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BioPharm INTERNATIONAL®

Resources and Tools to Accelerate Oligonucleotide Therapeutics to Market

- Oligo Trends and Insights
- Designation of Oligo Starting Materials
- Oligo Profiling and Impurity Control
- Oligo Identification and Quantitation

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Oligonucleotide Therapeutics: Trends and Insights

Q&A with Chris Rosenau, Product Manager, Nucleic Acid Therapeutics, Thermo Fisher Scientific

Chris Rosenau received his BS in Bacteriology and Zoology from the University of Wisconsin–Madison. In his 20+ year career, he has held a variety of R&D positions in molecular biology, ran the Blood Center of Wisconsin's Solid Organ Histocompatibility Transplant Laboratory, and managed cell-free DNA diagnostic products. Since mid-2020, he has been a Product Manager for Nucleic Acid Therapeutics at Thermo Fisher Scientific, responsible for materials used in development and manufacturing of oligonucleotide therapeutics and mRNA vaccines, such as phosphoramidites and modified nucleoside triphosphates.

What are oligonucleotide therapeutics?

Oligonucleotide therapeutics are short DNA or RNA molecules that modulate the expression of target RNA for the treatment of diseases. These oligos are about 20 nucleotides long, can be single- or double-stranded, and are traditionally chemically synthesized using phosphoramidites as building blocks. This drug family includes antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs) involved in RNA interference (RNAi), small activating RNAs (saRNAs), aptamers, and guide RNAs (gRNAs) involved in CRISPR gene editing technology. As of 2022, there are 14 such drugs currently commercialized worldwide. Twelve of these are approved in the United States, the majority of which are ASOs and siRNAs. They treat diseases including muscular dystrophy, polyneuropathy, and hypercholesterolemia, with many more therapeutics currently in development and other diseases being targeted

What are the main differences between ASO and RNAi technologies?

They both act on target RNA, resulting in RNA cleavage and ultimately preventing protein translation. However, their structure and mechanisms are different. ASOs are single-stranded oligos that recruit RNase H1 to cleave their RNA targets in the nucleus or the cytoplasm. On the other hand, RNAi involves double-stranded siRNAs that recruit the RNA-induced silencing complex (RISC) to cleave their RNA targets in the cytoplasm.

What are some trends and challenges in ASO and RNAi therapeutics?

The success of mRNA-based COVID-19 vaccines has brought renewed interest to other nucleic acid drugs, including ASO and RNAi therapeutics. In the next 5 years, the oligo therapeutics market is expected to grow by 25–30%; over 1,000 oligonucleotide molecules are currently in the pipeline. Major drugs such as ONPATTRO®, inclisiran, and others have shown that chemically synthesized oligonucleotide drugs have the potential to be best-in-class molecules, helping millions of patients around the world with genetic, cardiometabolic, and other diseases.

As both ASOs and siRNAs are short oligonucleotides, their stability in the body, immunogenicity to the host, specificity to their target sequences, and delivery to target organs have always been challenges.

As the building blocks for oligonucleotide therapeutics, modifications to phosphoramidite structures have been developed to improve performance and mitigate these issues. For example, 2′-O-methyl (2′-OMe) and 2′-fluoro (2′-Fl) modifications are commonly incorporated

into siRNA oligonucleotides to improve resistance to nucleases and thermal stability, and reduce off-target effects. For ASOs, 2′-O-methoxyethyl (2′-MOE)–modified amidites, among others, can be incorporated to produce similar effects. For delivery, encapsulating oligonucleotide therapeutics in lipid nanoparticles (LNPs) or conjugating them to N-acetylgalactosamine (GalNAc) moieties that have been developed in recent years facilitate improved durability and specificity of organ targeting for nucleic acid drugs.

What do customers care about when requesting phosphoramidites for oligonucleotide synthesis?

Customers look for quality assurance, documentation support, capacity, and batch-to-batch consistency in their products.

Customers commonly approach us with quality requirements. Providing quality products starts with solid manufacturing processes housed within a well-established quality system, which we have developed over decades in partnership with our customers, and the documentation that supports it. Thermo Fisher has been providing phosphoramidites on a global scale with leading quality systems and >40 years of nucleic acid chemistry innovation. Our manufacturing site is certified for ISO 9001:2015 for quality assurance, traceability, and documentation support.

