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Large-scale synthesis of high purity "Phos reagent" useful for oligonucleotide therapeutics ☆

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Abstract

A large-scale, cost-effective, and environmentally clean synthesis of high purity 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (Phos reagent) has been accomplished on a commercial scale. Treatment of PCl_3 with diisopropylamine followed by 3-hydroxylpropionitrile furnished the Phos reagent in excellent yield. The ^{31}P NMR of the Phos reagent prepared at large-scale show consistent purities >99% when several key factors are controlled. These controlling factors include sourcing high purity key raw materials, identification and elimination of critical impurities, stability and storage of Phos reagent.

Keywords: Phosphoramidites; Oligonucleotide; Phosphitylation

1. Introduction

Oligonucleotides are an emerging class of drugs with more than 40 candidates in various stages of clinical trials for the treatment of variety of cancers, viral infections, and inflammatory disorders [1]. Currently, six of these products are in late phase III clinical trials while one drug is a commercial product [1,2]. In 1998 FDA approved Vitravene™, the first antisense oligonucleotide drug for the treatment of cytomegalovirus retinitis in HIV patients [2]. Demand for these synthetic oligonucleotides is expected to reach metric tons per year [3]. Development of cost effective processes for the manufacturing of these drugs at the metric ton scale while meeting the high purity specifications is an important and substantial challenge for the industry. This task is especially monumental since the key raw materials are expensive.

Oligonucleotides are assembled in a step-wise fashion using solid-phase phosphoramidite chemistry (Scheme 1) [1]. Protected nucleosides react with a phosphitylation reagent to form a nucleosidic phosphoramidites (**3**), a key raw material for the synthesis of oligonucleotides. Currently, the 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite **1** (Phos reagent) is the reagent of choice for the phosphitylation reaction due to its inherent safety. Phosphoramidites **3** and solid-support **4** are the two critical raw materials that contribute >80% of the total raw material cost required for oligonucleotide synthesis. Particularly, the quality of **3** is very important for the overall product yields and oligonucleotide purity. The quality of phosphoramidites **3** prepared from

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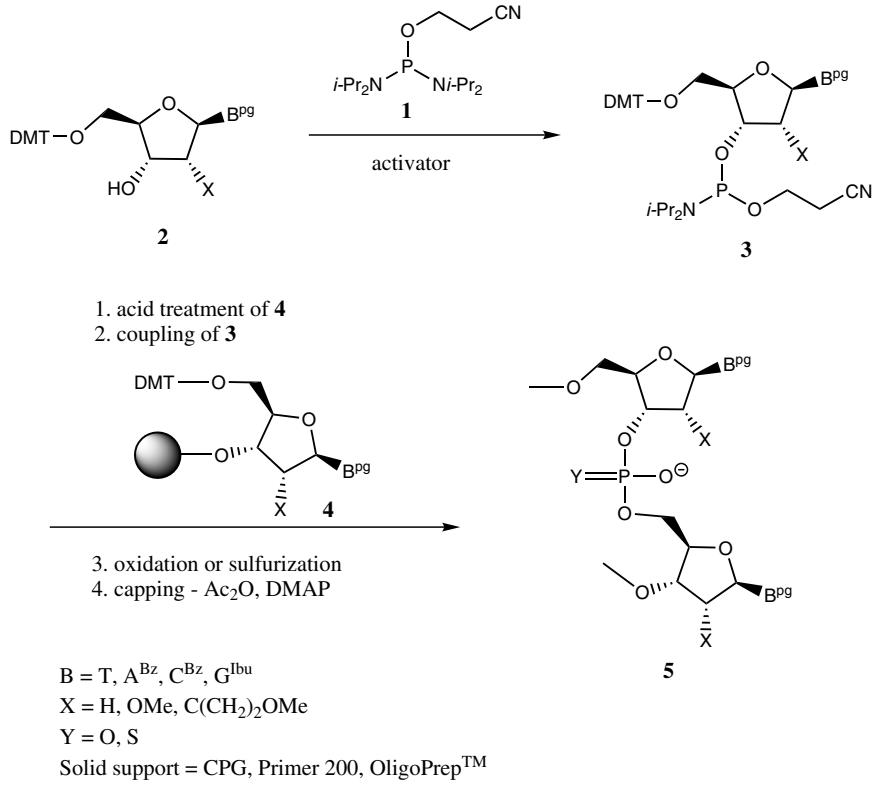


