

Unlocking P(V): Reagents for chiral phosphorothioate synthesis

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Phosphorothioate nucleotides have emerged as powerful pharmacological substitutes of their native phosphodiester analogs with important translational applications in antisense oligonucleotide (ASO) therapeutics and cyclic dinucleotide (CDN) synthesis. Stereocontrolled installation of this chiral motif has long been hampered by the systemic use of phosphorus(III) [P(III)]-based reagent systems as the sole practical means of oligonucleotide assembly. A fundamentally different approach is described herein: the invention of a P(V)-based reagent platform for programmable, traceless, diastereoselective phosphorus-sulfur incorporation. The power of this reagent system is demonstrated through the robust and stereocontrolled synthesis of various nucleotidic architectures, including ASOs and CDNs, via an efficient, inexpensive, and operationally simple protocol.

Molecular-based gene therapy is rapidly emerging as one of the most promising modes of disease amelioration, as evidenced by the swell of clinical trials employing microRNA, small interfering RNA, and antisense oligonucleotides (ASOs) (1). In a recent example, the ASO nusinersen (trade name Spinraza) (Fig. 1A) was approved by the U.S. Food and Drug Administration (FDA) as the sole available treatment of spinal muscular atrophy, the leading genetic cause of infant mortality (2). This breakthrough therapy comprises 18 nucleosides bound by phosphorothioate (PS) linkages (i.e., those in which one of the nonbridging oxygen atoms has been substituted with a sulfur). Compared with the native phosphodiester linkage, this simple modification confers a higher degree of metabolic stability and improved cellular uptake through an observation known as the thio effect (3–6). In fact, most FDA-approved ASOs incorporate this moiety, with numerous candidates under clinical evaluation (7). Although the PS alteration enhances the pharmacological profile of these systems, it comes at the expense of substantially increased structural complexity—each phosphorus atom is now an uncontrolled stereogenic center (8). Thus, in the case of Spinraza, patients receive a mixture of more than 100,000 discrete diastereoisomers that, in principle, may bear distinct three-dimensional structures and pharmaceutical properties (9, 10). Despite recent

studies suggesting that stereodefined systems may improve therapeutic efficacy, readily scalable preparation methods often disregard the stereochemistry at phosphorus, whereas most of the methods that enable stereocontrol are impractical for large-scale manufacturing (10–24). As outlined in Fig. 1B, three coupling paradigms have traditionally been employed to facilitate P–O bond formation, each relying on cumbersome P(III)-based reagent strategies. The first (Fig. 1B, i), which produces stereorandom PS ASOs, commences with a classic phosphoramidite loading step to append the first nucleoside, followed by deprotection, coupling to a second nucleoside, and then oxidative sulfurization of the resulting P(III) species to the P(V) state (11, 12). The second strategy (Fig. 1B, iii), capable of accessing stereo-defined PS linkages, uses a phosphoramidate containing a P(III)-centered chiral auxiliary to control the phosphorous stereochemistry but is otherwise identical to the first. The most mature manifestation of this method stems from work developed by Wada and co-workers (further refined by WAVE Life Sciences) and requires a total of seven steps for the preparation of the chiral auxiliary (10, 13–15). Finally, an approach pioneered by Stec and co-workers (Fig. 1B, ii) relies on a similar strategy that begins with a P(III)-oxathiaphospholane loading reaction, followed (again) by oxidative sulfurization to furnish a chiral P(V)-oxathiaphospholane (OTP) sulfide auxiliary; this stable OTP can then be coupled to form the requisite internucleotide linkage using a simple amine activator [typically 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU)] (16–18). However, by Stec's own admission, this strategy is scale limited, suffers poor coupling efficiency, requires resolution of stereorandom OTPs, and is extremely labor intensive (19). Taken together, the deficiencies of current methods point to an overreliance on low-valent P–O bond forming chemistry, as P(III) has historically been viewed as the sole gateway

for loading (and primary access for coupling) reactions. Reported herein is a major departure from that dogma: the invention of simple, P(V)-based reagents for phosphorus-sulfur incorporation (PSI, stylized hereafter as Ψ) (1) via reactions characterized by marked rapidity and scalability and complete stereocontrol at phosphorus.

