Kinetic Preference for the 3′−5′-Linked Dimer in the Reaction of Guanosine 5′-Phosphorylmorpholinamide with Deoxyguanosine 5′-Phosphoryl-2-methylimidazolide as a Function of Poly(C) Concentration

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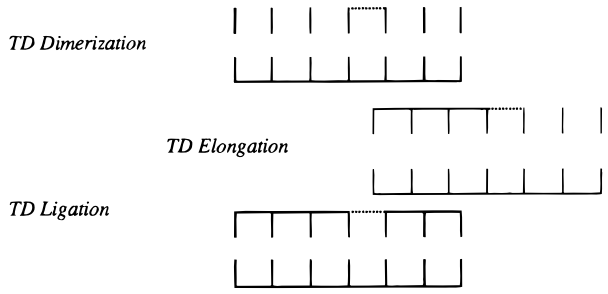
The formation of the internucleotide bond in diguanylate synthesis was studied in aqueous solution at pH 8 and 0.2 M Mg^{2+} in the presence and absence of polycytidylate, poly(C). The investigation was simplified by using guanosine 5′-phosphorylmorpholinamide, mor-pG, which can act only as a nucleophile, and deoxyguanosine 5′-phosphoryl-2-methylimidazolide, 2-MeImpdG, which can act only as an electrophile. The time-dependent product distribution was monitored by high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC/MS). In the absence of poly(C) the reaction between mor-pG and 2-MeImpdG yielded small amounts of the dimer mor-pGpdG with a regioselectivity of 2′−5′:3′−5′ = 3.5. In the presence of poly(C) dimer yields increased and a reversal in regioselectivity occurred; both effects were in proportion to the concentration of the polymer. The results can be quantitatively explained with the proposition that poly(C), acting as the template, catalyzes the reaction between template-bound monomers by about a factor of 4−5 over the reaction in solution and yields dimers with a regioselectivity of 2′−5′:3′−5′ ≈ 0.33. These findings illustrate the intrinsic preference of guanosine *monomers* to correctly self-assemble on the appropriate template.

Introduction

Nature has used enzymatic template-directed (TD) reactions, such as DNA replication, to catalyze and direct the synthesis of the biopolymers RNA and DNA. In an attempt to find prebiotic precursors of the extant DNA replicating systems, work pioneered by Orgel examined RNA-directed synthesis of polynucleotides in the absence of enzymes.[1−6] It was found that an RNA or a DNA single strand, a so-called template, typically directs and catalyzes the synthesis of its complementary strand from chemically activated monomeric or oligomeric nucleic acids (Chart 1). It has been shown that elongation, i.e., the incorporation of an activated mononucleotide, follows Watson−Crick base-pairing rules and is catalyzed by the template,[7] and in certain cases the formation of the 3′−5′-internucleotide linkage is preferred over the 2′−5′-linkage.[7] Similar effects of catalysis and regioselectivity are observed with TD ligation, i.e., the condensation of two oligonucleotides within a double helix.[8] However, the effect of the template on the initiation of the polymerization, i.e., the dimerization, which is the slowest step, has not been established.

Chart 1. Schematic Representation of TD Reactions[a]



[a] Both oligomers and monomers are template-bound. The internucleotide bond to be formed is shown as a broken line.

One of the more extensively studied nonenzymatic polymerizations is the poly(C)-directed oligoguanylate synthesis from guanosine 5′-phosphate 2-methylimidazolide, 2-MeImpG.[9a,10−13] This polymerization produces excellent yields of oligoguanylates up to 40 bases long,[9b] primarily 3′−5′-linked, and discriminates between guanosine and the other nucleobases by a factor of 500:1.[9a] In the absence of the template only small amounts of dimers and traces of trimers are detected. It was

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(9) (a) Inoue, T.; Orgel, L. E., *J. Mol. Biol.* **1982**, *162*, 201−218. (b) The fact that poly(C) induces the formation of long oligoguanylates is consistent with a large catalytic effect on the elongation and a small, if any, effect on the dimerization. If dimerization and elongation were catalyzed to a comparable degree, then the products would be short oligomers.

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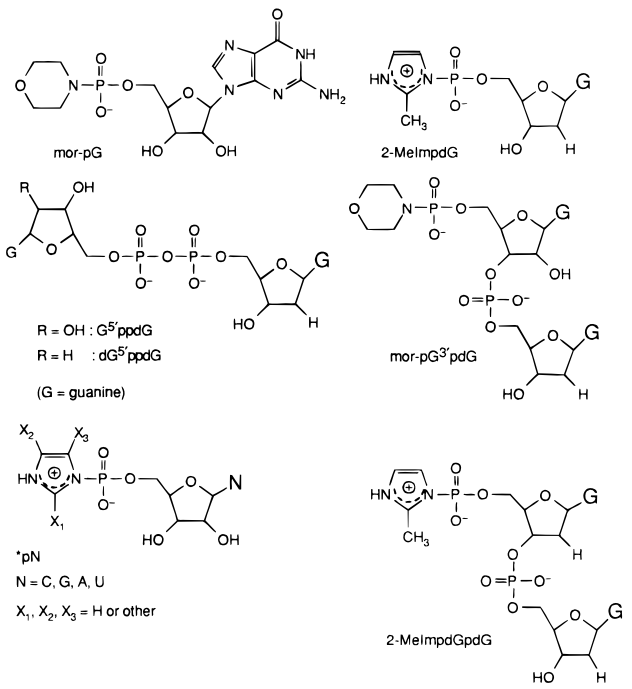
Chart 2



determined that the template catalyzes the elongation process by 20-fold, but enhances selectively the formation of the 3′−5′-linkage by 140-fold at 37 °C.[11] Initially, it was believed that the poly(C)/2-MeImpG helix is already melted at 20 °C[14] and hence conjectured that dimerization occurs in solution at 20 °C.[10] However, recent studies at 23 °C showed that 2-MeImpG as well as other guanosine monomers binds poly(C) with a free monomer concentration of 5.5 mM at half-occupancy of the template.[15] In addition, rates of 3′−5′-linked dimer formation were obtained as a function of 2-MeImpG concentration at a constant template concentration (0.05 M poly(C)), and a mechanism was proposed by which dimerization occurs within long stacks of template-bound monomers.[13] Nevertheless, the rate of dimer formation in the absence of poly(C) is not known, and the effect of template concentration on the yield and the regioselectivity of 2-MeImpG dimerization have not been quantified.

Studies of the dimerization of 2-MeImpG or other activated nucleotides in the presence of the complementary polymer are inherently difficult because 2-MeImpG acts both as a nucleophile and as an electrophile and because the produced dimers are rapidly consumed by the polymerization. To stop the reaction at the dimerization stage, the nucleophilic site has to be separated from the electrophilic site. This requirement has been met here by using mor-pG as the nucleophile and 2-MeImpdG (see Chart 2) as the electrophile. The choice of mor-pG was based on the observation that morpholine is a very poor leaving group in neutral aqueous solutions; it becomes reactive only at pH < 4.[16] Hence mor-pG can act practically only as a nucleophile. 2-MeImpdG was chosen because the deoxyribose is much less reactive than the ribose,[7a,17] and therefore this compound can act practically as an electrophile only. Indeed, the investigation of dimerization in the reaction of mor-pG with 2-MeImpdG allowed, for the first time, a correlation between template concentration, on one hand, and the yield and regioselectivity of diguanylate product distribution, on the other.

