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Which 3-Ribofuranosyl-Substituted Purine 5′-Phosphates Undergo Template-Directed Oligomerization?

Aubrey R. Hill, Jr.,[1] Shiv Kumar,[2] Vemanna D. Patil,[2] Nelson J. Leonard,[2] and Leslie E. Orgel[1]

[1] The Salk Institute for Biological Studies, Post Office Box 85800, San Diego, CA 92186-5800, USA
[2] School of Chemical Sciences, University of Illinois at Urbana–Champaign, 1209 West California Street, Urbana, IL 61801, USA

Summary. We have studied the oligomerization reactions of the 2-methylimidazolide derivatives of 3-isoisoguanosine 5′-phosphate (**2**) and 3-isoxanthosine 5′-phosphate (**5**) in the presence of a variety of homopolynucleotide templates. In no case did we observe a substantial template-facilitated production of long oligomers. Polyuridylic acid directed the synthesis of low molecular-weight products from both monomers. Polycytidylic acid, polyadenylic acid, polyinosinic acid, and polyguanylic acid were ineffective as templates in the systems that we investigated.

Key words. 3-Isoisoguanosine 5′-phosphate — 3-Isoxanthosine 5′-phosphate — Template-directed synthesis — Phosphoroimidazolides

Introduction

The facilitation of the oligomerization of activated monoribonucleotides by polyribonucleotide templates, which has been well documented (Joyce 1987) is not restricted to natural nucleotide substrates (Schwartz and Orgel 1985; Zielinski and Orgel 1985, 1987; Harada and Orgel 1990). The results are of interest for their possible relevance to simple genetic processes that might have preceded DNA and RNA replication (Joyce et al. 1987). A study of the template-directed oligomerization of the imidazolide of 3-isoadenosine 5′-phosphate (ImpiA) on poly(U) showed that the reaction of ImpiA is more efficient than the corresponding reaction of adenosine 5′-phosphoroimidazolide on poly(U), and produces mainly 3′–5′-linked oligomers (Hill et al. 1988). We have now extended the study to the template-directed reactions of the 5′-phosphates of two other 3-ribofuranosyl-substituted purines.

Offprint requests to: L.E. Orgel

Abbreviations: Poly(U), polyuridylic acid; poly(C), polycytidylic acid; poly(A), polyadenylic acid; poly(G), polyguanylic acid; poly(I), polyinosinic acid; iX, 3-isoxanthosine; piX, 3-isoxanthosine 5′-phosphate; 2-MeImpiX, the 2-methylimidazolide of isoxanthosine 5′-phosphate; iiG, 3-isoisoguanosine; piiG, 3-isoisoguanosine 5′-phosphate; 2-MeImpiiG, the 2-methylimidazolide of 3-isoisoguanosine 5′-phosphate; 1-MeIm, 1-methylimidazole; 2-MeIm, 2-methylimidazole; DCC, dicyclohexylcarbodiimide; FAB, fast atom bombardment; TEAB, triethylammonium bicarbonate; TLC, thin layer chromatography

Materials and Methods

Materials. Reagent grade chemicals were used throughout. Triphenylphosphine, 2,2′-dipyridyl-disulfide, 1-MeIm, and 2-MeIm were purchased from Aldrich; alkaline phosphatase and bovine pancreatic ribonuclease were from Boehringer Mannheim Biochemicals, and ribonuclease T_2 was from Sigma. Poly(U) was prepared by a published procedure (Steiner and Beers 1958). Poly(A), poly(C), poly(G), and poly(I) were purchased from Sigma Chemical Co. The 2-methylimidazolides of the 5′-phosphates (**2** and **5**) were obtained by a slight modification (Joyce et al. 1984) of a standard procedure (Mukaiyama and Hashimoto 1971).

