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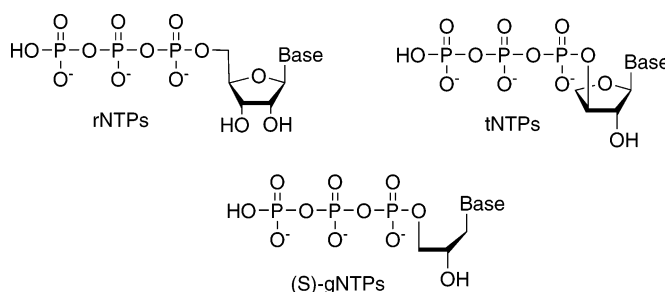
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ABSTRACT



The synthesis of (*S*)-glycerol nucleoside triphosphates (gNTPs) and the analysis of their substrate activities for enzymatic polymerization is described. NTPs with simplified carbohydrate backbones such as the tNTPs (α -L-threose-NTPs) are polymerase substrates and offer the potential to create non-natural aptamer sequences with simplified backbones through enzymatic means. The acyclic (*S*)-GNA was modeled after the shortened α -threofuranosyl backbone. Here we describe the synthesis of (*S*)-glycerol NTPs and initial enzymatic testing of this further simplified nucleic acid backbone.

Glycerol nucleic acid (GNA), which has a simple acyclic nucleic acid backbone, is of current interest¹ as a potential evolutionary progenitor of RNA. Simplified nucleic acid backbones have been the focus of recent study. Since RNA systems are structurally more complex, there are questions regarding the ability of RNA polymers to have evolved directly from a prebiotic environment.^{2,3} Eschenmoser et al. have synthesized and screened a number of nucleic acid systems based on alternative carbohydrate backbones and examined their ability to self-pair and to pair with RNA/DNA.⁴ The shortened and simplified α -L-threofuranosyl

backbone (TNA) possesses the ability to form both types of duplexes.^{5,6} Threose nucleoside triphosphates (tNTPs) were also shown to be remarkably efficient substrates for template-directed enzymatic polymerization by Terminator DNA polymerase; the investigation of TNA aptamers is now feasible.^{7,8}

Since TNA forms duplexes with RNA and DNA, the six-bond backbone repeat that is characteristic of RNA and DNA is not a critical requirement for progenitor nucleic acid systems. Polymers based on simpler backbones may also

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exhibit useful enzymatic activity. A further simplification of the TNA backbone involves opening the cyclic sugar to yield an acyclic or glycerol (GNA) backbone that conserves the five-bond backbone repeat of TNA (Figure 1). Oligomers

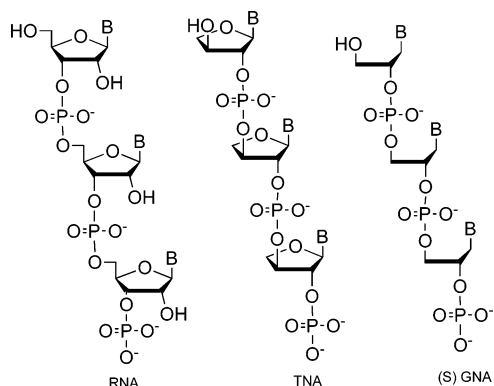


Figure 1. RNA, TNA, and (S)-GNA polymers. (S)-Glycerol nucleic acids are simplified nucleic acids containing the same 5-bond repeating, but acyclic, backbone as TNA.

based upon the GNA monomers have been prepared, and of the two possible enantiomers, (S)-GNA forms the more stable heteroduplexes with RNA.¹

The synthesis of three-carbon nucleic acid backbones was initially described by Holy⁹ and phosphoramidites were later described by Andrews.¹⁰ *R*-(+)-Glycidol was used to define the stereochemistry of the final product, obtained by ring opening of the chiral epoxide. The propane-2,3-diol phosphoramidite monomers were compatible with standard solid-phase coupling chemistries used to create GNA oligomers. Meggers employed these strategies to construct poly-A and poly-T GNA oligomers for their base-pairing studies.¹ Here we describe the syntheses of the acyclic glycerol-based gNTP monomers (Figure 2), which we have used for enzymatic incorporation into a primer/template complex.

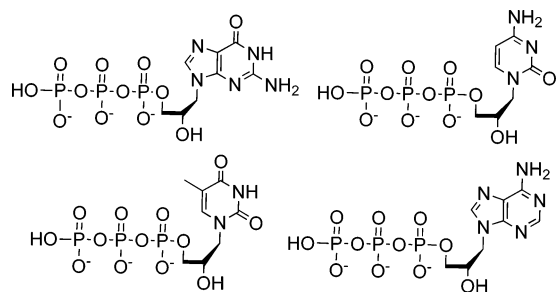
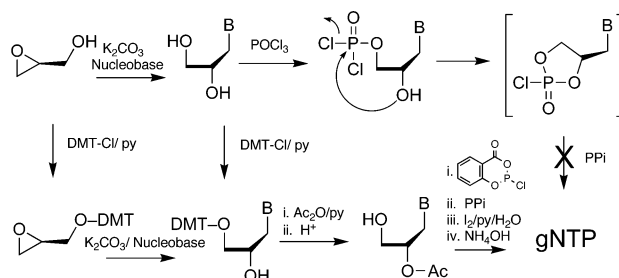


Figure 2. (S)-Glycerol nucleoside triphosphates (gNTPs) of the four common nucleobases.

