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# Artificial nucleotide codons for enzymatic DNA synthesis

Nazarii Sabat,<sup>a</sup> Andreas Stämpfli,<sup>b</sup> Marie Flamme,<sup>a</sup> Steven Hanlon,<sup>c</sup> Serena Bisagni,<sup>c</sup> Filippo Sladojevich,<sup>b</sup> Kurt Püntener<sup>c</sup> and Marcel Hollenstein<sup>a\*</sup>

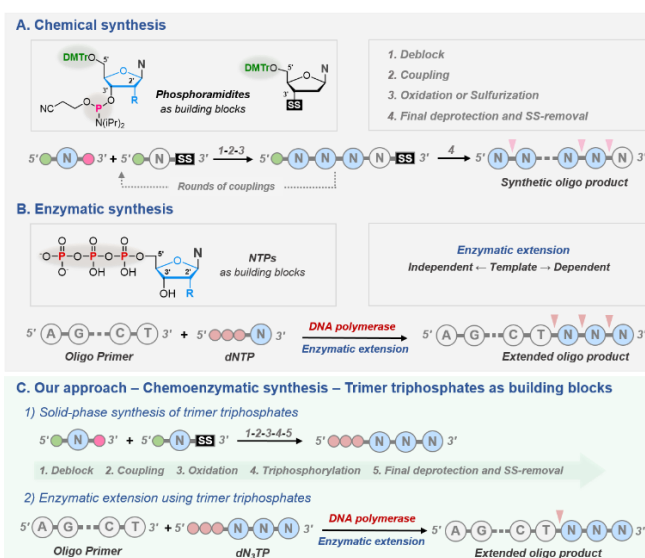
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Herein, we report the high-yielding solid-phase synthesis of unmodified and chemically modified trinucleotide triphosphates (dN<sub>3</sub>TPs). These synthetic codons can be used for enzymatic DNA synthesis provided their scaffold is stabilized with phosphorothioate units. Enzymatic synthesis with three rather than one letter nucleotides will be useful to produce xenonucleic acids (XNAs) and for *in vitro* selection of modified functional nucleic acids.

The ribosome converts the genetic information encoded in nucleic acids into proteins. Even though amino acids are assembled one by one, this decoding process requires Watson-Crick interactions between a trinucleotide codon on mRNA and a corresponding anticodon of a cognate aminoacyl-tRNA. The genetic code is degenerate and codons are recognized by one or multiple tRNAs due to a sequence flexibility mainly at the level of the third nucleotide.<sup>1, 2</sup> On the other hand, synthesis of nucleic acids is mediated by polymerases and proceeds by assembling individual, single-letter nucleotides on growing chains. Due to the differences in mechanisms, the error rates involved in the synthesis of these fundamental biopolymers vary substantially from very low for DNA replication to a more error-prone transcription of DNA into mRNA, and modest fidelity for the translation of mRNA into proteins (error rates of  $\sim 10^{-8}$ ,  $\sim 10^{-5}$ , and  $\sim 10^{-4}$ , respectively).<sup>3, 4</sup>

Both synthetic machineries can be tweaked to form chemical variants of proteins and nucleic acids via both genetic and chemical methods. For instance, additional, unnatural amino acid building blocks can be introduced into the scaffold of proteins by reassigning the amber stop codon to an orthogonal amino-acyl tRNA synthetase/tRNA (aaRS/tRNA) pair.<sup>5, 6</sup> Similarly, polymerases can incorporate base-, phosphate- and sugar-modified nucleoside triphosphates into DNA, RNA, and XNAs.<sup>7-16</sup> Proteins and nucleic acids both benefit from such an increase in chemodiversity. In the case of nucleic acids, the co-polymerization of modified nucleoside triphosphates enables Darwinian selection experiments to raise functional nucleic acids with enhanced properties,<sup>8, 17-19</sup> expand the genetic alphabet with orthogonal base pairs,<sup>11, 20, 21</sup> and functionally



**Fig. 1.** (A) Chemical synthesis of oligonucleotides with phosphoramidites; (B) Enzymatic synthesis of oligonucleotides with polymerases and nucleotides; (C) Proposed chemoenzymatic synthesis of oligonucleotides using trimer triphosphates (dN<sub>3</sub>TPs) as substrates.