Customers also want to make sure their phosphoramidite supplier is capable of meeting their capacity needs. We are committed to growing with our customers as their demand for oligo synthesis increases. We have recently expanded our phosphoramidite capacity, significantly adding to our already industrial-scale manufacturing suite to both serve our large customers and ensure that we have safety stock available for all customers.

“Thermo Fisher has been providing phosphoramidites on a global scale with leading quality systems and >40 years of nucleic acid chemistry innovation.”

Scale-up and future capacity are also important when our customers are starting with a custom modification but also looking toward their large-scale manufacturing down the road. At Thermo Fisher, our technical Process Development team develops their small-scale methods with eventual industrial scale in mind, shortening lead times when our customers require larger volumes. Scale-up and tech transfer are built in from the beginning of our process.

Batch-to-batch consistency is very important for our customers’ processes as well. We have the knowledge and experience to minimize batch-to-batch variation, starting from raw material qualification and incoming raw material testing, ensuring quality is

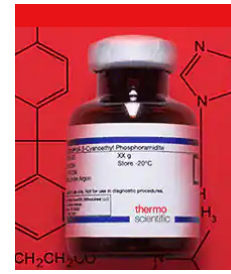
maintained in manufacturing via in-process testing, and eventually ensuring every product meets final product release specifications.

What are your organization's capabilities?

A broad range of nucleic acid chemistry options are available, including both off-the-shelf and custom phosphoramidites. Our phosphoramidite offerings include standard phosphoramidites, modified phosphoramidites with 2′ sugar modifications (e.g., 2′-OMe, 2′-Fl, and 2′-MOE) and various protection modifications, dye labels, structural moieties, linkers, and spacers. We have also partnered for >40 years to co-innovate with our customers, including custom manufacturing (e.g., GalNAc) and analytical services.

Thermo Scientific™ DNA and RNA phosphoramidites are suitable for oligonucleotide manufacturers for the development of therapeutic, diagnostic, and research applications. Thermo Scientific™ TheraPure™ phosphoramidites undergo additional quality control release testing compared to our standard phosphoramidites, helping ensure that impurities and residual solvents are controlled to the stringent levels required by our customers for their oligo therapeutics and diagnostic applications.

Outside of our phosphoramidite team, Thermo Fisher provides reagents for oligonucleotide synthesis and analysis tools for synthesized oligonucleotides, as well as the ability to synthesize multi-gram yields.



SPONSORED CONTENT

Oligonucleotide synthesis for therapeutics

How does your organization work with a customer who is interested in custom phosphoramidites?

It starts with a discussion between the customer and our business development team to get a clear understanding of the customer's needs, timeline, budget, quality requirements, potential scale-up, and so on. Our technical team then reviews details involving the technical specifications of the products, specifics regarding the processes we will use, whether there are any IP issues, etc. Our sourcing team reviews the raw material needs for the project and manages the supply chain. Our project management team oversees the project and communicates with the customer in collaboration with a dedicated internal technical team on inputs, design, planning, milestones, etc. In summary, we partner with our customers from day one to project completion so that they are informed of the project status and progress along the way, delivering the final product exactly as requested.

How is your phosphoramidite team contributing in this area?

Thermo Fisher provides global scale, service excellence, cost efficiency, co-

innovation, and high quality standards in phosphoramidite offerings.

- **Global scale:** We can provide raw materials on a global scale with a secure supply chain headquartered in the US. We have also expanded our manufacturing capacity in 2020 and 2021, with 5x additional capacity online in 2022.
- **Service excellence:** Our customer service team strives for a <48-hour response to orders and >98% delivery to customers by the promised date. We have phosphoramidites with standard and common modifications available in bulk, allowing for ease of purchase
- **Co-innovation:** We have partnered with our customers for >40 years on co-innovation and custom development projects, including supporting the manufacturing of customized GalNAc phosphoramidites.
- **High quality standards:** Our quality attributes include purity profiles, impurity profiles, materials-of-origin information, and traceability. We also provide our customers access to quality management and scientific staff for continual regulatory and technical support. We have also initiated multiple sustainability programs to further optimize our processes and reduce waste.