Challenging the lore of the field, we opted for an inventive approach with the mission of bypassing P(III) entirely; the reaction sequence should rely exclusively on a P(V)-based platform to forge all key P–heteroatom bonds. This would require the development of a readily accessible reagent with programmable stereochemistry, capable of achieving both loading and coupling steps with robust precision. The realization of this design required a systematic investigation of three key components: (i) an inexpensive chiral backbone to predictably impart stereocontrol, (ii) a safe source of P(V) to eliminate the previously unavoidable oxidative sulfurization step, and (iii) a reactive yet stable leaving group to enable a facile loading reaction. Ideally, a traceless approach would join the parent species in a direct and asymmetric fashion while liberating a single P(S)O unit with no evidence of the chemical method of installation remaining. These disparate attributes should harmoniously assemble in such a way as to appeal to both process and discovery chemists—traits such as scalability, stability, crystallinity, and rapid reaction times were deemed critical to success. A matrix of candidate backbones, PS sources, and leaving groups was systematically investigated, as summarized in Fig. 1C. The tactical combination of these three constituents proved essential in achievement of the goals set forth above, leading to invention of the Ψ reagent system (1; Fig. 1D). Featuring a rigid, limonene-derived backbone anchored to an OTP sulfide core and a stable perfluorinated thiophenol leaving group, this platform embodies an impressive balance of stability and reactivity. The Ψ reagents enable the rapid construction of stereopure nucleotide systems and boast an operational simplicity akin to iterative peptide assembly.

Both (R)-PS- and (S)-PS-linked oligonucleotides can be readily accessed through selection of the desired Ψ reagent enantiomer, (+)- Ψ and (–)- Ψ , respectively (Fig. 2A). Derived from limonene, an inexpensive chiral pool terpene and common food additive, these OTP sulfide reagents are produced after epoxidation and ring opening. In the figure, the loading step refers to the appendage of the first nucleoside to the Ψ reagent to deliver a stable OTP-adduct, whereas the coupling forms the chiral internucleotide linkage. A variety of nucleosides (4 to 7) can be loaded in excellent yield (76 to 96%; Fig. 2B) onto Ψ with complete stereocontrol, accompanied by loss of pentafluorothiophenol. When using Ψ in excess, no competitive displacement of the OTP-sulfur is observed, despite the fact that this entity is primed for a subsequent coupling step. The 30-min reaction is air and moisture tolerant, occurs at ambient temperature, and requires only DBU as an activator. Notably, the next nucleoside in the sequence can

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be coupled under identical conditions to deliver the dinucleotide as a single diastereoisomer, again in exceptional yield (70 to 91%). The intermediate loaded OTP nucleosides are generally crystalline, bench-stable species.

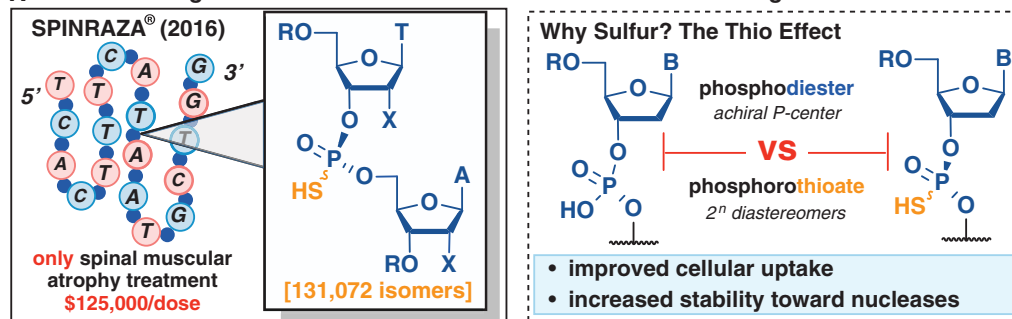
Dimeric combinations of the four native deoxynucleosides (A, T, G, and C) were prepared in high yields with complete stereocontrol; no differences in reaction performance were observed, regardless of base identity (Fig. 2C; **8** to **17**). In a testament to the power of these transformations, both enantiomers of the Ψ reagents were made on a multigram scale, as were all loaded and coupled compounds. Further, these reactions operate under complete reagent control, as evidenced by the stereochemical outcome in every case: (+)- Ψ leads to (*S*)-loaded and (*R*)-coupled products, whereas (–)- Ψ reactions are predictably reversed. In a feature distinct to these systems, one can simply invert the coupling sequence order to obtain the opposite P-stereochemistry using the same isomer of Ψ reagent (see supplementary materials for a graphical discussion).

