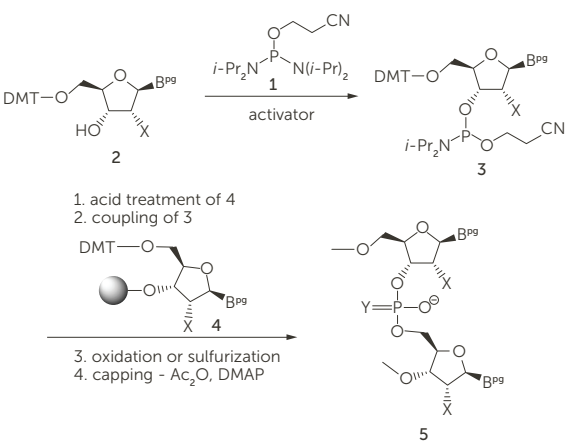
The Art of Phosphitylation

The preparation of new biologically active entities has generated immense study in the field of phosphorylation, specifically with oligonucleotides, modified oligonucleotides, phosphopeptides, carbohydrate phosphates, phospholipids and various bio-phosphates. Michalski et al.[1] and McMurray et al.[2] have published two excellent reviews on various phosphorylation techniques. One such class of drugs, oligonucleotides, is an emerging class of drugs in various stages of clinical trials for the treatment of a variety of cancers, viral infections, and inflammatory disorders.[3] In 1998 FDA approved Vitravene®, the first antisense oligonucleotide drug for the treatment of cytomegalovirus retinitis in HIV patients.[4] By mid-2022, 16 FDA approved oligionucleotide drugs[5] have been in development along with more than 200 in the clinical and pre-clinical pipeline.[6,7] Demand for these synthetic oligo-nucleotides is expected to reach metric tons per year.[4] The general synthesis is shown in Scheme 1.[4]

SCHEME 1



B = T, A^{Bz}, C^{Bz}, G^{Ibu}
X = H, OMe, $C(CH_2)_2OMe$
Y = O, S
Solid support = CPG, Primer 200, OligoPrep™

Phosphorylation is accomplished by introduction of a P^V reagent or by phosphitylation with P^{III} reagents followed by oxidation. Phosphitylation procedures with phosphoramidites and chlorophosphoramidites have been used most extensively from a wide variety of organophosphorus reagents, Figure 1.[2] One advantage of P^V reagents is the direct formation of the final P^V products, but suffers from low reactivity. The decreased reactivity can be overcome with more reactive P^{III} reagents with subsequent oxidation to the desired P^V phosphate. Phosphor-amidites are prepared from more reactive chlorophosphines. Highly reactive phosphorochlorodites are very sensitive to moisture and often require fresh preparation before usage. Phosphoramidites excel due to the lack of reactivity with alcohols and other H-nucleophiles unless activated.[1] Suitable protecting groups on the phosphorus can tune the efficiency of the procedure. Steric and electronic effects can fine-tune the coupling selectivity by prevention of multiple couplings. This review will focus on the use of P^{III} reagents and subsequent advantages in the selection of various protection and deprotection organic moieties on the P^{III} reagent.

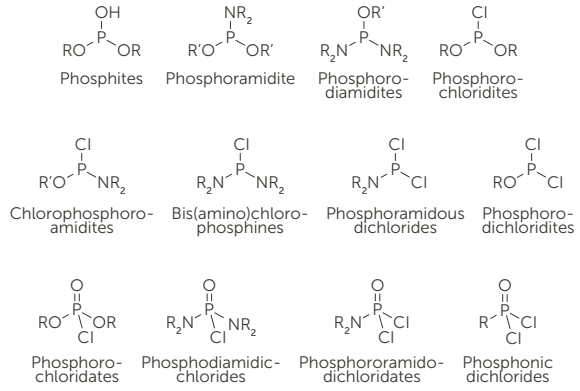
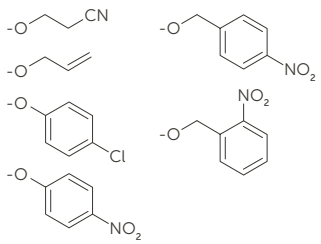


Figure 1. Various classes of P^{III} and P^V phosphorus reagents.

An immense effort has gone into phosphorylation via phosphoramidites. In 2001, a Beilstein computer search yielded 2550 compounds.[2] A 2022 SciFinder analysis of phosphorylated nucleosides prepared by phosphitylation show 5,048 compounds.[8] The main reasons for the plethora of compounds are yield, purity, and the method of deprotection of the coupled phosphoramidite or subsequent phosphate esters. Orthogonal deprotection can dictate the choice of alcohol or amine groups involved in the phosphorylation. Table 1 shows a listing of the more commonly used alcohol and amines in phosphitylation and phosphorylation. This list is not all encompassing.

