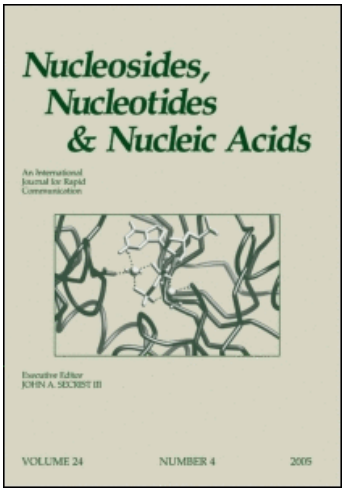

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SYNTHESIS AND ENZYMATIC CHARACTERIZATION OF METHYLENE ANALOGS OF ADENOSINE 5′-TETRAPHOSPHATE (P_4A)

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▫ *A new methodology for synthesis of biologically important nucleoside tri- and tetraphosphates containing a bisphosphonate moiety instead of the terminal pyrophosphate bond is described. The series consists of tri- and tetraphosphate analogs of adenosine, guanosine and 7-methylguanosine (characteristic for mRNA cap). We have adopted a two-step procedure that allowed us to insert a methylene bridge into the phosphate chain. Nucleoside mono- or diphosphates were first activated (as imidazole derivatives) and then used in coupling reactions with organic salts of bisphosphonate. The resulting synthetic method enabled us to obtain the desired compounds with high yields and does not require any protective groups. This makes it very useful for the synthesis of labile compounds such as those containing the 7-methylguanosine ring. The structures of the synthesized compounds were confirmed by NMR spectroscopy. They were tested as potential substrates and inhibitors of several hydrolases.*

Keywords Bisphosphonates, Adenosine 5′-Tetraphosphate, Nucleoside Tetraphosphatase

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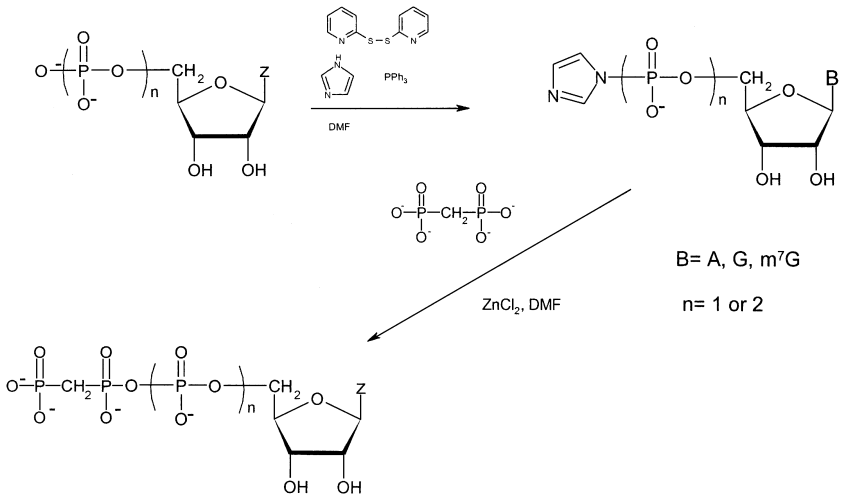
INTRODUCTION

Bisphosphonates are chemically stable analogs of inorganic pyrophosphate, and are resistant to chemical and enzymatic hydrolysis. Bisphosphonates that closely resemble pyrophosphate can lead to nonhydrolyzable analogs of NTP that may inhibit NTP-dependent intracellular enzymes. For the present studies the following compounds were synthesised: pCH_2ppG, pCH_2pppG, pCH_2ppA, pCH_2pppA by reaction of bisphosphonate with commercial nucleotides: GMP, GDP, AMP, and ADP, respectively. The nucleotide substrates were activated with imidazole and then coupled with bisphosphonate in DMF in the presence of $ZnCl_2$ as a catalyst (Scheme 1). Satisfactory yields (70–90%) of the coupling reaction were obtained (Figure 1). Moreover, pCH_2ppG and pCH_2pppG were methylated at position 7 of the guanine ring, yielding two new cap analogs: pCH_2ppm^7G and pCH_2pppm^7G. These nucleotides can be applied to the study of many enzymatic processes. In particular, the tri- and tetraphosphate analogues of 7-methylguanosine containing a bisphosphonate moiety could be very useful tools for elucidating decapping processes.

CHEMISTRY

Activation of the 5′-phosphate group was achieved by conversion to a 5′-phosphoroimidazolide derivative using triphenyl phosphine/2,2′-dithopdipyridine as a condensing agent. The activated compound was reacted with the triethylammonium salt of bisphosphonate in DMF. The reaction rate was greatly accelerated when it was carried out in the presence of an 8-fold excess of $ZnCl_2$.[1,2] The coupling efficiency of the activated nucleotide with bisphosphonate was similar



SCHEME 1 Coupling reaction of activated nucleotide with biphosphonate.