General Procedures. Template-directed reactions were carried out in 10-μl volumes with the following concentrations of reagents: 0.05 M template, 0.05 M 2-MeImpiiG (**3**) or 2-MeImpiX (**6**), 0.075 M $MgCl_2$, 0.2 M NaCl, and 0.2 M 1-MeIm·HCl (pH 8.0). The template, $MgCl_2$, and NaCl were evaporated to dryness in glass culture tubes (12 × 75 mm) under vacuum. A cold solution of 2-MeImpiiG or 2-MeImpiX and 1-MeIm was added and the reactants were mixed thoroughly. The tubes were capped and incubated at 0°C. Aliquots were taken for analysis at appropriate times. Prior to HPLC analysis, reactions containing poly(C) or poly(U) were usually subjected to enzymatic degradation with pancreatic ribonuclease, an enzyme that cleaves 3′,5′ internucleotide bonds adjacent to pyrimidine residues, by incubating a

mixture (50 μl) containing 1.0 ODU template, 0.06 M Tris·HCl (pH 7.4), and 2 units enzyme overnight at 37°C. HPLC analyses were performed using an RPC-5 analytical column as described in an earlier publication (Joyce et al. 1984). UV absorption was monitored at a wavelength of 254 nm for reaction mixtures containing piiG (2) and at a wavelength of 273 nm for those containing piX (5).

3-Isoisoguanosine 5'-phosphate (2). 3-Isoisoguanosine (3-β-D-ribofuranosylisoguanine, 1) prepared according to the procedure described by Schmidt and Townsend (1975), was 5'-phosphorylated by the method of Imai et al. (1969). 3-Isoisoguanosine (142 mg, 0.5 mmol) was suspended in 10 ml of *m*-cresol and cooled to 0°C in an ice-water bath. After 10 min, 0.2 ml of pyrophosphoryl chloride was added. The mixture was stirred under an argon atmosphere for 30 min, at which time a clear, yellowish solution had formed. Stirring was continued at 0°C for 2.5 h, then the reaction mixture was added dropwise to ice-cooled water (100 ml), which was extracted with three 25-ml portions of diethyl ether. The aqueous layer was adjusted to pH 3 by the dropwise addition of 1 N NaOH and was then applied to a column of DEAE-Sephadex A-25 (2.5 × 13 cm). Elution with a linear gradient of 0.01–0.5 M (600 ml each) of aqueous TEAB (pH 7.4) was monitored at 280 nm. The appropriate fractions containing the material were evaporated to dryness and coevaporated with methanol (3 × 25 ml). Dissolution in 10 ml of water and lyophilization gave 165 mg (71%) of pure 5'-phosphate (as the mono triethylammonium salt), R_f 0.3 (1 M LiCl, DEAE-Cellulose TLC plate). Low-resolution FAB mass spectrum: m/z 364.1 (MH^+) (as the free acid). High-resolution FAB mass spectrum: m/z 364.0700 ($C_{10}H_{15}N_5O_8P$ requires 364.0658). Compound 2 was then converted to the 2-methylimidazolide, 2-MeImpiiG (3).

3-Isoxanthosine 5'-Phosphate (5). This was obtained from 3-isoxanthosine (3-β-D-ribofuranosylxanthine, 4) (Schmidt and Townsend 1975) as described above for the preparation of piiG. The triethylammonium salt was converted to the Li^+ salt by passage through a column of Dowex (Li^+ form): yield 25%. Low-resolution FAB mass spectrum: m/z 365 (MH^+) and 371 (MH^+ as the mono Li^+ salt). High-resolution FAB mass spectrum: m/z 365.0499 ($C_{10}H_{14}N_4O_9P$ requires 365.0499); 371.0582 ($C_{10}H_{13}N_4O_9P$ Li requires 371.0580). Compound 5 was then converted to the 2-methylimidazolide, 2-MeImpiX (6).