TABLE 1. VARIOUS PROTECTING GROUPS COMMONLY USED IN PHOSPHORYLATION AND PHOSPHITYLATION REAGENTS

Alkoxy Groups (-OR)		Amine Groups (-NR_2)
R = -OMe -OEt -Ot–Bu	-OPh -OBn	R = Me Et *i*-Pr

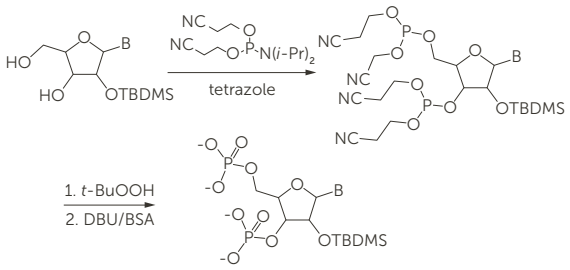


Variation within the amine group has been small compared to the alcohol group on the P^{III} reagents. The preferred amine group is *N,N*-diisopropylamino, –N(*i*-Pr)$_2$, due to increased stability of the reagents and intermediates. Phosphitylation agents such as ROP(NR_2)$_2$ are most popular in the synthesis of oligonucleotides and their thio-analogs due to their increased stability and allow for effective regio-selective phosphorylation by virtue of the steric hindrance, Scheme 1. According to the 2001 Beilstein search, more than 75% of the phosphitylation agents reported were N,N-diisopropyl amine derivatives.[1,2] In the 2022 SciFinder analysis, the percentage of N,N-diisopropyl amine derivatives increased to 90%.[8]

Variety in the alkoxy groups varies from the simple (-OMe, OEt, etc.) to the exotic. Simple alkoxides can be easily removed from P^V phosphate esters under hydrolysis conditions. The steric bulk of the O*t*-Bu group require more vigorous deprotection conditions with trifluoroacetic acid (TFA). Oxidation of phosphoramidites with traditional I_2/water causes extensive *t*-Bu cleavage while oxidation without deprotection can be accomplished with *m*-chloroperoxybenzoic acid (CPBA).[9]

Phosphoramidites that contain 2-cyanoethyl groups are used most often in nucleotide chemistry.[1] The recent SciFinder analysis showed 82% of phosphoramidites contained both -N(*i*-Pr)$_2$ and 2-cyanoethyl groups.[8] Removal of 2-cyanoethyl protection can be accomplished by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) by β-elimination. Phosphates that contain two 2-cyanoethyl protecting groups are converted into the secondary ester anion, whose electronic character prevents removal of the second 2-cyanoethyl group. The second 2-cyanoethyl group can be removed by expedient silylation of this anion by common silylation reagents and afford the deprotected primary phosphate.[10]

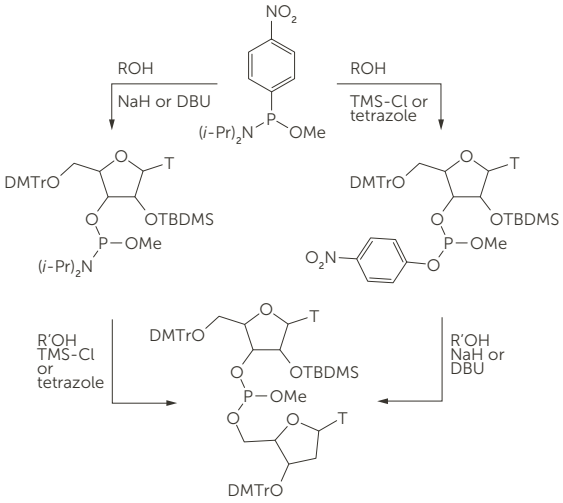
SCHEME 2



Phosphoramidites that contain aryloxy groups, $ArOP(NR_2)_2$ and $(ArO)_2P(NR_2)$, are resistant to acidic conditions used to activate amido groups for alcohol couplings. Alcohols and other nucleophiles under strong bases (DBU or NaH) can exchange aryloxy groups without affecting the amido group, Scheme 3. Interestingly, the anion of 4-nitrophenoxy can demethylate the phosphate after oxidation and can be performed as a one-flask procedure, Scheme 4.[11] Selective coupling can occur with two aryloxy groups of (*i*-Pr)$_2$NP(OAr)$_2$ (Ar = 4-nitrophenoxy) in a step-wise fashion with the appropriate alcohols, ROH and R'OH, and base (DBU).[12] This method leaves the amido group available for additional phosphitylation. Fluoro-phosphoramidites, ROP(F)NR'$_2$, can be prepared from phosphoramidites that contain a 4-nitrophenoxyl group by addition of tetrabutylammonium fluoride (TBAF).[13, 14]