Cyclic dinucleotides (CDNs) are macrocyclic natural products endowed with noteworthy biological activities and a storied history (25). Ample research indicates that these compounds play a number of prominent biological roles, most notably as secondary messengers in both the mammalian immune system and bacterial communication (26–31). Their role as agonists of innate immune response via binding and activation of the stimulator of interferon genes (STING) protein has led to a surge of interest across the pharmaceutical industry (26, 27, 32). As a consequence, numerous industrial and academic groups have targeted CDN constructs for evaluation as potential therapeutic agents. Just as the PS modification facilitates a marked improvement in ASO properties, a similar effect has been observed in CDNs, adding a layer of complexity to an already challenging class of natural products (8, 33–40). To be sure, the current impediment to rapid clinical progression of these compounds lies in the chemical synthesis. The modular strategies of traditional medicinal chemistry programs are stymied by both the poorly soluble nature of CDNs and the litany of engineered protecting-group schemes required to shield the array of heteroatoms found in the nucleobase, sugar core, and P(III)-based starting materials. As shown

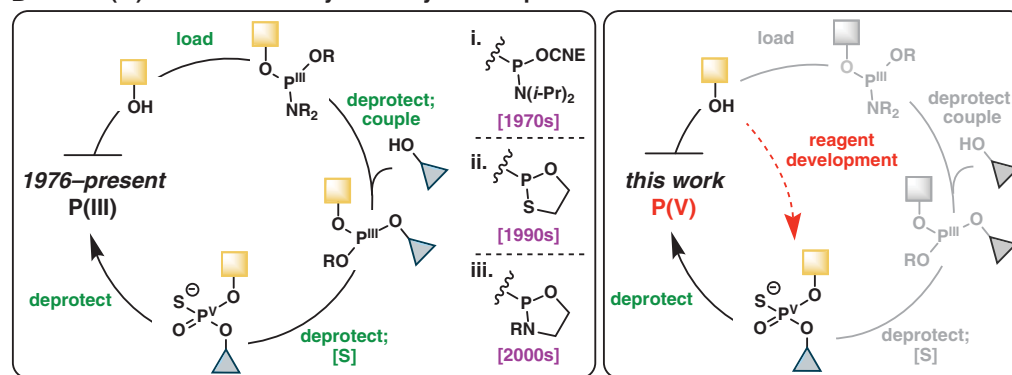
in Fig. 3A, nine steps are generally required from the parent nucleoside to prepare a single CDN as a mixture of all four possible diastereoisomers, with an observed ratio that appears to be substrate dependent (33–40). Of this arduous sequence, only four steps can be considered construction reactions, or those that contribute directly to P–O bond formation. The remainder account for various synthetic concessions, including

protecting-group manipulations and P(III)-to-P(V) oxidations. As compared with PS linkage integration in ASOs, the challenge of incorporating chiral PS linkages into CDNs becomes far more profound, as one is at the mercy of substrate bias to obtain diastereoselectivity at phosphorus. Modular, stereocontrolled CDN synthesis has so far proven elusive (Fig. 3A). In contrast, Ψ reagents enable a marked divergence from the

