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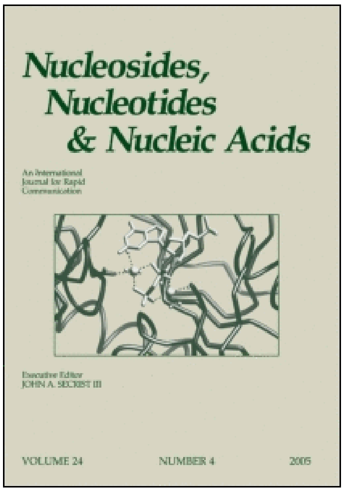

Nucleosides, Nucleotides and Nucleic Acids

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CHEMICAL SYNTHESIS OF CAP ANALOGUES: P^1,P^2-DINUCLEOSIDE-5'-DIPHOSPHATES

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Abstract

A procedure was developed for the chemical synthesis of P^1,P^2-dinucleoside-5'-diphosphates (N_1(5')pp(5')N_2) on a nanomolar scale. Reaction conditions for activating purine-5'-monophosphates (pA, pG, and pm^{7}G) by 1,1'-carbonyldiimidazole were studied and optimized in respect to solvents and amount of activating reagent used. Various dinucleoside-5'-diphosphates were synthesized in 62 - 98 % yield by incubating activated and non-activated purine-5'-monophosphates. Two unexpected by-products were formed by competition reactions: the imidazolidate of the non-activated nucleotide and the corresponding symmetrically substituted dinucleoside-5'-diphosphate. A mechanism is proposed to explain the observed side reactions.

About thirty years ago, Khorana[1] reported the synthesis of P^1,P^2-diadenosine-5'-pyrophosphates. This type of dinucleoside pyrophophate was shown to be an important intermediate in the chemical polymerisations of nucleotides[2] as well as in the enzymatic coupling reaction of NAD$^+$-dependent DNA-ligase[3] from E. coli. Similar linkages were found in important biochemical cosubstrates of redox reactions (e.g. NAD$^+$, NADP$^+$, FAD, and FMN) and in 5'-terminal "cap" structures (m^{7}G(5')ppp(5')N) of most viral and cellular messenger RNAs, of their precursors as well as of small nuclear RNAs[4]. The cap plays an important role in the formation of initiation complexes during protein synthesis since it promotes the attachment of ribosomes to mRNAs[5]. This complex formation is additionally facilitated by a specific cap binding protein[6] and by initiation factors. It has been shown by physico-chemical measurements that the very rigid conformation[7] of the P^1,P^3-

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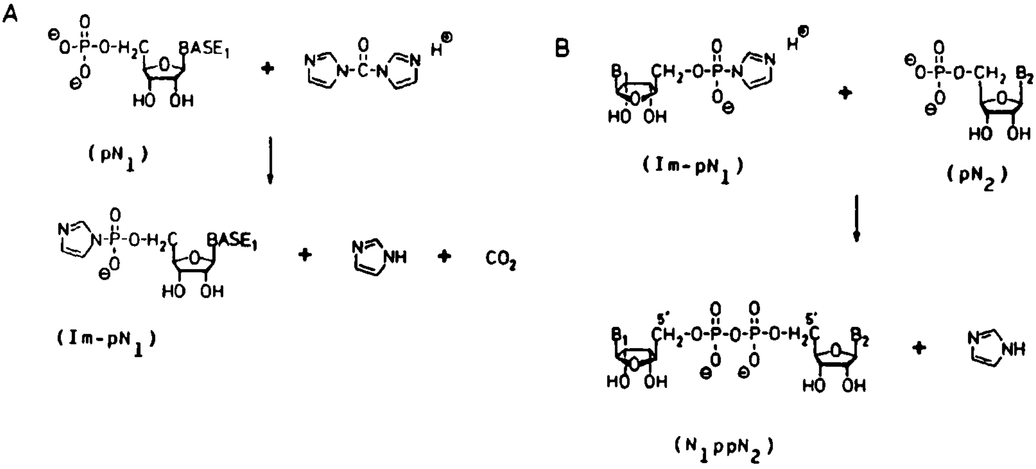
dinucleoside-5'-triphosphates $m^7G(5')ppp(5')N$, which is probably important for the biological function of the cap[8], is very similar to the conformation of the diphosphates[9] $m^7G(5')pp(5')N$. We have developed relatively mild reaction conditions for the chemical synthesis of P^1,P^2-dinucleoside-5'-diphosphates in high yields by using the imidazolidate reaction of Cramer and Neunhoeffer[10].