Here, we have explored the possibility of synthesizing DNA sequences three-by-three by combining trinucleotide 5'-triphosphate (dN<sub>3</sub>TP) building blocks and polymerase-assisted reactions. Inspired by the work of the Holliger lab who evolved RNA ribozymes capable of synthesizing RNA with rN<sub>3</sub>TPs as substrates,<sup>29, 30</sup> we synthesized dN<sub>3</sub>TPs consisting of natural and modified nucleotidic scaffolds (Fig. 1C) and questioned whether protein enzymes could be coerced to synthesize DNA with these building blocks. While unmodified DNA dN<sub>3</sub>TPs are degraded by polymerases, presumably due to their inherent phosphatase activity,<sup>31</sup> strengthening the phosphodiester linkage by replacement of the non-bridging oxygen with sulfur permits the use of dN<sub>3</sub>TPs for the synthesis of DNA.

We hypothesized that incorporating *three* rather than *one* letter at the time would further accelerate DNA synthesis which is highly sought after especially in the context of storage of digital information applications. In addition, trinucleotides could serve as Trojan horses to sneak in chemical modifications past polymerases by simply changing the nature of the internal nucleotide.<sup>32</sup> Lastly,

<sup>a</sup>Institut Pasteur, Université Paris Cité, CNRS UMR3523, Department of Structural Biology and Chemistry, Laboratory for Bioorganic Chemistry of Nucleic Acids, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France.

<sup>b</sup>Pharma Research and Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Grenzacherstrasse 124, 4070, Basel, Switzerland.

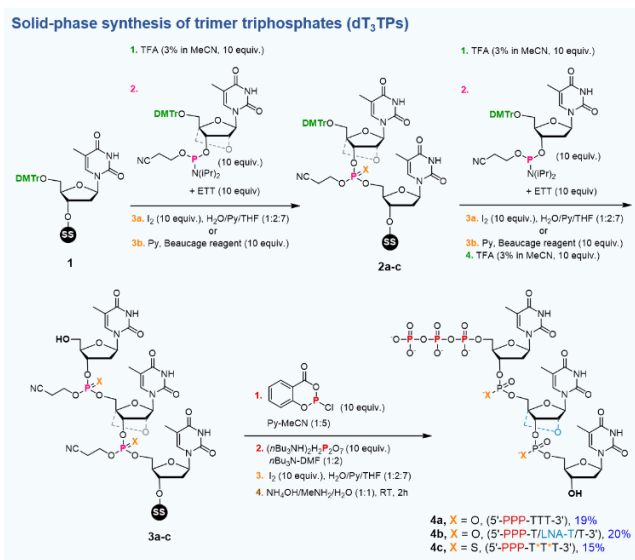
<sup>c</sup>Pharmaceutical Division, Synthetic Molecules Technical Development, Process Chemistry & Catalysis, F. Hoffmann-La Roche Ltd, 4070 Basel, Switzerland.

tag DNA and RNA.<sup>22-24</sup> Despite well-established protocols, most processes still rely on the stepwise incorporation of modified nucleotides by polymerases (Fig. 1).<sup>25</sup> Chemoenzymatic methods that would favor poly- rather than mono-nucleotide incorporation events would be particularly useful, especially for the production of long RNA and XNA sequences.<sup>26-28</sup>

from a process development viewpoint, truncated N-3 impurities originated from incomplete couplings, would be much easier to deplete in downstream purification steps – resulting in potential higher isolated yields and lower costs.