Results

General. Experiments were conducted in aqueous solutions in the presence of 0.5 M HEPES buffer (pH ≈ 8.0), 0.2 M $MgCl_2$, and 1 M NaCl. The same conditions had been used for the TD 2-MeImpG oligomerization[9a,13] and for the binding experiments of mor-pG and 2-MeImpG on poly(C).[15] In the present study concentrations varied in the range 31.5 mM ≤ [mor-pG] + [2-MeImpdG] ≤ 148 mM and 7.6 mM ≤ [poly(C)] ≤ 55.3 mM with poly(C) expressed in monomer equivalents. Reaction mixtures were incubated at 23 °C for up to 6 days until about 90% of 2-MeImpdG had reacted. Aliquots were obtained at regular intervals and analyzed by HPLC. Representative samples were analyzed also by LC/MS. All guanosine monomers exhibit similar extinction coefficients, and therefore product distributions are obtained directly as percent of the total HPLC area corresponding to the initial substrate(s).

Self-Condensation of 2-MeImpdG. In the absence of poly(C) and with 0.2 M Mn^{2+} three products were observed:[18] the hydrolysis product, 5′dGMP, the product coming from substitution with the buffer, HEPES-pdG, and dideoxyguanosine 5′-pyrophosphate, dG5′ppdG (Chart 2) all eluting ahead of the starting material (see Figure 1A; identification is described in the Experimental Section). After 148 h of incubation, a 0.104 M 2-MeImpdG solution at pH 7.81 yields 48.2% 5′dGMP, 13.9% HEPES-pdG, 22.4% dG5′ppdG, and 10.9% of unreacted 2-MeImpdG (Figure 1A). The determination of the kinetics of this condensation will be described below. In the presence of 0.05 M poly(C) and 0.2 M Mg^{2+} and after 152 h of incubation, a 0.107 M 2-MeImpdG solution at pH 7.90 yields 33.7% 5′dGMP, 7.8% HEPES-pdG, 15.2% dG5′ppdG, 22.4% of unreacted 2-MeImpdG, and 13.2% products presumed to be the internucleotide-linked dimers, pdGpdG and 2-MeImpdGpdG (see Chart 2 and Figure 1B). The condensation of 2-MeImpdG in the presence of poly(C) was not investigated any further.

Reaction of Mor-pG with 2-MeImpdG in the Absence of Poly(C). In addition to 5′dGMP, HEPES-pdG, and dG5′ppdG expected from the reaction of 2-MeImpdG above, reaction mixtures of mor-pG and 2-MeImpdG yielded two new products. The new products elute after mor-pG on HPLC and were shown to be mor-pG2′pdG and mor-pG3′pdG (see the Experimental Section). The total yield of dimers amounts to 5.5% after 6 days of incubation, when most of 2-MeImpdG has been consumed. The product ratio *R*, referring here to the reaction in solution, $R = R(S) =$ [mor-pG2′pdG]:[mor-

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(18) The self-condensation of 2-MeImpdG was studied in the presence of Mn^{2+} instead of Mg^{2+}. Unpublished results indicate that Mn^{2+} and Mg^{2+} yield very similar product distributions.

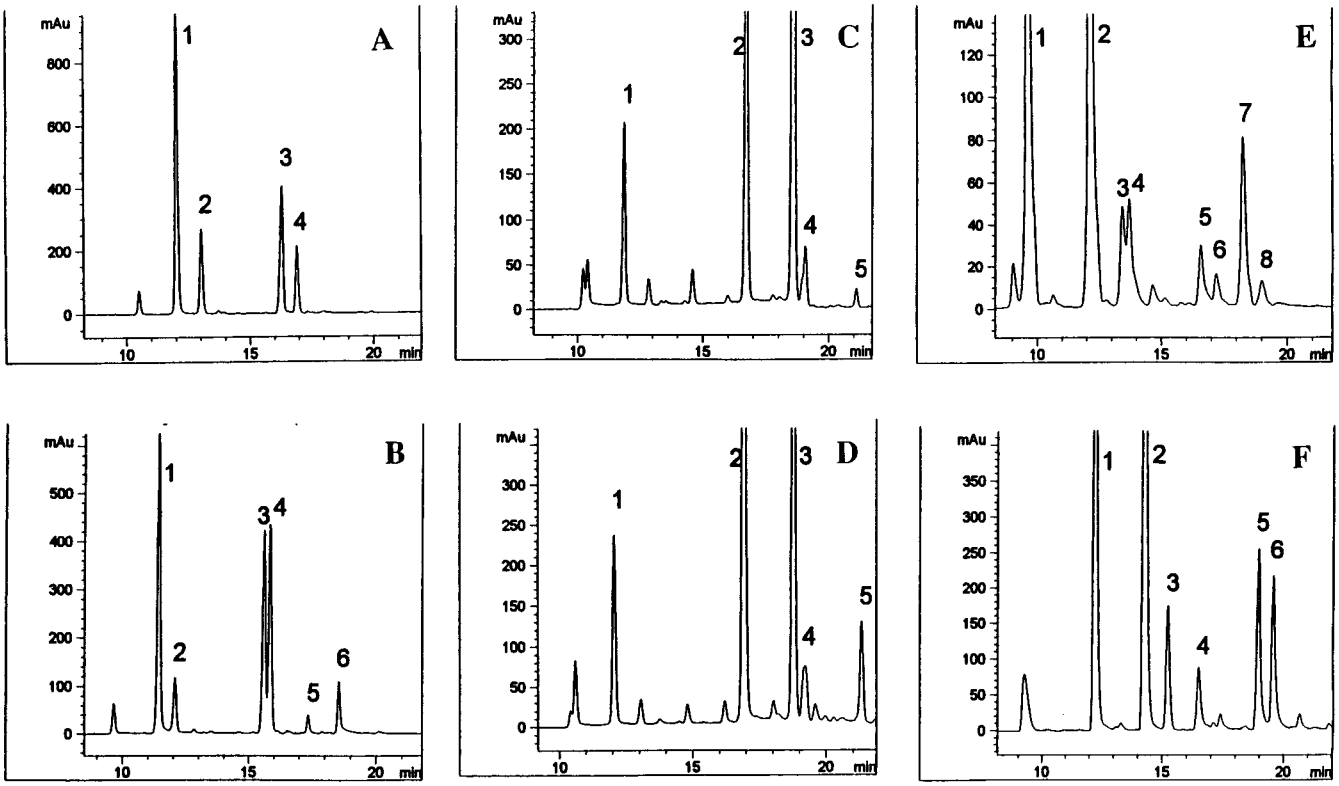


Figure 1. Reverse-phase HPLC profiles of selected reaction mixtures incubated under conditions exemplified in the Results section. Samples A, C, D, and E were analyzed with TFA, sample B was analyzed with 1-HSA chromatography (both chromatographies at 1.0 mL/min), and sample F was analyzed with HCOOH chromatography used for the LC/MS analysis (at 0.5 mL/min). Chromatographies are detailed in the Experimental Section. (A) 0.10 M 2-MeImpdG incubated for 148 h. Identification of peaks: 1, 5′dGMP; 2, HEPES-dpG; 3, dG$^{5'}$ppdG; 4, 2-MeImpdG. (B) 0.107 M 2-MeImpdG with 0.05 M poly(C) incubated for 29.5 h. Identification of peaks 1−4 as under A. Peaks 5 and 6, tentatively assigned to pdGpdG and 2-MeImpdGpdG, respectively. Slight differences in retention times between this HPLC profile and the one under A are due to the different chromatography used. (C) Reaction of 0.0251 M 2-MeImpdG with 0.05 M mor-pG incubated for 48 h in the presence of 0.0076 M poly(C). Identification of peaks: 1, 5′dGMP; 2, 2-MeImpdG; 3, mor-pG; 4, mor-pG$^{2'}$pdG; 5, mor-pG$^{3'}$pdG. (D) Reaction of 0.0808 M 2-MeImpdG with 0.0673 M mor-pG incubated for 23 h in the presence of 0.0475 M poly(C). Identification of peaks is the same as under C. (E) Reaction of 0.072 M 2-MeImpdG with 0.076 M mor-pG incubated for 72 h and subsequently partially acid hydrolyzed (see the Results section). Identification of peaks: 1, 5′GMP; 2, 5′dGMP; 3, HEPES-pdG; 4, pG$^{2'}$pdG; 5, dG$^{5'}$ppdG; 6 pG$^{3'}$pdG; 7, mor-pG (leftover); 8, mor-pG$^{2'}$pdG (leftover). Here 2′−5′:3′−5′ = sum of areas of peaks 4 and 8/area of peak 6. (F) Reaction of 0.0681 M 2-MeImpdG with 0.0442 M mor-pG incubated for 172 h in the presence of 0.0404 M poly(C); subsequently completely acid hydrolyzed (see the Results section). For identification of peaks 1−6 see under E. Here 2′−5′:3′−5′ = area of peak 4 /area of peak 6. Differences in retention times between this HPLC profile and the one under E are due to the different chromatography used.