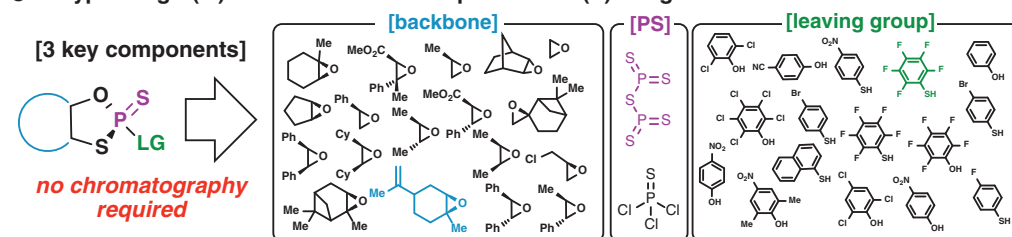
A Antisense Oligonucleotides: A Monumental Stereochemical Challenge



B The P(III) Scaffold: Primary Gateway to Phosphorothioates



C Bypassing P(III): The Quest to Develop an Ideal P(V) Reagent



D PSI Reagent: A Simple, Redox-Economic Alternative

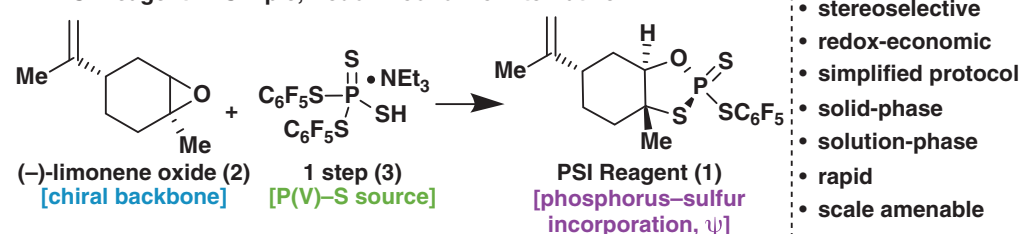
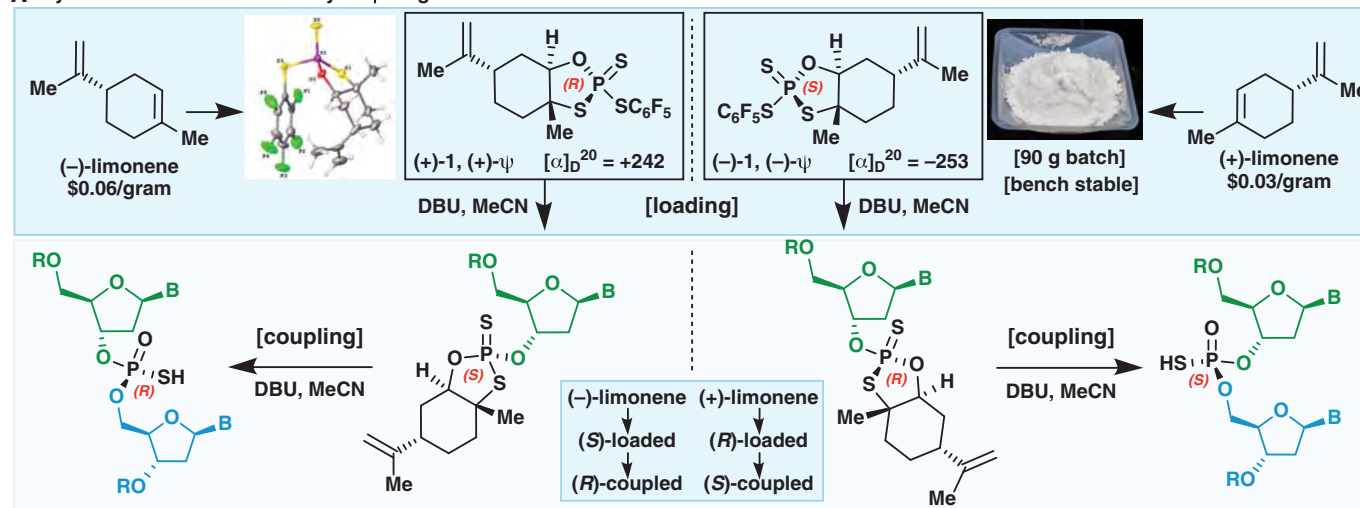
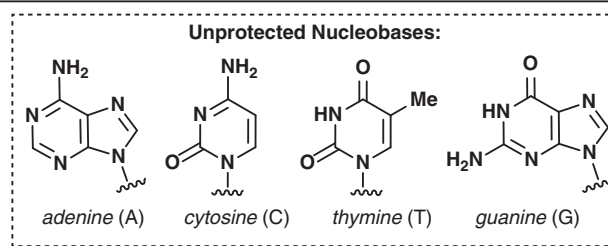
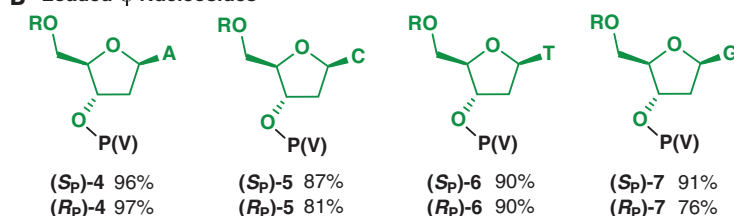
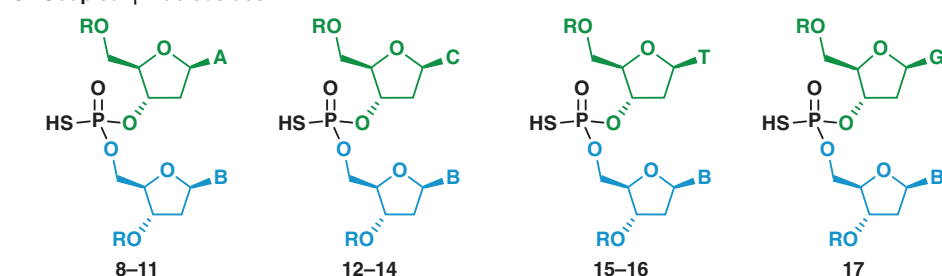