In order to evaluate whether DNA polymerases would accept polynucleotide 5'-triphosphates (dN<sub>3</sub>TPs), we started our study with the synthesis of trideoxythymidine triphosphates (dT<sub>3</sub>TPs) as model substrates. In order to produce larger amounts of dT<sub>3</sub>TPs, we designed and constructed an efficient manual column synthesis device (Fig. S11, ESI) that allowed us to proceed with higher loadings of solid support (> 50 μmole per column) which in turn yielded the expected products in sufficient (i.e. 10-15 mg) quantities. We next optimized the synthetic cycles for the preparation of trimer 5'-d(TTT)-3' **3a** starting from thymidine immobilized on solid support **1** (Fig. 2). Particularly, we minimized the excess of reagents and removed the capping steps without impairing the high coupling yields typically observed in phosphoramidite chemistry (>95% conversion).<sup>33</sup> Oxidation to P(V) was carried out using standard iodine in pyridine solution, while the sulfurization steps were performed with the Beaucage reagent.<sup>34</sup> After optimization of the conditions for manual, solid phase synthesis of trinucleotides, we carried out triphosphorylation on the solid support-bound trimer **3** by application of the Ludwig-Eckstein protocol.<sup>35</sup> Finally, global deprotection and removal from the solid support followed by HPLC purification resulted in trimer triphosphates **4a-c** in 15-20% isolated total yields (Fig. 2). With this general synthetic approach, we were able to obtain trimer triphosphates containing either natural (**4a**), or chemically modified sequences (locked nucleic acid (LNA)-containing **4b** and phosphorothioated **4c**).

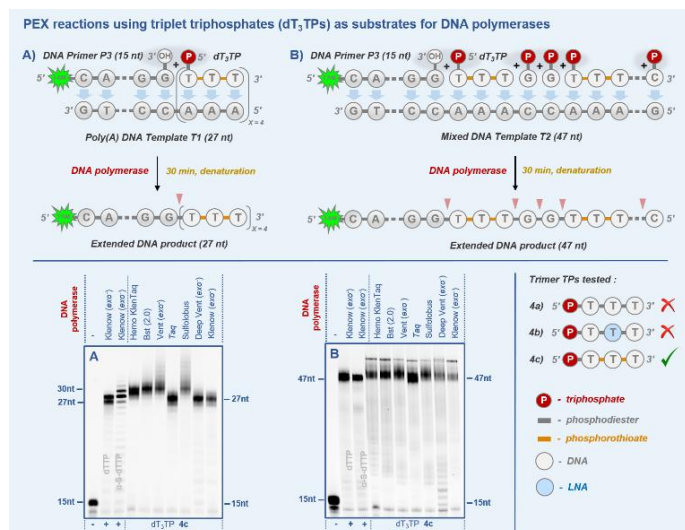
Next, having our model dT<sub>3</sub>TPs **4a-c** at hand, we evaluated their capacity at acting as substrates for various DNA polymerases in primer extension (PEX) reactions. Firstly, we assessed the substrate capacity of dT<sub>3</sub>TPs **4a-c** with the terminal deoxynucleotidyl transferase (TdT)<sup>36, 37</sup> and the Poly(U) Polymerase (PUP)<sup>26, 38</sup> which both incorporate nucleotides at the 3'-termini of ssDNA and RNA primers, respectively. Hence, we incubated dT<sub>3</sub>TPs **4a-c** along with 5'-FAM-labelled DNA primer **P1** (19 nt), 5'-FAM-labelled RNA primer **P2** (18 nt), (Fig. S12-3, ESI) along with these polymerases. Unfortunately, we did not observe any incorporation of the trinucleotides **4a-c** into DNA or RNA unlike control reactions with the mononucleoside triphosphates dTTP, UTP, and LNA-TTP.