ThermoFisher SCIENTIFIC

TheraPure phosphoramidites: the ongoing pursuit of excellence

Discover the value of Thermo Scientific™ TheraPure™ phosphoramadites

- Designed for oligonucleotide manufacturers requiring **highly defined impurity profiles** and **strictly controlled processes**
- Manufactured with **expertise** in protection chemistries, modifications, manufacturing scale-up, and innovative purification strategies
- Produced in our state-of-the-art Milwaukee, Wisconsin facility, with an **ISO 9001:2015–certified** quality management system

World-class manufacturing

- Expanded manufacturing capacity with metric-ton capability
- Regionally diverse supply chain
- Phosphoramidites available in bulk packaging

Customer-focused technologies

- Nucleic acid chemistry and custom chemistry expertise for co-innovation and custom development projects
- State-of-the-art manufacturing facility
- Sustainability program to optimize processes and reduce waste

Dedicated customer service

- <48-hour* response to orders
- >98%* delivery to customer at promised date
- Standard synthesizer packaging and custom packaging options available, ready for prompt delivery

Continually improved quality system with extensive testing

- ISO 9001–compliant quality management system refined over decades of partnership with our customers
- Impurity profiling, control of critical impurities, and traceability for materials of origin
- High-quality TheraPure phosphoramidite offerings

Analytical capabilities

- Analytical method development
- Method verification/validation
- Test method transfer
- Stability studies

* May not be applicable in all regions.

thermo scientific

Ordering information

Product	Cat. No.
TheraPure DNA phosphoramidites	
TheraPure dA β-Cyanoethyl Phosphoramidite	27-2030
TheraPure dC β-Cyanoethyl Phosphoramidite	27-2032
TheraPure dG β-Cyanoethyl Phosphoramidite	27-2034
TheraPure DMF-dG Phosphoramidite	27-1737
TheraPure dT β-Cyanoethyl Phosphoramidite	27-2036
TheraPure RNA phosphoramidites	
TheraPure Bz-rA Phosphoramidite	27-1903
TheraPure Ac-rC Phosphoramidite	27-1805
TheraPure iBu-rG Phosphoramidite	27-1906
TheraPure rU Phosphoramidite	27-1804
TheraPure 2′-OMe phosphoramidites	
TheraPure 2′-OMe-Bz-A Phosphoramidite	27-2042
TheraPure 2′-OMe-Ac-C Phosphoramidite	27-2043
TheraPure 2′-OMe-iBu-G Phosphoramidite	27-2046
TheraPure 2′-OMe-U Phosphoramidite	27-2044
TheraPure 2′-MOE phosphoramidites	
TheraPure 2′-MOE-A Phosphoramidite	27-1019
TheraPure 2′-MOE-5mC Phosphoramidite	27-1020
TheraPure 2′-MOE-G Phosphoramidite	27-1022
TheraPure 2′-MOE-T Phosphoramidite	27-1021
TheraPure 2′-fluoro phosphoramidites	
TheraPure 2′-Fluoro-Bz-A Phosphoramidite	27-1601
TheraPure 2′-Fluoro-Acetyl-C Phosphoramidite	27-1604
TheraPure 2′-Fluoro-iBu-G Phosphoramidite	27-1607
TheraPure 2′-Fluoro-U Phosphoramidite	27-1602
Fast deprotect DNA phosphoramidites	
PAC-dA Phosphoramidite	27-1723
iPrPAC-dG Phosphoramidite	27-1726
iBu-dC Phosphoramidite	27-1725
Ac-dC Phosphoramidite	29-1727
TheraPure DMF-dG Phosphoramidite	27-1737
Structural phosphoramidites	
dU Phosphoramidite	27-1738
dI Phosphoramidite	27-1744
N6-Me-dA Phosphoramidite	27-1746
5-Me-dC Phosphoramidite	27-1748
N4-Ethyl-dC Phosphoramidite	27-1743