3-β-D-Ribofuranosylisoguanosine 5'-Diphosphate (3-Isoisoguanosine 5'-Diphosphate). 3-β-D-ribofuranosylisoguanosine 5'-monophosphate (2) (91 mg, 0.25 mmol) was dissolved in 4 ml of *t*-butanol and 4 ml of water. Morpholine (0.6 ml) was added, and the mixture was heated at reflux. A solution of DCC (900 mg) in *t*-butanol (14 ml) was added in small portions over 3 h. The reaction was monitored by TLC on cellulose plates, with 2:1 2-propanol and 0.25 M ammonium bicarbonate as the developing solvent. After additional reflux for about 3 h, complete conversion of the monophosphate to the faster running phosphomorpholidate was observed. The mixture was cooled, and the solvent was removed *in vacuo*. The residue was dissolved in 25 ml of water and washed three times with 25 ml of diethyl ether. The aqueous layer was evaporated *in vacuo*.

The resulting solid was dissolved in dry pyridine (5 ml) and added to a solution of tributylammonium phosphate [prepared from 98 mg of phosphoric acid (crystals) and 1 equivalent of tributylamine in pyridine, then dried by repeated evaporation of pyridine and dissolved in 10 ml pyridine]. The mixture was stirred at room temperature in a stoppered flask for 3 days and then evaporated *in vacuo*. The residue was dissolved in water (400 ml) and applied to a DEAE-Sephadex A-25 column (2.5 × 13 cm). Elution with a 0–0.7 M gradient of TEAB (pH 7.5) was monitored at 280 nm, and the appropriate fractions gave, after evaporation and coevaporation with methanol (3 × 40 ml), a cream-colored solid, R_f 0.27 (cellulose plate, 2:1 2-propanol–0.25 M NH_4HCO_3): yield ~75%. Low-resolution FAB mass spectrum: m/z 444 (MH^+). High-resolution FAB mass spectrum: m/z 444.03360 ($C_{10}H_{16}N_5O_{11}P_2$ requires 444.03215).

3-Isoxanthosine 5'-diphosphate and 3-isoadenosine 5'-diphosphate were prepared similarly from their monophosphates by the Moffatt and Khorana (1961) method.

3-Isoxanthosine 5'-Diphosphate. R_f 0.32 (2:1 2-propanol–0.25 M NH_4HCO_3). Low-resolution FAB mass spectrum: m/z 445.3 (MH^+). High-resolution FAB mass spectrum: m/z 445.01740 ($C_{10}H_{15}N_4O_{12}P_2$ requires 445.01618).

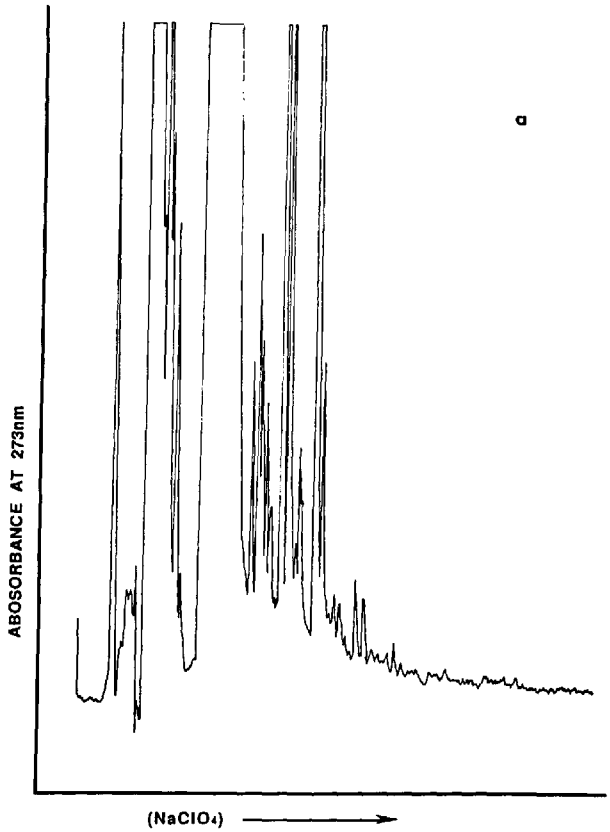
3-Isoadenosine 5'-Diphosphate and Poly(3-isoA). R_f 0.40 (2:1 2-propanol–0.25 M NH_4HCO_3). Low-resolution FAB mass spectrum: m/z 428.1 (MH^+). High-resolution FAB mass spectrum: m/z 428.03880 ($C_{10}H_{16}N_5O_{10}P_2$ requires 428.03725) (Leonard and Laursen 1965). This was converted to poly(3-isoA) as previously described (Michelson et al. 1966).

