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A Pre-RNA Candidate Revisited: Both Enantiomers of Flexible Nucleoside Triphosphates are DNA Polymerase Substrates

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Simplified alternatives to natural nucleic acids may have served in molecular evolution as a bridge between prebiotic chemistry and the RNA world. While RNA itself currently fulfills both genotypic and phenotypic roles as a carrier of genetic information and catalyst, molecular complexity brings into question its suitability as the first informational molecule. Candidate structures for the latter include PNA,[1] TNA,[2] and GNA.[3]

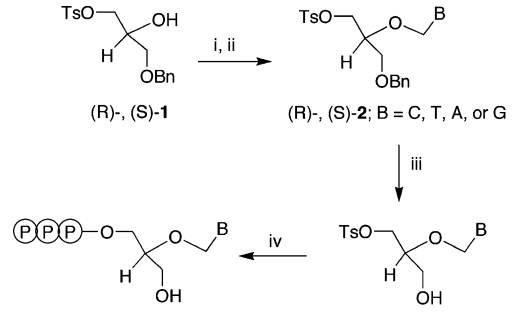
One of the earliest proposed pre-RNA candidates is the flexible nucleic acid (FNA) structure based on the mixed acetal aminal of formyl glycerol.[4] FNA achieves dramatic stereochemical simplification by formally deleting the 2′-CHOH group of RNA, thereby replacing the four RNA stereocenters with a single (pro)stereocenter. This simplicity comes at the price of increased backbone flexibility and possibly less optimal physical properties. Indeed, stability measurements on DNA/FNA chimeric duplexes have shown weak or no hybridization.[5] Yet, an important predicted feature of FNA is insensitivity to chirality[4]—in relation to both itself and other molecules. Despite the documented inability of FNA to form stable duplexes with DNA, flexible nucleoside triphosphates (fNTPs) are substrates for DNA polymerases. Consistent with early predictions, FNA chirality is unimportant as polymerases accept both (*S*)- and (*R*)-fNTP antipodes. These results provide a renewed basis for positing FNA as a viable informational molecule.

Syntheses of both the (*S*)- and (*R*)-enantiomers of all four fNTPs are shown in Scheme 1. The syntheses began with alcohol **1**, available from commercial (*S*)- and (*R*)-glycidyl tosylates.[6] Chloromethylation of **1**,[7] followed by a Vorbruggen reaction[8] with silylated bases, yielded benzyl-protected nucleosides **2**. Debenzylation with Pd/cyclohexene[7] and then displacement of the tosylate with butylammonium triphosphate[9] gave the triphosphates **4**.

Terminating acyclonucleoside triphosphates related to acyclovir lacking the hydroxyl group required for chain extension have been employed as an alternative to dideoxynucleotides in the Sanger DNA sequencing method.[10] Due to the efficiency with which 9°N A485L exo- (Therminator) DNA polymerase has accepted terminating acyclonucleoside substrates,[11] we used it initially in conjunction with primed template **1** to assess incorporation and extension of fNTPs. The results, displayed in Figure 1, panel b, show multiple incorporations of flexible nucleotides in the case of racemic, (*R*)-, and (*S*)-fCTPs. Maximal incorporation is seen with the "non-natural" configuration found in the (*R*)-enantiomer, leading mainly to the incorporation of four nucleotides with longer bands visible, followed closely in efficiency by the racemic and antipodal triphosphates. As shown in panel c of Figure 1, a wider screen of polymerases did not yield results superior to those obtained with Therminator, although many other polymerases showed activity.

The preference of B family *archaeal* polymerases[11] for chain terminating acyclonucleotides led us to compare the activities of Therminator, Vent exo⁻, and Deep Vent exo⁻ using templates **1**−**4** and both antipodes of the complete fNTP series, as shown in Figure 2a. Of the three polymerases, Therminator shows notable efficiency in primer extension with all four fNTPs of both antipodes, incorporating up to 7 nt (full-length product) with (*S*)- and (*R*)-fATP. For the series of higher temperature incubations (73 °C, panels i−iii), Therminator polymerase extends the primer by up to 5 nt and incorporates monomers in the "natural" (*S*)-series with a preferred ordering of A ≈ T > C ≈ G. In contrast, Vent exo⁻ and Deep Vent exo⁻ polymerases extend a maximum of 2 nt and show less marked base preferences. At lower incubation temperature (55 °C, panel iv), primer extension by Therminator increases to 7 nt (full-length product) in the case of fATP, and monomer incorpora-

Scheme 1. Synthesis of fNTPs[a]