Product	Cat. No.
TheraPure locked nucleic acids	
TheraPure Locked Nucleic Acid A (Bz) Phosphoramidite	27-1340
TheraPure Locked Nucleic Acid 5-Me-C (Bz) Phosphoramidite	27-1348
TheraPure Locked Nucleic Acid G (DMF) Phosphoramidite	27-1347
TheraPure Locked Nucleic Acid T Phosphoramidite	27-1346
Standard DNA phosphoramidites	
dA β-Cyanoethyl Phosphoramidite	27-1730
dC β-Cyanoethyl Phosphoramidite	27-1732
dG β-Cyanoethyl Phosphoramidite	27-1734
T β-Cyanoethyl Phosphoramidite	27-1736
Standard RNA phosphoramidites	
Bz-rA Phosphoramidite	27-1403
Ac-rC Phosphoramidite	27-1405
iBu-rG Phosphoramidite	27-1406
rU Phosphoramidite	27-1404
Standard 2′-OMe phosphoramidites	
2′-OMe-PAC-A Phosphoramidite	27-1822
2′-OMe-iPrPAC-G Phosphoramidite	27-1826
2′-OMe-Bz-A β-Cyanoethyl Phosphoramidite	27-1842
2′-OMe-Ac-C β-Cyanoethyl Phosphoramidite	27-1823
2′-OMe-iBu-G β-Cyanoethyl Phosphoramidite	27-1846
2′-OMe-U β-Cyanoethyl Phosphoramidite	27-1825
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DyLight DY647 Phosphoramidite	SY6334
DyLight DY677 Phosphoramidite	SY6336
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Phosphorylating Phosphoramidite	27-1794
Reverse Abasic Phosphoramidite	27-1998

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Perspectives on the Designation of Oligonucleotide Starting Materials

By William F. Kiesman,[1] Andrew K. McPherson,[2] Louis J. Diorazio,[3] Leo Van den Bergh,[4] Peter D. Smith,[5] John M. Northall,[6] Alec Fettes,[7] Tiejun Wang,[8] Martin Mehlmann,[9] Syed Raza,[10] and Gary Held[11]

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ABSTRACT

The designation of starting materials (SMs) for pharmaceuticals has been a topic of great interest and debate since the first ICH quality guidance was published. The increase in the number and variety of commercialized oligonucleotides (antisense oligonucleotides—ASOs, small interfering RNAs—siRNAs, etc.) in recent years has reignited dialogue on this topic because of the unique complexity of the monomeric nucleotides and other contributory materials used to manufacture oligonucleotides. The SM working group in the European Pharma Oligonucleotide Consortium (EPOC) was formed to help establish simple, risk-based criteria to guide the justification of oligonucleotide SMs. This article provides a description of the common types of SMs, classes of SM impurities, and control strategies that will be helpful to maintain manufacturing consistency.

INTRODUCTION

A harmonized approach for the designation and justification of starting materials (SMs) for new chemical entities (NCEs) has been outlined in recent regulatory guidance [1,2] and proposals from industry groups [3]. These risk-based approaches provide insights into how SMs can impact drug substance quality and also mechanisms for control of critical attributes of SMs that may impact drug substance quality.

As chemically synthesized active ingredients, oligonucleotides have the potential to share similar risk-based justifications as more traditional, small molecule NCEs. This anticipation is hindered, however, by the lack of recognized standards and the small numbers of approved oligonucleotide products. Such a situation could lead to justification of SMs for oligonucleotide products being subject to inconsistent expectations by agencies in different regions or, indeed, by sponsor companies.

The European Pharma Oligonucleotide Consortium (EPOC) [4] was created in 2018 to address this and similar situations. EPOC is a collaboration between multiple pharma companies with the aim of sharing chemistry, manufacturing and control (CMC) knowledge, and strategies to enable harmonization of oligonucleotide development and commercialization practices. The consortium will publish science-based recommendations for the development of oligonucleotide therapeutics in a series of technical white papers. These draw on its collective subject matter expertise, complementing that in the literature and will serve as a reference for industry practice and to help establish development principles for oligonucleotides. The consortium aims to be proactive and inclusive, and anticipates initiating wider discussion on oligonucleotide CMC practice and policy to expedite access to potentially life-changing medicines.

Within EPOC, the Oligonucleotide SMs Working Group was launched to examine member company practices and propose risk-based strategies for more uniform oligonucleotide SM justification packages.

