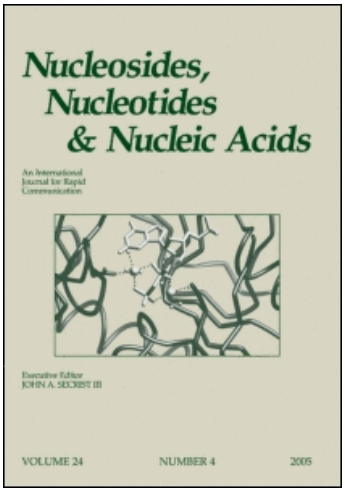

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Synthesis and Properties of P^1, P^2-, P^1, P^3- and P^1, P^4-Dinucleoside Di-, Tri- and Tetraphosphate mRNA 5'-Cap Analogues

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SYNTHESIS AND PROPERTIES OF P^1,P^2-, P^1,P^3- AND P^1,P^4-DINUCLEOSIDE DI-, TRI- AND TETRAPHOSPHATE mRNA 5'-CAP ANALOGUES

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Abstract: Chemically synthesized dinucleoside P^1,P^2-di-, P^1,P^3-tri- and P^1,P^4-tetraphosphates, derivatives of 5'-linked 7-methylguanosine and guanosine were characterized with respect to their structural properties and functional effect on eukaryotic translation inhibition.

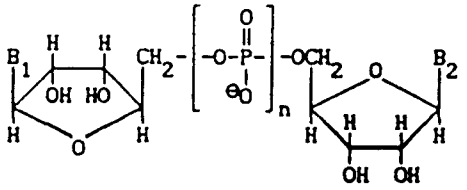
Dinucleotide oligophosphates with 5',5' linkages are of considerable biochemical interest. One of them is the 5'-terminal "cap", m7G(5')ppp(5')N, a structure common to most eukaryotic mRNAs. We have focused our attention[1,2] on the synthesis of cap analogues and their physicochemical and biological properties.

In the present communication we report the synthesis of dinucleoside analogues linked by a 5',5'-di-, tri- and tetraphosphate bridges. In addition, a new approach to the introduction of 7-methyl group into dinucleoside cap analogues has been demonstrated.

RESULTS AND DISCUSSION

The pyrophosphate bond formation in di-, tri- and tetraphosphate cap analogues, which structures are shown in FIG. 1, was achieved by the method introduced recently by Sawai *et al.*[3]. Prepared that way GppG(1), GpppG(4) and GppppG(7) were employed as starting materials for a one-pot synthesis of appropriate mono- and double-7-methylated analogues (compounds: 2 and 3; 5 and 6; 8 and 9). Some of the analogues had been previously synthesized using other methodology[4]; their properties proved to be identical.

The structures of the products (1 - 13) were verified by ^1H and ^{31}P NMR spectroscopy. Selected examples of the ^1H NMR spectra analysis are collected in TABLE 1 and 2. The dinucleoside phosphates with the same nucleoside units show only singular set of



1 GppG: $B_1 = B_2 = G^*$, n = 2
2 m^7GppG: $B_1 = m^7G^*$, $B_2 = G$, n = 2
3 m^7Gpp(m^7G): $B_1 = B_2 = m^7G$, n = 2
4 GpppG: $B_1 = B_2 = G$, n = 3
5 m^7GpppG: $B_1 = m^7G$, $B_2 = G$, n = 3
6 m^7Gppp(m^7G): $B_1 = B_2 = m^7G$, n = 3
7 GppppG: $B_1 = B_2 = G$, n = 4
8 m^7GppppG: $B_1 = m^7G$, $B_2 = G$, n = 4
9 m^7Gpppp(m^7G): $B_1 = B_2 = m^7G$, n = 4
10 $m^{2}_{2}{}^{,7}$GpppG: $B_1 = m^{2}_{2}{}^{,7}G^*$, $B_2 = G$, n = 3
11 $m^{2,2,7}_{3}$GpppG: $B_1 = m^{2,2,7}_{3}G^*$, $B_2 = G$, n = 3
12 $m^{2}_{2}{}^{,7}$GppppG: $B_1 = m^{2}_{2}{}^{,7}G$, $B_2 = G$, n = 4
13 $m^{2,2,7}_{3}$GppppG: $B_1 = m^{2,2,7}_{3}G$, $B_2 = G$, n = 4

*G = *Guanine*, m^7G = *7-Methylguanine*, $m^{2}_{2}{}^{,7}G$ = *N^2,7-Dimethylguanine*, $m^{2,2,7}_{3}G$ = *N^2,N^2,7-Trimethylguanine*

FIG. 1. Structures of the synthesized mRNA 5'-cap analogues.