The reaction conditions for the activation step (Scheme 1A) were investigated for pA, pG, and pm^7G. Using a 5 molar excess of 1,1'-carbonyldiimidazole in dimethylformamide (DMF), the tri-n-butylammonium-(TNBA)-salts of the monophosphates pA and pG were activated to Im-pA and Im-pG in approximately 85 % yield after 30 min . Longer incubation times increased the yields of imidazolidates only slightly. The symmetrically substituted dinucleoside-5'-diphosphate was formed as a by-product of the side reaction between the activated and the non-activated nucleotide (starting compound) in approximately 10 % yield. The odd nucleotide pm^7G, which is part of the 5'-terminal cap structure of eukaryotic mRNAs, is insoluble in DMF probably due to the positive charge at N7. However, after conversion of pm^7G into the di-TNBA-salt solubility in DMSO improved. In general, imidazolidation of nucleotides in DMSO gave rise to a lower yield compared to that obtained in DMF therefore the reaction time had to be extended to 5 h in order to achieve formation of $Im\text{-}pm^7G$ in 50 - 60 % yield.

P^1,P^2-dinucleoside-5'-diphosphates (Scheme 1B) were synthesized on a nanomolar scale by incubating TNBA-salts of activated and non-activated purine-5'-



SCHEME 1: Synthesis of P^1,P^2-dinucleoside-5'-diphosphates by the imidazolidate procedure. A) Activation of nucleotides with 1,1'-carbonyldiimidazole and B) Coupling between activated and non-activated nucleotide.

monophosphates in DMF. Excess 1,1'-carbonyldiimidazole had to be removed by decomposition with methanol before coupling to the non-activated nucleotide could be started. When residual 1,1'-carbonyldiimidazole was present during the coupling reaction the added non-activated nucleotide became easily activated and the symmetrical dinucleoside-5'-diphosphate of the non-activated nucleotide was formed. Our conditions did lead to a complete destruction of excess 1,1'-carbonyldiimidazole and were thus more suitable than those described previously[11,12]. Side reactions on other nucleophilic groups, such as the 2'-OH and 3'-OH groups of the ribose moiety[13] were suppressed due to the reaction conditions employed.

Coupling of Im-pA to ^{14}C-pG resulted in AppG formation (FIG. 1 and TABLE 1). AppG was resistant to alkaline phosphatase but sensitive to snake venom phosphodiesterase which produced 5'-monophosphates. Unsymmetrically substituted AppG can also be synthesized from Im-pG and pA, although with slightly reduced yields (TABLE 1). Two-dimensional thin-layer co-chromatography of the products from both reactions (Im-pA + pG and Im-pG + pA) provided further evidence for the chemical identity of the newly formed dinucleotides. In the product analysis (FIG. 1A) small