This article summarizes general approaches to the justification packages that include the following:

- Determination of the criticality of SM impurities
- Illustration using deoxy phosphoramidites with typical quality attributes of SMs and analytical methods used for controls
- Application of justification to more complex phosphoramidites, for example, 2′-(methoxyethoxy)ribose (MOE) and locked nucleic acids (LNAs)
- Extension to convergent syntheses of oligonucleotides from smaller oligonucleotides such as dimers/blockmers
- Approaches for components of conjugates (linkers and ligands)

A broad range of SMs has been applied to the manufacture of therapeutic oligonucleotides and it is not feasible to cover all of the options. Rather than provide hard and fast rules, this report illustrates principles to consider for simpler SMs and elaborates this as the perceived complexity increases. In this way, sponsor companies can adapt and apply these principles to justification and specification of oligonucleotide SMs in the context of their own drug projects and corporate approaches to regulatory filing.

Oligonucleotide therapeutics are becoming more prevalent in the global marketplace and manufacturing scales for these complex products are increasing as larger volume indications become legitimate targets. A flexible, harmonized risk-based SM justification approach shared by regulators and developers will help ensure sustainable patient access to affordable, high-quality products.

DISCUSSION

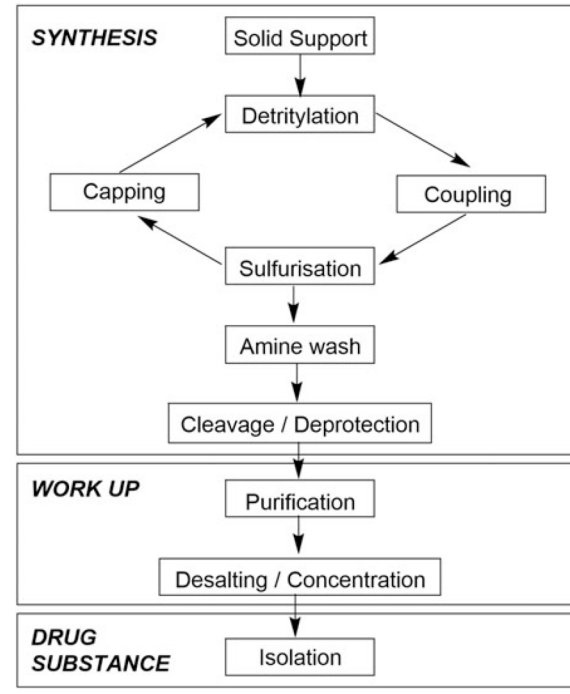
The manufacturing process

Before discussing SMs, it is necessary to understand the oligonucleotide manufacturing process to provide a context for justification of certain materials as SMs. The range of operations that are conducted may act as purging steps for SM-derived impurities and must be considered in any justification.

As an end-to-end manufacturing process, the various operations can be grouped into three main activities that are conceptually similar to those employed in standard small-molecule preparation. These are Synthesis, Work-up (often referred to as Downstream Processing for oligonucleotides), and Drug Substance Isolation (FIGURE 1).

When examined in more detail, however, the oligonucleotide process is different from small molecule manufacturing. The chemical synthesis of a therapeutic oligonucleotide is most often carried out on a functionalized solid support using an automated synthesizer. The oligonucleotide chain is extended through iterative synthetic cycles where each cycle results in the incorporation

FIGURE 1: Process overview of oligonucleotide manufacturing operations.



of one additional nucleotide unit (FIGURE 2). The cycle consists of four successive steps:

- **Detritylation**: removal of a 4,4′-dimethoxytrityl (DMT) protecting group at the site where chain elongation will occur
- **Coupling:** reaction with an activated phosphoramidite-functionalized building block to enable introduction of a single-nucleotide unit into the growing oligonucleotide chain
- **Sulfurization/Oxidation:** introduction of a sulfur or oxygen atom at the newly created internucleotide phosphotriester linkage, resulting in conversion from P(III) to P(V)
- **Capping:** addition of a reactive acylating reagent to effect capping of any unreacted hydroxyl center remaining as a result of incomplete coupling or undesired deprotection side-reactions and reduce propagation of such impurities

