Classification and characterization of impurities in phosphoramidites used in making therapeutic oligonucleotides

Risk mitigation strategies for entering clinical phases

Introduction

As advances in gene expression modulation by siRNA technologies continue, the reality of RNAi therapeutics edges closer. Oligotherapeutic development programs exist at almost all major pharmaceutical companies, with over $6B invested in oligotherapeutics programs since 2002.*

DNA and RNA oligonucleotides are synthesized using nucleic acid monomers called phosphoramidites. Phosphoramidites have a dimethoxytrityl (DMT) protection group on the 5′-OH and a β-cyanoethyl-N,N′-diisopropylamino-phosphoramidite (CEP) group on the 3′-OH of the dexoxyribose (DNA) or ribose (RNA). RNA phosphoramidites have a 2′-OH protection group, most commonly a *tert*-butyldimethylsilyl (TBDMS) or a methyl group.

The nucleobase exocyclic amines of both RNA and DNA phosphoramidites will be protected by any of several moieties, most commonly benzoyl and isobutyryl. In some circumstances, such as when mild deprotection is required to prevent depurination, other protecting groups are used.

Oligosynthesis is performed by sequential addition of individual phosphoramidites by coupling of the ribose sugar 3′-OH of the new base to the ribose sugar 5′-OH of the previous base. The synthesis cycle involves four chemical reactions: detritylation, coupling, capping, and oxidation. These cycles are repeated until the desired nucleotide sequence has been achieved.

The repetitive nature of oligosynthesis can amplify the risk of phosphoramidite impurities, resulting in poor quality of the final oligonucleotide. For example, if a single phosphoramidite used in synthesis of a 20-mer contains a critical impurity at a concentration of 0.2%, and this phosphoramidite is added 8 times in the sequence, the resulting oligonucleotide will contain 1.6% of this impurity.

The ability of phosphoramidite manufacturers to control phosphoramidite impurities is critical to oligotherapeutic supply chain management.

Impurity classification

Potential impurities in phosphoramidites are commonly classified in three categories.

- Nonreactive and noncritical
- Reactive but noncritical
- Reactive and critical

Critical impurities are defined as those that are incorporated into the oligonucleotide during synthesis such that oligos containing these impurities are difficult or impossible to separate from the desired synthesis product. There are also critical impurities that, when incorporated into an oligonucleotide, are difficult or impossible to detect.

The first class of impurities is nonreactive and noncritical. This class includes compounds having no phosphorus, compounds with phosphorus, hydrolyzed nucleoside H-phosphonates, and nucleoside dimer triesterx. They are not incorporated into oligonucleotides during synthesis and, by definition, are noncritical.

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* Asia Tides presentation by Gary Carter of Agilent Technologies.

The second class of impurities is reactive but noncritical. These are impurities that may become incorporated into oligonucleotides during synthesis but they are easily detected. Oligos containing these impurities are easily separated from the desired synthesis product. This class of impurities includes phosphoramidites with modifications on the 5′-OH other than DMT, phosphoramidites with different 3′-aminoalkyl protecting groups, and phosphoramidites with different base protecting groups.

The third class of impurities is reactive and critical. This class of impurities is of most concern to oligonucleotide manufacturers.

Critical impurity characterization

In an effort to provide further clarification and risk mitigation to our partners, we have further divided critical impurities into four classes.

- **Class 1:** Includes amidites with unnatural base modifications.
- **Class 2:** Includes 2′-3′ bis-ribose or deoxyribose amidites present either as monomers or dimers.
- **Class 3:** Includes DMT-amino nucleobases, unprotected nucleobases, 5′-ribose protection other than DMT and, in the case of RNA, ribose 2′-OH protection other than TBDMS, 2′-O-Me, or 2′-F.
- **Class 4:** Composed of structural isomers and pose perhaps the greatest theoretical impurity risk. These include alpha-anomers of the nucleobases and inversion of the DMT and amidite sugar protection to create the 3′-DMT-5′-amidite isomer form. An inversion seen in RNA is of the 2′ and 3′ ribose protecting groups to yield 3′-TBDMS-2′-amidite.

Phosphoramidite quality and control of critical impurities

The high expense associated with failed development programs and rework of key studies necessitates tight quality control of supply chain for raw materials and key components. For these reasons, Thermo Fisher Scientific takes a comprehensive approach to its own supply chain management, quality systems, and control and risk mitigation. The intrinsic benefits of this rigorous process are passed on directly to our many partners.

Thermo Scientific™ TheraPure™ DNA and RNA phosphoramidites are made with the highest standards of supply chain and manufacturing control to minimize or eliminate impurities of concern to oligotherapeutic developers.

A core management philosophy of Thermo Scientific phosphoramidite manufacturing is full integration of operations management with quality and business systems. Methods for managing quality are intrinsic to day-to-day operations and workflow management.