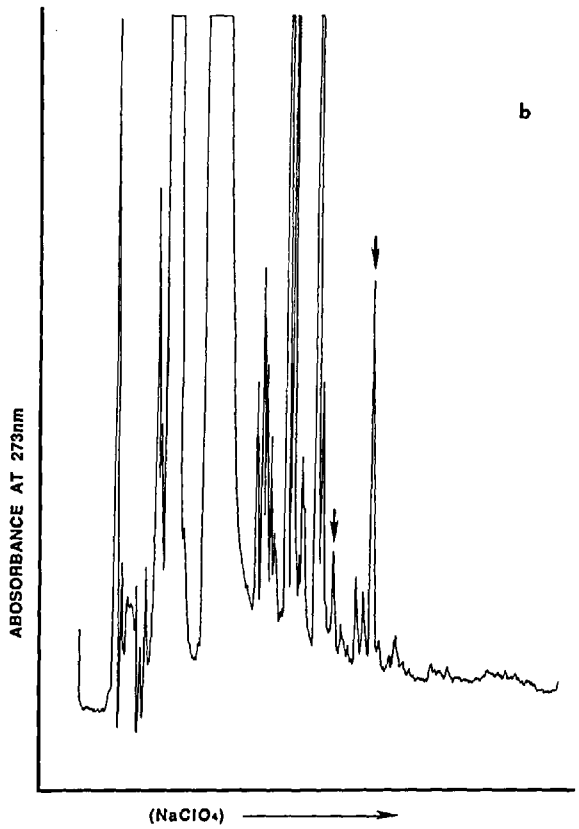
Results

Oligomerization of 2-MeImpiX and 2-MeImpiiG

Preliminary experiments showed that the HPLC elution profiles of the products formed by the oligomerization of 2-MeImpiX (6) were almost unaffected by the presence of poly(U), poly(C), poly(A), poly(G), or poly(I) in the reaction mixtures, provided the products were analyzed prior to digestion with pancreatic ribonuclease (Fig. 1c). In the case of poly(U) and poly(C) templates, we further analyzed the products after digesting the polymers with pancreatic ribonuclease. We were surprised to find that one large and one much smaller peak appeared when poly(U) was used as template (Fig. 1b) that were absent when poly(C) replaced poly(U) (Fig. 1a). Comparison with the products formed in the absence of a template showed that the major new peak did not appear in the elution profile of the products from the template-free reaction (Fig. 1c). (The peaks that disappear after pancreatic ribonuclease treatment are surviving 5'-phosphorimidazolides that hydrolyze nonenzymatically to 5'-phosphates when the reaction mixture is incubated at 37°C overnight).

A very similar, but less extensive, series of experiments using 2-MeImpiiG (3) as substrate gave very similar results. Poly(U), poly(C), and poly(A) had little effect on the pattern of products observed when reaction mixtures were analyzed prior to digestion with pancreatic ribonuclease (data not shown). After pancreatic ribonuclease hydrolysis, major and minor products were formed in the presence of poly(U) (Fig. 2b) that were absent when poly(C) replaced poly(U) (Fig. 2a). The major product is not represented in the elution profiles of the template-free reaction mixture.





