An Improved P(V) Oligonucleotide Synthesis Platform

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ABSTRACT: Three critical advances in simplifying the adoption of P(V)-based stereopure, phosphorothioate-containing oligonucleotide synthesis are reported. A more inexpensive phosphorus-sulfur incorporation reagent (Ψ^{Br}) is introduced, a robust linker system was developed, and a systematic study of common nucleobase protecting groups performed to significantly reduce the barrier to adoption of this technology.

Oligonucleotide-based medicines are an exciting and proven therapeutic modality whereby the production of specific proteins can be controlled through selective binding to mRNA using Watson-Crick-Franklin base pairing.[1-3] In principle, this approach can tackle the root causes of disease, offer prolonged therapeutic benefits, and be effective against seemingly intractable indications. For example, nusinersen (Spinraza), the first oligonucleotide FDA-approved drug for the treatment of spinal muscular atrophy, consists of 18 nucleosides linked by phosphorothioate (PS) bonds[4], enhancing its metabolic stability and cellular uptake (known as the "thio-effect").[4] Now considered an essential component of therapeutic oligonucleotides, the PS modification is present in nearly every FDA-approved oligonucleotide therapeutic.[5] Through traditional solid-phase oligonucleotide synthesis (SPOS), PS linkages are introduced with uncontrolled stereochemistry, creating 2^n isomers (where n is the number of PS linkages), often resulting in mixtures of over 100,000 diastereoisomers.[6-9]

This long-standing synthetic challenge in the field was the inspiration to develop a new approach to oligonucleotide synthesis using the P(V)-oxidation state featuring exquisite control of stereochemistry. In contrast to widely used P(III)-based protocols (Figure 1A), P(V) reduces the step count per synthesis cycle and provides complete stereocontrol rather than stereorandom or stereoenriched products. Although initial findings in this area were promising, the developed synthesis cycle suffered from the use of an expensive reagent, an unstable linker system, and bespoke nucleobase protecting groups (Figure 1B). This letter describes several improvements to this technique that ameliorate those shortcomings thus paving the way to more widespread adoption of the P(V)-platform for the discovery and development of therapeutic oligonucleotides (Figure 1C).

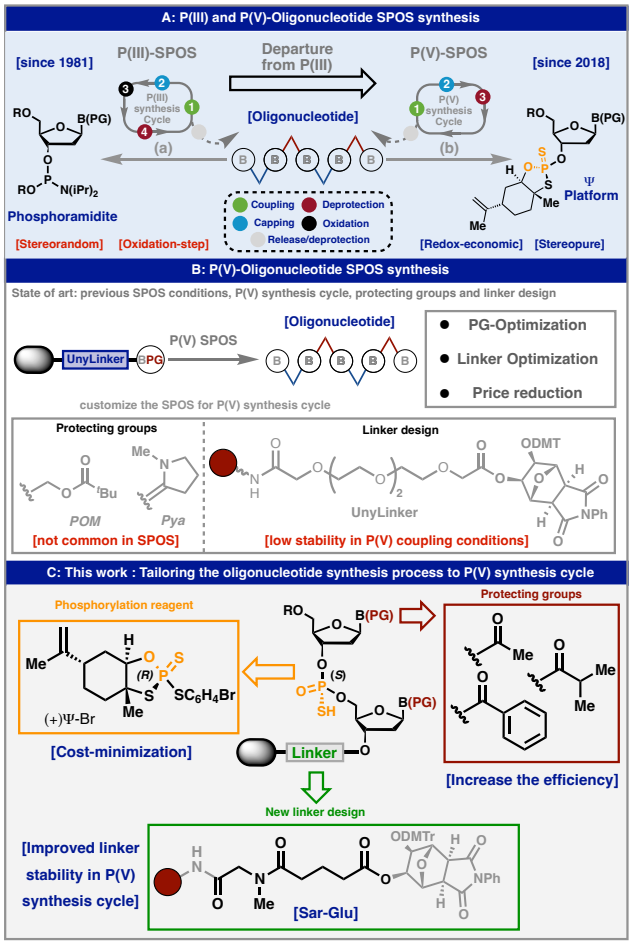

Figure 1. (a) Comparing P(III)- and P(V)-based oligonucleotide synthesis; (b) P(V)-Oligonucleotide SPOS cycle; (c) Tailoring the oligonucleotide synthesis process to P(V) synthesis cycle.