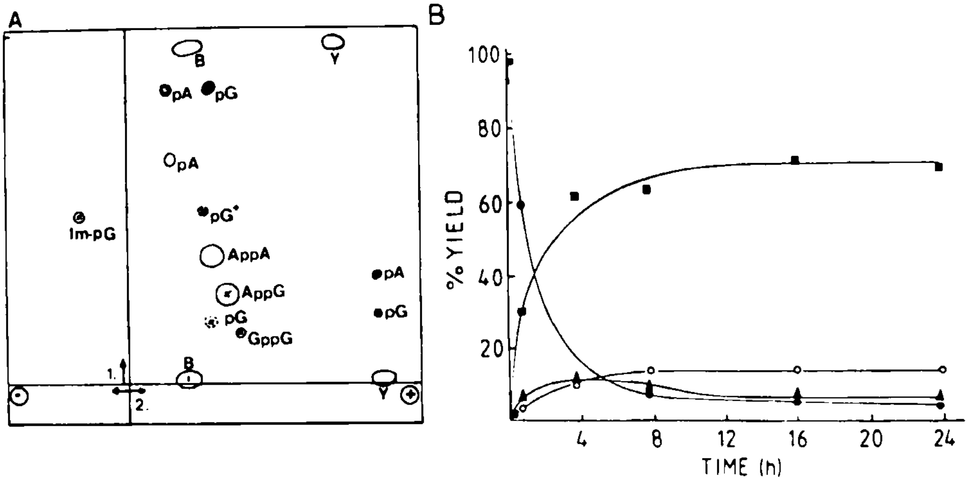


FIG. 1: Chromatographic (A) and kinetic (B) analysis of AppG formation. pA was activated by 1,1'-carbonyldiimidazole and incubated with ^{14}C-pG as described in Experimental. Aliquots, corresponding to 20 nmoles of ^{14}C-pG, were removed at the indicated times and analysed by two-dimensional TLC/TLE. Radioactive spots were marked with "x" in the UV pattern (A). Yields were determined by quantifying the radioactivity of each spot (B). The reference substances pA and pG (hatched spots) were applied on the thin-layer plate at the right in the first dimension and at the left in the second dimension. B and Y correspond to the blue (xylencyanole FF) and yellow (orange Y) markers, respectively. Im-pG is partly decomposed to pG$^+$. (●): pG; (▲): Im-pG; (■): AppG; (○): GppG.

TABLE 1: Yields of dinucleoside-5'-diphosphate formation

educts	ratio	products	yield (%)
Im-pA + pG	4 : 1	AppG	63
Im-pA + pG	2 : 1	AppG	73
Im-pA + pG	1.2 : 1	AppG	57
Im-pG + pA	2 : 1	AppG	62
Im-pm^{7}G + pA	2.4 : 1	m^{7}GppA	52
Im-pA + pA	4 : 3	AppA	98
Im-pG + pG	4 : 3	GppG	62

Yields of symmetrical products were calculated in relation to total added nucleotide and yields of unsymmetrically substituted products were calculated as described in Experimental.

amounts of activated Im-pG were detected which allowed the synthesis of the symmetrically substituted dinucleotide GppG. AppG formation reached a plateau after 12 - 16 h (FIG. 1B) when a 2-fold excess of activated mononucleotide was used. In several different experiments yields of 64 - 73 % AppG were obtained (TABLE 1). A 2 molar excess of the activated mononucleotide seems to be optimal for the synthesis of AppG (TABLE 1). A higher excess of the imidazolidate increased the formation of Im-pG in a competition reaction. Coupling of Im-pA and pG is also possible in DMSO and formamide but the yield of AppG was reduced to 39 % and 3 % respectively.

The low solubility of the TNBA-salt of pm^{7}G in DMF is disadvantageous for the synthesis of dinucleoside-5'-diphosphates containing pm^{7}G. Fortunately, pm^{7}G can be activated by 1,1'-carbonyldiimidazole in DMSO in acceptable yields. Since the imidazolidate of pm^{7}G dissolved slowly in DMF, this solvent was used in the coupling of a 2.4-fold excess of Im-pm^{7}G with non-activated ^{3}H-pA. m^{7}GppA was formed im 52 % yield as the main product (TABLE 1). Non-reacted pA (45 %), small amounts of Im-pA (approximately 1 %) and in some experiments also AppA (less than 1 %) were found after thin-layer electrophoresis (TLE).

During the coupling reaction two unexpected by-products were formed from the non-activated nucleotide: the corresponding imidazolidate and the symmetrically substituted dinucleotide (FIG. 1). In control experiments the possibility was excluded that residual 1,1'-carbonyldiimidazole was the reason for the activation of the added non-activated nucleotide because under our conditions 1,1'-carbonyldiimidazole was completely destroyed by methanol.

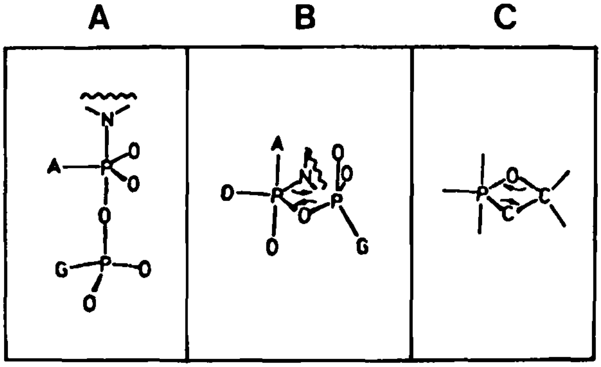


FIG. 2: Proposed reaction mechanism for the imidazole group transfer from Im-pA to pG. A) Transition state of AppG during coupling of Im-pA and pG. B) Proposed transition state for the transfer of the imidazole residue from Im-pA to pG. C) Transition state of the Wittig reaction.