Fig. 1. Background and development. (A) Inspiration, (B) historical context, (C) development, and (D) invention of the Ψ reagent platform. *i*-Pr, iso-propyl; Me, methyl; Ph, phenyl; Cy, cyclohexyl; Et, ethyl; LG, leaving group.

A Synthesis and stereochemistry of Ψ reagentsB Loaded Ψ NucleosidesC Coupled Ψ Nucleosides

dinucleotide	yield (d.r.)
8, dA-dA (<i>R_P</i>)	65% (>99:1)
9, dA-dC (<i>S_P</i>)	91% (>99:1)
10, dA-dT (<i>S_P</i>)	61% (>99:1)
11, dA-dG (<i>S_P</i>)	79% (>99:1)
12, dC-dC (<i>R_P</i>)	73% (>99:1)
13, dC-dT (<i>S_P</i>)	76% (>99:1)
14, dC-dG (<i>R_P</i>)	82% (>99:1)
15, dT-dT (<i>S_P</i>)	72% (>99:1)
16, dT-dG (<i>S_P</i>)	67% (>99:1)
17, dG-dG (<i>R_P</i>)	86% (>99:1)

Fig. 2. Formalisms for the use of Ψ reagents in dinucleotide synthesis. (A) Stereochemical assignments. (B) Loading of nucleoside monomers. (C) Coupling to produce stereopure PS dinucleotides. R, TBDPS (*tert*-butyldiphenylsilyl). Loading:

nucleoside [1 equivalent (equiv.)], Ψ reagent (1.3 equiv.), DBU (1.3 equiv.), MeCN, 25°C, 30 min. Coupling: nucleoside-P(V) (1.0 equiv.), coupling partner (2.0 equiv.), DBU (3.0 equiv.), MeCN, 25°C, 30 min.