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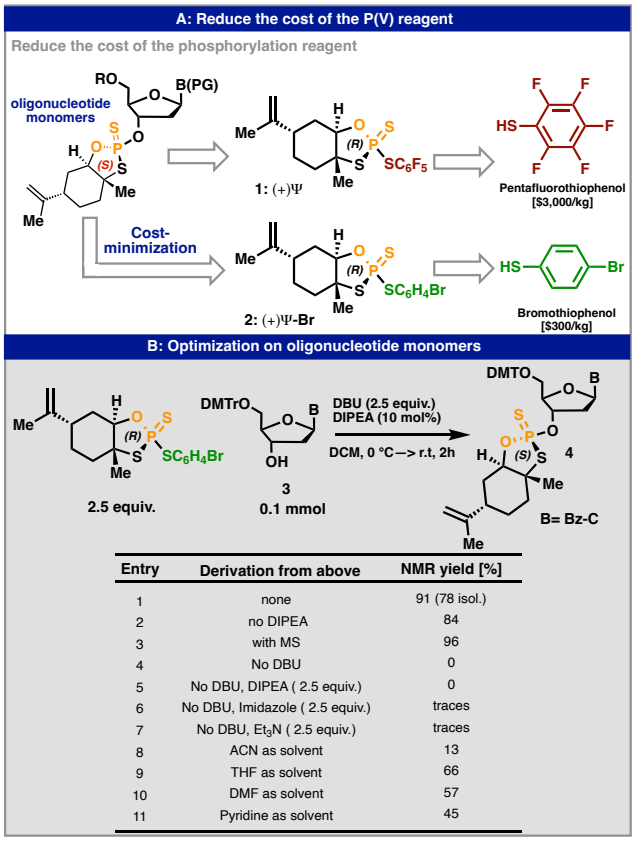
Nature's approach of creating oligonucleotides utilizes the P(V) rather than the P(III) oxidation state to form the phosphate linkages. Although early laboratory techniques for forming these P–O bonds also employed P(V), the approach was largely abandoned in the 1970s in favor of the superior coupling efficiency provided by P(III)-based reagents. It wasn't until nearly a decade later that Beaucage and Caruthers (1981) enhanced the coupling efficiency of P(III) reagents, leading to the development of what is now known as "Phosphoramidite" chemistry that can be summarized in 4 synthetic steps (1) coupling, (2) capping, (3) oxidation and (4) deprotection (Figure 1A).[10-12] In 2018, the introduction of the Phosphorus-Sulfur Incorporation (PSI, Ψ) platform simplified the control of stereochemistry in oligonucleotides, and reduced the SPOS cycle to 3 steps. The platform quickly garnered attention from the scientific community and gained popularity in the oligonucleotide and medicinal chemistry fields.[13-22] this innovation has since enabled access to stereodefined CDNs, nucleotides, and phosphonates.[23-28]



Entry	Derivation from above	NMR yield [%]
1	none	91 (78 isol.)
2	no DIPEA	84
3	with MS	96
4	No DBU	0
5	No DBU, DIPEA (2.5 equiv.)	0
6	No DBU, Imidazole (2.5 equiv.)	traces
7	No DBU, Et_3N (2.5 equiv.)	traces
8	ACN as solvent	13
9	THF as solvent	66
10	DMF as solvent	57
11	Pyridine as solvent	45

Figure 2. (a) Bromothiophenol as an alternative, less expensive leaving group in place of pentafluorothiophenol; (b) Monomer loading optimization.

In 2021, the Ψ-platform was expanded to install a wide variety of phosphate linkages into oligonucleotides.[24] This approach can install stereodefined and racemic phosphorothioates (PS) as well as native phosphodiester (PO) and phosphorodithioate (PS_2) linkages into oligonucleotides utilizing an automated oligonucleotide synthesizer albeit with non-canonical base protecting groups [Pivaloyloxymethyl (Pom) and 1-methylpyrrolidin-2-imine (Pya)] and a modified linker support to improve stability towards DBU(Figure 1B), none of which are commercially available. It was found that the P(V) platform resulted in lower isolated yields when using a sequence- and linkage-agnostic protocol. In direct comparison, the P(III) synthesized constructs were generally produced with double the yield. This is not surprising as P(III)- methods have undergone decades of evolution and optimization. Indeed, commercial oligonucleotide synthesizers are already tailored to the P(III) platform. In this context, our efforts focused on optimizing three key components of the P(V) oligonucleotide platform whilst preserving all the advantages of the Ψ-platform: nucleobase protecting group chemistry, linker support optimization, and reducing the cost of the phosphorylation reagent, (Figure 1C).

