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RESEARCH ARTICLE

Mechanochemical synthesis of short DNA fragments

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Abstract: We demonstrate the first mechanochemical synthesis of DNA fragments by ball milling, enabling the synthesis of oligomers of controllable sequence and length using multi-step, one-pot reactions, without bulk solvent or the need to isolate intermediates. Mechanochemistry allowed for coupling of phosphoramidite monomers to the 5′-hydroxyl group of nucleosides, iodine/water oxidation of the resulting phosphite triester linkage, and removal of the 5′-dimethoxytrityl (DMTr) protecting group *in situ* in good yields (up to 60% over three steps) to produce DNA dimers in a one-pot manner. *H*-phosphonate chemistry under milling conditions enabled coupling and protection of the *H*-phosphonate linkage, as well as removal of the 5′-DMTr protecting group *in situ*, enabling a one-pot process with good yields (up to 65% over three steps, or ca. 87% per step). Sulfurization of the internucleotide linkage was possible using elemental sulfur (S_8) or sulfur transfer reagents, yielding the target DNA phosphorothioate dimers in good yield (up to 80% over two steps). This work opens the door to creation of solvent-free synthesis methodologies for DNA and RNA therapeutics.

Introduction

Oligonucleotides (ONs) have become an exciting new class of therapeutics with multiple already approved for treating a wide range of genetic diseases.[1] With increasing numbers of ON therapeutics nearing the market, the manufacturing process for ONs is assuming increased importance, with consideration of building block (monomer, dimer, trimers, etc.) availability, solvent use (waste management cost), product yield, purity, and scalability. The demand for synthetic ONs has reached an all-time high and will only continue to grow.[2]

Solid-phase ON synthesis is well established and takes advantage of well-developed solution-based chemistry using phosphoramidite building blocks on insoluble polymer supports[3] such as controlled pore glass (CPG) (**Figure S1**).[4] Many different studies have focused on the use of soluble ionic tags,[5] soluble polymer supports,[6] and block coupling[7] to address some of the challenges of solid-phase synthesis.[2, 8] Whereas this approach to ON targets is based on surface immobilization of growing chains, the chemistry itself is done through solution processes that are among the most solvent-demanding synthesis procedures, demanding an enormous amount of acetonitrile as solvent per kilogram of target, making the assembly of these valuable targets a major environmental challenge.

Mechanochemistry has recently emerged as a versatile approach for the synthesis of a wide range of molecular targets and materials, through grinding, milling or shearing without the need for bulk solvents or elevated temperatures.[9] Performed either by neat milling or promoted by a small amount of liquid phase (liquid-assisted grinding, LAG), mechanochemistry can provide access to chemical reactions that are not only rapid, scalable and void of bulk solvents, but also yield products, catalytic transformations and reaction selectivities that are difficult or not at all observable in solution.[10] The amount of liquid in LAG synthesis is expressed by η, the ratio of liquid volume to the weight of reactants, and lies between 0-2 μL/mg.[11] While mechanochemistry was shown to be applicable for the synthesis of nucleosides, nucleotides and related materials by several groups, including those of Vyle and of Roy, the area remains under-developed.[12] For instance, recent studies have focused on protecting group chemistry,[13] phosphitylations,[14] pyrophosphate formation,[15] and dinucleoside 5′,5′-polyphosphates.[16] Encouraged by this prior work, and the growing need for rapid, simple and efficient approach to synthetic ONs, we envisioned the use of mechanochemistry as a general platform to synthesize ON structures in a solvent-less environment. Herein, we report the first strategy for the ball millling synthesis of 3′-5′-linked di- and trinucleotides based on both phosphoramidite and *H*-phosphonate chemistry.

