Title: Mechanochemical synthesis of short DNA fragments

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Abstract:
We report the general and rapid synthesis of short DNA fragments of controllable sequence and length using multi-step, one-pot mechanochemical reactions, without bulk solvent or the need to isolate intermediates. We demonstrate, for the first time, the multi-step ball milling synthesis of DNA dimers and trimers via phosphoramidite and H-phosphonate chemistries. The use of 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′-dimethoxy (DMTr) protecting group *in situ* in good yields (up to 60% over three steps) to produce DNA dimers in one-pot manner. Sulfurization of phosphite triester linkages was possible using elemental sulfur yielding the corresponding phosphorothioate DNA dimers in good yield (up to 80% over two steps). By using H-phosphonate chemistry under milling conditions, it was possible to couple, protect the H-phosphonate linkage, and remove the 5′-DMTr protecting group *in situ*, enabling a one-pot process with good yields comparable to existing solvent-based procedures (up to 65% over three steps, or ca. 87% per step). This work opens the door to creation of solvent-free methodologies for the assembly of complex DNA and RNA therapeutics.

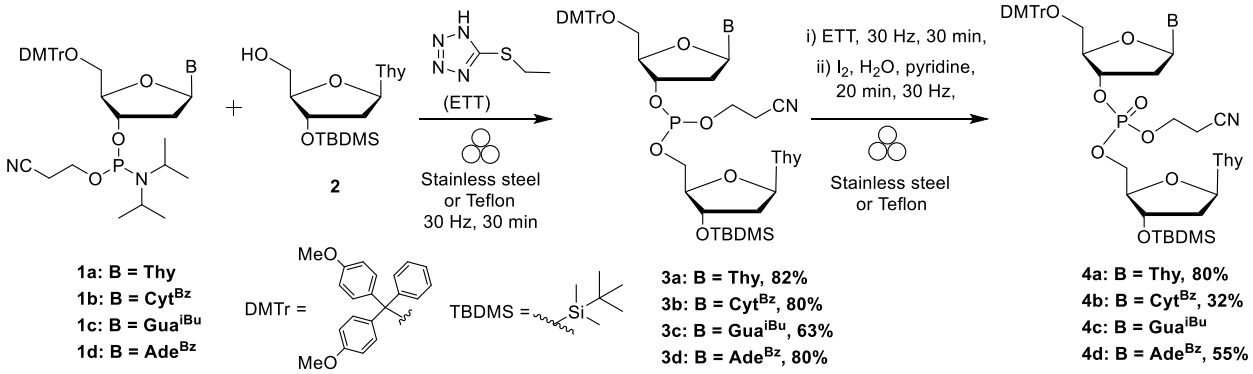
Main text:
Oligonucleotides (ONs) have become an exciting new class of therapeutics with multiple already approved for treating a wide range of genetic diseases.[1-3] With increasing numbers of ON therapeutics nearing the market, the manufacturing process for ONs is assuming increased importance, with 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.[4]
Solid-phase ON synthesis is well established and takes advantage of well-developed solution-based chemistry using phosphoramidite building blocks on insoluble polymer supports[5] such as controlled pore glass (CPG) (**Figure S1**).[6-9] Many different studies have focused on the use of soluble ionic tags,[10, 11] soluble polymer supports,[12, 13] and block coupling[14] to address some of the challenges of solid-phase synthesis.[4, 15] 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 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 mechanical agitation, in the form of grinding, milling or shearing, without the need for bulk solvents or elevated temperatures.[16-24] Performed either by neat milling or promoted by a small amount of liquid phase in liquid-assisted grinding (LAG), mechanochemistry was shown to provide access to chemical reactions that are not only rapid, scalable and void of bulk solvents, but can also lead to products, catalytic transformations and reaction selectivities that are difficult or not at all observable in solution.[25-29] The amount of

liquid in mechanochemical LAG syntheses is expressed through η, the ratio of liquid volume to the weight of reactants, and lies in the range of 0-2 μL/mg.[30] Importantly, 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 relatively under-developed.[31, 32] Specifically, while recent studies have focused on protecting group chemistry,[33] phosphitylations,[34, 35] and pyrophosphate formation,[36, 37] Roy and co-workers have recently demonstrated the synthesis of dinucleoside 5′,5′-polyphosphates via phosphorimidazolide intermediates.[38] 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.