pG$^{3'}$pdG] = 2′−5′:3′−5′ = 3.5, was determined for two experiments with total guanosine concentration of 0.091 and 0.148 M after 171 h of incubation. In contrast to 2-MeImpdG, which is consumed by hydrolysis and reaction with the buffer, only a small percent of mor-pG is consumed during the 6 day incubation. This is because mor-pG is a poor electrophile and is not subject to any other reaction besides incorporation into the dimers.

Six experiments (one at pH 7.76 and five at pH 8.20 ± 0.03) were performed with approximately equimolar concentration of mor-pG and 2-MeImpdG (Table 1). Total monomer concentration ranges from 0.0315 to 0.148 M. These six experiments are subdivided into two groups: low substrate concentration (the first three) and high substrate concentration (the last three). The percent dimer yield of these experiments is reported in Table 1 at two representative times *S* (stands for short incubation) and *L* (stands for long incubation). The *S* column shows that the dimer yield increases with increasing concentration, although the incubation time was shortened from 20 ± 2 h (first group) to 9 ± 2 h (second group). A similar trend is observed in the *L* column, where the incubation time varied from 92 ± 2 h (first group) to 70 ± 2 h (second group). The observation of higher percent yield of dimers with increasing substrate concentration suggests that the reaction is second-order, i.e., first-order in mor-pG and first-order in 2-MeImpdG. The fact that there is still an increase in the percent yield going from 0.10 to 0.148 M suggests that the reaction is approximately second-order even at the highest concentration of material. The relatively small, 2-fold only, increase in the percent dimer yield for an almost 5-fold increase in substrate concentration may be attributed to the fact that dimerization is a minor reaction compared to 2-MeImpdG hydrolysis and reaction with HEPES, which are the primary modes for substrate consumption. The formation of dimers is substantially faster at the onset of the reaction than at later stages because of 2-MeImpdG depletion and perhaps also because of the accumulation of unreactive material which can form complexes with the substrates.

Kinetic Determinations. Dimerization rate constants, k_d, were obtained as a function of the concentration of the guanosine monomers. By adopting the

Table 1. Percent Dimer Yield at Two Representative Times *S* and *L* as a Function of Initial Concentration of Starting Materials in the Reaction of Mor-pG with 2-MeImpdG at pH 8.20 ± 0.03 at 23 °C in the Presence of 0.5 M HEPES, 0.2 M $MgCl_2$, and 1.0 M NaCl

$[G]_{tot}$,[a] M	[mor-pG], M	[2-MeImpdG], M	incubation time *S*,[b] h	% yield *S*[c]	incubation time *L*,[b] h	% yield *L*[c]	k_d,[d] M^{-1} h^{-1}
0.0395[e]	0.0297	0.0098	21.5	1.60	93.4	3.56	0.070
0.0315	0.0150	0.0165	21.0	1.34	92.8	2.85	0.069
0.0471	0.0240	0.0231	17.8	1.41	89.7	3.73	0.057
0.1000	0.0503	0.0495	7.0 (23.0)	1.70 (2.64)	69.4	4.69	0.077
0.0910	0.0436	0.0473	6.75	1.64	69.1	4.36	0.091
0.1480[f]	0.0760	0.0720	11.3	2.82	72.2	5.55	0.051
2-MeImpdG Self-Condensation (i) with and (ii) without poly(C) at pH 7.85 ± 0.04							
(i) with 0.05 M poly(C) and 0.2 M Mg^{2+}	0.107		6.5 (23)	2.7 (9.0)[g]	152	28.5[g]	
(ii) with 0.2 M Mn^{2+}	0.104		3.5	1.1[h]	148	22.4[h]	0.017

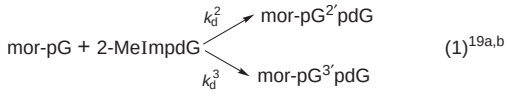
[a] $[G]_{tot}$ = [mor-pG] + [2-MeImpdG]. [b] *S* stands for short, *L* for long incubation time. [c] Percent yield based on the initial $[G]_{tot}$, expressed in monomer equivalents; it is not corrected for hypochromicity. [d] k_d, bimolecular rate constant determined as described in the Results section. [e] At pH 7.76. [f] Cloudy. [g] One of the dimers formed is dG[5′]ppdG; the others are presumed to be 2-MeImpdGpdG and pdGpdG. [h] The only dimer detected is dG[5′]ppdG.

Table 2. Percent Dimer Yield and 2′−5′:3′−5′ Ratio (*R*) in the Reaction of Mor-pG with 2-MeImpdG as a Function of Poly(C) Concentration after 23 h and after 171 h of Incubation at 23 °C and pH 7.90 ± 0.02 in the Presence of 0.2 M $MgCl_2$ and 1.0 M NaCl

[poly(C)],[a] M	[mor-pG],[b] M	[2-MeImpdG],[b] M	$[G]_{free}$,[c] M	% yield,[d] 23 h	% yield,[e] (23 h) TD	% yield,[d] 171 h	% yield,[f] (171 h) TD	*R* (R_{calc} at 23 h, 171 h)[g]
0.0076[h]	0.0500	0.0251	0.0674	3.3	1.3	6.5	2.5	1.37 (1.40, 1.30)
0.0100	0.0493	0.0402	0.0800	4.5	2.5	9.8	4.8	1.00 (1.00, 1.00)
0.0200	0.0470	0.0440	0.0710	6.5	4.5	13.5	8.5	0.71 (0.78, 0.75)
0.0306	0.0474	0.0596	0.0764	9.3	7.3	19.2	14.2	0.61 (0.64, 0.58)
0.0404	0.0442	0.0681	0.0716	8.9	6.9	21.8	16.8	0.53 (0.65, 0.54)
0.0553	0.0522	0.0616	0.0587	11.8	10.4	23.5	19.5	0.50 (0.53, 0.46)

[a] Poly(C) concentration is given in monomer equivalents. [b] [mor-pG] + [2-MeImpdG] = $[G]_{tot}$. [c] $[G]_{free} = [G]_{tot} - [poly(C)]$. [d] Percent dimer yield is based on $[G]_{tot}$; it is expressed in monomer equivalents, and it is not corrected for hypochromicity. [e] Percent yield (TD) after 23 h of incubation is calculated from the observed % yield by subtracting 1.4% for reaction with $[G]_{free} < 0.06$ M and 2.0% for reaction with $[G]_{free}$ in the range 0.06 M ≤ $[G]_{free}$ ≤ 0.08 M. These estimations are made by interpolation of the data in Table 1. [f] Percent yield (TD) after 171 h of incubation is calculated from the observed % yield by subtracting 4% for reaction with $[G]_{free} < 0.07$ M and 5% for reaction with $[G]_{free}$ in the range 0.07 M ≤ $[G]_{free}$ ≤ 0.08 M. These estimations are made by extrapolation of the data in Table 1. [g] *R* = 2′−5′:3′−5′ experimentally determined after 171 h of incubation; R_{calc} at 23 h is the calculated value based on the % yield after 23 h of incubation, *R*(S) = 3.5 for the solution reaction and *R*(TD) = 0.40 for the TD reaction; R_{calc} at 171 h is based on the % yield after 171 h of incubation, *R*(S) = 3.5 and *R*(TD) = 0.29 (see the Discussion section). [h] At pH 8.20 ± 0.02.

following methodology, such k_d values could be determined even though 2-MeImpdG participates in other reactions besides dimerization (eq 1). The methodology is based on eq 3, which is an approximation of eq 2 for the interval $t_2 - t_1$. k_d is the sum of the individual rate constants k_d^2 and k_d^3 for formation of the 2′−5′-linked and 3′−5′-linked isomer, respectively (see eq 1); $\Delta t = t_2 - t_1$; $[2\text{-MeImpdG}]_{ave}$ and $[\text{mor-pG}]_{ave}$ are the average concentrations during the interval $t_2 - t_1$, and Δ[dimers] is the concentration of the dimer(s) formed within the same time interval (see eq 3).