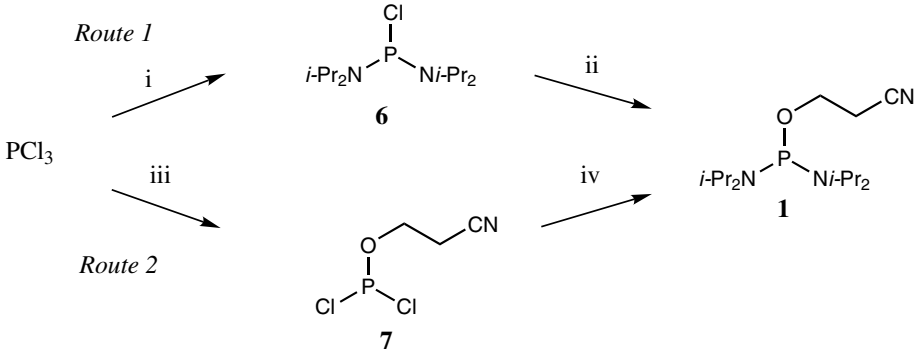
Scheme 1.

commercial grade Phos reagent was about 97% (^{31}P NMR) [3]. Recently, it was demonstrated that use of >99% pure **3** resulted in improved synthesis efficiency and reduction of various impurities in the final oligonucleotide products [4].

This report will focus on the large-scale manufacture of pharmaceutical grade Phos reagent of purities >99% based on ^{31}P NMR that permits synthesis of >99% pure **3**. The impact of key raw materials on the overall purity of Phos reagent and its storage, stability, and quality control is discussed.

2. Results and discussion

The preparation of pharmaceutical grade Phos reagent can be accomplished by one of the two routes (Scheme 2): (i) initial reaction of PCl_3 with $(i\text{-Pr})_2NH$ (2 equiv) followed by the reaction of **6** with 3-hydroxypropionitrile (1 equiv) [5], alternatively (ii) by addition of 3-hydroxypropionitrile (1 equiv) to PCl_3 followed by the addition of $i\text{-Pr}_2NH$ (2 equiv) to the intermediate **7** [6]. Anhydrous acetonitrile is the preferred solvent for the couplings with 3-hydroxypropionitrile, which



Reagents and Conditions: i, $i\text{-Pr}_2NH$ (2 equiv), THF, -10 °C; ii, $HOCH_2CH_2CN$ (1 equiv), MeCN, 0 °C; iii, $HOCH_2CH_2CN$ (1 equiv), MeCN, 0 °C; iv, $i\text{-Pr}_2NH$ (2 equiv), THF, -10 °C

Scheme 2.

produce faster reactions and cleaner purity profiles compared to reactions performed in toluene. The use of anhydrous THF in the reactions with $(i\text{-Pr})_2NH$ allowed for the removal of hydrochloride salts by filtration. Although both routes provide Phos reagent with acceptable purities of >99% by ^{31}P NMR, route 2 performed more consistently in our hands during large-scale operation.

The sourcing of high purity $(i\text{-Pr})_2NH$, 3-hydroxypropionitrile, THF and acetonitrile is critical for synthesis of Phos reagent consistently above 99% purity. Each reagent must pass stringent Quality Control criterion, which consists of GC, ^{1}H, ^{13}C and ^{31}P NMR. These tests allow for the strict monitoring of various amine and alcohol impurities common in $i\text{-Pr}_2NH$ and 3-hydroxypropionitrile (Fig. 1).

The consequence of the presence of trace amounts of **8**–**11** has been investigated (Scheme 3) during synthesis of the Phos reagent. For example, impurities **12** and **13** arising from the presence of **8** and **9** in $(i\text{-Pr})_2NH$, cannot be removed via distillation and are equally effective phosphitylation reagents in the preparation of phosphoramidites **3**. The residual presence of **10** can produce **15** and **16**, while residual primary alcohol impurities can produce impurity **17**. The monitoring of trace levels of these impurities in the Phos reagent is problematic and makes the product purity profiles inconsistent from a cGMP standpoint. The impurities **16** and **17** are more critical in the Phos reagent and must be avoided at all costs as they can be incorporated into the oligonucleotide products. Also, expensive nucleoside bases are consumed if these impurities are present. (see Fig. 3).

Impurities that can arise form from incomplete reactions during the preparation of Phos reagent are shown in Fig. 2. Presence of the impurity **18** can produce the

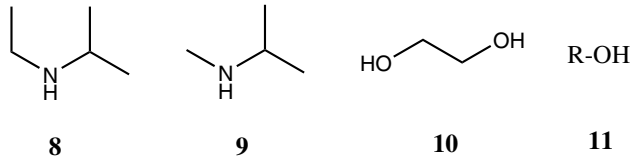


Fig. 1. Common impurities in $(i\text{-Pr})_2NH$ and 3-hydroxypropionitrile.