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.

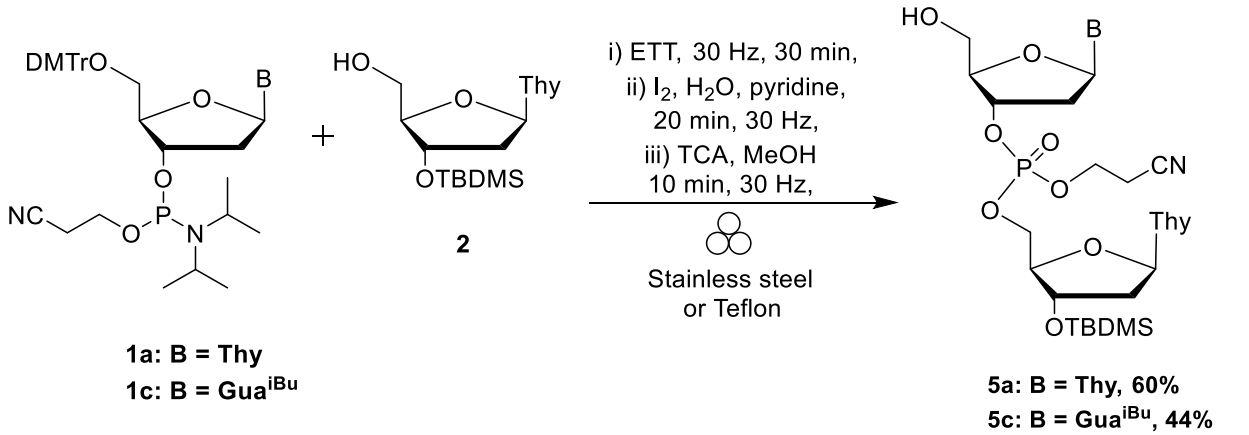


Scheme 1. Mechanochemical synthesis of DNA dimers via phosphoramidite chemistry

Thus, **1a-d** (2 equivalents) were coupled with 3′-*O*-*tert*-butyldimethylsilyl thymidine (**2**),[39] 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 three-circle symbol proposed by Rightmire and Hanusa).[40] The ^{31}P NMR spectrum of the crude product displayed two sets of signals at 140 ppm[41, 42] 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**). After purification, **3a-d** were produced in good yields (63-80%), and were identical to standards prepared in solution (see Supplemental Information).

The next step in the cycle is the oxidation reaction is required to convert the reactive phosphite triester P(III) species to the more stable phosphate triester P(V) species.[8, 41] While this is accomplished in standard solid-phase synthesis through I_2 in aqueous pyridine/THF solution, we found that the transformation is 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),[43] however, provided **4c** in 36% yield, again observing partial removal of the DMTr group from this dimer during its isolation.