The base for the quality systems is the ISO 9001-2015 standard. However, our quality system exceeds the ISO 9001 standards with breadth of an FDA-registered system and elements of cGMP compliance. Note: we are not currently registered with the FDA.

Our supply chain is controlled at both the supplier level and the item level. Suppliers are regularly audited, subject to change control notification and formal complaint resolution systems. We maintain, wherever possible, multiple, qualified sources of supply and contingency plans for alternate-site manufacturing for supply chain reliability and in the event of catastrophe at any one site.

On the item level, key raw materials are qualified prior to use in manufacturing. We employ a risk-based assessment including Certificate of Analysis review, physicochemical analysis, and functional performance. We analyze multiple lots for confirmation of lot-to-lot consistency.

TheraPure phosphoramidite manufacturing processes are engineered to minimize or eliminate reaction conditions that could result in amidite impurities. Rigorous, documented process control is maintained. QC testing using advanced analytical technologies including HPLC, ^{31}P NMR, and LC-UV-MS is performed at intermediate steps. State-of-the-art purification methods are used to obtain the highest amidite purity possible.

TheraPure DNA and RNA phosphoramidites are manufactured to provide high overall purity and control of critical impurity levels, with excellent batch-to-batch reproducibility required for synthesis of oligonucleotides as active pharmaceutical ingredients.

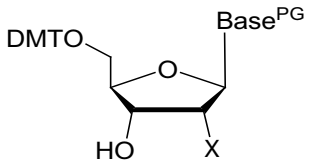
In summary, the comprehensive process, quality, and systems control employed provide a higher level of confidence and reduced risk and exposure for our clients as their oligotherapeutic development programs enter critical phases in the clinic. We believe that the combination of these measures makes for a better development outcome for our clients and their programs.

Table 1. Potential impurities in DNA, 2′-OMe, and RNA phosphoramidites.

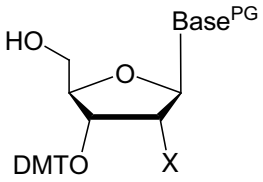
Compound number	Name	Classification
1	5′-DMT-3′-OH-nucleoside	Nonreactive and noncritical
2	3′-DMT-5′-OH-nucleoside	Nonreactive and noncritical
3	5′,3′-Bis-DMT-nucleoside	Nonreactive and noncritical
4	5′-TBDMS-2′(3′)-TBDMS-nucleoside	Nonreactive and noncritical
5	5′-DMT-3′,2′-bis-TBDMS-nucleoside	Nonreactive and noncritical
6	5′-DMT-bis-CE-phosphite	Nonreactive and noncritical
7	5′-DMT-3′-CE-H-phosphonate	Nonreactive and noncritical
8	5′-DMT-3′-CE-H-phosphonoamidate	Nonreactive and noncritical
9	5′-DMT-3′-amidate	Nonreactive and noncritical
10	5′-DMT-3′-phosphonoamidate	Nonreactive and noncritical
11	5′-modified-trityl-3′-amidite	Reactive but noncritical
12	5′-DMT-3′-modified-aminoalkyl-amidite	Reactive but noncritical
13	5′-DMT-N-modified-base-protection-3′-amidite	Reactive but noncritical
14	5′-DMT-3′-methoxy-amidite	Reactive and critical
15	5′-DMT-(CE-amiditoethyl)-phosphite	Reactive and critical
16	5′-DMT-amiditoethyl-amidite	Reactive and critical
17	Bis-nucleoside-amidite	Reactive and critical
18	Bis-nucleoside-CE-phosphite	Nonreactive and noncritical
19	Bis-nucleoside-amiditoethyl-amidite	Reactive and critical
20	5′-DMT-3′-ethoxy-ethoxy-amidite	Reactive but noncritical
21	3′,3′-Bis-diisopropylamine-amidite	Reactive and critical
22	CNET-pyrimidine-3′-amidite	Reactive and critical
23	5′-DMT-base-unprotected-3′-amidite	Reactive and critical
24	1′-Bis-nucleoside dimer-3′-amidite	Reactive and critical
25	5′,N-Bis-DMT-nucleoside-3′-amidite	Reactive and critical
26	5′,2′-Bis-TBDMS-3′-amidite	Reactive and critical
27	3′,2′-Bis-TBDMS-5′-amidite	Reactive and critical
28	3′-DMT-5′-amidite	Reactive and critical
29	5′-DMT-1′-alpha-anomer-3′-amidite	Reactive and critical
30	5′-DMT-3′-TBDMS-2′-amidite	Reactive and critical

Table 2. Chemical structures of potential impurities (X = H, OMe, or OTBDMS).