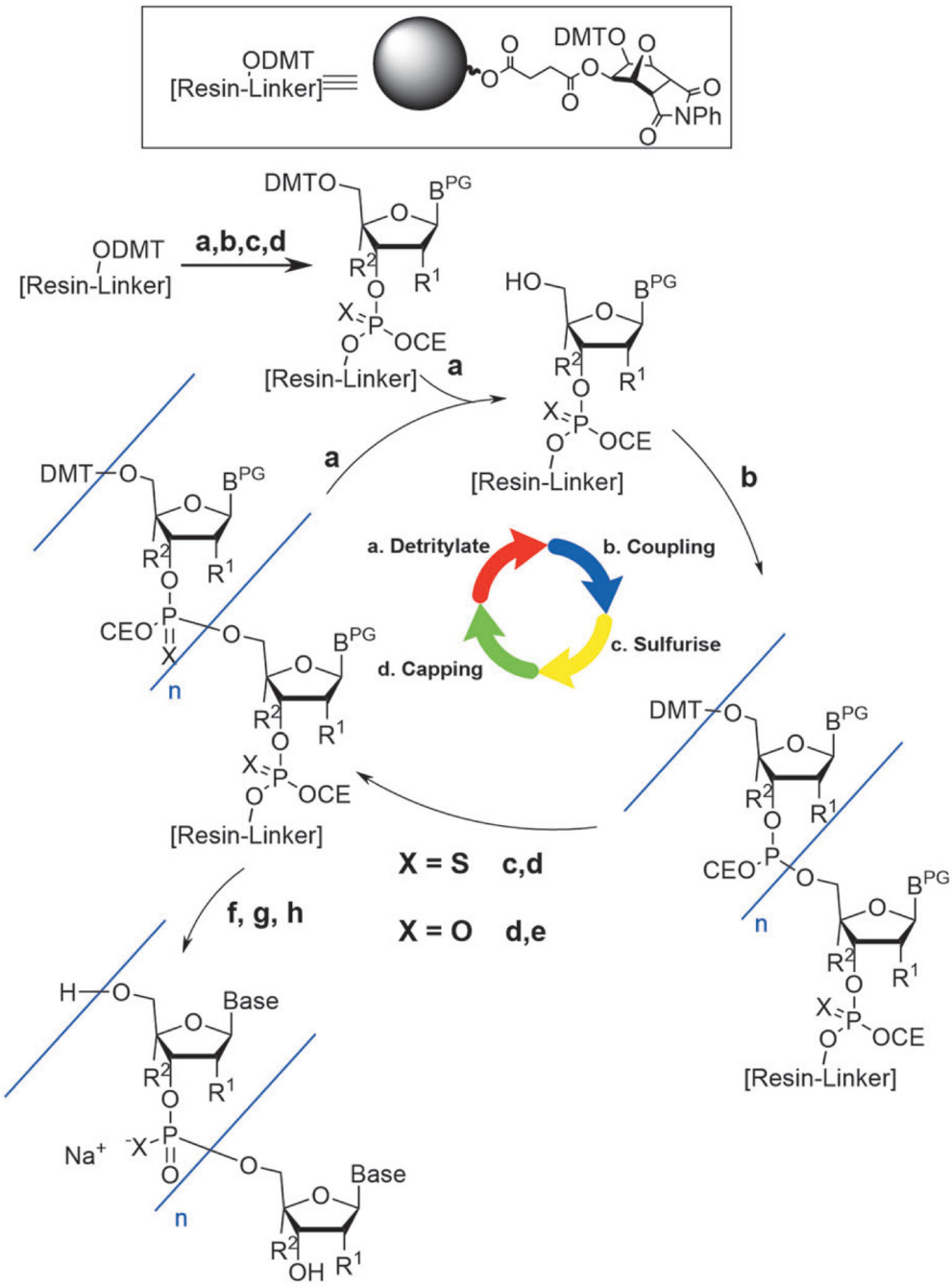
Each step is highly selective and very high yielding and the solid support is thoroughly washed with solvent between each successive operation in the cycle. In combination with the high solubility of excess reagents and associated byproducts, this ensures that there is no carry-over of reagents, building blocks, and nontethered impurities between the different synthetic steps.*

The solid-supported synthesis is carried out as a single continuous operation. For a 20-mer oligonucleotide, this means a total of ~80 synthetic steps carried out sequentially without pause in the process or isolated intermediates. The solid-supported synthesis is performed on a packed column with all reagents, solvents, and building blocks delivered as solutions under computer-programmed control. When combined with the advantages already mentioned (robust, high yielding, and highly selective chemistry with extensive column washing steps), this results in a highly controlled and predictable outcome for the synthesis phase.