(1)[19a,b]

$$d[\text{dimers}]/dt = k_d[\text{2-MeImpdG}]_t[\text{mor-pG}]_t \quad (2)$$

$$\Delta[\text{dimers}]/\Delta t = k_d[\text{2-MeImpdG}]_{ave}[\text{mor-pG}]_{ave} \quad (3)$$

$$\text{where: } [\text{dimers}] = [\text{mor-pG}^{2'}\text{pdG}] + [\text{mor-pG}^{3'}\text{pdG}] \quad (4)^{19b}$$

Two initial rate determinations according to eq 3 were performed for each reaction mixture, and the k_d values, obtained as the average, are reported in Table 1. Rate determinations were only made at the onset of the reaction, because the rate of dimer formation was slowed down with time. The k_d values obtained from these five experiments are, within experimental error, the same and provide an average $k_d = 0.07 \pm 0.02$ M^{-1} h^{-1} at pH 8.20.

The k_d value obtained at pH 8.20 is identical to the $k_d = 0.07 \pm 0.02$ M^{-1} h^{-1} determined at pH 7.76, indicating the pH independence of the dimerization in this pH range. Similarly, experiments in the presence of poly(C) (not reported) do not show a measurable pH effect in the range 7.8 ≤ pH ≤ 8.20. Thus for practical purposes we will assume that the experiments performed in the absence of poly(C) at pH 8.20 ± 0.03 (Table 1) are directly comparable to the experiments performed in the presence of poly(C) at pH 7.90 ± 0.03 (Table 2). The k_d value for the self-condensation of 2-MeImpdG ($k_d = 0.017 \pm 0.005$ M^{-1} h^{-1}) was obtained in a similar manner by applying eqs 1−4 after replacing [mor-pG] with [2-MeImpdG] in eqs 2 and 3 and [mor-pGpdG] (both 2′ and 3′) with [dG[5′]ppdG] in eqs 1 and 4. The rate constant for hydrolysis of 2-MeImpdG and reaction with HEPES, k_H, in the presence of 0.2 M Mg^{2+} and 0.5 M HEPES and at pH 8.20 ± 0.03 was determined by applying eq 5, $k_H = 0.008 \pm 0.001$ h^{-1} ([5′dGMP]:[HEPES-pdG] ≈ 6:1, data not

(19) (a) Values of k_d^2 and k_d^3 can be calculated from k_d and *R*(S) based on the relationships $k_d = k_d^2 + k_d^3$ and $R(S) = k_d^2/k_d^3 = 3.5$ being the corresponding product ratio for the solution reaction. (b) After long incubation times or after acidic hydrolysis a fraction of the dimers has hydrolyzed to [pG[2′]pdG] + [pG[3′]pdG], so that under such conditions eq 1 and eq 4 need to be modified to incorporate the hydrolyzed dimers, e.g., [dimers] = [mor-pG[2′]pdG] + [mor-pG[3′]pdG] + [pG[2′]pdG] + [pG[3′]pdG].

shown). In contrast to k_d, which was only determined at the onset of the reaction, k_H did not vary with time and therefore was obtained as the average value of measurements that spanned up to two half-lives. In the presence of 0.02 M poly(C) and 0.091 M total guanosine, $k_H = 0.007 \pm 0.001$ h^{-1} (data not shown), indicating that the template has no significant effect on the hydrolysis of 2-MeImpdG.

$$\Delta\{[5'\text{dGMP}] + [\text{HEPES-pdG}]\}/\Delta t = k_H[\text{2-MeImpdG}]_{ave} \quad (5)$$

Reaction of Mor-pG with 2-MeImpdG in the Presence of Poly(C). Most experiments with poly(C) were performed at pH 7.90 ± 0.03 with approximately equimolar concentrations of mor-pG and 2-MeImpdG, a total concentration of guanosine, $[G]_{tot}$, close to 0.1, and 0.0076 M ≤ [poly(C)] ≤ 0.0553 M. The product distribution was monitored with HPLC, and the percent dimer yield at representative times, i.e., 23 h (early) and 171 h (late), is reported in Table 2 and so are ratios of the 2′−5′- vs the 3′−5′-linked dimer, R = 2′−5′:3′−5′.

Discussion

Self-Condensation of 2-MeImpdG. These experiments were run as a control for the reaction of mor-pG with 2-MeImpdG. The P−N bond in 2-MeImpdG exhibits reactivity similar to that established for other phosphoimidazolide-activated nucleotides, abbreviated *pN (see Chart 2).[20] The main reaction pathways are (i) P−N bond hydrolysis, which for 2-MeImpdG yields 2-methylimidazole and 5′dGMP, (ii) reaction with the buffer HEPES, which here leads to HEPES-pdG by replacing 2-methylimidazole with the HEPES moiety, and (iii) dimerization, which in the case of 2-MeImpdG could lead to the formation of one internucleotide-linked dimer, 2-MeImpdGpdG, and formation of dideoxyguanosine 5′-pyrophosphate, dG$^{5'}$ppdG (see Figure 1A). In the absence of poly(C), no peak was detected that could have been attributed to 2-MeImpdGpdG; this is consistent with the low reactivity of the deoxyribose compared to that of ribose.[7a,17]

dG$^{5'}$ppdG is likely to be formed via two pathways, in analogy with the mechanism proposed for the formation of pyrophosphate dimers in the self-condensation reaction of *pN such as 2-MeImpC and 2-MeImpU (see Chart 2).[20] The first pathway is dimerization of 2-MeImpdG by reaction of the oxyanion of the phosphate moiety of one molecule with the P−N bond of another molecule. The second pathway involves reaction of the hydrolysis product, 5′dGMP, after it accumulates, with 2-MeImpdG. The contribution of this second pathway can easily explain the observed acceleration of pyrophosphate formation with incubation time. Both pathways involve the oxyanion of the phosphate moiety as the nucleophile, except in the latter pathway the nucleophile has a pK_a of about 7, whereas in the first pathway the nucleophile has a pK_a of about 1.5. The dimerization rate constant, $k_d = 0.017$ M^{-1} h^{-1} in the presence of 0.2 M Mn^{2+} (Table 1, last entry),[18] refers to the pathway with two molecules of 2-MeImpdG, because the rate determinations were performed at the onset of the reaction, i.e., before substantial amounts of 5′dGMP were formed.