TABLE 1. ^{1}H-NMR chemical shifts (δ in ppm) of the selected cap analogues.

Compound		H8	H1'	H2'	H3'	H4'	H5'	H5"	7-CH_3	N^2-CH_3
2	m^7G	*a*	5.820	4.43*b*	4.40*b*	4.40*b*	4.34*b*	4.20*b*	4.025	---
	G	7.935	5.735	4.580	4.43*b*	4.40*b*	4.34*b*	4.20*b*	---	---
3	m^7G	*a*	5.865	4.510	4.420	4.38*b*	4.38*b*	4.220	4.105	---
6	m^7G	*a*	5.940	4.540	4.460	4.385	4.375	4.265	4.110	---
8	m^7G	*a*	5.965	4.600	4.485	4.37*b*	4.37*b*	4.280	4.075	---
	G	8.055	5.735	4.650	4.530	4.37*b*	4.26*b*	4.26*b*	---	---
9	m^7G	*a*	6.015	4.645	4.535	4.400	4.380	4.280	4.120	---
12	$m^{2,7}$G	*a*	5.975	4.570	4.470	4.37*b*	4.37*b*	4.275	4.065	2.870
	G	8.045	5.855	4.710	4.520	4.37*b*	4.27*b*	4.27*b*	---	---
13	$m^{2,2,7}$G	*a*	5.995	4.600	4.465	4.38*b*	4.360	4.285	4.065	3.090
	G	8.015	5.775	4.655	4.500	4.38*b*	4.26*b*	4.26*b*	---	---

a - exchanged for ^{2}H;
b - approximate values due to signals overlapping.

TABLE 2. ^{1}H-^{1}H and ^{1}H-^{31}P coupling constants (in Hz) and conformational parameters for 5'-cap analogues.

Compound		J(i,j)								Conformation		
		1',2'	2',3'	3',4'	4',5'	4',5"	5',5"	5',P	5",P	%N	%+sc	%ap
2	m^7G	2.9	*a*	*a*	*a*	*a*	*a*	*a*	*a*	70*b*	*a*	*a*
	G	5.9	5.4	*a*	*a*	*a*	*a*	*a*	*a*	40*b*	*a*	*a*
3	m^7G	3.9	4.9	5.4	*a*	2.5	12.0	*a*	5.0	58	85*b*	75*b*
6	m^7G	3.8	5.0	5.2	2.7	3.2	12.2	4.7	5.8	58	78	73
8	m^7G	3.5	5.0	5.5	*a*	2.5	12.0	*a*	4.5	61	85*b*	75*b*
	G	6.1	5.1	3.3	4.0	4.0	*a*	6.0	6.0	35	57	65
9	m^7G	4.0	4.8	5.1	2.7	2.1	11.8	3.5	5.7	56	89	80
12	$m^{2,7}G$	3.7	5.1	5.2	*a*	2.5	12.0	*a*	5.9	59	85*b*	70*b*
	G	6.6	5.1	2.9	4.0	4.0	*a*	6.5	6.5	31	57	61
13	$m^{2,2,7}G$	3.6	4.9	5.3	*a*	2.5	12.0	*a*	5.0	60	85*b*	75*b*
	G	6.2	5.3	3.2	4.0	4.0	*a*	6.5	6.5	34	57	61

a - value not determined due to signals overlapping;
b - approximate value due to lack of the suitable coupling constants.

^{1}H NMR signals due to symmetry. The nucleoside phosphates with different nucleoside units show the characteristic ^{1}H NMR patterns for each of them. A ^{31}P NMR spectrum of a typical dinucleoside triphosphate shows a doublet of two-phosphorus intensity at -12.2 ppm (P1 and P3) and a triplet at -22.8 ppm (P2); the ^{31}P-^{31}P coupling constant being 19.5 Hz.