[a] Reaction conditions: (i) trioxane, HCl(g), CH_2Cl_2, 0°; (ii) *N*,*O*-bis-TMS-acetamide, nucleobase (A, G, C, or T), TMSOTF, CH_3CN; (iii) EtOH or MeOH, cyclohexene, Pd(black), 80°; (iv) $[(Bu)_4N]_5P_3O_{10}$, CH_3CN, room temperature.

a)
primer: 5'dCGC CAG GGT TTT CCC AGT CAC GAC G3'
template 1: 3'dGCG GTC CCA AAA GGG TCA GTG CTG C***GG GGG GG***5'
template 2: 3'dGCG GTC CCA AAA GGG TCA GTG CTG C***AA AAA AA***5'
template 3: 3'dGCG GTC CCA AAA GGG TCA GTG CTG C***TT TTT TT***5'
template 4: 3'dGCG GTC CCA AAA GGG TCA GTG CTG C***CC CCC CC***5'
template 5: 3'dGCG GTC CCA AAA GGG TCA GTG CTG~
~***CCC GCT CCG CAT CCC TTC ACT TAC CAG TCC***5'

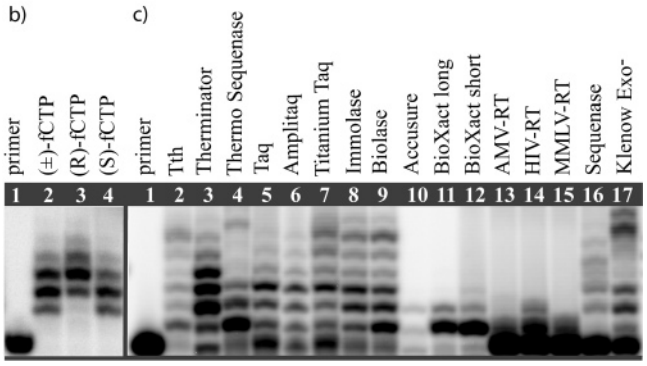


Figure 1. (a) Primer and template sequences; italicized text refers to template coding regions. (b) Primer extension using template **1** with (±)-, (*S*)-, and (*R*)-fCTP as indicated, 2U Therminator DNA polymerase, 1U Tth pyrophosphatase, 75 °C, 24 h. (c) Primer extension using template **1** with (*S*)-fCTP. Reactions were conducted with 1× reaction buffer (as supplied by manufacturer), 200 μM (*S*)-fCTP, 1 μL of polymerase as supplied, 74 °C (lanes 2−12), and 37 °C (lanes 13−17), 24 h.

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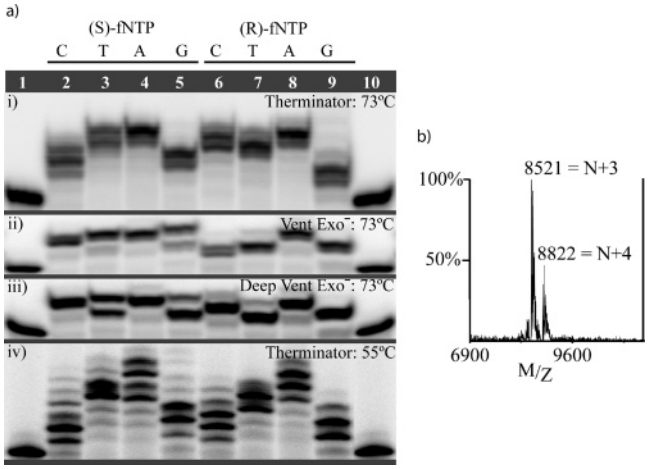

Figure 2. (a) Primer extension with ^{32}P 5′-labeled primer, templates **1**−**4**, and (*S*)- and (*R*)-fNTPs as indicated. In addition to polymerase, all reactions included 1U Tth pyrophosphatase. Lanes 1 and 10 contain radiolabeled primer only. (b) MALDI-TOF MS of polymerization products formed under conditions as in panel (a), inset (i) with (*S*)-fATP. Calculated mass for $[(N+3) + H^+] = 8519$, $[(N+4) + H^+] = 8821$.

tion efficiency takes on a new ordering of A > T > G > C. For Therminator, antipodal efficiencies may be summarized as (*S*)-fG > (*R*)-fG, (*S*)-fA ≈ (*R*)-fA, (*S*)-fT ≈ (*R*)-fT, (*S*)-fC < (*R*)-fC at both incubation temperatures. When integrated over all four nucleotides, these results indicate that on average antipodal fNTPs are not distinguished. A mass spectrum of the products from a preparative polymerization with primed template **3** and (*S*)-fATP is given in Figure 2b.