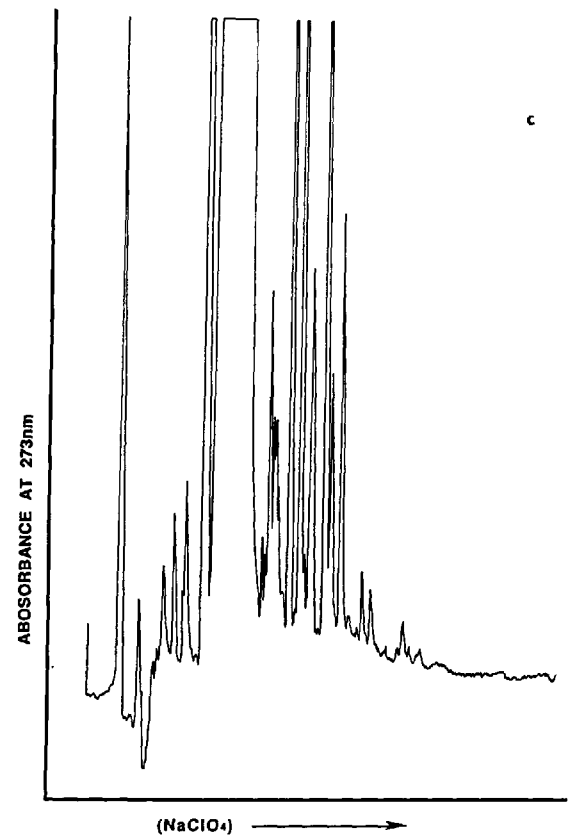


Fig. 1. HPLC elution profiles of the products from the condensation of 2-MeImpiX after 1 week: **a** in the presence of poly(C)—the template has been digested with pancreatic ribonuclease; **b** in the presence of poly(U)—the template has been digested with pancreatic ribonuclease; **c** in the absence of a template. Almost identical patterns of products (Fig. 1c) were obtained when poly(U), poly(C), poly(A), poly(G), or poly(I) was present, provided the samples were not treated with pancreatic ribonuclease. The additional peaks in **b** are indicated by arrows.