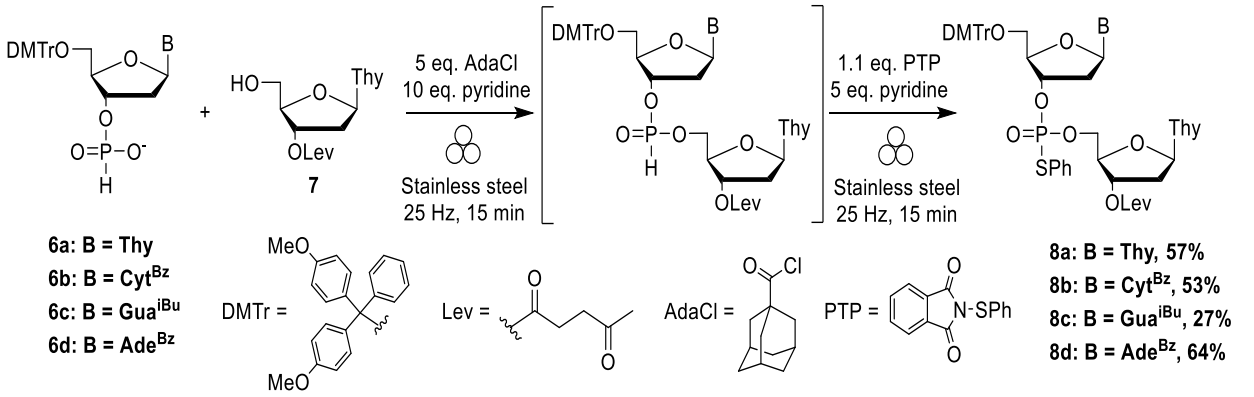


Scheme 2. One-pot, three-step mechanochemical synthesis of DNA dimers via phosphoramidite chemistry

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. Indeed, coupling of **1a** with **2** (30 min, 30 Hz), followed by oxidation with I_2 crystals and wet pyridine (20 min, 30 Hz, η = 0.07 μL/mg), and detritylation with solid trichloroacetic acid (TCA) and methanol (MeOH, 10 min, 30 Hz, η = 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 promoted 5′-*O* protonation, as well as release and quenching of the trityl cation, driving the detritylation reaction to completion. When this three-step procedure was applied to the synthesis of dGpT, **5c** was synthesized in the same manner in 44% yield (three steps from **1c**; **Scheme 2**).

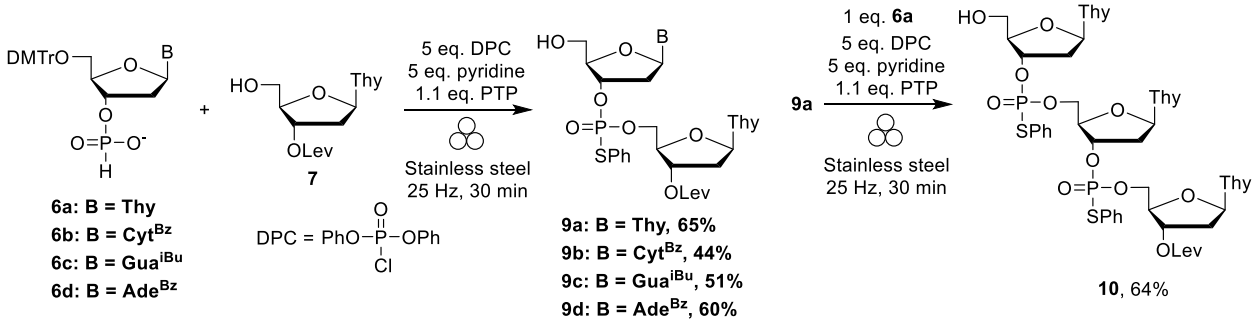
H-phosphonate Chemistry.

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.[44, 45] Nucleotide H-phosphonate couplings are rapid and are most often performed in pyridine in the presence of a carbonyl chloride or chlorophosphate activator.[46-49] 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**). 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**).[46] 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 (η = 0.39 μL/mg), we observed in the crude ^{31}P NMR spectrum complete consumption of **6a** and formation of two new peaks around 7-9 ppm, 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.[50, 51]



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

For this reason, we turned to a modified H-phosphonate approach, using thiophosphoric esters as protecting groups for the linkage.[49, 52] 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 (η = 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. 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. These compounds 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 11-14, **Table S1**). Similarly to the phosphoramidite chemistry, we observed premature detritylation and problems in isolation for dGpT (**8c**) (entry 13, **Table S1**).