Results and Discussion

Phosphoramidite Chemistry. The first aim of the current study was to recreate the phosphoramidite synthesis cycle in a ball millling environment, with dinucleotides as targets. Thus, **1a-d** (2 equivalents) were coupled with 3′-*O*-*tert*-butyldimethylsilyl thymidine (**2**),[17] in the presence of 5-(ethylthio)-1*H*-tetrazole (ETT, 3 equivalents) by milling for 30 minutes in a steel or a Teflon® assembly, at a frequency of 30 Hz (**Scheme** 1, the mechanochemical conditions indicated by the symbol proposed by Rightmire and Hanusa).[18] The ^{31}P NMR spectrum of the crude product displayed two sets of signals at 140 ppm[19] corresponding to the expected diastereomeric phosphite triesters (**3a-d**), and another set of signals at ca. 8 ppm, assigned to the *H*-phosphonate by-product resulting from the hydrolysis of excess **1a-d** (**Figure S3**). Formation of the *H*-phosphonate by-product was reduced by drying the reaction vessels as well as reagents under high vacuum prior to performing the reaction. After purification, **3a-d** were produced in good yields (63-80%) and were identical to standards prepared in solution (see Supplemental Information).

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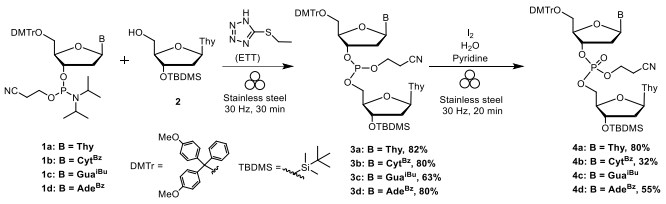
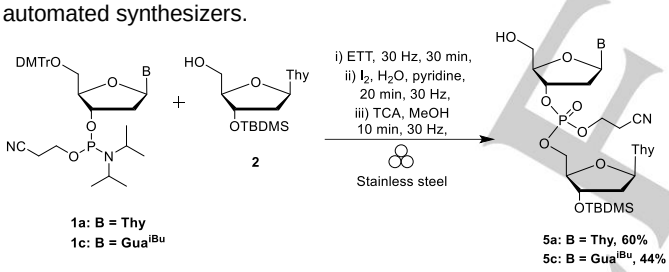


Scheme 1. Mechanochemical synthesis of DNA dimers via phosphoramidite chemistry.

The next step in the cycle is the oxidation reaction required to convert the reactive phosphite triester P(III) species to the more stable phosphate triester P(V) species.[4c, 19a] While this is accomplished in standard solid-phase synthesis through I_2 in aqueous pyridine/THF solution, we found that the transformation was readily achieved by one-pot mechanochemistry from **1a-d** and **2**, by adding solid iodine, water and pyridine (LAG, $\eta \approx 0.1$ µL/mg) into the milled reaction mixture and milling for an additional 20 minutes (**Scheme 1**). As a result, compounds **4a, 4b** and **4d** were produced with variable yields (32-80%). In the case of dGpT **4c**, the dimer could not be isolated, partly due to premature loss of the 5′-*O*-dimethoxytrityl (DMTr) group during the milling and purification steps. Switching the oxidant from I_2 to *meta*-chloroperbenzoic acid (mCPBA),[20] however, provided **4c** in 36% yield, again observing partial removal of the DMTr group from this dimer during its isolation.

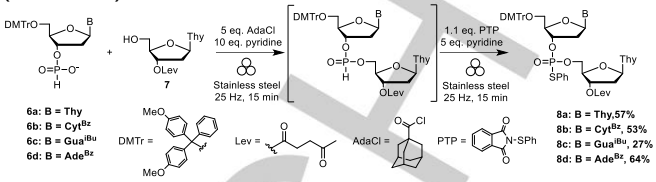
To deal with the challenge of premature detritylation of **4c**, we attempted to perform coupling, oxidation and detritylation steps in a one-pot sequence without isolation of intermediates (**Scheme 2**), effectively recreating the synthesis cycle currently used on automated synthesizers.



Scheme 2. One-pot, three-step mechanochemical synthesis of DNA dimers via phosphoramidite chemistry.