**Fig. 2.** Solid-phase synthesis of trimer triphosphates (dT<sub>3</sub>TPs).

We then focused on template-dependent enzymatic DNA synthesis. Therefore, we evaluated the substrate acceptance of dT<sub>3</sub>TPs **4a-c** in PEX reactions using a 5'-FAM-labeled DNA primer **P3** (15 nt) on two DNA templates **T1** (27 nt) and **T2** (47 nt) in conjunction with seven different DNA polymerases (Fig. 3). Gel analysis (PAGE 20%) revealed that the products of the PEX reactions obtained with dT<sub>3</sub>TP **4a** bearing phosphodiester linkages on DNA template **T1** with a dA<sub>12</sub> tail consisted of primer extended by a single nucleotide (N+1 product) or a distribution of multiple (N+1 to N+3) products reminiscent of the pattern observed for TdT-mediated tailing reactions (Fig. S14A, ESI). These results suggested degradation of the starting dT<sub>3</sub>TP **4a** at the level of connecting phosphates most probably due to the esterase activity of DNA polymerases.<sup>31</sup> While such an outcome was expected with dT<sub>3</sub>TPs **4a**, we believed that the presence of an LNA building block would reduce hydrolysis by the phosphodiesterase activity of polymerases.<sup>39</sup> However, analysis of the PEX reactions carried out with trinucleotide **4b** revealed a similar trend as that observed with unmodified **4a** (Fig. S14B, ESI). Hence, we decided to replace standard phosphodiester bonds with phosphorothioates (PS) in order to strengthen the trinucleotide moieties against polymerase-mediated hydrolysis.<sup>40</sup> In order to evaluate the effect of PS bonds on the stability of the dT<sub>3</sub>TP, we performed PEX reactions of 5'-FAM-labeled DNA primer **P3** (15 nt) with dT<sub>3</sub>TP **4c** on the dA<sub>12</sub>-containing DNA template **T1** (27 nt) capable of accommodating up to four trimer sequences (Fig. 3A). PAGE analysis revealed the formation of extended oligonucleotide products with all of the seven DNA polymerases that were tested.

With the aim of further demonstrating the compatibility of dT<sub>3</sub>TP **4c** with polymerase-mediated DNA synthesis, we carried out an LC-MS analysis of the resulting PEX reaction products. To do so, we performed preparative scale PEX reactions with *Taq* and *Klenow* (*exo*<sup>+</sup>) DNA polymerases and further analyzed these by LC-MS (Tables S11, ESI). In parallel, we also ran positive control experiments using dTTP and instead of dT<sub>3</sub>TP **4c**.



**Fig. 3.** Primer extension reactions (30 minutes) using triplet triphosphates ( $dT_3TP$ s) **4a-c** (200  $\mu M$  final) as substrates for DNA polymerases. **(A)** PEX reactions with the  $dA_{12}$ -containing DNA template **T1**; **(B)** PEX reactions with the mixed DNA template **T2**.

LC-MS analysis of the product stemming from a large scale PEX reaction with dTTP showed that together with the expected (N+12, 27 nt) reaction product, (N+13/14/15) products were also formed which occur *via* non-templated addition or strand slippage on the homopolymeric template (Tables S11, ESI).<sup>41, 42</sup> On the other hand, we only observed the formation of the expected, fully extended N+12 (27 nt) product when triplet triphosphate **4c** was used in the reaction mixtures. Interestingly, we could not detect any product corresponding to shorter, truncated products suggesting the absence of hydrolysis of trinucleotide **4c** under these conditions. Encouraged by these results, we next sought to further evaluate the compatibility of trinucleotide **4c** with enzymatic synthesis of more complex sequences. Thus, we constructed a mixed DNA template **T2** (47 nt) consisting of mixtures of dG, dC, and dT interspaced with five  $dA_3$  repeats (Fig. 3B). PEX reactions were carried out with the same DNA primer **P3** and with the conditions used for the template containing a  $dA_{12}$  tail. Gel electrophoresis analysis revealed the formation of the fully extended products with all seven DNA polymerases. We also performed preparative scale reactions with *Taq* and Klenow (*exo*) DNA polymerases followed by LC-MS analysis, which confirmed the integrity of the formed product (Tables S12, ESI). Noteworthy, together with the desired products we also observed the partial formation of products with an additional dT (Klenow(*exo*)) or  $dT_3$  (*Taq*) overhang. These findings also explain the slightly different band mobilities observed with different polymerases in Fig. 3A and 3B. In addition, a similar observation was made with the positive control reaction carried out with natural dTTP where three to four nucleotides were added in a non-templated manner (Tables S12, ESI).<sup>41, 42</sup>