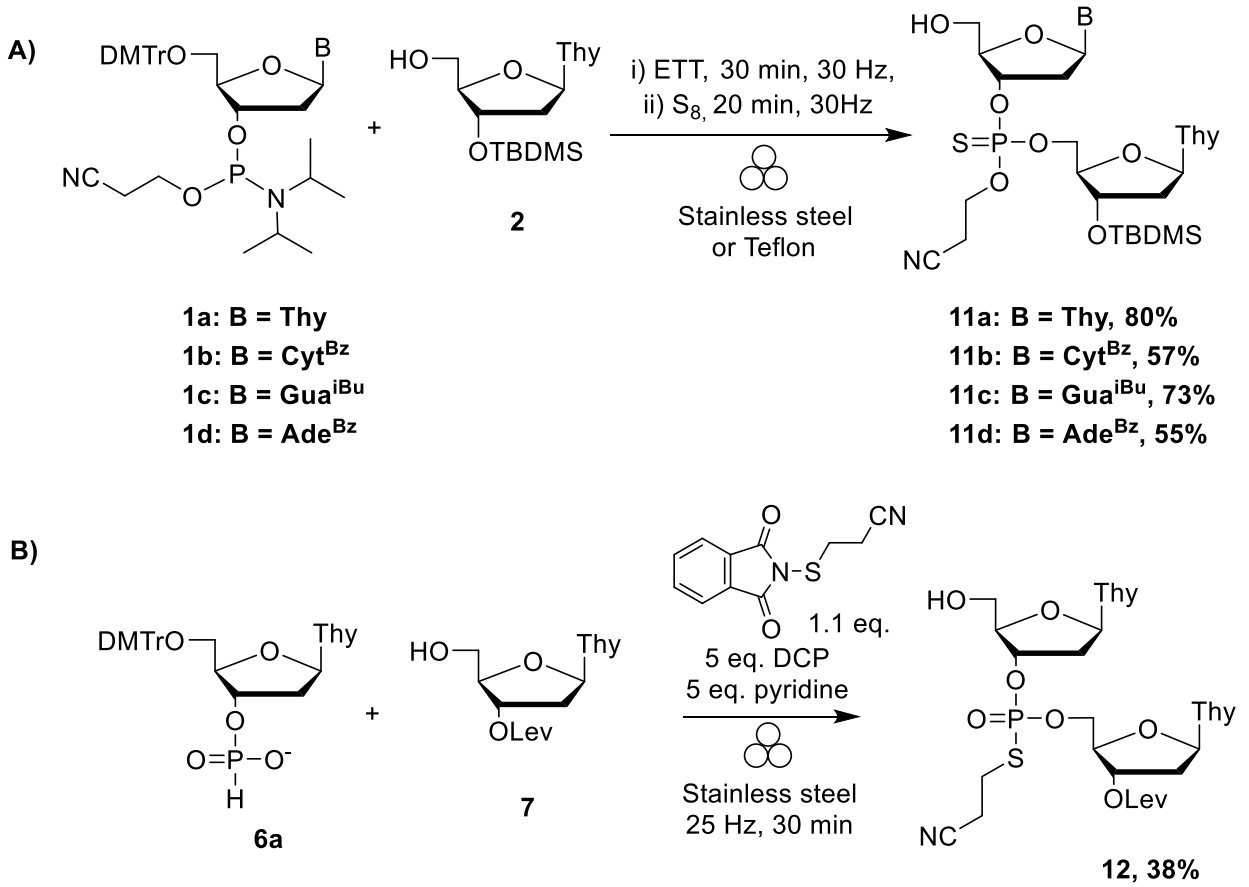
Scheme 4. One-pot, single-step synthesis of partially-protected DNA dimers and trimer via H-phosphonate chemistry

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.

Phosphorothioate formation.

One of the most widely-used phosphate modifications in oligonucleotide therapeutics is the phosphorothioate (PS) linkage.[53-55] PS linkages alleviate a major challenge associated with using

oligonucleotides *in vivo* by providing resistance against a variety of extra- and intracellular nucleases.



Scheme 5. Synthesis phosphorothioate dimers via phosphoramidite and H-phosphonate chemistries

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 S28 and S29**).
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**).

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. 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.[10, 11] Furthermore, we see no reason why the herein presented reaction sequences should not be compatible with industrial-scale roller mills and/or continuous mechanochemical manufacture via twin screw extrusion,[56] as has recently been done in the context of small organic molecule targets and metal-organic frameworks.[57, 58] 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[59], and Hernández and co-workers[60].

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