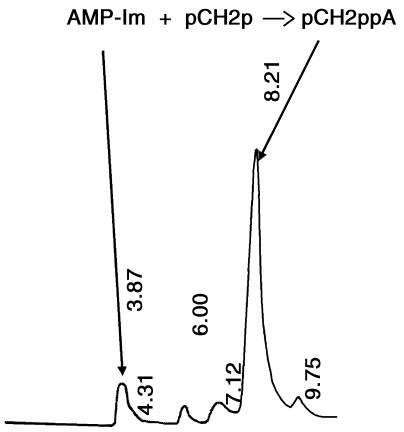
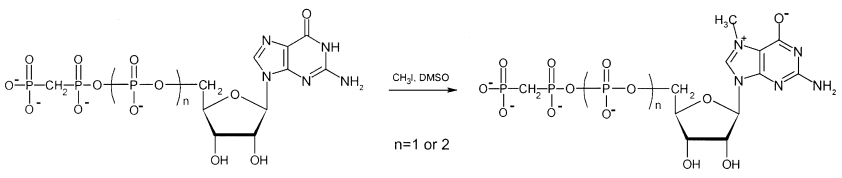


FIGURE 1 HPLC profile (ion-exchange column) of the reaction mixture. Activated adenosine 5′-monophosphate is coupled with bisphosphonate in DMF.

to the coupling with pyrophosphate.[3] The reaction was completed after 2.5 h. The 3-fold molar excess of bisphosphonate led exclusively to the mononucleotide product (pCH_2ppN); the dinucleotide product ($NppCH_2ppN$) was not observed. However, when a 2-fold excess of bisphosphonate was used, the reaction mixture contained ~10% of the dinucleotide product. When the activated nucleotide was in excess, the dinucleotide was the main product. The overall yield of the two steps (activation and coupling) was 78% in the case of pCH_2ppA and 74% in the case of pCH_2ppG. For the 5′-tetraphosphates, pCH_2pppA and pCH_2pppG, the yields were lower by 5 to 10%. A similar procedure was used to synthesize pCH_2ppm^7G and pCH_2pppm^7G, the mRNA cap analogs. The activation of 7-methylguanosine 5′-monophosphate and 7-methylguanosine 5′-diphosphate and coupling with bisphosphonate were less efficient (20–33%) than in the case of the parent non-methylated nucleotides. Higher overall yields were obtained when the methyl group was introduced at the final step of the reaction. Methylation of pCH_2ppG or pCH_2pppG at the 7-position of guanosine were performed in DMSO using a 4-fold excess of MeI and gave pCH_2ppm^7G and pCH_2pppm^7G with 49% and 45% overall yields, respectively (Scheme 2). The structures of final products were confirmed by MS spectrometry and ^1H and ^{31}P NMR spectroscopy (Table 1).



SCHEME 2 N-7 methylation of modified quanosine nucleotides.

TABLE 1 ^{1}H NMR and ^{31}P NMR Spectra of the Final Products were Run on a Varian UNITYplus 400 MHz Spectrometer at 25°C, and Concentration of 2.5 mg/mL in $2H_2O$

	pCH_2ppG	pCH_2pppG	pCH_2ppA	pCH_2pppA	pCH_2ppm^7G	pCH_2pppm^7G
H2	–	–	8.264	8.273	–	–
H8	8.112	8.121	8.538	8.557	–[b]	–[b]
H1′	5.932	5.927	6.155	6.152	6.077	6.078
H2′	4.79[a]	4.85[a]	4.78[a]	4.82[a]	4.667	4.702
H3′	4.565	4.591	4.573	4.616	4.531	4.565
H4′	4.355	4.365	4.406	4.415	4.405	4.415
H5′	4.22[a]	4.22[a]	4.25[a]	4.29[a]	4.33[a]	4.36[a]
H5″	4.21[a]	4.25[a]	4.23[a]	4.21[a]	4.26[a]	4.29[a]
CH_3	–	–	–	–	4.125	4.131
CH_2	2.311 (J = 20.40)	2.340 (J = 20.20)	2.330 (J = 20.38)	2.295 (J = 20.20)	2.314 (J = 20.20)	2.354 (J = 20.37)
Pα	−3.827	4.149	−3.831	−4.145	−3.871	−4.150
Pβ	16.405	−15.510	16.093	−15.406	16.473	−15.480
Pγ	21.327	16.544	21.549	17.749	21.312	16.389
Pδ		21.345		20.566		21.477

^{1}H NMR chemical shifts in ppm (± 0.001) *vs.* internal sodium 3-trimethylsilyl- [2,2,3,3-$^{2}H_4$]-propionate and ^{31}P NMR chemical shifts in ppm (± 0.01) *vs.* external H_3PO_4.

[a]Approximate value due to overlapping.

[b]Exchangeable proton.

ENZYMATIC STUDIES

The newly synthesized compounds have been tested as potential substrates and inhibitors of several hydrolases. The following analogs of adenosine-5′-tetraphosphate (ppppA): pCH_2pppA, pCH_2pppG and pCH_2pppm^7G were substrates for neither the specific nucleoside tetraphosphatase from yellow lupin seeds nor the yeast exopolyphosphatase (EC 3.6.1.11) that is able to dephosphorylate p_4Ns to the corresponding NTPs. Interestingly, the analogs did not inhibit these hydrolases either. However, they were strong inhibitors of both the asymmetrical and symmetrical Ap_4A hydrolases.

The Enzymes

Nucleoside tetraphosphatase (EC 3.6.1.14) from yellow lupin seeds,[4] exopolyphosphatase (EC 3.6.1.11) from bakers' yeast,[5] (*asymmetrical*) diadenosine tetraphosphate hydrolase (EC 3.6.1.17) from lupin[6] and (*symmetrical*) diadenosine tetraphosphate hydrolase (EC 3.6.1.41) from *Escherichia coli*[7] were obtained as described earlier.

Enzyme Assays

All the enzymes were assayed by thin-layer chromatography of the corresponding reaction mixtures.[4–7] Substrates were separated from (potential) products on silica plates containing a fluorescent indicator (from Merck) by developing the chromatograms in a mixture of dioxane:concentrated ammonia:

water (v/v 6:1:4) for the Ap_4A hydrolases, and dioxane:concentrated ammonia: water (v/v 6:1:6) for p_4A degrading enzymes, for 60–90 min. The nucleotide spots were visualized under short ultraviolet light. Quantification was based on the use of labeled substrates, [^{3}H]-p_4A or [^{3}H]-Ap_4A.

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