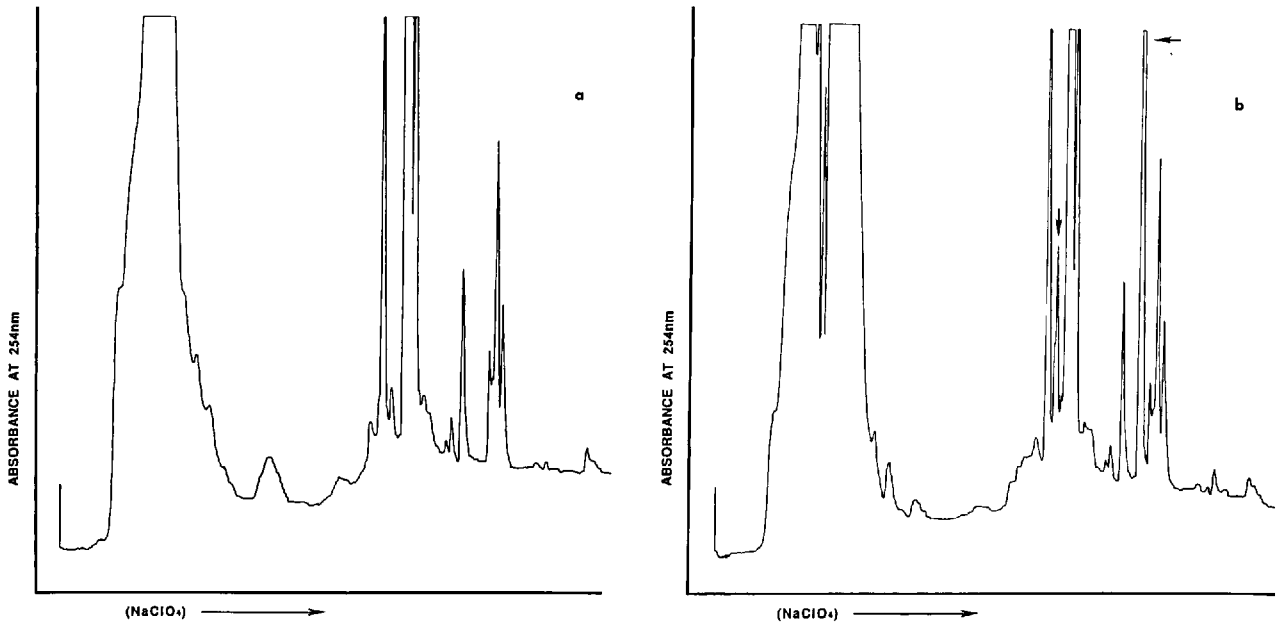


Fig. 2. HPLC elution profiles of the product from the condensation of 2-MeImpiiG after 2 weeks: **a** in the presence of poly(C)—the template has been digested with pancreatic ribonuclease; **b** in the presence of poly(U)—the template has been digested with pancreatic ribonuclease. The additional peaks in **b** are indicated by arrows.

The appearance of previously undetected products after pancreatic ribonuclease digestion of the templates was unexpected, so we carried out a further experiment to establish that the separate mononucleotides piX and piiG had been incorporated into the appropriate products. We took aliquots of reaction mixtures calculated to contain roughly equal amounts of the major new products from the 2-MeImpiX and 2-MeImpiiG reactions, and coinjected them for HPLC analysis. The new peaks, although they had very similar retention times, were clearly resolved. This established that the new peaks do not correspond to degradation products of impurities in the poly(U). We also considered the possibility that the new peaks were formed by the pancreatic ribonuclease digestion of a product of the reaction of the activated nucleotide with an impurity in the poly(U) preparation. We repeated the experiment using 2-MeImpG as substrate in the presence of the same poly(U) preparation. We did not detect a comparable new peak in this case.

In other experiments (data not presented), we found that poly(3-isoadenylic acid) did not act as a template for the oligomerization of 2-MeImpU, 2-MeImpC, 2-MeImpA, 2-MeImpG, or 2-MeImpI.

Discussion

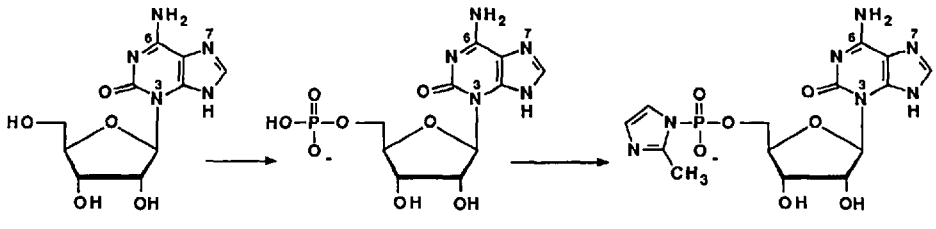
Synthesis

The synthesis of piiG (3-β-D-ribofuranosylisoguanine 5′-phosphate, **2**) was effected by phosphorylation of unprotected iiG (**1**) (Schmidt and Townsend 1975) with pyrophosphoryl chloride in *m*-cresol (Imai et al. 1969). Compound **2** was converted to the 2-methylimidazolide **3** by a general method described previously (Joyce et al. 1984).

A similar sequence was employed for the conversion of iX (**4**) to the 5′-phosphate **5** and thence to the 2-methylimidazolide **6.** In this sequence, piX (**5**) was characterized as the Li^+ salt. Compound **5** had been obtained earlier enzymatically by treatment of xanthine with PP-ribose-P in the presence of a pyrimidine ribonucleotide pyrophosphorylase from beef erythrocytes (Hatfield and Wyngaarden 1964).