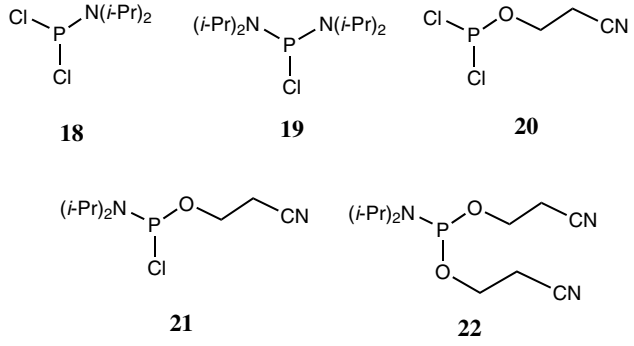
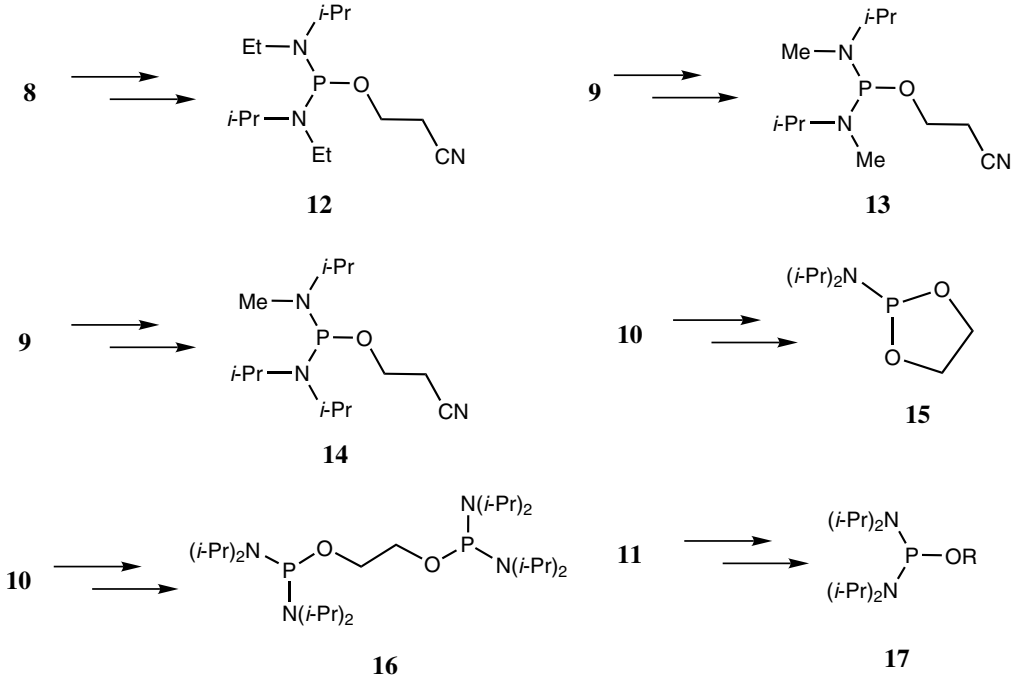
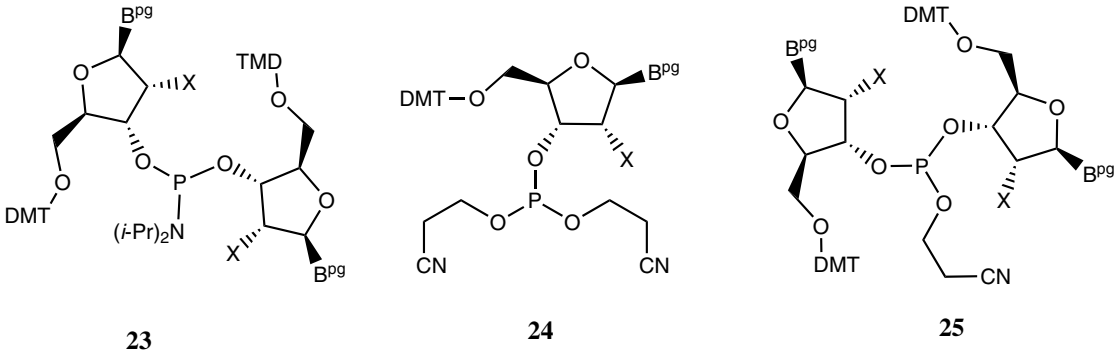


Fig. 2. Impurities from incomplete Phos reagent preparations.



Scheme 3.