Phosphoramidites with benzyloxy groups have been used with the purpose of deprotection by heterogenolysis with palladium catalysts. Similarly, phosphoramidites with allyloxy groups can be deprotected by transition metal catalyzed isomerization (Pd, Rh, Ir). Elegant orthogonal deprotection has been reported in the preparation of phosphopeptides that contain both groups. In the preparation of monobenzyl-protected amino acids, Fmoc-protected serine and threonine allylic esters were phosphitylated with either $(BnO)_2PN(i\text{-}Pr)_2$ or $(i\text{-}Pr_2)NP(OBn)(O\text{-allyl})$ and oxidized, Schemes 5 and 6. The allyl group can be removed by Bu_3SnH and $Pd(PPh_3)_4$ in HOAc in the presence of the benzyloxy group. Single benzyloxy deprotection can be accomplished cleanly with NaI.[15, 16]

SCHEME 3



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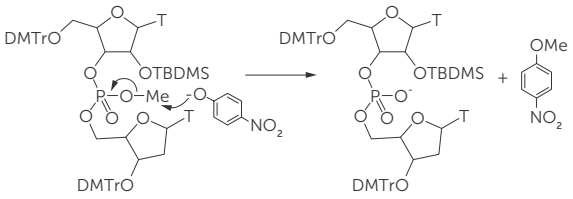
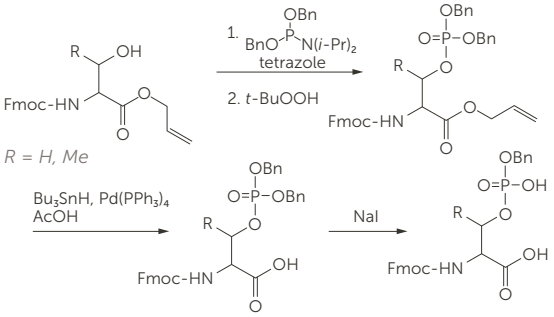
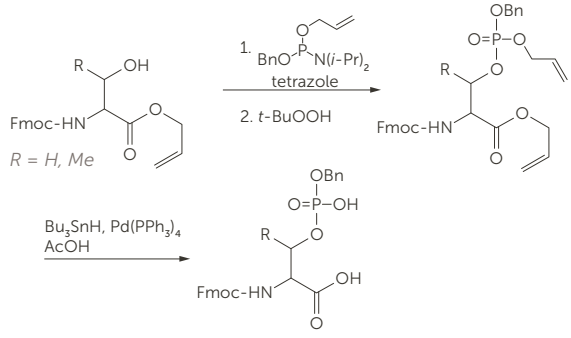


SCHEME 5



Phosphorochloridites, chlorophosphoramidites, and bis(amino)chlorophosphines have been used as phosphitylation agents, which add rapidly to hydroxyl groups without activation by displacement of the chloride. As stated previously, these reagents are moisture sensitive and require fresh preparation prior to use. These reagents, along with the dichloro-analogs, have been used to generate new phosphitylation reagents for phosphorylation, some of which are shown in Figure 2. SATE groups have been used in phosphopeptide pro-drugs. The theory is that the highly polar phosphate group cannot cross the cell membrane without protection as the triester. Once the SATE-protected phosphopeptide crosses the membrane, esterase-mediated cleavage of the S-acyl bond generates an unstable thioethyl phosphate triester. Elimination of episulfide (the sulfur equivalent oxirane) by intramolecular nucleophilic substitution of the -SH group results in C-O bond cleavage to the deprotected phosphate.[17 – 20]

SCHEME 6



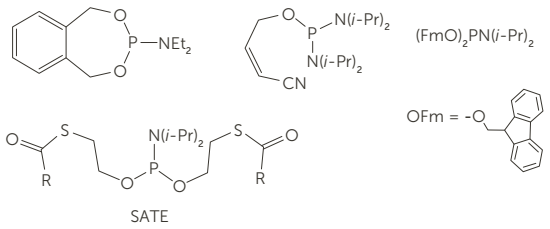
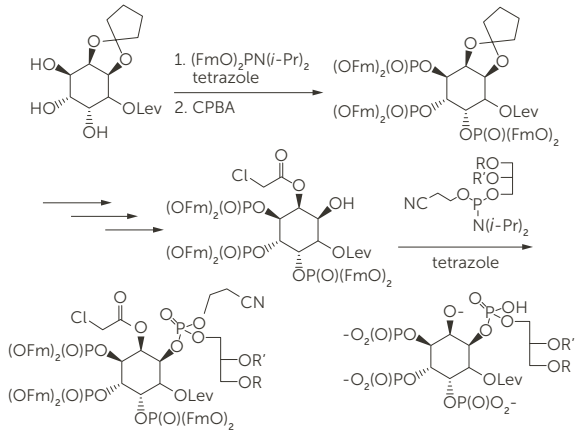


Figure 2. Phosphoramidite reagents derived from chlorophosphoramidites and analogs.