Once the oligonucleotide sequence has been completed, deprotection steps are required before the work-up/downstream processing steps (purification, desalting, and concentration). The first operation is an amine wash to effect removal of the phosphorous backbone protecting group (typically 2-cyanoethyl, CE) and results in a global backbone deprotection of the phosphate/phosphorothioate esters, giving the triethylamine salt of the resin-bound oligonucleotide.

Treatment of the resin-supported oligonucleotide with aqueous ammonia removes various amine protecting groups on the nucleobases, as well as triggering cleavage of the resin linker, resulting in

FIGURE 2: Typical oligonucleotide synthesis process.



Reagents a) Detritylation (e.g. dichloroacetic acid, toluene); b) Coupling (nucleotide phosphamidite, coupling agents e.g. 4,5-dicyanoimidazole, *N*-methylimidazole, MeCN); c) Sulfurization (e.g. xanthane hydride, pyridine) d) Capping (e.g. acetic anhydride, MeCN, *N*-methylimidazole, pyridine);e) Oxidation (e.g. iodine, water, pyridine); f) Backbone Deprotection (e.g. triethylamine, MeCN); g) Cleavage and Deprotection (e.g. concentrated ammonia solution); h) Downstream Processing – purification, desalting, *in situ* detritylation, ultrafiltration)

release of the oligonucleotide from the solid support (often referred to as cleavage and deprotection). Subsequent filtration and washing (to remove the solid support) result in a solution of crude, 5′-DMT protected oligonucleotide, ready for purification.

The crude oligonucleotide solution is purified by liquid chromatography, typically strong anion ion exchange (SAX). Impurities not closely related to the active pharmaceutical ingredient (API; eg, by virtue of significant difference in chain length—shortmers/longmers) are readily separated during the chromatographic purification step; however, full-length (and close to full length) oligonucleotide impurities will not be removed during this step. The eluate is progressed forward to the desalting/concentration step.

Depending on the precise nature of the process, the 5′-DMT group can be removed during the solid-supported synthesis before amine treatment, during chromatography, or as a standalone postchromatography operation.

In the desalting/concentration step the counter ion is exchanged (if needed) and the oligonucleotide solution is concentrated. This can be achieved by ultrafiltration/diafiltration, for example, through use of a tangential flow filtration apparatus equipped with membranes. During this step, residual organic solvents, salts, and low-molecular weight impurities are removed according to the pore diameter cutoff size of the membrane. Alternatively, it is possible to carry out a sequence of ethanol-based oligonucleotide precipitations and subsequent reconstitutions from water as a means to remove low-molecular weight impurities and concentrate the oligonucleotide.

After the desalting and concentration operations, the API can be provided directly as an aqueous concentrate [5] or isolated as a solid—typically by lyophilization.

The repetitive nature of this overall oligonucleotide synthesis process where individual cycles apply similar conditions to substrates and reagents of broadly similar reactivity delivers a predictable outcome and is routinely used as the method of choice for Good Manufacturing Practice (GMP) manufacture of therapeutic oligonucleotides. In this respect, the high degree of automation, absence of in-process testing, and robust performance are reminiscent of a well-understood, well-defined commercial manufacturing process, even for preclinical manufactures.

Although oligonucleotide therapeutics are explicitly excluded from the scope of ICHQ6A (and by reference to ICHQ11), there are concepts in the quality guidelines

that are currently being generally applied to oligonucleotide manufacturing with the most relevant being ICH M7 (regarding mutagenic impurities) [6], ICH Q3C (residual solvents) [7] and ICH Q3D (elemental impurities) [8], and ICH Q7 (Good manufacturing practices) [9]. One notable diversion from the guidance relates to ICH Q3A (Impurities in New Drug Substances [10]). While the general concepts of reporting limits, identification limits, and qualification limits still apply, higher thresholds have been accepted for oligonucleotides than for small molecules, to date [11].

