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Acceleration of the Template-Directed Reactions of Nucleoside 5′-Phosphorimidazolides by Acylation

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Summary. Nucleoside-5′-phosphorimidazolides react readily with acylating agents to give N-substituted products that are highly activated. In most cases these acylated derivatives undergo rapid hydrolysis to give nucleoside 5′-phosphates, whether or not a complementary template is present. However, guanosine 5′-phosphorimidazolide reacts with diethyl pyrocarbonate to give a derivative that oligomerizes rapidly and efficiently in the presence of polycytidylic acid and Pb^{2+}. The reaction is complete in about 1 h, whereas the corresponding reaction in the absence of an acylating agent takes several days. However, the final yield of long oligomers is lower when diethyl pyrocarbonate is present.

Key words: Guanosine 5′-phosphorimidazolide — Diethyl pyrocarbonate — Poly(C)-directed condensation — Acylation

Introduction

Activated derivatives of guanylic and adenylic acid such as the 5′-phosphorimidazolides, ImpG and ImpA, undergo a number of efficient template-directed reactions. However, at temperatures sufficiently low to permit the formation of helical complexes involving monomeric nucleotides, reactions are complete only after several days (Joyce 1987). The development of a system that would permit the completion of a template-directed reaction at 0°C in a short time would be very useful in developing models of a number of aspects of template-catalyzed chemistry.

In principle, rapid reactions could be achieved by using a sufficiently activated nucleotide but, in practice, such compounds can be stored and handled only with considerable difficulty. We decided, therefore, to explore the possibility of generating strongly activated derivatives in situ. We had previously shown that, by using excess of 1-Me imidazole as a buffer, it is possible to drive the replacement of imidazole in ImpA by the N-substituted derivative, and hence to accelerate substantially the rate of reaction (Orgel and Lohrmann 1974). It seemed reasonable, therefore, to study the effect of acylating agents that could react rapidly with the unsubstituted N-atom of the imidazole moiety.

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Abbreviations: pG, guanosine 5′-monophosphate; pA, adenosine 5′-monophosphate; ImpG, guanosine 5′-phosphorimidazolide; ImpA, adenosine 5′-phosphorimidazolide; 2-MeImpG, (guanosine 5′-phosphor)-2-methylimidazolide; 2-MeImpA, (adenosine 5′-phosphor)-2-methylimidazolide; poly(C), polycytidylic acid; poly(U), polyuridylic acid; PRNase, pancreatic ribonuclease

Materials and Methods

Materials. Reagent-grade materials were used throughout. Diethyl pyrocarbonate, acetyl chloride, and tetrabutyl ammoniun hydroxide were purchased from Aldrich; dimethyl pyrocarbonate and di-*t*-butyl pyrocarbonate from Fluka; acetic anhydride and poly(C) from Sigma; benzoyl chloride from Mallinkrodt; and 2,6-lutidine from Matheson, Coleman and Bell. Poly(U) was prepared using a procedure published by Steiner and Beers (1958), the activated imidazolides according to Joyce et al. (1984), and the N-carboxyanhydrides of L-alanine, L-valine, and L-leucine following Katakai and Iizuka (1985). PRNase was purchased from Boehringer Mannheim.

Chromatography. Paper chromatography was conducted on Whatman 3MM paper by the descending technique, using *n*-propanol, concentrated ammonia, and water (55:10:35) as eluent. Prior to application of the samples on the paper, samples containing poly(C) or poly(U) were treated with PRNase at 37°C in the presence of Tris (0.5 M, pH 7.5) for 2 h to hydrolyze the

template. The identity of the products of hydrolysis of the imidazolides was established by cochromatography with authentic samples of the nucleotides, pG and pA.

HPLC was carried out on an RPC-5 column as described by Lohrmann and Orgel (1980). The samples were eluted at pH 12 in the presence of 0.002 M Tris, with a linear gradient of sodium perchlorate that increased from 0.0 M to 0.04 M over a period of 60 min. The flow rate was 1 ml/min and the pressure was approximately 1900 PSI.

The rate of hydrolysis of the activated nucleotides in the presence of the dialkyl pyrocarbonates was monitored by HPLC using a C_{18} reverse-phase, semipreparative column with an initial phosphate buffer (pH 6.5), and a linear gradient of 0.005 M tetrabutyl ammonium hydroxide in methanol.