Activation of pG must have occured during the incubation of Im-pA with non-activated pG. This observation might be explained by the following hypothesis: the reaction between Im-pA and pG is a nucleophilic substitution which probably proceeds via a transition state where entering and leaving groups are located at the apices of a bipyramidal-trigonal structure (FIG. 2A). The dinucleotide AppG is formed after exit of the imidazole residue. It is conceivable that the substituents in the bipyramidal-trigonal transition state rearrange and form a conformation in which the imidazole residue could be transferred from pA to pG (FIG. 2B). Such rearrangements of binding electrons in a four membered cycle are principally possible; they have been also postulated for the transition state of the Wittig reaction between triphenylphosphine alkylenes and carbonyl compounds (FIG. 2C).

Our detailed investigation of the activation step for the synthesis of P^1,P^2-dinucleoside-5'-diphosphates resulted in the use of milder reaction conditions than those previously described[11,12,14]. By varying the solvent and the molar excess of the reaction components it was possible to synthesize m^7GppA which is a "cap" analogous dinucleoside-5'-diphosphate. The yield was several fold higher and the reaction time considerably shorter than previously described for a similiar reaction[14]. P^1,P^2-dinucleoside-5'-triphosphates can also be synthesized by the imidazolidate method but a broader spectrum of reaction products with a reduced yield is obtained as we and others have noticed independently[15].

Experimental

Imidazolidation of purine nucleotides:

In general, reactions were carried out with freshly distilled and carefully dried solvents avoiding moisture completely. Tri-n-butlyamin (TNBA)-salts were prepared as previously described[11,12]. The mono-TNBA-salts of pA and pG were dissolved in dimethylformamide (DMF) at a concentration of 100 nmoles/15 μl and treated with a 5 molar excess of 1,1'-carbonyldiimidazole. The di-TNBA-salt of pm^7G had to be converted into the imidazolidate in dimethylsulphoxide (DMSO) because of solubility problems. Imidazolidation was stopped by addition of 0.1 vol of tri-n-butylamine and 0.1 vol of water, when the activated nucleotides were to be isolated. Solvents were evaporated under vacuum.

Preparation of P^1,P^2-dinucleoside-5'-diphosphates:

Excess 1,1'-carbonyldiimidazole from the activation reaction was destroyed at room temperature with 15 μl methanol/100 nmoles of nucleotide for 30 min and the solvent was evaporated under vacuum. The imidazolidate was dissolved in DMF (200nmoles/ 30 μl) and a solution of the non-activated nucleotide in DMF (100 nmoles/15 μl) was added. The molar ratio of activated to non-activated nucleotide was varied (TABLE 1). Reactions were stopped after 16 h of incubation at room temperature by addition of 0.1 vol tri-n-butylamine and 0.1 vol of water. Subsequently, the solvents were evaporated under vacuum. The non-activated nucleotide was radioactively labeled (^{14}C-pG: 400 nCi/μmol; ^{3}H-pA: 1.25 μCi/μmol), and its quantity was taken to calculate the reaction yields. All experiments were performed in a 20 to 500 nmolar range.

Analytical procedures:

Reaction products were dissolved in 0.2 M triethylamine and analysed on cellulose plates (Merck, 20 x 20 cm) by ascending chromatography (1. dimension) using solvent A[10]: n-propanol - concentrated NH_4OH (25 % w/v) - water (60 : 12 : 28, v/v/v) and electrophoresis (2. dimension) using 5 % acetic acid (v/v) adjusted to pH 4.0 with morpholine. TLE was performed for 2.5 h at a constant current of 19 mA until orange Y had migrated for approximately 13 cm. Nucleotides were localized on thin-layer plates by UV-light at 254 nm or by a spark chamber (Betacamera BF 290 HR, Berthold, Wildbad, FRG) when they were radioactively labeled. After elution with 0.2 M triethylamine the recovered nucleotides (40 nmoles) were incubated at $37^{o}C$ for 90 min either with 0.13 units of alkaline phosphatase (calf intestine, Boehringer Mannheim) in 10 μl of 0.1 M ammonium bicarbonate buffer (pH 8.5) containig 1 mM $MgCl_2$, or with 1.3 μg phosphodiesterase (VPH, Worthington) in the same buffer but at an increased Mg^{2+} concentration of 15 mM.

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