23 24 25

Fig. 3. Downstream impurities as a consequence of impurities **18**–**22**.

nucleoside dimer **23**. Similarly, the impurities **20** and **22** can produce the non-reactive nucleosides **25** and **24**, respectively. Recently, Gaus et al. have identified the presence of these nucleoside amidites **23**–**25** in the commercial lots of amidites **3** [4]. It is imperative to eliminate the formation of by-product **22** and minimize the presence of possible reactive intermediates such as **18** and **20** during synthesis of Phos reagent. These impurities (**18**–**22**) are not easily removed from the Phos reagent by conventional distillation methods. Furthermore, presence of impurities **18**–**20** would consume expensive nucleoside starting materials. Impurity **26** forms from Phos reagent in the presence of adventitious water, whether from the solvent, reagents, or poor storage conditions, and can lead to the formation of branched oligonucleotides (Fig. 4).

As a result, use of high purity raw materials permits the synthesis of pharmaceutical grade Phos reagent. Additionally, all raw materials are handled and stored under nitrogen to eliminate moisture and formation of colored oxidative impurities of $(i\text{-Pr})_2NH$ and PCl_3.

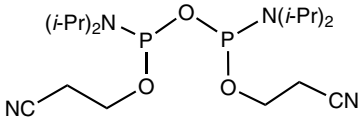


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Fig. 4. Hydrolysis impurities of Phos reagent.

Table 1 shows the levels of possible impurities in the Phos reagent with respect to their acceptable limits.

Phos reagent is clear colorless liquid at room temperature that is moisture, air and temperature sensitive. The quality of Phos reagent slowly degrades at room temperature (20–25 °C), when stored for extended period of time. Gratifyingly, cold storage below 2 °C eliminates the slow degradation of Phos reagent. Long-term storage studies (Table 2) of the Phos reagent under nitrogen at 2 °C confirmed that the reagent is perfectly stable without compromising the overall quality.

Table 1
Purity profile of Phos reagent prepared at multi-kilogram scale

Compounds	^{31}P Resonance (ppm)	Purity by ^{31}P NMR[a]	
		Acceptable limits (%)	Experimental (%)
Phos reagent (**1**)	122–123	⩾98.5	99.4
Combined P(III) impurities	124–181	⩽1	0.48
Combined P(V) impurities	0–70	⩽1	0.12
Individual P(III) impurities			
13 and **14**	125–137	⩽0.3	0.16
19	138–141	⩽0.2	0.16
16	142–144	⩽0.15	nd[b]
22	148–151	⩽0.25	0.07
21	178–181	⩽0.1	0.09

[a] ^{31}P NMR analysis: 800+ acquisitions, 3 μs (30° pulse), sweep range = −250 to +250 ppm, C_6D_6.
[b] Not detected.

Table 2
Stability of Phos reagent at <2 °C under nitrogen[a]

Compounds	^{31}P resonance (ppm)	Time (months)			
		t = 0 (%)	t = 1 (%)	t = 3 (%)	t = 9 (%)
Phos reagent (**1**)	122–123	99.43	99.40	99.45	99.20
Combined P(III) impurities	124–181	0.39	0.37	0.50	0.62
Combined P(V) impurities	0–70	0.17	0.23	0.04	0.19

[a] Based on ^{31}P NMR: 800+ acquisitions, 3 μs (30° pulse), sweep range = −250 to +250 ppm, C_6D_6.

3. Conclusions and outlook

Phos reagent has been synthesized from PCl_3 by two routes varying the mode of addition. Although both routes produce Phos reagent with >99% purity, the addition of 3-hydroxypropionitrile followed by reaction with $(i\text{-Pr})_2NH$ provided the most consistent purity profiles. This technique has been used to prepare Phos reagent at the ton scale per year with purities >99%. The high purity can be maintained with proper storage under nitrogen and at 2 °C.

More importantly, it is prudent to eliminate the impurities from the raw materials during early part of the synthesis when it is easier and cheaper compared to the final oligonucleotides product. Use of high purity Phos reagent is essential for the preparation of oligonucleotide drugs of better quality, lower cost, improved yields and reduced waste.

References

[1] Y.S. Sanghvi, M. Andrade, R.R. Deshmukh, L. Holmburg, A.N. Scozzari, D.L. Cole, in: G. Hartmann, S. Endres (Eds.), Kluwar Academic Publishers, Norwell, MA, 1999, p. 3.
[2] L. Jarvis, Chemical Marketer, Reporter, 7 June 2004, p. 7.
[3] Y.S. Sanghvi, Z. Guo, H.M. Pfundheller, A. Converso, Org. Proc. Res. Dev. 4 (2000) 175.
[4] C. Rental, P. Olsen, A. Krotz, J. McArdle, H. Gaus, Tides 2003, Las Vegas, NV, 28 April 28–1 May 2003.
[5] C. Harris, S. Jackson, D.J. Wilson, in: 16th International Conference on Phosphorus Chemistry, Birmingham, UK, 4–9 July 2004, PS2-020.
[6] J. Nielsen, O. Dahl, Nucl. Acids Res. 15 (1987) 3626.