First, attention was focused on the P(V) reagent itself, due to the high price and limited availability of Pentafluorothiophenol ($3,000/kg, Figure 2A). Fortunately, 4-bromothiophenol ($300/kg) was identified as a suitable alternative that is much easier to source on large scale and that ultimately reduced the cost of the leaving group by ten-fold. More importantly the P(V) reagent derived from this leaving group (Ψ^{Br}), was found to be competent in the preparation of nucleoside monomers (Figure 2A.), while maintaining the same predictable and reproducible stereochemical outcome as the previously published PSI reagents (see SI for x-ray crystal structure of Ψ^{Br}). A series of conditions were evaluated to understand the loading of (Ψ^{Br}) onto nucleosides to afford P(V) monomers (Figure 2B). For this study, Benzoyl protected deoxycytidine (**Bz-dC**) was used as the model substrate. As expected, it was found that DBU plays a crucial role and cannot be replaced by other common bases such as DIPEA, imidazole, or Et_3N (Figure 2B, Entries 4-7). Various solvents were tested (ACN, THF, DMF and Pyridine, entries 8-11), revealing that DCM provides the best yield (Entry 1). Additionally, the use of molecular sieves slightly increased the yield; however, azeotropic drying of the starting materials with THF three times achieved a similar effect. Ultimately, the use of 10 mol% DIPEA along with DBU (2.5 equiv) proved ideal.

With these optimized conditions established, the performance of differentially protected nucleoside P(V) monomers in SPOS (Figure 3A) was evaluated. To do this, a set of PSI monomers were prepared using the (+) and (–)-PSI^{Br} reagents. Starting with protected 5'-O-DMTr-Nucleosides (A, C, T and G). Products (**4**-**10**) were obtained in 39–78% yields with perfect diastereoselectivities, on scales ranging from 0.1 to 2 mmol.

During the initial work on the P(V), it was found that the standard succinate-based CPG linker (**12**) was unstable to DBU. Presumably, the amide N-H bond is deprotonated by DBU followed by cyclization onto the ester, cleaving the UnyLinker and prematurely liberating the oligonucleotide from the solid-support. Based on previous reports on the instability of succinate linkers to DBU, more base stable linkers were pursued.[29] Strategies evaluated included blocking the free N-H (N-Me linker **11**) and extending the distance between the amide (linkers **13**, **14**) and the resin. This set of CPG linkers was then subjected to concentrations of DBU to mimic that encountered in oligonucleotide couplings steps and the % DMTr remaining was measured by

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absorbance (Figure 3B). As previously reported, the commercial, succinate-based CPG (**12**) was rapidly cleaved with no discernable DMTr signal after several cycles. The Q-linker (**13**) also demonstrated poor stability with only 48% remaining. It was found that a *Sar-Glu*-based linker (**11**) exhibited the most favorable properties showing a 94% recovery and stability after 120 coupling cycles. The *PEG₄*-based linker (**14**) was also found to be relativelystable under the same conditions with a recovery of almost 80% after 120 cycles. To verify that linker (**11**) was amenable to SPOS and not just incubation with DBU, a 1µmol P(III) control synthesis between linker (**11**) and the commercially available succinate-based CPG (**12**) (see SI) was performed. Yields were comparable, 28% for (**11**) and 25.4% for (**12**), with no major differences in detected impurities.