After ring opening of the *R*-(+)-glycidol with the four common nucleobases, initial attempts to synthesize the

triphosphates from these dihydroxy monomers with POCl₃ as the phosphorylating agent met with limited success.^{11,12} After POCl₃ phosphorylation, rapid intramolecular cyclization by the secondary hydroxyl, prior to pyrophosphate addition, generated the cyclic phosphodiester, likely as the result of both proximity effects and backbone flexibility (Scheme 1). To block this reaction pathway, the nucleobases

Scheme 1. Syntheses of gNTPs



and secondary hydroxyls of the GNA monomers were protected. Treatment of the monomers with DMT-Cl followed by acetic anhydride placed the trityl group on the primary hydroxyl and acetylated the secondary hydroxyl and, when present, the nucleobase amino groups. Removal of the trityl groups was accomplished by using 3% dichloroacetic in dry dichloromethane and the products were purified by flash chromatography with use of dry dichloromethane for three column volumes before eluting with a methanol:dichloromethane mixture. With a protected secondary hydroxyl (and nucleobase), we could phosphorylate using Eckstein's¹³ phosphite method and these procedures resulted in excellent overall yields of the desired gNTPs (Scheme 1).

Single and multiple nucleotide incorporation assays were conducted to test substrate activities for gNTP monomers with various polymerases. Primer extensions were carried out by using radio-labeled primer annealed to appropriate templates and incubated with a polymerase and one or more gNTPs. The reactions were quenched by using stop buffer and analyzed by 20% denaturing PAGE (Figure 3).

Polymerases and reverse transcriptases from differing families (Therminator, Vent, Deep Vent, AMV-RT, M-MuLV, HIV-RT, KF, KF_{exo-}, Bst, and Taq) were tested for polymerization activity on DNA primers and templates. These enzymes exhibited the ability to readily incorporate one gNTP, with Therminator DNA polymerase accomplishing the *N* + 1 extension in the shortest time, but we have not observed further extension of GNA attached to the DNA or RNA primers.

The difficulty in forming longer strands may be due to a number of factors: (i) loss of contacts between the primer/template and polymerase as the growing strand incorporates

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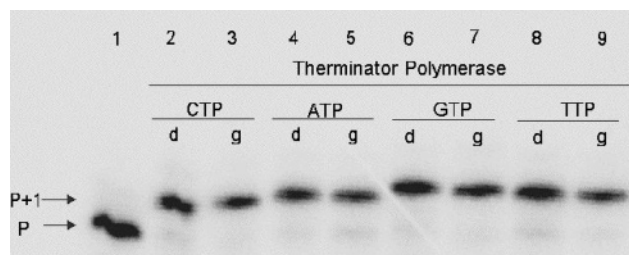


Figure 3. Single nucleotide incorporations of 2'-deoxy- (d) and (S)-gNTPs (g) by Terminator DNA polymerase. Lane 1: 20 μ L of 250 nM radiolabeled 23-mer primer. Lanes 2–9: 20 μ L of 250 nM primer annealed to an appropriate template, 74 μ M Terminator DNA polymerase, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris-HCl, 2 mM MgSO_4 , 0.1% Triton X-100, 0.1 mg/mL BSA, 2.5 mM DTT, at pH 8.8 and incubated at 55 $^\circ\text{C}$. Lanes 2, 4, 6, and 8 contained 0.5 μ M dNTP and were stopped after 1 min. Lanes 3, 5, 7, and 9 contained 500 μ M gNTP and were incubated for 30 min. Reactions were quenched with 8 M urea and 100 mM EDTA and analyzed by 20% denaturing PAGE.

a greater number of GNA monomers; (ii) alignment of the primer 2'-hydroxyl for attack at the α phosphate of the gNTP after a GNA monomer has been added could be unfavorable due to the smaller size, greater flexibility, or increased rotational freedom along the glycerol backbone; and (iii) loss of duplex stability with increased GNA content may result in uncontrolled denaturation. On the other hand, no significant differences in the elongations were observed for DNA vs RNA templates. GNA has been reported to form a more stable duplex with RNA than with DNA, but this did not improve the extent of primer extension. Temperatures at or below the reported T_m for the heteroduplexes also did not result in improvement, though reduced enzyme efficiency at lower temperatures may be a contributing factor.

The structure of a GNA/RNA(DNA) duplex has yet to be determined, so the conformation of a GNA-containing duplex may be significantly different from that of "normal", A- or B-like structures, further reducing contacts available for

polymerase binding and function. Incorporation of the initial gNTP indicated that the monomers are sufficiently accommodated within the active site for successful incorporation within a broad range of polymerases so substrate recognition by the active site does not appear to play a limiting role. Residues associated with duplex binding may be interesting candidates for mutation for directed evolution of polymerases capable of more efficient synthesis of long GNA oligomers.^{14,15}

Glycerol nucleic acids are promising molecules for investigation of potential RNA progenitors. GNA's simplicity offers potential as an intermediate catalytic and/or information storage polymer between the "Prebiotic" and "RNA" worlds. However, a plausible prebiotic synthesis of GNA has yet to be demonstrated. This structural simplicity also offers ease of synthesis and functionalization which may lend GNA to more diverse biochemical applications. Enzymatic systems able to transcribe into or replicate long sequences of GNA are necessary to fully investigate GNA sequence space and their oligomer properties. To date, we have not identified an enzyme system that possesses the ideal characteristics, but we identified several polymerases that are candidates for further evolution. The availability of synthetic GNA oligomers and now all four gNTPs provides the tools needed to evolve the enzymatic machinery.

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Supporting Information Available: Experimental procedures and characterization of all compounds along with NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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