A ^{31}P NMR spectrum of a typical dinucleoside tetraphosphate has an AA'XX' pattern with the chemical shifts and coupling constants as follows: $\delta(P1) = \delta(P4) = -10.9$ ppm, $\delta(P2) = \delta(P3) = -22.8$ ppm, $J(P1,P2) = J(P3,P4) = 15$ Hz, $J(P2,P3) = 19.4$ Hz, $J(P1,P3) = J(P2,P4) = 2.8$ Hz. Vicinal coupling constants were used to follow conformation of the analogues by the methodology described earlier[4]. All the dinucleotide cap analogues studied are conformationally similar to one another and to those studied hitherto[4]. The 7-substituted nucleotides show characteristic 55 to 70% populations of the N (C3' *endo*) form of sugar ring, more than 85% populations of the *+sc* conformer about the C4'-C5' bond *i.e. gauche* orientation of O5' to O4' and C3', and 70 to 80% populations of the conformer with a transoidal orientation of C4' and P about the C5'-O5' bond. The unsubstituted guanine moieties prefer the S type sugar puckering (55 to 70%) and a less marked predominance of *+sc* (55 - 70%) and *ap* (*gauche-trans*, 60 to 73%) conformations about the C4'-C5' and C5'-O5' bonds, respectively.

Fluorescence and UV-absorption studies of the obtained compounds have been accomplished by the methodology described previously[5]. Fluorescence maxima (λ max), acidity constants (pK) and quantum yields at pH 5.02 and 9.07 were determined. In addition, for intramolecular base stacking evaluation the fluorescence intensities were measured as a

function of temperature. The following conclusions may be drawn: (i) compounds 3, 6 and 9 exhibit no self-stacking at pH 5.02 due to electrostatic repulsion between the two positively charged 7-methylguanine rings; (ii) for compounds 12 and 13 a considerable (about 40%) decrease in stacking is noted in relation to their triphosphate counterparts (compounds 10 and 11, respectively); the analogous difference between 8 and 5 is less marked.

In a continuation of our previous studies[2,6], the synthesized analogues were assayed as competitive inhibitors of rabbit globin mRNA translation in a rabbit reticulocyte cell-free system[7]. We observed that the dinucleotides bearing two 7-methylguanine residues (3, 6 and 9) are equivalent to the analogues with one 7-methyl group (2, 5 ad 8) containing the same number of phosphates. Moreover, the inhibitory effect increases together with the number of phosphates, for example 9 is more (about 4 times) potent inhibitor than 6, and significantly more (about 15 times) potent than 3. Non-methylated analogues (1, 4 and 7) exhibit considerably weaker activity as inhibitors than their 7-methylated counterparts.

EXPERIMENTAL

Compounds 1, 4 and 7 were prepared from guanosine 5'-phosphorimidazolide (P-ImGMP) and GMP, GDP and GTP, respectively, in water solution in the presence of manganese(II) ions[3] Analogously, appropriate imidazole-activated N^2- and 7-methylated nucleotides, P-Im($m^{2,7}$)GMP and P-Im($m^{2,2,7}$)GMP, prepared from $m^{2,7}$GMP and $m^{2,2,7}$GMP as previously reported[2,4], were coupled with GDP or GTP to afford compounds 10 - 13.

Compound 1 was methylated with methyl iodide (three molar excess) in DMSO at room temperature[1] and, the reaction progress was monitored by HPLC (Supelco LC-18-T reverse phase column, linear gradient of methanol from 0 to 25% in 0.1 M KH_2PO_4, pH 6, within 15 min. The samples were analyzed at 1 hr intervals and, the reaction was quenched when concentrations of m^7GppG and m^7GppG(m^7) were equal (about 40% each, as revealed by monitoring at 260 nm). The resulting mixture was subjected to ion exchange column chromatography on DEAE-Sephadex (A-25, HCO_3^- form) and eluted with a linear gradient of triethylammonium bicarbonate (pH 7.4) from 0 to 1 mol/l. The obtained fractions, in order of appearance: m^7GppG(m^7) (3), m^7GppG (2) and unreacted 1, were pooled, concentrated and converted into corresponding sodium salts as described earlier[1,4]. In a similar manner compounds 5 and 6, as well as 8 and 9, were prepared from 4 and 7, respectively.

^{1}H NMR spectra were recorded on a Jeol GX 400 and a Bruker AM 500 instruments in ^2H_2O. ^{31}P NMR spectra were run on a Varian XL 200 apparatus. The absorption spectra were recorded on a Cary 3E spectrophotometer. The fluorescence excitation and emission spectra were measured on a Perkin Elmer LS-50 spectrometer.

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