Indeed, coupling of **1a** with **2** (30 min, 30 Hz), followed by oxidation with I_2 crystals and wet pyridine (20 min, 30 Hz, $\eta = 0.07$ µL/mg), and detritylation with solid trichloroacetic acid (TCA) and methanol (MeOH, 10 min, 30 Hz, $\eta = 0.06$ µL/mg) produced **5a** in 60% yield (over three steps; **Scheme 2**). The presence of a small amount of MeOH as a LAG additive was necessary, since TCA alone failed to cleave the DMTr group. We hypothesize that LAG promotes 5′-*O* protonation, as well as releases and quenches the trityl cation, driving the detritylation reaction to completion. When this three-step procedure was applied to the in-situ synthesis of dGpT, **5c** was synthesized in the same manner in 44% yield (three steps from **1c**; **Scheme 2**).

***H*-Phosphonate Chemistry.** While phosphoramidite chemistry is the method of choice for typical solid-phase oligonucleotide synthesis, *H*-phosphonate chemistry has also been shown to be an effective strategy.[21] Nucleotide *H*-phosphonate couplings are rapid and are most often performed in pyridine in the presence of a carbonyl chloride or chlorophosphate activator.[22] We first attempted to mechanochemically couple protected *H*-phosphonate **6a** with **7** using pivaloyl chloride (PivCl) as an activator in the presence of stoichiometric amounts of pyridine (**Scheme 3**).



Scheme 3. One-pot, two-step mechanochemical synthesis of fully protected DNA dimers via *H*-phosphonate chemistry

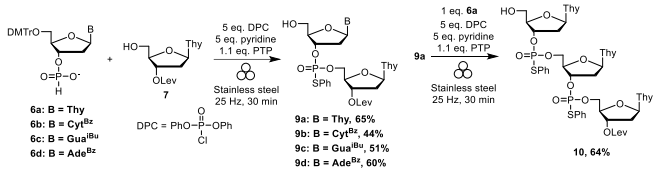
Despite screening a wide range of conditions, including reactant ratio, time, and milling frequency, no formation of *H*-phosphonate diesters was observed according to ^{31}P NMR (**Figure S2a and b**).[22a] However, using adamantoyl chloride (AdaCl) as an activator, upon 15 minutes milling of equimolar amounts of **6a** and **7** in the presence of 5 equivalents of AdaCl and 10 equivalents of pyridine ($\eta = 0.39$ µL/mg), we observed complete consumption of **6a** and formation of two peaks around 7-9 ppm in the ^{31}P NMR spectrum, corresponding to the two diastereomers of the *H*-phosphonate diester (**Figure S2c**). However, the product was difficult to isolate due to low stability of *H*-phosphonate diesters under basic conditions.[23]

For this reason, we turned to a modified *H*-phosphonate approach, using thiophosphoric esters as protecting groups for the linkage.[22d, 24] From our initial studies, we knew that *H*-phosphonate diesters could be prepared by ball milling in 15 minutes. Gratifyingly, upon subsequent addition of the sulfur-transfer reagent *N*-(phenylthio)phthalimide (PTP) and pyridine ($\eta = 0.34$ µL/mg) to the crude reaction mixture, the protected dimers **8a-d** (**Scheme 3**) were obtained in 15 minutes under the same milling conditions. This was evidenced by appearance of two new peaks around 24 ppm in the ^{31}P-NMR spectrum (**Figure S2d**), corresponding to the thiophosphoric ester, and the disappearance of the peaks of the *H*-phosphonate diester. Despite conducting these reactions under basic conditions, the previously mentioned hydrolysis was avoided by using dry pyridine and drying reaction vessels and reagents under high vacuum prior to performing the reactions. These compounds (**8a-d**) were much more stable, and easily isolable by simple column chromatography in modest yields (27-65%; or, average yield of 52-80% per step; entries 1-4, **Table S1**). Similarly, to the phosphoramidite chemistry, we observed premature detritylation and problems in isolation for dGpT (**8c**) (entry 3, **Table S1**).

Screening of various activators for the coupling reaction revealed that the coupling and sulfurization steps could be carried out in one-pot simultaneously, using diphenyl phosphoryl chloride (DPC) as the activator in place of AdaCl.[22d] Additionally, by varying the amount of base used it was also possible to remove the 5′-DMTr protecting group during the same reaction, yielding a strategy to synthesize partially protected dimers **9a-d** in one step (**Scheme 4**). We were similarly able to isolate these compounds by column chromatography in 44-65% yields, corresponding to 76-87% per step (entries 5-8, **Table S1**).