Reaction of Mor-pG with 2-MeImpdG in the Absence of Poly(C). The reaction of mor-pG with 2-MeImpdG yielded two new products, even though, based on the reactivity of *pN, we had anticipated the formation of three dimers: mor-pG$^{2'}$pdG, mor-pG$^{3'}$pdG, and G$^{5'}$ppdG (either in the free form or as a morpholine derivative). However, only traces of G$^{5'}$ppdG (Chart 2)—not to be confused with dG$^{5'}$ppdG—were detected (see the Experimental Section). Thus, there seems to be a difference between *pN- and morpholine-activated nucleotides in that the pyrophosphate dimer is a condensation product with the former but not with the latter. This difference could be attributed to a steric hindrance of reaction by the oxygen anion originating from the substantially bulkier morpholine moiety compared to the "flat" 2-methylimidazole. The observed ratio of mor-pG$^{2'}$pdG:mor-pG$^{3'}$pdG = 3.5 (Figure 1E; here R = 2′−5′:3′−5′ = (area of peak 4 + area of peak 8)/area of peak 6) is practically the same as $R \approx 3$ determined earlier with other nucleotides such as 2-MeImpC, 2-MeImpU, and 2-MeImpG.[21]

On the basis of the observation that the percent dimer yield increases with increasing total substrate concentration, the reaction of mor-pG with 2-MeImpdG is postulated to be roughly second-order with $k_d = 0.070$ M^{-1} h^{-1} at pH 8.20, 23 °C, and in the presence of 0.2 M Mg^{2+} (see Table 1). The corresponding rate constant for adenosine 5′-phosphoimidazolide, ImpA, dimerization[22] $k_d = 0.011$ M^{-1} h^{-1} determined at pH 8.0, 25 °C, and in the presence of 0.075 M Mg^{2+} is substantially lower, partly due to the lower magnesium concentration and partly due to the different leaving group employed.

Effect of Poly(C) on Dimer Yield and Product Distribution. Table 2 reports the percent dimer yield, expressed in monomer equivalents and uncorrected for hypochromicity, observed after 23 h and after 171 h of incubation. The dimer yield increases with poly(C) concentration and after 171 h of incubation amounts to 23.5% with 0.0553 M poly(C), compared to about 5.5% in the absence of poly(C). Furthermore, R decreased with poly(C) concentration starting at R = 1.37 with 7.6 mM poly(C) and reached R = 0.50 with the highest poly(C) concentration used (compare Figure 1C with 1D). The decrease of R with increasing poly(C) concentration indicates that poly(C) selectively favors the 3′−5′-linked dimer over the 2′−5′-linked dimer. Overall, there is a reversal in the product distribution from 3.5:1 favoring the 2′−5′-linked in the absence of poly(C) (Figure 1E: 2′−5′:3′−5′ = sum of areas of peaks 4 and 8/area of peak 6) to an apparent 2:1 favoring the 3′−5′-linked dimer with concentrations of poly(C) equal or higher than 0.03 M (Figure 1F: 2′−5′:3′−5′ = area of peak 4/area of peak 6). Both the change in the ratio 2′−5′:3′−5′ and the increase in dimer yield seem to level off at the higher concentrations of poly(C).

Is Dimerization a Template-Directed Reaction? The prevailing view of the effect of poly(C) on catalyzing reaction among guanosine substrates at pH >7 is that it does it within a double-helical complex.[9a] Evidence for such a complex and the catalysis it provides for elonga-

(20) Kanavarioti, A. *Origins Life Evol. Biosph.* **1997**, *27*, 357−376.

(21) An earlier study (Lohrmann, R.; Orgel, L. E. *Tetrahedron* **1978**, *34*, 853−855) reported that 2′−5′:3′−5′ = 6−9, but recent work shows that this ratio is closer to 3 with both 2-methylimidazole and imidazole as the activating groups.[20]

(22) Kawamura, K.; Ferris J. P. *J. Am. Chem. Soc.* **1994**, *116*, 7564−7572.

Table 3. Efficiency and Rate of Dimer Formation, d[D]/d*t*, as a Function of Poly(C) Concentration

[poly(C)],[a] M	$[G]_{tot}$,[b] M	efficiency[c]	[3′-Dimer](TD),[d] M	d[D]/d*t*,[e] M h^{-1}	k_d(TD),[f] h^{-1}
0.0076	0.075	0.28	4.20×10^{-4}	1.83×10^{-5}	2.41×10^{-3}
0.0100	0.090	0.50	9.70×10^{-4}	4.22×10^{-5}	4.22×10^{-3}
0.0200	0.091	0.44	1.77×10^{-3}	7.70×10^{-5}	3.85×10^{-3}
0.0306	0.107	0.57	3.37×10^{-3}	1.47×10^{-4}	4.80×10^{-3}
0.0404	0.112	0.54	3.33×10^{-3}	1.45×10^{-4}	3.60×10^{-3}
0.0553	0.114	0.46	5.11×10^{-3}	2.22×10^{-4}	4.01×10^{-3}

[a] Poly(C) concentration in cytidine equivalents. [b] $[G]_{tot}$ = [mor-pG] + [2-MeImpdG]. [c] Efficiency calculated according to eq 6 (see text). [d] Concentration of the 3′−5′-linked dimer formed on the template after 23 h of incubation (calculated via eq 7; see text). [e] Rate of dimer formation, d[D]/d*t* = ([3′-Dimer](TD))/23. [f] First-order rate constant for 3′−5′-internucleotide bond formation between mor-pG and 2-MeImpdG on the template: k_d(TD) = (d[D]/d*t*)/[poly(C)] (see text).

tion and ligation reactions has been reviewed already.[7−9,13] Below we will show that the results reported in Table 2 are compatible with a dimerization that is partially occurring in solution and partially on the template. Under the experimental conditions, it can be assumed that practically all C-sites are occupied with guanosine monomers,[23] because [mor-pG] + [2-MeImpdG] ≫ [poly(C)]. Hence the concentration of template-bound monomer, $[G]_{temp}$, is given by $[G]_{temp}$ = [poly(C)], and the concentration of free guanosine, $[G]_{free}$, is given as $[G]_{free}$ = $[G]_{tot}$ − [poly(C)]; these values are listed in Table 2. Based on the data reported in Table 1, an estimate has been made of the percent dimer yield that is contributed by $[G]_{free}$ from the reaction in solution (see footnote in Table 2 for estimations). The estimated contribution is subtracted from the observed yield in order to determine the percent yield due to the template reaction, % yield TD, that is also reported in Table 2 both at 23 and 171 h.

The effect of poly(C) in affecting regioselectivity is most easily seen in the data reported with 0.010 M poly(C) (second experiment in Table 2). In this experiment after 23 h of incubation there is 4.5% of dimer observed, from which about half (2.5%) is coming from the TD dimerization. Similarly, in the same experiment but after 171 h of incubation the total dimer yield amounts to 9.8%, of which half (4.8%) is due to the TD reaction. Since approximately half of the yield with 0.01 M poly(C) is coming from the solution reaction and the other half from the TD reaction and taking into account that for the same experiment a value of R = 2′−5′:3′−5′ = 1.0 is observed, the conclusion is reached that the TD reaction must have regioselectivity opposite of that of the solution reaction, i.e., R(TD) = 1/R(S) = 0.29. The best fit to the experimentally observed R is obtained by using R(TD) = 0.4 for the yields at 23 h and R(TD) = 0.29 for the yields observed at 171 h. Calculated product ratios, R_{calc}, are also reported in the last column of Table 2 and exhibit very good agreement with the observed R values. Therefore the observed reversal in regioselectivity (Table 2) is quantitatively consistent with a TD dimerization that favors the formation of the 3′−5′-linked dimer 2.5−3.5 times over the 2′−5′-linked one. The preference of 2.5−3.5 for the 3′−5′-linkage deduced for this dimerization is substantially lower than that observed in TD ligation with imidazole as the activating group (3′−5′:2′−5′ = 10−15).[8] This could be attributed to a better positioning of the reactive complex when the double helix is formed from oligomers instead of monomer stacks.