Reaction Conditions. Reactions were carried out in 0.65-ml presiliconized Eppendorf tubes. The total reaction volume was 50 μl. In template-directed reactions, a sufficient volume of an aqueous solution of poly(U) or poly(C) to give a final concentration of 0.2 M was added to the tube. The solution was evaporated to dryness. In control reactions, these steps were omitted. Next, sufficient 2,6-lutidine buffer (pH 7.0) and solutions of NaCl, $MgCl_2$, and the activated nucleotides were added to give final concentrations of 0.4 M, 1.2 M, 0.19 M, and 0.1 M, respectively. $Zn(NO_3)_2$ or $Pb(NO_3)_2$ solution, if required, was then added with vigorous shaking, followed by enough water to bring the volume to 50 μl. If Zn^{2+} or Pb^{2+} was present, the final concentration was 0.01 M. The acylating agents were added as liquids or solids, with vigorous stirring. When ImpG was used, a gel formed after the addition of some of the activating agents; with ImpA the reaction mixture sometimes deposited a white precipitate. Measurements of pH were made with a pH microelectrode before and at various times after the addition of the acylating agent.

In order to follow the time course of the reaction, 10 reaction mixtures were prepared for each experiment. The reaction mixture was sampled before addition of the acylating agent and at times that ranged from 10 s to several days. To stop the reaction after the desired time, excess EDTA (0.5 M, pH 8.0) was added to quench the divalent cations, and the reaction mixture was then transferred to a capped, sterile polypropylene tube (12 × 75 mm) and diluted to 2.5 ml (50-fold) with pH 12 buffer containing 0.002 M Tris. Solutions were stored at −78°C prior to HPLC analysis.

In the case of the reaction of diethyl pyrocarbonate with ImpG, a large-scale reaction mixture (0.8 ml) was prepared and monitored for temperature changes upon addition of the acylating agent. This experiment showed that no significant temperature changes occurred either upon addition of diethyl pyrocarbonate or during the first 2 h of reaction.

Results

In a series of preliminary experiments we surveyed a range of acylating agents for their effect on the rate of hydrolysis of the phosphorimidazolides ImpA, 2-MeImpA, ImpG, and 2-MeImpG, and on the yield of oligomeric products formed from these compounds in the absence of a template. We monitored the pH of the reaction mixture in each case. These preliminary reactions were carried out with the same concentrations of reagents in the presence of the same concentrations of buffer as were used in the template-directed reactions.

Acetyl chloride or benzoylchloride (0.1 M) brought about a rapid lowering of the pH of the reaction mixture by 3–4 pH units, no doubt because they generate two equivalents of acid on hydrolysis. No oligomeric products were detected. Essentially the same results were obtained with acetic anhydride. The N-carboxyanhydrides of L-alanine, L-valine, and L-leucine, although they had little effect on the pH of the reaction mixture, brought about only a modest acceleration in the rate of hydrolysis of the phosphorimidazolides. None of these reagents, therefore, merited further study.

The most satisfactory acylating agents discovered in these preliminary experiments were the dialkyl pyrocarbonates. Dimethyl and diethyl pyrocarbonate caused rapid hydrolysis of all four phosphorimidazolides without bringing about a significant change in pH. In addition, substantial amounts of oligomeric products were generated on treating ImpG with diethyl pyrocarbonate. We decided, therefore, to study the reactions induced by the dialkyl pyrocarbonates in more detail.

In control experiments we found that the half-lives for the hydrolysis of ImpG and 2-MeImpG in the presence of diethyl pyrocarbonate were 7 min and 10 min, respectively. In the case of ImpA and 2-MeImpA in the presence of diethyl pyrocarbonate, we found half-lives of 9 and 13 min, respectively. In the presence of dimethyl pyrocarbonate, half-lives of about 15 min were observed for all the monomers, whereas in the presence of di-*t*-butyl pyrocarbonate half-lives of about 10 h were observed throughout. Under the same conditions, very little hydrolysis of the phosphorimidazolides was detected after 1 day in the absence of an acylating agent.

We carried out a substantial number of experiments using acylating agents in the presence of a template with or without Zn^{2+} or Pb^{2+} ions. In general, we found that the reactions were completed much more rapidly in the presence of dimethyl or diethyl pyrocarbonate than in the absence of an acylating agent. However, except in the case of the oligomerization of ImpG in the presence of Pb^{2+}, we could not detect oligomeric products longer than pentamers. We decided, therefore, to study the effect of acylation on the template-directed reactions of ImpG in the presence of Pb^{2+} in detail.

In Fig. 1 we present HPLC profiles of the products obtained after various times from ImpG in the presence of poly(C) and Pb^{2+} with or without dialkyl pyrocarbonates. In the presence of diethyl pyrocarbonate the reaction is essentially complete after 1 h. When no acylating reagent is present, the reaction progresses much more slowly (Lohrmann and Orgel 1980)—the formation of oligomers as long as those obtained in 15 min with diethyl pyrocarbonate occurs only after about 1 day in its absence.