traditional P(III) approach, requiring only four or five steps from a starting nucleoside to arrive at a stereopure CDN. Two general protocols for CDN synthesis have been devised, as outlined in Fig. 3B. The stepwise approach ensures complete transfer of stereochemical information from the Ψ reagent by forging the CDN macrocycle one P–O bond at a time. To this point, CDN **21** was constructed as a single diastereoisomer in 14% overall yield via an operationally simple sequence: First, the desired Ψ reagent was loaded onto a nucleoside at either the 3' or 5' position and was then coupled to a resin-bound nucleoside (itself anchored to the solid support). The dinucleotide was then cleaved from the solid support to reveal the remaining free alcohol with no purification required. The final coupling reaction was achieved within 10 minutes to afford the desired

CDN target. Conversely, the concerted protocol accomplishes macrocyclization directly from the linear dinucleotide diol, as in **20**. Notably, CDN **21**, procured in stereopure form via the stepwise approach, was accessed in 44% yield via the concerted method, albeit with a 3:1 diastereomeric ratio (d.r.), a result of the aforementioned competitive 3' versus 5' Ψ loading in the penultimate bond formation (Fig. 3C, path A versus path B). Though step-economic, the concerted sequence effects high d.r. only when the reactivity of the 3' and 5' alcohols is sufficiently differentiated to enable selective reactivity with the Ψ reagent. As with the synthesis of linear dinucleotides (Fig. 2C), the order of assembly can be used to program the resulting phosphorus stereochemistry; thus, **18** was synthesized as three distinct stereoisomers, each with perfect stereocontrol,

whereas CDN **19** was obtained in 24% overall yield as a single diastereoisomer. Both approaches can be conducted with similar facility in solution and as well as in solid phase.

Beyond dimeric nucleotides, fascination surrounding P(III) in the context of general solid-phase oligonucleotide construction persists. This intrigue has resulted in a staggering number of publications and an entire industry of specialized equipment built to accommodate the complex reactivity and sensitivity of P(III)-derived systems. Perhaps unsurprisingly, reports of P(V) approaches to solid-phase oligonucleotide synthesis (SPOS) of PS-analogs remain conspicuously absent. To this end, an unoptimized automated load-deprotect-couple P(V) cycle (Fig. 4) was employed, using equipment designed for P(III) chemistry, to furnish the homo-dT pentamer (**23**) as a single