How efficient is the template? The efficiency of the template can be defined as the fraction of template that yields complementary product, i.e., dimer (expressed in monomer equivalents). The efficiency can be calculated from eq 6. In eq 6 h is the hypochromicity correction factor for guanosine dimers with h = 1.15,[24] and % dimer yield (TD) corresponds to 171 h of incubation (eighth column in Table 2) when most of the substrate is consumed. As defined in eq 6 an efficiency of 1 indicates that the template is capable of producing an equivalent amount of dimer; in this system turnover is not expected because diguanylate binds tightly on poly(C).[25]

$$\text{Efficiency} = [\text{Dimer}]/[\text{poly(C)}] = \frac{[G]_{tot}}{[\text{poly(C)}]}\, h\, \frac{\%\ \text{yield (TD)}}{100} \quad (6)$$

Except for the first experiment with the lowest poly(C) concentration, all the other experiments show an efficiency of 0.50 ± 0.07 (Table 3), which indicates that only half of the template is effective in directing dimer formation. Efficiencies substantially lower than 1 are most likely the result of the high concentration of monomers used in this study, which leads to accumulation of unreactive byproducts such as 5′dGMP and HEPES-pdG that compete for the C-sites. The alternative possibility that the complex formed between poly(C) and mono(G) is a triplex (2C:1G) is also consistent with an efficiency = 0.50 and cannot be excluded on the basis of these data. However, related studies under similar conditions strongly argue in favor of a double helix and against a triple helix.[13] The efficiency of the reaction with 0.0076 M poly(C) is only 0.33. This lower efficiency may be attributed to the fact that a substantial fraction of dimer is formed in the solution, and that, as soon as this dimer is formed, it binds on C-sites and prevents the complexation of monomers with the template. In this way, the dimer formed in solution inhibits the template from reaching full efficiency.

How strongly does the template catalyze the dimerization? One way of determining the catalytic effect of

(23) An earlier study revealed strong association of mor-pG with poly(C) (with an association constant for binding adjacent to an occupied position K_a = 180 M^{-1})[15] in the range 1.78−7.55 mM poly(C), which is lower than the range of poly(C) employed here. In addition, the guanosine monomer concentration is at least a factor of 10 higher in the present study as compared to the earlier study. The affinity of 2-MeImpdG has not been determined. It is expected to be of the same magnitude or perhaps somewhat lower than the affinity of mor-pG. The observation that in the presence of poly(C) 2-MeImpdG yields condensation products that were not detectable in the absence of poly(C) also argues in favor of moderately strong binding of 2-MeImpdG on poly(C). The above arguments imply that monomer saturation of poly(C) should be complete in all the experiments reported here.

(24) Diguanylates exhibit a hypochromicity correction factor of h = 1.15 in neutral and acidic solutions (from T. Brian Hurley, Senior Thesis for the Degree of B.S. in Chemistry, 1993, University of California at Santa Cruz) and h = 1.34 in basic solutions.[13]

(25) Lipsett, M. N. *J. Biol. Chem.* **1964**, *239*, 1256.

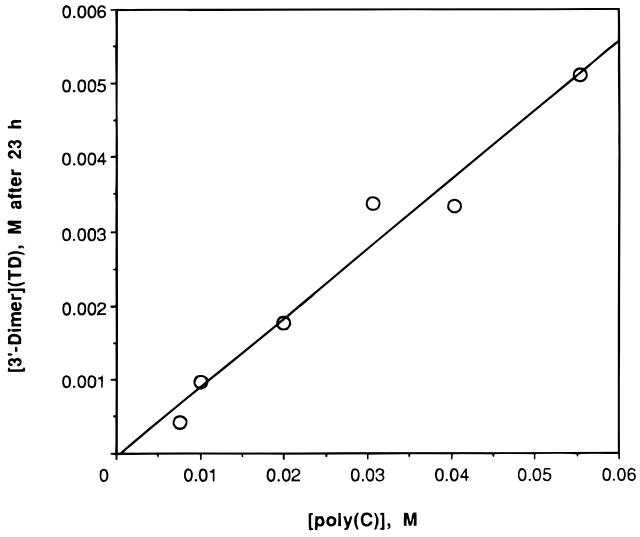


Figure 2. Concentration of mor-pG$^{3'}$pdG produced by the TD reaction, [3′-Dimer](TD), as a function of poly(C) concentration after 23 h of incubation.

poly(C) is by comparing experiments where $[G]_{tot}$ in the absence of poly(C) is the same as $[G]_{temp}$; note that $[G]_{temp}$ = [poly(C)]. For example, the experiment with $[G]_{tot}$ = 0.0315 M (second in Table 1) can be compared with the experiment with 0.0306 M poly(C) (fourth entry in Table 2). With the former, percent dimer yield = 1.34 (after 21 h), whereas with the latter, percent yield (TD) = 7.3 (after 23 h). This yield increase corresponds to a factor of 5.4 for the catalysis provided by poly(C). A similar comparison can be made between experiment with $[G]_{tot}$ = 0.0395 M (first in Table 1) and experiment with 0.0404 M poly(C) (fifth in Table 2); it suggests a factor of 4.3 catalysis by the template. The conclusion that poly(C) catalyzes dimerization by a factor of 4−5 over the reaction in solution is consistent with the observation that dimer yield in the presence of the template reaches up to 23.5%, whereas in its absence dimer yield is at most 5.5%. It should be noted that the catalytic effect of poly(C) is surprisingly small in our system compared to the 1000-fold catalysis of the ImpA dimerization induced by the presence of Na^+−montmorillonite.[22]

The concentration of the 3′−5′-dimer formed by the TD reaction, [3′dimer] (TD), can be calculated via eq 7 and is reported in Table 3 for the 23 h incubation time.

$$[3'\text{dimer}]\,(\text{TD}) = [\text{G}]_{\text{tot}}\frac{\%\ \text{yield (TD)}}{100}\frac{h}{2}\frac{1}{1+R(\text{TD})} \quad (7)$$

In eq 7 h = 1.15 (as in eq 6) and the factor $\{1/(1 + R(\text{TD}))\}$ = 0.75 with R(TD) = 0.35 (taken as the average of the values 0.40 and 0.29, see above) corrects for the fact that not all of the dimer formed on the template is 3′−5′-linked. The concentration of 3′-dimer (TD) accumulated after 23 h of incubation is plotted as a function of poly(C) concentration in Figure 2. Although there is some scatter in the data, the plot exhibits a linear relationship between the concentration of 3′−5′-linked dimer formed by the TD reaction and the concentration of poly(C), strongly supporting the proposition that the reaction between mor-pG and 2-MeImpdG is a TD dimerization.

The rate of 3′−5′-linked dimer formation, d[D]/dt, has been calculated from [3′dimer] (TD) after 23 h of incubation; d[D]/dt = [3′dimer] (TD)/23. Such d[D]/dt values for each poly(C) experiment are included in Table 3. As one would expect, they exhibit the same dependence with poly(C) concentration as the dimer concentration plotted in Figure 2. By normalizing d[D]/dt values of the present study with the corresponding poly(C) concentration, an intrinsic rate constant of dimerization, k_d(TD), with k_d(TD) = d[D]/dt/$[G]_{temp}$ = d[D]/dt/[poly(C)] can be calculated. k_d(TD) represents the first-order rate constant for 3′−5′-internucleotide bond formation between mor-pG and 2-MeImpdG on the template. These values are listed in Table 3 and, with the exception of the k_d(TD) obtained with the first experiment, are practically independent of [poly(C)]. They provide an average $k_d(\text{TD}) = 4.10 \times 10^{-3}$ h^{-1} to be compared with another intrinsic rate constant of dimerization $k_d(\text{TD}) = 1.8 \times 10^{-2}$ h^{-1} obtained from the dimerization of 2-MeImpG. The latter was obtained by analysis of the rate of dimer formation as a function of 2-MeImpG concentration in the range 0.005 M ≤ [2-MeImpG] ≤ 0.045 M in the presence of 0.05 M poly(C);[13] the analysis corrects for the fact that the template is not fully occupied. Comparison of the intrinsic rate constants of TD dimerization in the systems mor-pG/2-MeImpdG and 2-MeImpG suggests that the latter is more than 4 times faster than the former. The 4-fold difference in reactivity between these two systems that share identical nucleophile and electrophile could be attributed to a statistical factor. This is because both 2-MeImpdG and mor-pG have only one reactive site, whereas 2-MeImpG has two reactive sites for each of the participating molecules, thus resulting in a statistically 4-fold faster intrinsic rate constant of dimerization.