9-Fluorenylmethyl phosphoramidites, $(FmO)_2PN(i\text{-}Pr)_2$, were used in the exhaustive deprotection of phosphoinositol compound $PI(3,4,5)P_3$, Scheme 7. Other protecting groups such as benzyloxy, 2-cyanoethoxy, 2-(trimethylsilyl)-ethoxy, which are removed by β-elimination or β-fragmentation, were found to be unsuitable.[21]

SCHEME 7



R' = arachidonyl ($C_{17}H_{35}CO_2$-) R = stearoyl ($C_{19}H_{31}CO_2$-)

In 1995, the use of chiral oxazaphospholidine were employed in preparation of stereoselective phosphoramidite synthons, which were converted to chiral oligonucleoside phosphorothioates.[22] Chiral chloro-oxazaphospholidine synthons are based on ephedrine, prolinol, and substituted prolinols, Figure 3.[23] The P-Cl bond reacts with DMT-protected nucleoside to form the initial phosphoramidite as a single diastereomer at phosphorus, Scheme 8. Tetrazole-initiated reaction of solid-supported nucleoside forms a chiral-enriched dinucleoside phosphite. Chiral-enriched oligonucleoside phosphorothioates were prepared after sulfurization, capping, removal of the DMT protection, and removal of the solid support.[24] This work has inspired many groups to pursue this approach.

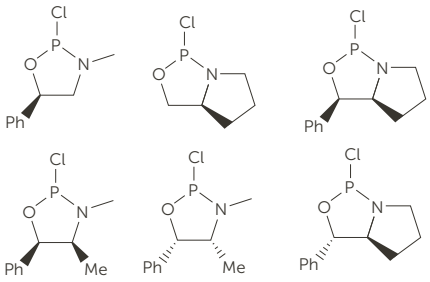
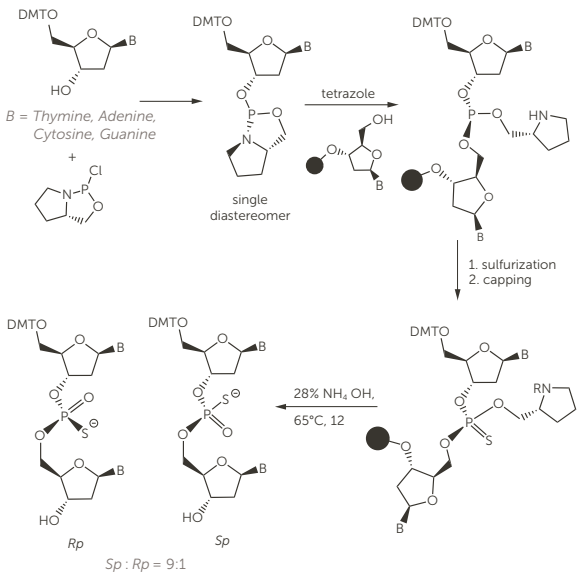
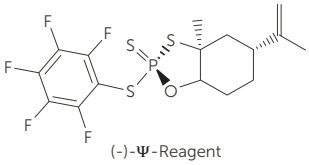


Figure 3. Several reported chiral chloro-oxazaphospholidines.

SCHEME 8



A new approach towards chiral P^V phosphorothioates has been developed with enantiomerically pure sulfur-limonene based reagents know as Ψ-Reagent. This suite of reagents has successfully made coupled dinucleotides and methylphosphonothioates with high stereo-control (>20:1 dr).[25,26]



(-)-Ψ-Reagent

Tetracoordinate P^V compounds are indispensable in the mechanism of life. The study of these compounds in the nature and as biologically active drugs has been immense. Historically, P^{III} phosphoramidites has surpassed the use of P^V reagents; however, new sulfur-based technologies are opening new pathways to oligonucleotides.

About the Author

Scott A. Laneman, Ph.D., Director, Innovation, supports Entegris' Specialty Chemicals and Engineered Materials division.

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