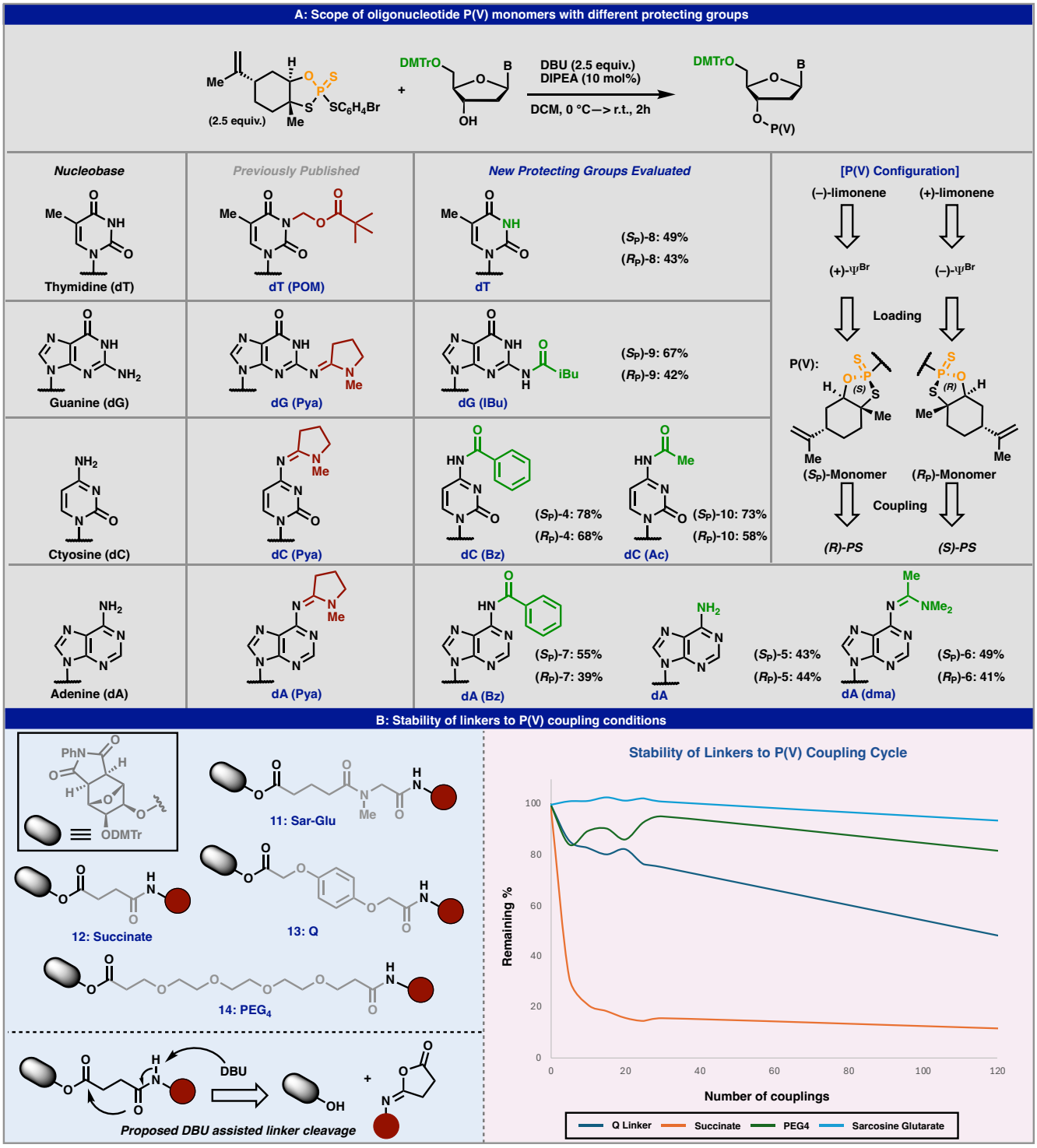

Figure 3. (a)Scope of oligonucleotide P(V) monomers with different protecting groups; (b) Stability of candidate linkers to P(V) coupling conditions. Linker decomposition was monitored via absorbance at A_{495}.

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With an improved set of P(V) reagents (+ and - Ψ^{Br}), a process for preparing the P(V)-monomers and an SPOS-compatible linker (with more resistance to DBU) in hand, attention turned to evaluating the performance of the differentially base protected monomers in SPOS.

Preliminary protecting group compatibility tests were performed on a pentamer alternating each individual test monomer with standard POM-dT-PSI (Figure 4A), followed by a mixed base sequence. For simplicity sake, this was performed on both isomers of the oligonucleotide but only the R-isomer is shown (see SI for S isomer). Each synthesized oligonucleotide was cartridge purified, followed by LC/MS analysis to determine if full-length product was observed