Conformations

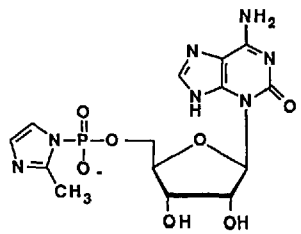
The conformations of iX and iiG have not been determined directly. However, both 3-isoadenosine (Kumar et al. 1988) and 3-isoinosine, which occurs as the 7H-tautomer (Kumar et al. 1989), in the solid state, adopt a conformation about N-Cl′ with the sugar *syn* to the 2-H and *anti* to the 5-membered ring. This suggests strongly that iX and iiG are also likely to adopt the conformation shown for isoxanthosine in **4** (also **5** and **6**) and for isoisoguanosine in **1** (also **2** and **3**). This is not inconsistent with ability of the beef phosphorylase enzyme to catalyze the formation of the nucleotide from iX and PP-ribose-P. Beef phosphorylase normally catalyzes syntheses in which the 2-CO group of a pyrimidine is *anti* to the sugar ring in the product. It is possible that the iX and iiG nucleotides are initially formed in this less stable conformation and reorient once



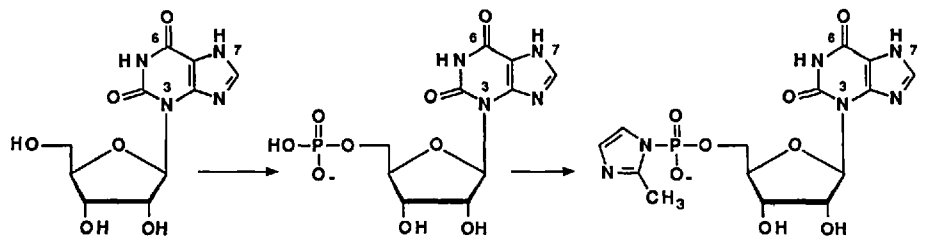
3-Isoisoguanosine (iiG)
1

piiG
2

2-MeImpiiG
3



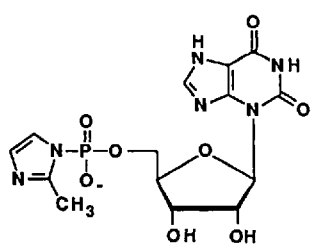
3a



3-Isoxanthosine (IX)
4

pIX
5

2-MeImpIX
6



6a

they are released from the enzyme. It should be noted that the conformation which we anticipate to be more stable is incompatible with a hydrogen-bonding scheme that has recently been proposed (Wächtershäuser 1988).

Intermolecular Hydrogen Bonding

What are the possibilities for the binding of iiG (**1**) to polyribonucleotide templates? If it were to behave as a 5,6-disubstituted cytidine (Wächtershäuser 1988), Watson–Crick binding to poly(G) would be predicted (Fig. 3a). If it were to behave like 3-isoadenosine (Michelson et al. 1966), modified Hoogsteen binding (modified because purine substitution is at N3 rather than N9) to poly(U) would be predicted (Fig. 3b). For the case of iX (4), if it were to behave like a 5,6-disubstituted uridine (Wächtershäuser 1988), Watson–Crick binding to poly(A) would be predicted (Fig. 4a), and if it were to behave like 3-isoinosine, modified Hoogsteen binding to poly(C) would be expected (Fig. 4b). Earlier work (Davies and Davidson 1971; Davies 1976) showed that monomeric 3-methylxanthine, corresponding to iX, does indeed participate in both Watson–Crick and modified Hoogsteen hydrogen-bonding to poly(A) in the 1:2 complex.