Having confirmed the integrity of the expected products, we next questioned the mechanism of product formation, in particular, we wanted to ascertain that synthesis proceeded *via* incorporation of trinucleotides rather than by partial hydrolysis followed by integration of smaller fragments. Hence, we carried out PEX reactions using Klenow (*exo*) on template **T1** with limiting

concentrations of  $dT_3TP$  **4c** (i.e. 10-50  $\mu M$ ), and short reaction times (from 1 to 15 min), without supplementing the reaction mixtures with unmodified dNTPs to contain the reaction to single triplet incorporations. Gel analysis (PAGE 20%) revealed the formation of N+6 (21 nt) product already at 10  $\mu M$  concentration of **4c** after 1 minute of reaction time. Nonetheless, together with the N+6 product we also observed a slight formation of other (namely N+5/N+7) products suggesting the partial degradation of trinucleotide triphosphate or already formed product by polymerase under low triphosphate concentration conditions (Fig. S15, ESI).

We then used higher concentrations of  $dT_3TP$  **4c** and found out that starting from 50  $\mu M$  after short reaction times (1-15 minutes) the full-length (N+12, 27 nt) product was formed with very small levels (<5%) of degradation (Fig. S15, ESI). Finally, we performed the same concentration and time experiments with  $dT_3TP$  **4c** on mixed DNA template **T2** where the first sequence on the template was  $dA_3$  followed by  $dG_2$ , expected to accommodate only one trinucleotide. Gel analysis (PAGE 20%) confirmed the formation of (N+3, 18 nt) product with a similar band shifts as for a positive control reaction and no signs of (N+1/2) distribution (Fig. S16, ESI). Analysis of these time course experiments reveals the predominant formation of the expected full-length products. However, under low triphosphate concentrations, some products corresponding to partial hydrolysis are formed. While additional optimization experiments will be needed, particularly with different sequence compositions on the trinucleotides, we believe that under higher concentrations of trinucleotide triphosphates (50 to 200  $\mu M$ ), the degradation reaction catalyzed by polymerases is very slow and the predominant mechanism is formation of phosphodiester linkages between primer and trinucleotide that is very fast process (additionally accelerated by pre-assembly of trinucleotides on the template). This observation is further confirmed by analytical ion exchange HPLC monitoring of the stability of **4c** under PEX-like reaction conditions (Fig. S17-10, ESI). Conversely, under lower (<50  $\mu M$ ) triphosphate concentrations and on homopolymeric templates, the phosphatase activity of polymerases competes with polymerization albeit to a lesser extent.

In conclusion, we have developed a convenient method for manual solid phase synthesis of natural and modified trinucleotide triphosphates. We have then demonstrated that  $dT_3TP$ s act as excellent substrates for several DNA polymerases *provided* that the internucleosidic linkages are stabilized with PSs moieties to prevent hydrolysis. Moreover, this approach has a great potential to be further extended to longer lengths of shortmers (3-10 nt). We believe that this approach could be useful for the preparation of long XNA sequences that are accessible only with engineered polymerases, for the incorporation of nucleotides that are not compatible with polymerases or with unstable triphosphates (such as phosphorodiamidate morpholino (PMO)<sup>43, 44</sup> or peptide nucleic acids (PNA)<sup>45</sup>), for the synthesis of oligonucleotides with stereo-controlled PS bonds,<sup>46</sup> and for the inclusion of multiple base-modifications in SELEX for the selection of chemically modified functional nucleic acids.<sup>17, 27</sup> The complexity of synthesizing all sequence variants ( $4^4 = 64$  possibilities) that might be required for

such practical applications can be partially alleviated by applying existing synthetic methods.<sup>47</sup>

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### Conflicts of interest

There are no conflicts to declare.

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