The specificity of fNTP incorporation by Therminator polymerase was assessed by primer extension experiments with templates **1**−**4** and mismatched fNTPs (Figure 1S). A comparison was made of primer extension for pyrimidine−purine mismatches fC:A, fT:G, fA:C, fG:T and the matched pairs fC:G, fT:A, fA:T, fG:C. The results show minimal primer extension of mismatched fNTP/template combinations; primary products in these cases are the result of incorporating zero or one fNMP.

To further assess fNTP activity and probe the necessity of the A485L mutation, a series of polymerizations were conducted with primed template **5** based on progressive replacement of natural dNTPs by fNTPs using both 9°N A485L exo- (Therminator) and 9°N E143D (9°N$_m$)[12] polymerases (Figure 3). For Therminator, progressive replacement of dNTPs by fCTP, then fCTP and fTTP, followed by fCTP, fTTP, and fATP, gave apparent full-length product in the first two cases, less than full-length product in the third case at 72 °C, but apparent full-length product for the latter at 55 °C.[13] The corresponding number of FNA incorporations through the series are 3, 7, and 14 nt. The experiment using 9°N$_m$ at 55 °C gave similar results to Therminator for lanes with fNTPs present but dissimilar results in lanes where fNTPs were absent (i.e., lanes 4, 6, and 8 versus lanes 3, 5, 7, and 9). Evidently, the 3′-5′ exonuclease activity associated with 9°N$_m$ degraded components of all reactions incubated for 24 h in the absence of fNTPs (lane 2 is a positive control), in support of fNMP incorporation in lanes where the latter are present. It is further clear from these results that the A485L mutation of 9°N is not essential for activity.

In summary, both enantiomers of fNTPs are substrates for polymerases. Moreover, the resulting syntactic (*S*)- or (*R*)-FNA oligomers interact sufficiently with a complementary DNA template to enable serial chain extension. These results are consistent with earlier nonenzymatic template-directed synthesis of RNA on atactic FNA.[14] The inconsequence of FNA chirality likely derives from a combination of backbone flexibility and single stereogenic center

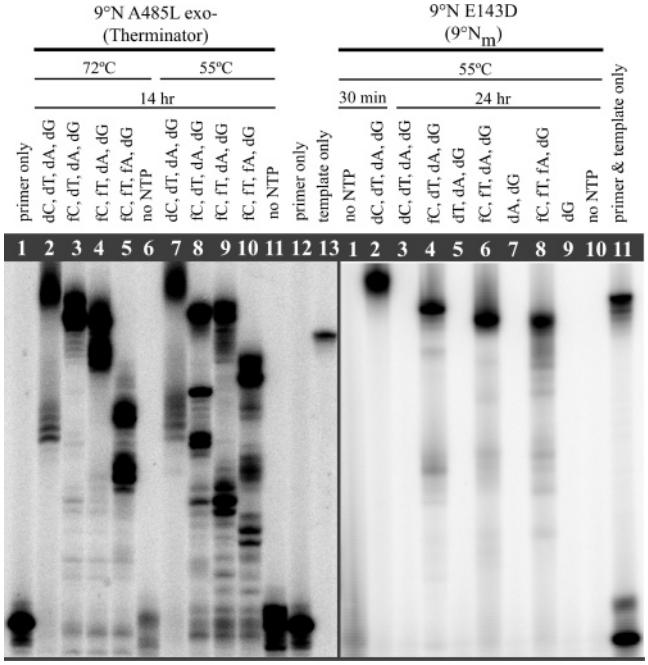

Figure 3. Primer extension on template **5** with progressive replacement of the dNTP pool with fNTPs ((*R*)-fCTP, (*S*)-fTTP, and (*S*)-fATP), as indicated. In addition to polymerase, all reactions included 1U Tth pyrophosphatase.

with the result being functionally equivalent conformational space shared by the two possible configurations. Intriguingly, this may include the unnatural antipode adopting the equivalent of an α-anomeric configuration.[4,15] The absence of enantiomer cross-inhibition[16] seen with FNA may have implications for the pre-RNA world and molecular evolution. Future work will be directed toward derivation of mutant polymerases with enhanced activity to support in vitro evolution[17] of functional FNAs.

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Supporting Information Available: Experimental procedures, including spectroscopic information. This material is available free of charge via the Internet at http://pubs.acs.org.

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