These two coupling strategies give us solventless routes to synthetically useful DNA dimer building blocks simply based on the choice of activator, equivalents of base, and the order of

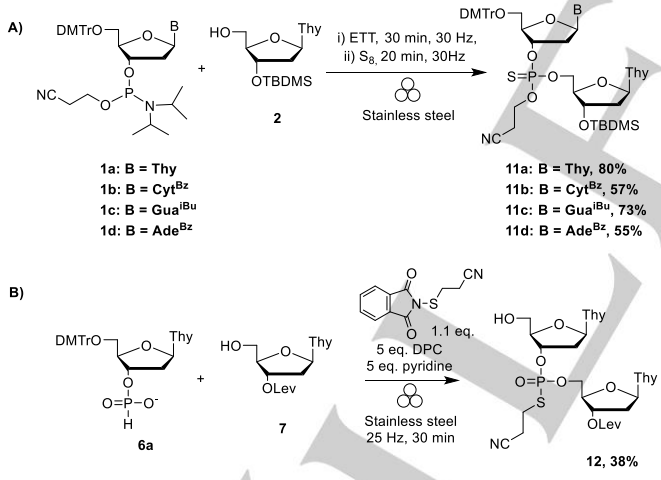
addition of reagents. It was also possible to prepare trimer **10** by coupling **9a** with monomer **6a** (**Scheme 4**). Trimer **10** was isolated by column chromatography in 64% yield, corresponding to an average yield of 86.2% per step.



Scheme 4. One-pot, single-step mechanochemical synthesis of partially-protected DNA dimers and trimer via *H*-phosphonate chemistry

Phosphorothioate formation. One of the most widely-used phosphate modifications in oligonucleotide therapeutics is the phosphorothioate (PS) linkage.[25] PS linkages alleviate a major challenge associated with using oligonucleotides *in vivo* by providing resistance against a variety of extra- and intracellular nucleases.

To produce the desired PS backbone, two strategies were pursued. First, **1a-d** were coupled to **2** (30 min, 30 Hz), and the resulting phosphite triester intermediates were treated *in situ* with yellow sulfur powder (20 min, 30 Hz) to give **11a-d** (55-80% over two steps; **Scheme 5A**). Compound **11a** was identical to a sample prepared by reacting phosphite triester **3a** directly with S_8 at 30 Hz for 5 min. In this case, the PS-TpT dimer was obtained as a mixture of PS-TpT (triester, **11a**) and PS-TpT (diester) in 8:2 ratio, respectively; the diester most likely results from the premature removal of the beta-cyanoethyl protecting group (^{31}P-NMR, **Figures S30 and S31**).



Scheme 5. Mechanochemical synthesis of phosphorothioate dimers *via* phosphoramidite and *H*-phosphonate chemistries.

Secondly, equimolar amounts of **6a** and **7** in the presence of five equivalents of diphenyl phosphoryl chloride (η = 0.8 µL/mg), *N*-[2-(cyanoethyl)thio]phthalimide, and pyridine (η = 0.3 µL/mg) were allowed to react at 25 Hz for 30 minutes (**Scheme 5B**). Notably, all three reactions: coupling, sulfurization, and detritylation, readily occur in one pot under these conditions. After work up, the crude product was precipitated and purified by column chromatography to afford **12** as a pair of diastereoisomers (^{31}P NMR δ_P in $CDCl_3$, 26.8, 27.3 ppm; 38% overall yield, Figure **S33**).