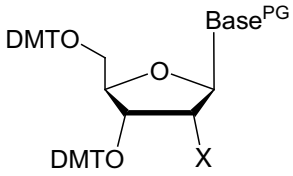
1: 5′-DMT-3′-OH-nucleoside



2: 3′-DMT-5′-OH-nucleoside

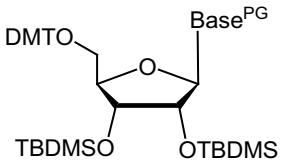


3: 5′,3′-Bis-DMT-nucleoside

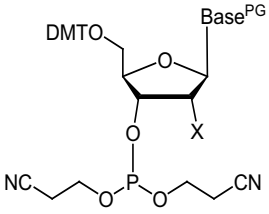


4: 5′-TBDMS-2′(3′)-TBDMS-nucleoside

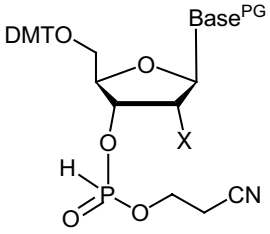
5: 5′-DMT-3′,2′-bis-TBDMS-nucleoside



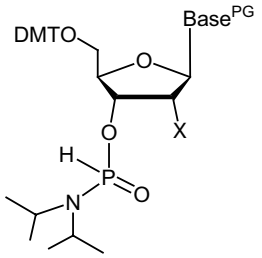
6: 5′-DMT-bis-CE-phosphite



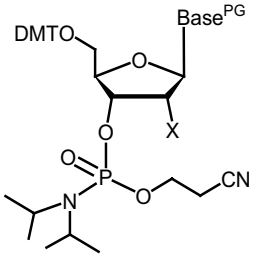
7: 5′-DMT-3′-CE-H-phosphonate



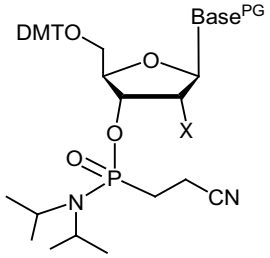
8: 5′-DMT-3′-CE-H-phosphonoamidate



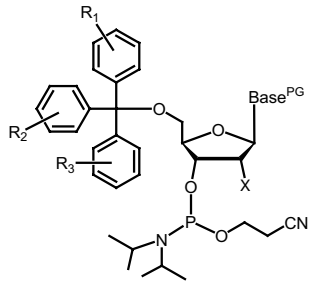
9: 5′-DMT-3′-amidate



10: 5′-DMT-3′-phosphonoamidate

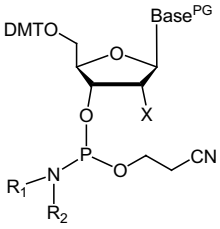


11: 5′-modified-trityl-3′-amidite



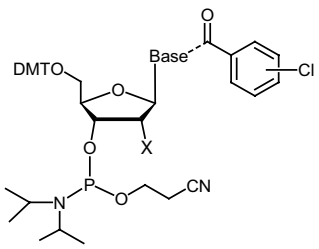
R_1, R_2, R_3 = OCH_3, OCH_2CH_3, Cl, OH, NH_2, etc.

12: 5′-DMT-3′-modified-aminoalkyl-amidite

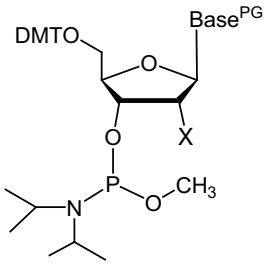


R_1, R_2 = CH_3, CH_2CH_3, $CH(CH_3)_2$, $CH_2CH_2CH_3$, etc.

13: 5′-DMT-N-modified-base-protection-3′-amidite



14: 5′-DMT-3′-methoxy-amidite



15: 5′-DMT-(CE-amiditoethyl)-phosphite

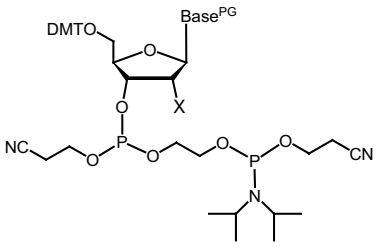
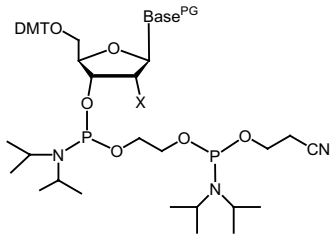
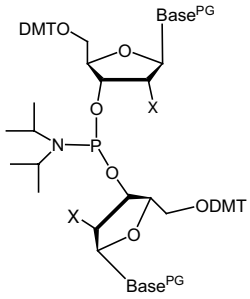


Table 2. Chemical structures of potential impurities (X = H, OMe, or OTBDMS). (continued)