Conclusions

Reaction of mor-pG with 2-MeImpdG in aqueous solution at pH 8 yields two internucleotide linked dimers, i.e., mor-pG$^{2'}$pdG and mor-pG$^{3'}$pdG, in a ratio of about 3.5 to 1. The rate of dimer formation is enhanced and the total dimer yield rises from about 5.5% to almost 24% as a function of poly(C) concentration. More importantly, the 2′−5′:3′−5′ ratio changes from 3.5 in the absence of poly(C) to 0.5 in the presence of [poly(C)] ≥ 0.030 M. The data are consistent, in a quantitative sense, with a template-directed mechanism of dimerization. The results suggest that reaction between template-bound monomers is 4−5 times faster than its solution counterpart, and there is a reversal in selectivity from 2′−5′:3′−5′ = 3.5 for the solution reaction to a 3′−5′:2′−5′ = 2.5−3.5 for the poly(C)-directed dimerization. These findings demonstrate that guanosine mononucleotides self-assemble on an RNA template and form dimers with enhanced reactivity and 3′−5′ regioselectivity. The enhanced reactivity and regioselectivity for RNA linkages at the *dimer* stage adds to the repertoire of advantages for selection of RNA in a prebiotic world.

Experimental Section

Materials. Reagent grade chemicals were used throughout. Solvents were HPLC quality. Water from a Millipore Unit operating at 18 mMW was filtered through a 0.25 μm filter. Poly(C) potassium salt and the free acids of 5′GMP and of 5′dGMP were purchased from Sigma. Morpholine and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and tris(hydroxylmethyl)aminomethane (TRIZMA) were purchased from Aldrich. Morpholine was distilled before use. RNase T_1 from *Aspergillus oryzae* was purchased from Boering-

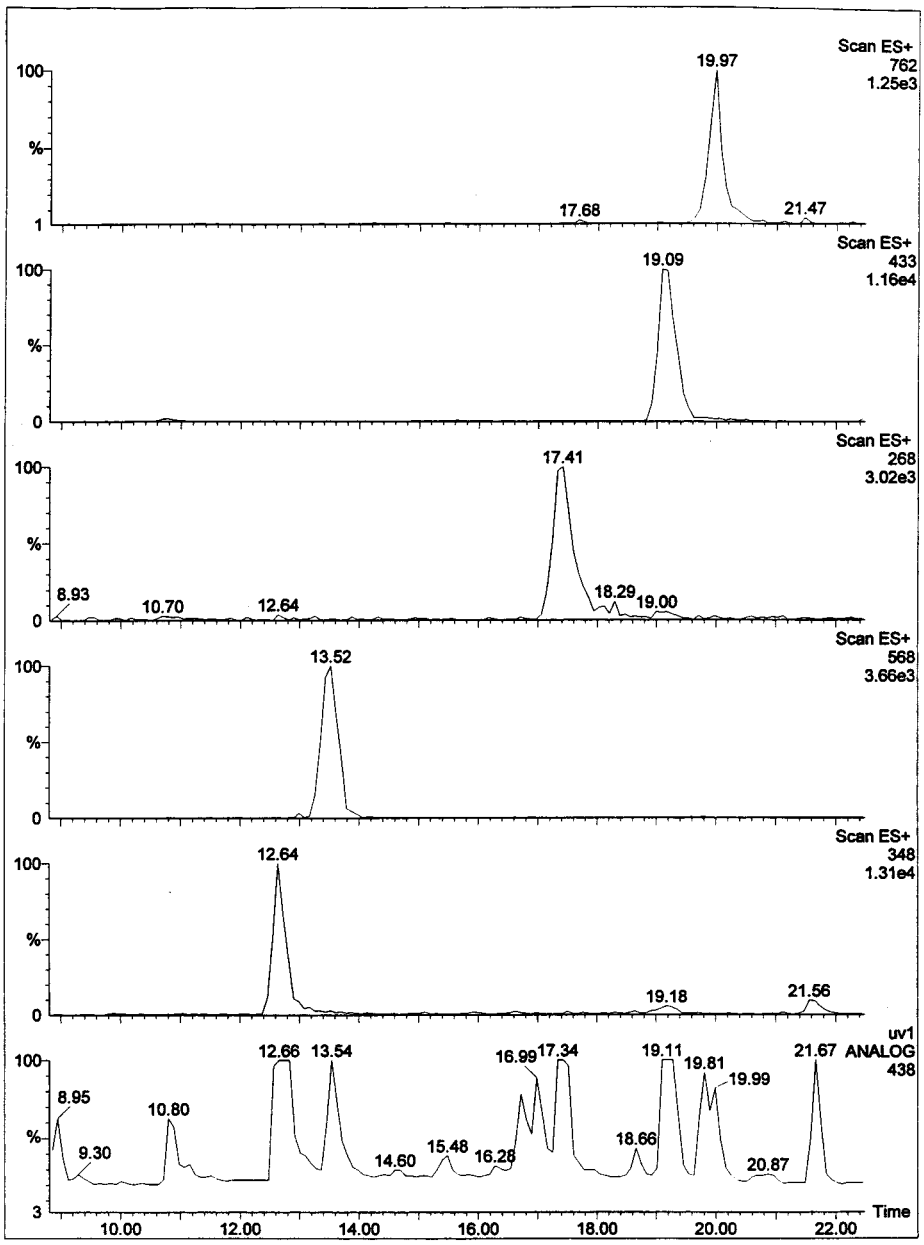


Figure 3. LC/MS data of a sample of mor-pG with 2-MeImpdG after 171 h incubation at 23 °C in the presence of poly(C). Sample was subject to RNase T_1 digestion. The lower trace represents the HPLC profile of the sample monitored at 254 nm. The other traces represent the abundance of product of a specific molecular ion detected by the mass spectrometer. Values *m*/*z*, reported on the right side of each scan, correspond to M + 1 for all the compounds of this study (M = molecular weight). Conditions for the LC/MS analysis are given in the Experimental Section. From bottom to top: Peak eluting at 12.64 min with $m/z = 348$ is attributed to 5′dGMP. Peak eluting at 13.52 min with $m/z = 568$ is attributed to HEPES-pdG. Peak eluting at 17.41 min with $m/z = 268$ is attributed to deoxyguanosine. Peak eluting at 19.09 min with $m/z = 433$ is attributed to mor-pG and peak eluting at 19.97 min with $m/z = 762$ is attributed to mor-pGpdG.

er Mannheim. The lithium salt of mor-pG was prepared indirectly from 2-MeImpG;[16] the sodium salt of 2-MeImpdG, from 2-methylimidazole and 5′dGMP.[16] Mor-pG was 92% pure by HPLC: λ_{min} at 224 nm, λ_{max} at 253 nm with extinction coefficient ϵ 12 070 in a pH 7.6 solution (5 mM TRIZMA). Contaminants were 3.2% 5′GMP and 4.7% 2-MeImpG and traces (less than 0.1%) of G-absorbing unknown species. LC/MS of mor-pG free acid showed M + 1= 439.1. 2-MeImpdG was determined to be 95.5% pure by HPLC: λ_{min} at 228 nm, λ_{max} at 252 nm with ϵ 12 070 in a pH 7.6 solution with 5 mM TRIZMA buffer. Analysis by HPLC shows one unidentified contaminant at 4% eluting earlier than 5′dGMP, which is only present at 0.5%. LC/MS of the free acid of 2-MeImpdG showed M + 1= 434.1.