Conclusion

In summary, we have demonstrated the ability to prepare synthetically useful DNA building blocks of controlled length and sequence *via* ball milling mechanochemistry, in the absence of bulk solvents. These dimers and trimers can be applied to traditional solid-phase synthesis of oligonucleotides in order to reduce the total number of synthesis cycles. This proof-of-principle study not only demonstrates that complex oligonucleotide structures can be assembled in a controlled way under ball milling conditions, but also that such structures readily form by multi-step reactions in one-pot fashion. Whereas the presented work has focused on the synthesis of dimers and trimers, the herein presented strategies are generally applicable and we are confident that the synthesis of longer sequences *via* block coupling under mechanochemical conditions, combined with ionic-tags to selectively precipitate intermediate products should also be possible, eliminating the use of chromatography for purifications of products.[5] Furthermore, we see no reason why the herein presented reaction sequences should not be compatible with industrial-scale mills and/or continuous mechanochemical manufacture via twin screw extrusion,[26] as has recently been done in the context of small organic molecule targets and metal-organic frameworks.[27] Finally, we note that the ability to obtain functional oligonucleotide structures without a bulk solvent might suggest the potential role of solid-state transformations in the early stages of chemical evolution, as recently highlighted by Trapp and co-workers,[28] and Hernández and co-workers.[29]

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Keywords: Mechanochemistry • DNA • Phosphoramidite • *H*-Phosphonate • Green Chemistry

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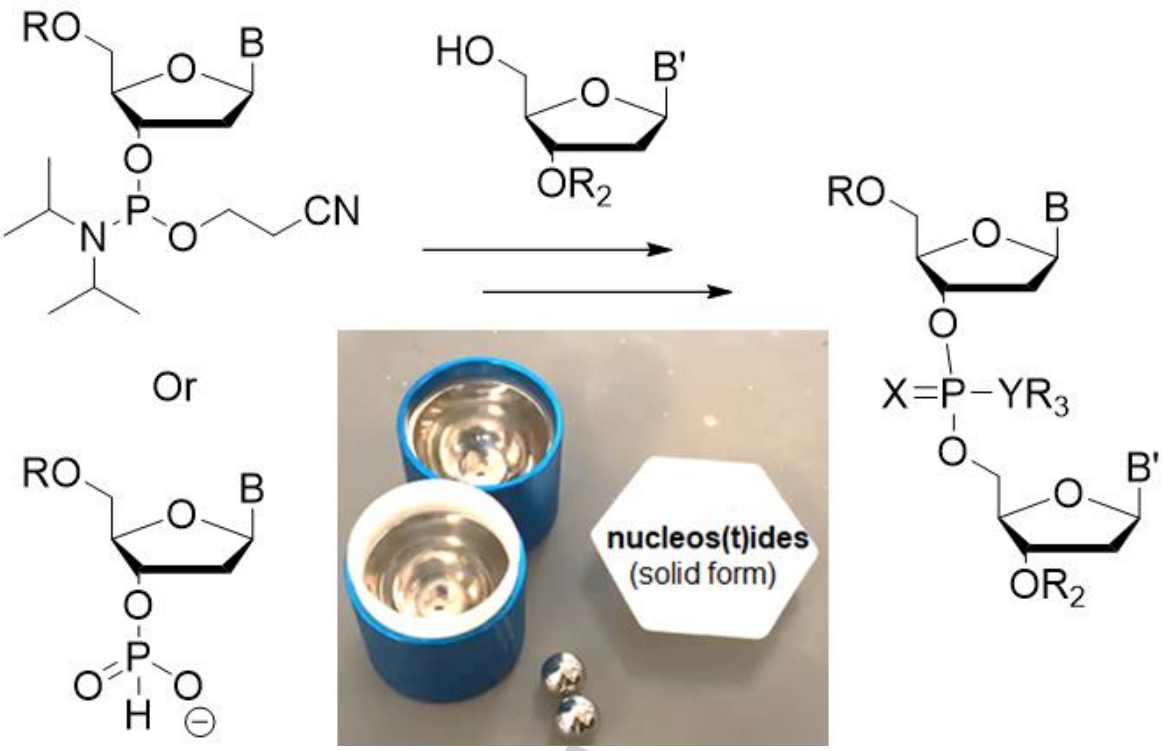

Shake to make DNA. Utilizing mechanochemistry to make DNA fragments using both phosphoramidite and *H*-phosphonate chemistries under minimal solvent conditions.

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