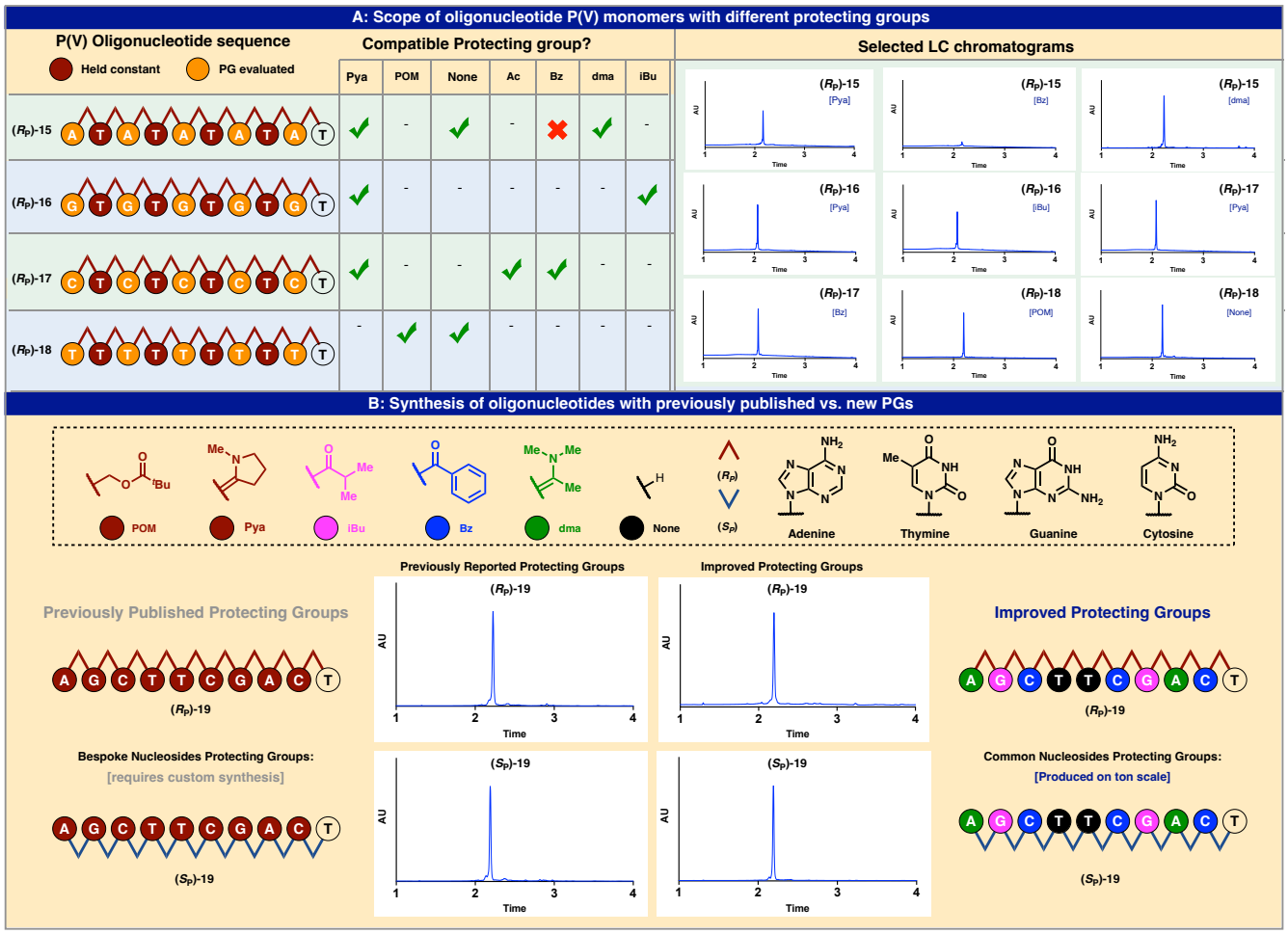

Figure 4. (A) Synthesis of model oligonucleotides using differentially nucleobase protected PSI monomers; (B) Full length, mixed-base oligonucleotide synthesis comparing previously published protecting groups with the improved set.

A systematic screening of adenosine protecting groups via **(R_P)-15** revealed that Pya, dimethylacetamidine (dma), and the non-protected (none) base are compatible with solid-phase oligonucleotide synthesis (SPOS) with similar yields. In contrast, Benzoyl (Bz)-protecting groups were found to exhibit lower compatibility with P(V) SPOS synthesis. (Figure 4A). The instability of Bz protected adenosine monomers to DBU has also been reported recently.[20]

For the guanosine base, by synthesizing **(R_P)-16** it was found that the isobutyrl (iBu) protecting group is compatible with P(V) conditions. This offers the advantage of being a commercially available nucleoside and a more cost-effective starting material (DMTr-G-iBu is [$2/g]) compared to the Pya protecting group, which is not commercially available. Through a similar process, it was found that cytosine monomers with Acetyl (Ac) and Bz protecting groups can both be utilized to prepare **(R_P)-17** in nearly identical quality and yield compared to the Pya comparator. Lastly, it was found that **(R_P)-18** synthesized using thymidine monomers with and without protection with equivalent yield and purity, supporting the notion that the there is no need for protection on thymidine bases.

Having identified each base protecting group in a stepwise and systematic fashion, a full length, mixed-base oligonucleotide (**19**) was prepared and directly compared to the previously published protecting groups. It was found that both **(R_P)-19** and **(S_P)-19** synthesized from monomers with the new protecting groups were formed in similar in yield and quality to those made via the previously published monomers.

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This study has optimized three key parameters that simplify the adoption of P(V)-based, phosphorothioate containing stereopure oligonucleotide synthesis. As such, a more seamless application of these techniques in industrial and academic settings using commercial oligonucleotide synthesizers is anticipated.

Associated Content

The Supporting Information is available .

Experimental details, materials, methods, characterization data, NMR spectra, for all new compounds.

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