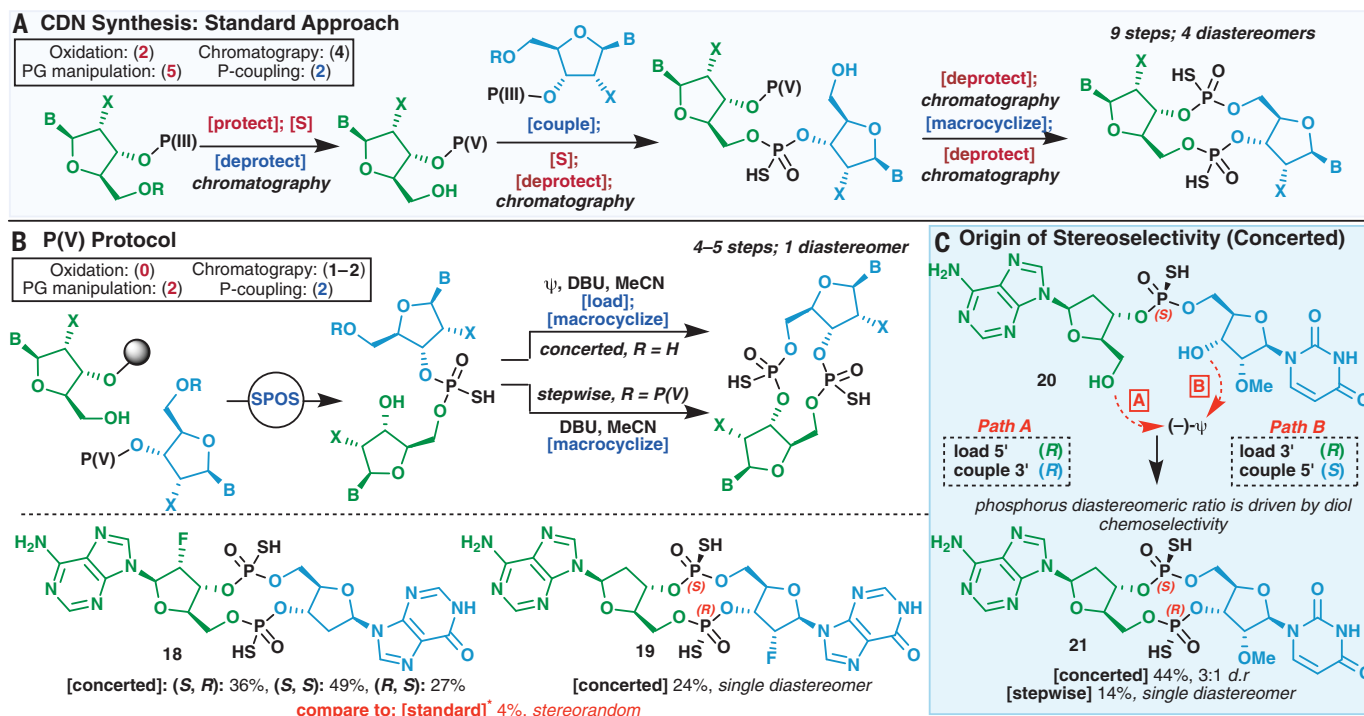


Fig. 3. Applications of the Ψ reagent platform to the simplified synthesis of CDNs. (A) Prior approach. (B) Stepwise and concerted macrocyclization using Ψ . *See (40) for the literature method. (C) Origin of observed stereochemistry in concerted protocol. PG, protecting group.

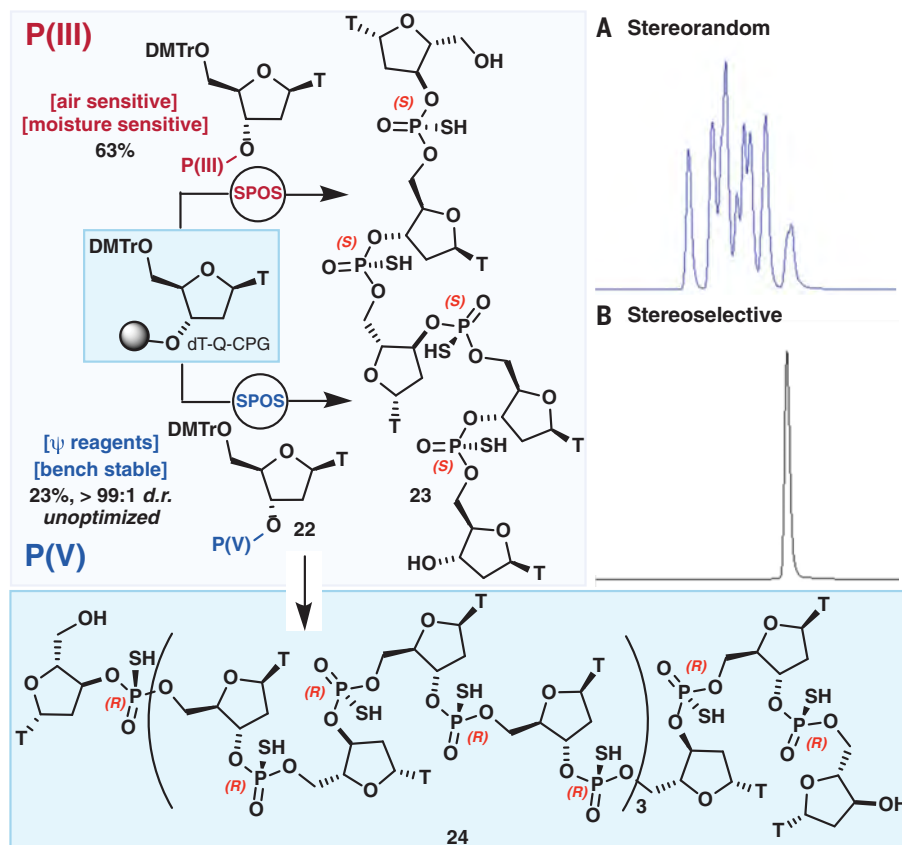


Fig. 4. Automated synthesis of PS oligonucleotides. (A) Crude HPLC trace of pentamer **23** (16 diastereoisomers) synthesized under standard P(III) automated conditions. (B) Crude HPLC trace of pentamer **23** (1 diastereoisomer) synthesized under unoptimized Ψ automated conditions. DMTr, dimethoxytrityl.

