 7,8-dihydro-8-hydroxy-7-methylguanosine, which is decomposed to both N^5- and N^6-formylated ring-opened structures. Although the ring-opening undoubtedly proceeds via this intermediate, we were unable to detect it by ^{1}H NMR spectroscopy even at the very early stages of the reaction, i.e. when less than 10 % of the starting material had been consumed. Morover, we could not obtain any evidence for the appearance of N^6-formyl intermediates. It is also quite clear that deformylation does not compete with deribosylation, an alternative that was not strictly excluded earlier.

EXPERIMENTAL

Materials. P^1-Guanosine-5' P^3-7-alkylguanosine-5' triphosphates, 1a-e, were prepared from P^1-S-phenyl-P^2-guanosine-5'-pyrophosphorothioate and appropriately substituted 7-alkylguanosine 5'-monophosphates by the method of Nakagawa et al.,[8] and purified by column chromatography on a DEAE-Sephadex resin (A-25, HCO_3^- form) as follows. The crude product (0.4 mmol) was applied to the column (3.5x70 cm), washed with 1 dm^3 of distilled water, and eluted with a

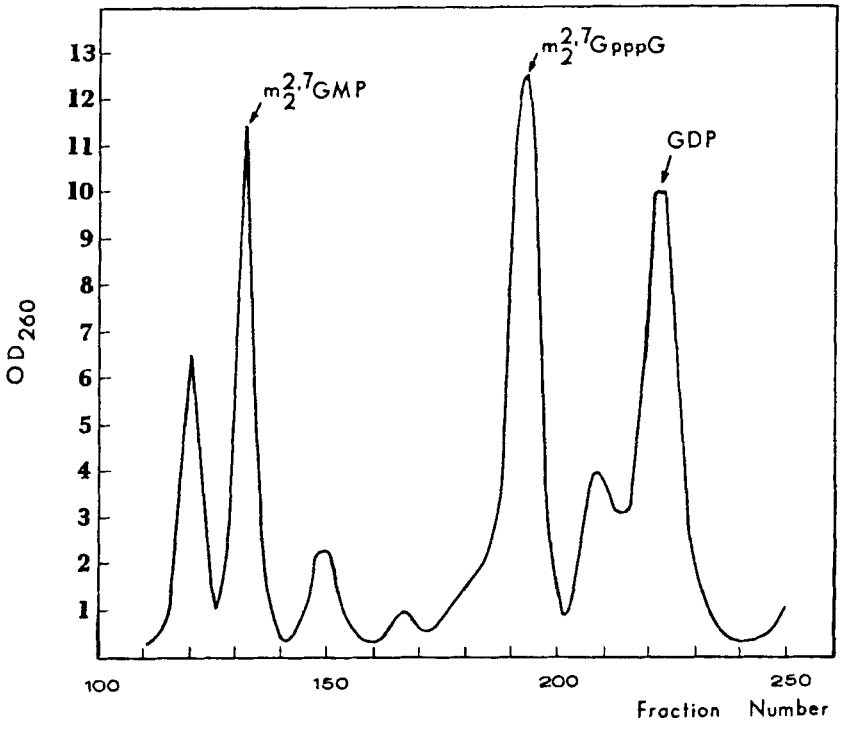


Fig. 3: An elution curve for the chromatographic purification of P^1-N^2,7-dimethylguanosine-5' P^3-guanosine-5' triphosphate, 1b, on DEAE-Sephadex (A25, HCO_3^- form).

linear gradient of triethylammonium bicarbonate (pH 7.5, from 0 to 1 mol dm^{-3}, total volume 4 dm^3). Fig. 3 shows an illustrative example of the elution pattern. Only the 7-benzyl derivative, 1e, needed further purification on Whatman 3 MM paper using a 1:1 (v/v) mixture of aqueous ammonium acetate (1 mol dm^{-3}) and ethanol as eluant. The isolated synthetic products were finally converted to their sodium salts by passing them through Dowex 50WX4 resin (50/100 mesh, Na^+ form), precipitated by ethanol and dried over P_2O_5. The yields, R_F values and UV spectroscopic parameters are listed in Table 7, and NMR spectroscopic data in Tables 1 and 2.