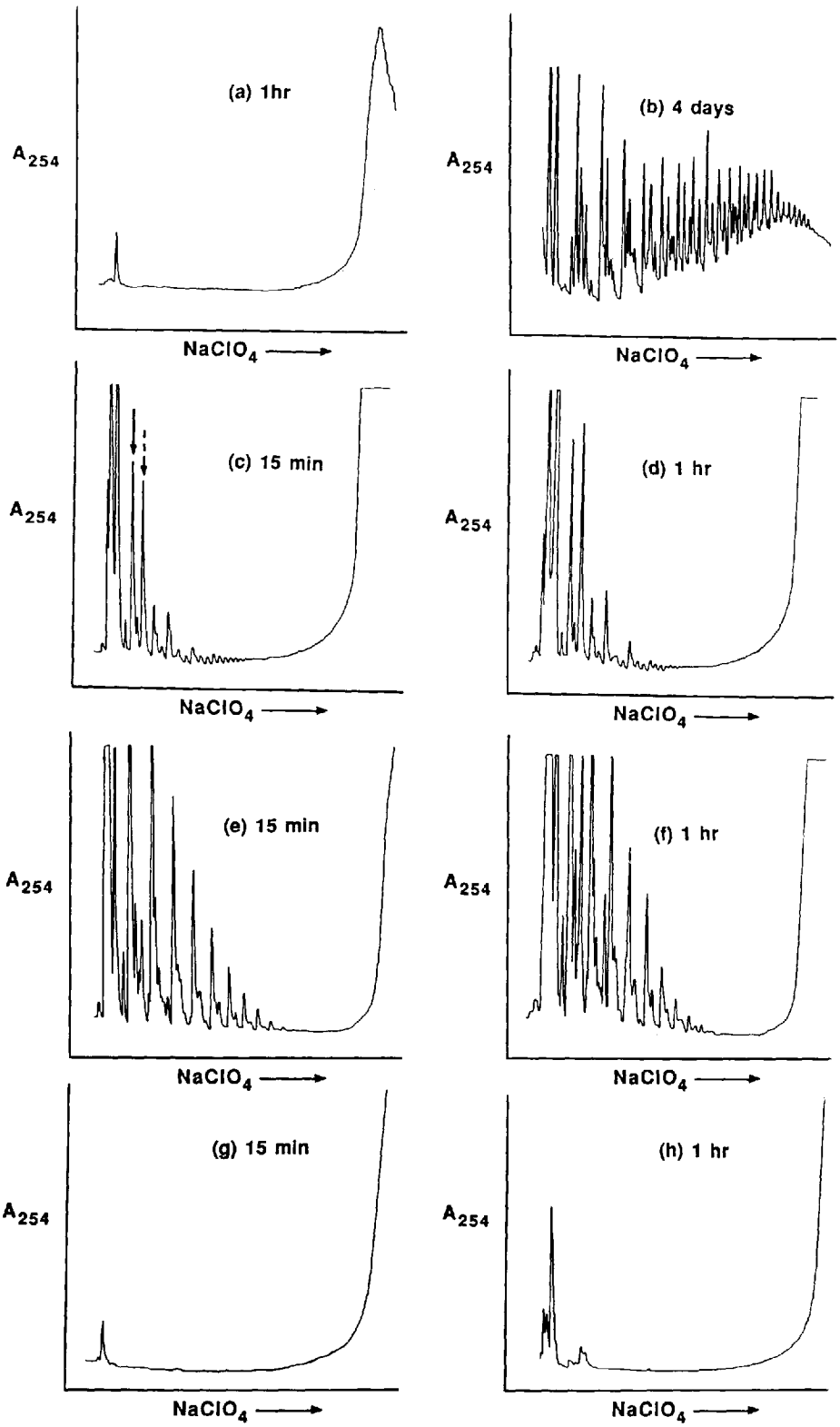
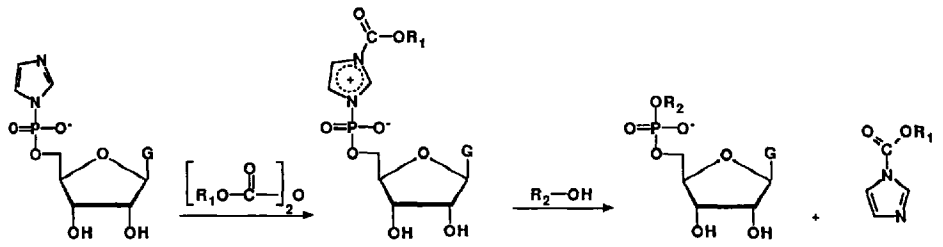