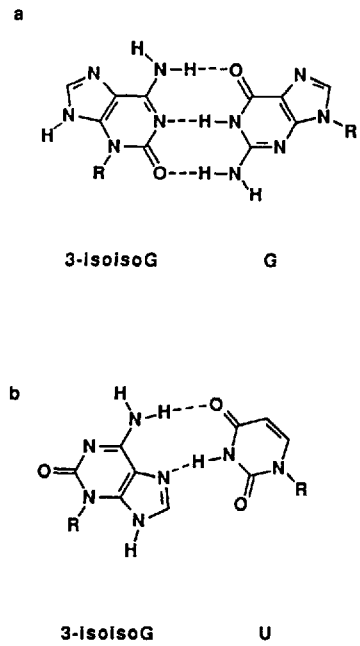


Fig. 3. Base-pairing schemes for iiG

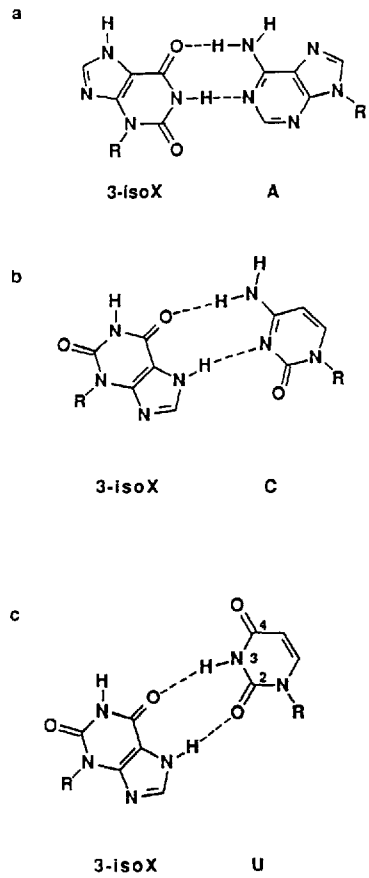


Fig. 4. Base-pairing schemes for iX

Template-Directed Synthesis

Our major experimental finding is that none of the attempted template-directed reactions gave yields of long oligomeric products substantially in excess of those obtained without a template. In all cases except those involving poly(U) the template had very little effect on the nature of the products. The results obtained with poly(U) as template are hard to interpret. Because peaks different from those obtained in template-free reactions appear only *after* ribonuclease hydrolysis of the poly(U), the products must be associated covalently or otherwise with the template throughout chromatography at pH 12. Tight binding of the template to products of molecular weight no greater than that of a tetramer at pH 12 by hydrogen-bonding is surprising, whereas covalent linkage of products to the template has not been observed in other similar reactions.

In the light of the surprising nature of this finding, our interpretation of the positive results is tentative. If we suppose that poly(U) orients both iiG and iX in such a way as to facilitate condensation, we must propose the hydrogen-bonding schemes in Figs. 3b and 4c. Modified Hoogsteen bonding between iiG and U (Fig. 3b) would lead to an antiparallel arrangement of the two strands. However, these arguments would also lead us to expect poly(C) to form an antiparallel helix with iX, but poly(C) does not facilitate the formation of oligo(iX)s. To explain any oligomerization of 2-MeImpiX on poly(U), a different modified Hoogsteen pairing would be needed (Fig. 4c). The proposed double-helix is antiparallel and is related to that suggested in Fig. 3b in the same way that a G:U wobble pair is related to a G:C Watson–Crick base pair.

The results described above illustrate the difficulty of predicting the outcome of template-directed oligomerization reactions on the basis of hypothetical base-pairing schemes. It is first necessary to assign the correct tautomeric structure and conformation to the components of the reaction mixture. This is not always easy when nucleoside analogues are involved that have not been studied experimentally. Even when these aspects of the problem can be dealt with satisfactorily, it is not possible to predict the outcome of experiments with any degree of confidence. Small changes of structure in the activated nucleotide have a profound influence on the efficiency and regiospecificity of the condensation reaction (Inoue and Orgel 1981).

The failure of a particular template-directed reaction can always be attributed to an inappropriate method of activation—perhaps the use of activated nucleotides different from 2-methylimidazolide derivatives or a buffer different from 1-MeIm would have worked better with iiG and iX, for example, and would have yielded higher molecular-weight products on appropriate templates. However, the failure of a variety of plausible template-directed

reactions, particularly that of 2-MeImpiX on poly(A), under the same conditions that would have led to the formation of oligomers at least 5–10 units long from activated A or G derivatives on poly(U) or poly(C) templates, respectively, is sufficient cause to accept the speculations of Wächtershäuser (1988) and other similar speculations with the utmost caution. Experimental evidence for such novel template-directed reactions would be of great interest in the context of the origins of life (Orgel 1986).

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