diastereoisomer in 23% yield. Notably, **23** was constructed without rigorous exclusion of air and moisture, in stark contrast to the operational constraints of traditional P(III) SPOS. Under traditional automated phosphoramidite conditions, the same oligonucleotide was obtained as a mixture of 16 diastereoisomers in 63% yield. In a promising demonstration, the 16-nucleotide oligomer **24** was successfully constructed via the unoptimized Ψ approach, though with an expected drop in crude purity (see supplementary materials for details). Indeed, the introduction of Ψ reagents engenders an advantageous approach to oligonucleotide synthesis enabled by a simple, reagent-based strategy capable of similarly effective solution- and solid-phase chemistry.

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ACKNOWLEDGMENTS

We thank B. Fink and A. Cernijenko for insightful discussions; J. Chen (TSRI) for assistance with analysis; D.-H. Huang and L. Pasternack (TSRI) for assistance with nuclear magnetic resonance spectroscopy; and A. L. Rheingold, C. E. Moore, and M. Gembicky (UCSD) for x-ray analysis. **Funding:** Financial support for this work was provided by NIH (grant GM-118176), Bristol-Myers Squibb, and NSF GRFP (J.N.D.). **Author contributions:** K.W.K., J.N.D., M.A.S., M.D.E., and P.S.B. conceived of the work. K.W.K., J.N.D., M.A.S., B.Z., J.C.V., C.K., S.E.M., I.M.M., Y.Z., C.H., J.Z., C.Y., and Q.W. designed and executed all experiments and performed all data analysis. R.E.O. and P.P. provided specific insight and direction for

the ASO and CDN studies, respectively. K.W.K., J.N.D., and P.S.B. wrote the manuscript. M.A.S., M.D.E., R.E.O., and I.M.M. assisted with editing. **Competing interests:** A provisional U.S. patent application on this work has been filed (application number 62657551), with K.W.K., J.N.D., M.A.S., B.Z., M.D.E., I.M.M., R.E.O., and P.S.B. listed as inventors. P.S.B. is a paid consultant for Bristol-Myers Squibb. **Data and materials availability:** Crystallographic data are available free of charge from the Cambridge Crystallographic Data Centre under reference numbers CCDC 1840250, 1849772, and 1849773. Experimental procedures, frequently asked questions, extensive optimization data, ¹H nuclear magnetic resonance (NMR) spectra, ¹³C NMR spectra, ³¹P NMR spectra, analytical high-performance liquid chromatography (HPLC) traces, and mass spectrometry data are available in the supplementary materials.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/361/6408/1234/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S4
Tables S1 to S5
References (41–48)
NMR Spectra

4 June 2018; accepted 12 July 2018
Published online 2 August 2018
10.1126/science.aau3369



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Science, **361** (6408), .

DOI: 10.1126/science.aau3369

A swift citrusy path to chiral phosphorus

The phosphates in the backbones of DNA and RNA are often drawn like crosses but are in fact tetrahedral. Sulfur is sometimes substituted for one of the phosphate oxygens during development of nucleotide-based drugs. Because of the geometry, this swap can lead to two different isomers. Knouse *et al.* report a pair of phosphorus reagents that conveniently produce either isomer selectively. This ability depended on the configuration of appended limonene substituents that are subsequently jettisoned. In addition to simplifying the route to sulfur-substituted oligonucleotides, these reagents will enable more precise studies of each isomer's distinct bioactivity.

Science, this issue p. 1234

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