Fig. 1. Template-directed self-condensation of ImpG in the presence of: **a** and **b**, Pb^{2+}; **c** and **d**, Pb^{2+} and dimethyl pyrocarbonate; **e** and **f**, Pb^{2+} and diethyl pyrocarbonate; **g** and **h**, Pb^{2+} and di-*t*-butyl pyrocarbonate. Reaction conditions: 0°C, pH 7.0, 0.2 M poly(C), 1.2 M NaCl, 0.19 M $MgCl_2$, 0.01 M $Pb(NO_3)_2$, 0.4 M 2,6-lutidine buffer, 0.1 M ImpG, 0.1 M dialkyl pyrocarbonate. In c two peaks corresponding to isomeric trimers are present. The solid arrow indicates the peak corresponding to the oligomer with the larger number of 2′-5′-linked bonds.

The major products produced in the presence of diethyl pyrocarbonate, like those formed in its absence, are 2′-5′-linked, as shown by the identity of their retention times with those of the major products produced in the absence of an acylating agent (Lohrmann and Orgel 1980). The final yield of long products is much greater when no acylating agent is used as may be seen by comparing Fig. 1b with Fig. 1f.

When dimethyl pyrocarbonate was substituted for diethyl pyrocarbonate, the reaction proceeded more slowly and the terminal yield of long oligomers was substantially reduced (Fig. 1c and d). The long even-spaced series of peaks in Fig. 1e, with only



Scheme 1.

small subpeaks indicates that the reaction accelerated by diethyl pyrocarbonate is regiospecific. In Fig. 1c the appearance of a pair of closely spaced peaks of roughly equal height in the trimer region shows that when dimethyl pyrocarbonate is substituted for diethyl pyrocarbonate, the reaction is no longer regiospecific.

When di-*t*-butyl pyrocarbonate was used as the acylating agent, oligomerization proceeded at a slow rate (Fig. 1g and h). After 1 day of reaction, the final yield of oligomers (picture not shown) was comparable to the amount of oligomers generated in the reaction containing diethyl pyrocarbonate after 15 min (Fig. 1e), but the regiospecificity of the reaction was low and no long oligomers could be detected.

Examination of reaction mixtures containing ImpG and each of the different dialkyl pyrocarbonates indicate that in the presence of diethyl and dimethyl pyrocarbonate the oligomerization was complete within 1 h. For the case of di-*t*-butyl pyrocarbonate, oligomerization was complete within 1 day. Reaction in the absence of dialkyl pyrocarbonates continued generating longer oligomers for periods as long as 14 days.

Discussion

Imidazole derivatives, in general, are good nucleophiles and can be acylated efficiently and rapidly in aqueous solution. The acylation of a phosphorimidazolide generates a product that is far more active than the starting material toward nucleophiles attacking the phosphorus atom. The substantial increase in the rates of hydrolysis and oligomerization that we observe are, therefore, easily explained (Scheme I).

We already know that alkyl substitution on the imidazole moiety of a nucleoside-5'-phosphorimidazolide profoundly influences the efficiency and regiospecificity of its template-directed reactions (Inoue and Orgel 1981). Unfortunately, it is not possible to predict the effect of particular substitutions. Thus, although we can be confident that acylation will increase the rate of reaction, only direct experimentation can indicate the nature of the products of our reactions.

It is interesting that the Pb^{2+}-catalyzed reaction of the carbethoxy derivative, like the corresponding reaction of the original phosphorimidazolide (Lohrmann and Orgel 1980), yields long 2'-5'-linked products, whereas the corresponding methyl and *t*-butyl derivatives yield only a mixture of short oligomers containing comparable amounts of 2'-5' and 3'-5' linkages. This effect of the alkyl group on the regiospecificity of the reaction, although not so extreme, is reminiscent of the effect of 2-alkyl substitution on the reaction of phosphorimidazolides in the absence of Pb^{2+} ions (Inoue and Orgel 1981).

Our results show that, with the help of acylating agents, template-directed reactions can be completed in at most an hour. We plan to explore the use of other acylating agents with the hope of finding reagents that permit the rapid and efficient synthesis of long 3'-5'-linked oligomers. This would make it practical to carry out experiments on chemical evolution involving many rounds of template-directed synthesis in a reasonably short time.

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