Preparation of Reaction Mixtures and Analysis. Substrates were weighed into small centrifuge test tubes, the solid was dissolved in water at half the volume of the final solution, and buffer containing the salts was added last. Typical volumes were 40−100 μL. Solutions with poly(C) were typically clear and remained clear during incubation. Solutions without poly(C) with the exception of one (sixth entry in Table 1) were clear, but during incubation precipitation occurred. The pH was measured with a microelectrode probe from Microelectrodes, and it remained stable during incubation. Aliquots (1 or 2 μL) of samples were diluted and quenched with EDTA before HPLC analysis. HPLC was run on a 1090 LC from Hewlett-Packard equipped with a diode array detector and an Alltima C18 column 4.6 × 250 mm 5μ

from Alltech used at 1.0 mL/min flow. Three chromatographies were used for analysis. The first one, abbreviated TFA, is solvent A, 0.02 M KH_2PO4 with 0.2% w/v trifluoroacetic acid (TFA) pH 2.5, and solvent B, 30% CH_3CN in water v/v with 0.2% w/v TFA; 0−15% B in 10 min; isocratic at 15% B for 4 min and then 15−30% B in 15 min. Order of elution and typical retention times with TFA at 1.0 mL/min are 5′GMP, 10.00 min; 5′dGMP, 11.85 min; HEPES-pdG, 12.63 min; 2-MeImpG, 13.54 min; pG$^{2'}$pdG, 14.31 min; G$^{5'}$ppdG, 15.05 min; 2-MeImpdG, 16.10 min; dG$^{5'}$ppdG, 16.58 min; mor-pG$^{3'}$p, 16.70 min; pG$^{3'}$pdG, 17.31 min; mor-pG, 18.59 min; mor-pG$^{2'}$pdG, 19.78 min; mor-pG$^{3'}$pdG, 21.79 min. Deoxyguanosine elutes very close to pG$^{3'}$pdG, and guanosine 3′,5′-diphosphate elutes as a broad peak ahead of 5′-GMP.

The second chromatography, abbreviated 1-HSA, was solvent A, 0.05 M KH_2PO4 with 0.2 mM 1-hexanesulfonic acid (1-HSA) pH 2.2, and solvent B, 30% CH_3CN in solvent A; 0−25% B in 13 min; isocratic at 25% B for 7 min. The third chromatography used formic acid as additive and is described below. All three chromatographies resulted in similar HPLC profiles; that is, the elution order did not change from one chromatography to the other. Compounds elute somewhat earlier with 1-HSA as compared to TFA. It was possible to use for a specific sample either the 4.8 × 250 mm Alltima column at 1.0 mL/min or the 3.2 × 250 mm Alltima column (see below) at 0.5 mL/min with the same gradient and obtain very similar HPLC profiles.

LC/MS Analysis. Representative samples were analyzed with an LC/MS Quattro II, i.e., a liquid chromatograph (1100 Hewlett-Packard LC) coupled with a quadrupole mass spectrometer from Micromass. Removal of solvent and ionization was done by positive electrospray. Accumulation of data was performed in the centroid mode. Samples were analyzed with an Alltima C18 solvent minimizer column 3.2 × 250 mm 5μ from Alltech at 0.5 mL/min flow. The solvent coming out of the column was split about 50:50 and led in parallel to the UV detector and to the MS probe. MS temperature probe was set at 120 °C (higher temperatures led to decomposition). Solvents used for LC/MS were as follows: solvent A, 0.2% formic acid in water, and solvent B, 30% CH_3CN in solvent A. Gradient: 0−25% B in 10 min and 25−43% in 10 min. Mass spectrometric determinations provided (M + 1)/z values (M = molecular weight, z = charge at pH 2, see Figure 3). The MS probe exhibits higher sensitivity with HCOOH than with TFA.

Product Identification. For most of the products identification was achieved both by coelution with a standard and by molecular weight determination by LC/MS. Standards were available for 5′GMP and 5′dGMP. HEPES-pdG, was characterized by running the reaction at 0.001 M 2-MeImpdG concentration in a 0.5 M and in a 1.0 M HEPES buffer and identifying the only product peak that doubles in size by doubling the concentration of the buffer. A solution containing a large amount of HEPES-pdG was used as a standard. Reaction of 2-MeImpG with 2-MeImpdG was used in order to make the three pyrophosphate dimers, i.e., G$^{5'}$ppG, dG$^{5'}$ppdG, and G$^{5'}$ppdG, in good yield. The first and last pyrophosphate were available as standards made by a published method.[26] Elution order on C18 is G$^{5'}$ppdG after G$^{5'}$ppG and ahead of dG$^{5'}$ppdG, which is consistent with the structure. Additional evidence for identification of G$^{5'}$ppdG was provided by LC/MS. It should be noted that LC/MS does not distinguish among isomeric dimers, such as pG$^{2'}$pdG, pG$^{3'}$pdG, and G$^{5'}$ppdG, whereas analysis on C18 can, as described earlier.

Characterization of the Two Isomeric mor-pGpdG (M + 1 = 762). The half-life of morpholine derivatives of nucleotides is approximately 2 h at pH 3 and 37 °C.[16] Hence, samples were brought to pH 3 by addition of sufficient HCl and incubated at 37 °C and the acidified samples were repeatedly analyzed by HPLC. The two unknown products disappeared, and two equivalent peaks with earlier retention times appeared, one eluting after HEPES-pdG and the other after dG$^{5'}$ppdG. A standard of G$^{5'}$ppdG (made as described above) was not found to coelute with any detectable peak. This suggested that G$^{5'}$ppdG is not one of the new hydrolysis products. The hydrolyzed product that elutes last was assigned to pG$^{3'}$pdG because it was degraded by RNase T_1. As a corollary, the other hydrolyzed product was assigned to pG$^{2'}$pdG. Reaction solutions devoid of poly(C) contained practically only mor-pG$^{2'}$pdG, which by acidic hydrolysis yielded pG$^{2'}$pdG. Comparison between samples with poly(C) and without poly(C) before and after hydrolysis facilitated the identification of all four dimers, i.e., mor-pGpdG and pGpdG.

RNase T_1 Digestion. RNase T_1 hydrolyzes 3′−5′-linkages at guanosine positions and yields 3′-phosphomonoesters. In 150 μL of a diluted sample (2 mM in guanosine) containing EDTA were added 20 μL of 1 M TRIZMA buffer pH 7.95 and 2 μL (200 units) of the enzyme; the sample was incubated for 2 h at 55 °C. Digestion of a hydrolyzed sample with RNase T_1 led to decomposition of pG$^{3'}$pdG and formation of deoxyguanosine and guanosine 3′,5′-diphosphate, which were identified by coelution with standards and by LC/MS (Figure 3). Mor-pG$^{3'}$pdG was partially degraded by RNase T_1 to mor-pG$^{3'}$p (M + 1 = 513) and deoxyguanosine (M + 1= 268), both detected by LC/MS. Both mor-pG$^{2'}$pdG and pG$^{2'}$pdG were left intact by RNase T_1 digestion.

Determination of Ratios 2′−5′:3′−5′ (R). The R values were obtained from samples that were acidified with HCl to pH 3.5 and incubated at 37 °C for 24 h in order to hydrolyze both the morpholine and the imidazolide moieties (see Figures 1E and 1F). The R values obtained in this manner were in agreement with R values obtained from the ratio of {[mor-pG$^{2'}$pdG] + [pG$^{2'}$pdG]}:{[mor-pG$^{3'}$pdG] + [pG$^{3'}$pdG]}, i.e., before hydrolysis.

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