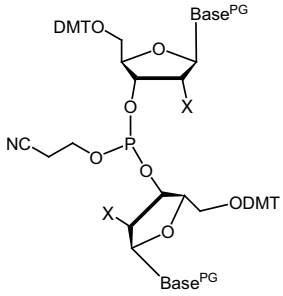
16: 5′-DMT-amiditoethyl-amidite



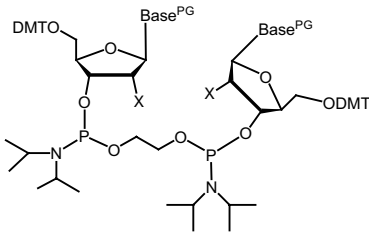
17: Bis-nucleoside-amidite



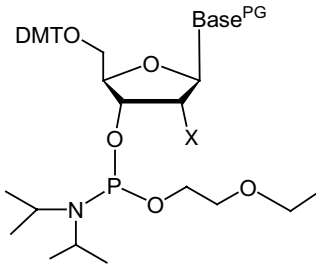
18: Bis-nucleoside-CE-phosphite



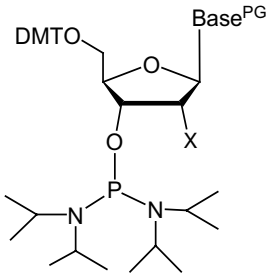
19: Bis-nucleoside-amiditoethyl-amidite



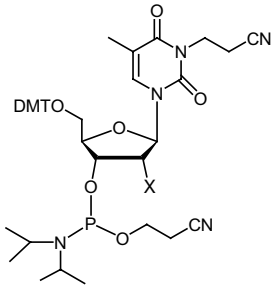
20: 5′-DMT-3′-ethoxy-ethoxy-amidite



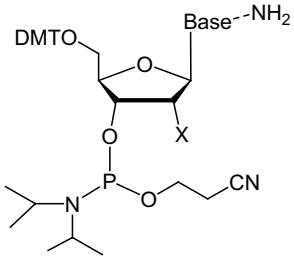
21: 3′,3′-Bis-diisopropylamine-amidite



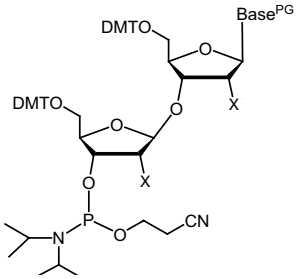
22: CNET-pyrimidine-3′-amidite



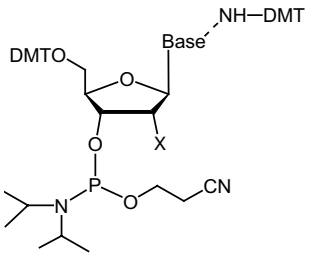
23: 5′-DMT-base-unprotected-3′-amidite



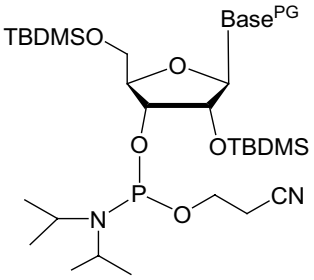
24: 1′-Bis-nucleoside dimer-3′-amidite



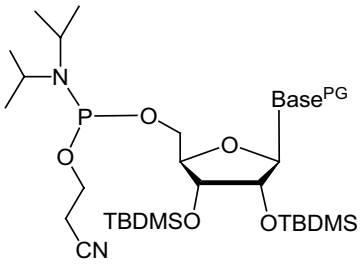
25: 5′,N-Bis-DMT-nucleoside-3′-amidite



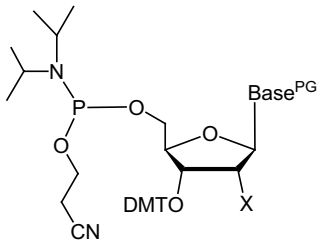
26: 5′,2′-Bis-TBDMS-3′-amidite



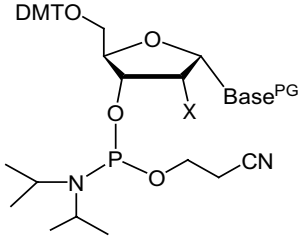
27: 3′,2′-Bis-TBDMS-5′-amidite



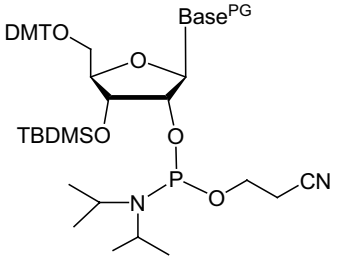
28: 3′-DMT-5′-amidite



29: 5′-DMT-1′-alpha-anomer-3′-amidite



30: 5′-DMT-3′-TBDMS-2′-amidite



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