Table 7: Yields, R_F values and UV spectroscopic data for the P^1,P^3-dinucleoside triphosphates prepared.[a]

Compd.	Yield	R_F		λ_{max}/nm		lg ε[b]
		c	d	pH 2	pH 7	
1a	20	0.49	0.04	259(233)[e]	255	4.35
1b	25	0.48	0.06	258(232)	256	4.37
1c	28	0.47	0.09	261(236)	258	4.42
1d	23	0.48	0.05	257(231)	255	4.34
1e	11	0.40	0.12	258(233)	256	4.25

[a]For NMR data see Tables 1 and 2. [b]At pH 7. [c]On cellulose F_{254} plates (Merck) with a mixture of aqueous ammonium sulfate (sat.), 2-propanol and potassium hydrogenphosphate (0.1 mol dm^{-3}, pH 7.4), 79:2:19 (v/v/v). [d]On cellulose plates with a 1:2 (v/v) mixture of aqueous ammonium sulfate (1 %, w/v) and 2-propanol. [e]Wavelength of absorption minimum in parentheses.

N^2-Methyl- and N^2,N^2-dimethyl-guanosine, employed as starting materials, were prepared from commercially available 5-amino-1-(β-D-ribofuranosyl)-4-imidazolecarboxamide (Sigma) via 2-thioinosine and inosine-2- sulfonate, as described by Yamazaki et al.[26] The nucleosides obtained were converted to their 5'-monophosphates with phosphorus oxychloride in trimethylphosphate.[27] It should be noted, however, that a prolonged treatment (12 h at 4 °C) was needed to phosphorylate N^2,N^2-dimethylguanosine. The nucleotides were finally methylated with methyl iodide in DMSO to their N7-methyl derivatives, and purified on a DEAE-Sephadex column by the procedure described previously for 2a.[28] The preparation of the other 7-alkylguanosine 5'-monophosphates employed has been described previously.[7] They were converted to the corresponding nucleosides by dephosphorylation with bacterial alkaline phosphatase.[29]

NMR spectroscopy. The ^1H and ^{31}P NMR spectroscopic data used for the conformational analysis of 1a-e were obtained on a Bruker AM 500 spectrometer at 298.2 K. The substrate concentration was about 5 10^{-3} mol dm^{-3} in ^2H_2O, and the pH

was adjusted to about 7.5. The chemical shifts, to an accuracy of ± 0.005 ppm, were measured relative to internal 2,2,3,3-tetradeuterio-3-tri-methylsilylpropanesulfonic acid sodium salt (TSP). The coupling constants were determined at an accuracy of ± 0.1 Hz. The ^{1}H and ^{13}C NMR spectroscopic characterization of the intermediates and products of the alkaline cleavage of $m^{7}G$ was carried out on a JEOL GX-400 spectrometer at 298.2 K. The shifts were measured relative to external TMS.

pK_a values. The acidity constants for the base moiety of 2a-n were determined spectrophotometrically by the method described previously.[18]

Kinetic measurements First-order rate constants for the alkaline cleavage of 1a-e, 2a-o and the nucleoside analogs of the latters were determined spectrophotometrically as described previously.[18]

Isotopic labeling. The release of [^{14}C]formate ion from 7-methyl-[8-^{14}C]guanosine during its alkaline decomposition in the absence and presence (0.1 mol dm^{-3}) of unlabelled formate ion was followed by passing the alkaline aliquots through a strong anion exchange resin (Dowex 1X4, mesh 100/200, Cl^- form).[24] It was verified that free formate ion was completely retained in the resin, while the starting material, intermediates and products were eluted through the column as neutral compounds. It is noteworthy that the hydroxide ions are exchanged with the chloride ions, and hence the alkaline aliquots are neutralized on passage through the resin.

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