ICH Q11 provides a science-driven, risk-based framework addressing the propensity for SMs to influence the quality of the drug substance. This requires a thorough understanding of actual and potential impurities, as well as their fate in downstream processing gained from knowledge of the synthetic route coupled with risk assessments. Although ICH Q11 explicitly states that oligonucleotides are out of scope, the ethos for SM selection outlined in ICH Q11 remains applicable, but should be considered with an appreciation of oligonucleotide processing. Working in this way, a number of principles can be considered in the designation of oligonucleotides SMs:

- A defined and stable structure with characteristic chemical and physical properties
- A significant structural fragment toward the structure of the drug substance
- Oligonucleotides with related sequences or size typically possess similar physical properties; so purging of impurities with the same or similar number of nucleotides as the desired product (full length impurities) is challenging.
- Effective SM specifications supported by detailed understanding of the fate and control of impurities in phosphoramidites or other SMs are vital aspects of the overall oligonucleotide control strategy.
- Analysis of the risk of impurity carryover across the unit operations is of greater priority than number of chemical transformations to reduce the risk of contamination and support the control strategy throughout the product lifecycle.
- The operation of many steps without interspersed analysis during solid-supported synthesis does not support application of traditional stepwise impurity fate and purging approaches.
- Ideally, the SM can be sourced as a commodity with controlled quality

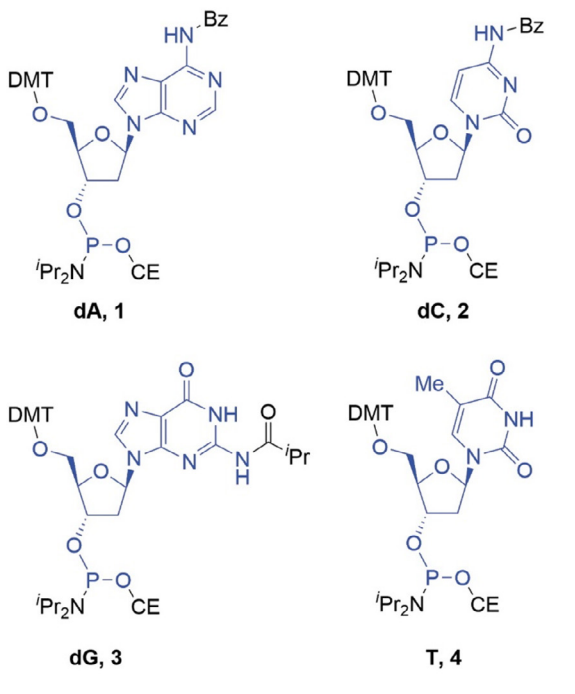
Phosphoramidite building blocks (henceforth described as amidites) are manufactured using standard chemical manufacturing technology and are well controlled. Several amidites are widely available from third-party commercial suppliers with controlled quality. To date, 2′-deoxyribose amidites (deoxyamidites) and 2′-(2-methoxyethoxy) ribose amidites (MOE amidites) have been accepted as appropriate SMs for

oligonucleotides, demonstrating that amidites can be acceptable in accordance with the principles of ICH Q11 guidelines. In this situation, the application of GMPcontrols for oligonucleotide drug substance manufacturing processes starts from the amidite SMs with an appropriate control strategy to ensure quality of the finished oligonucleotide.

Deoxyamidites

The deoxyamidites, for example, 1–4 (FIGURE 3), whose core structures are found naturally in DNA, are the most widely used building blocks in oligonucleotide APIs. As such, they provide a convenient introduction for how regulatory guidance can be applied to oligonucleotide SMs before introducing more complex examples.

FIGURE 3: Deoxyamidites (atoms marked in blue are incorporated into the oligonucleotide).



Deoxyamidites are incorporated into the oligonucleotide as significant structural fragments and therefore fulfill the most basic requirement for SMs. The most prevalent examples are the 2-cyanoethyl-*N*,*N*-di*iso*propylaminophosphoramidites, which were first applied in oligonucleotide synthesis in 1984 [12]. They were rapidly adopted as the standard approach due to supporting highly efficient coupling following appropriate activation. Supply of these materials has increased to a point where they are commercially available in large quantities (up to hundreds of kg batch size) from multiple vendors worldwide with multiton annual capacity available in the market. In addition, there are many more vendors capable of supplying medium- to small-scale amounts of material.

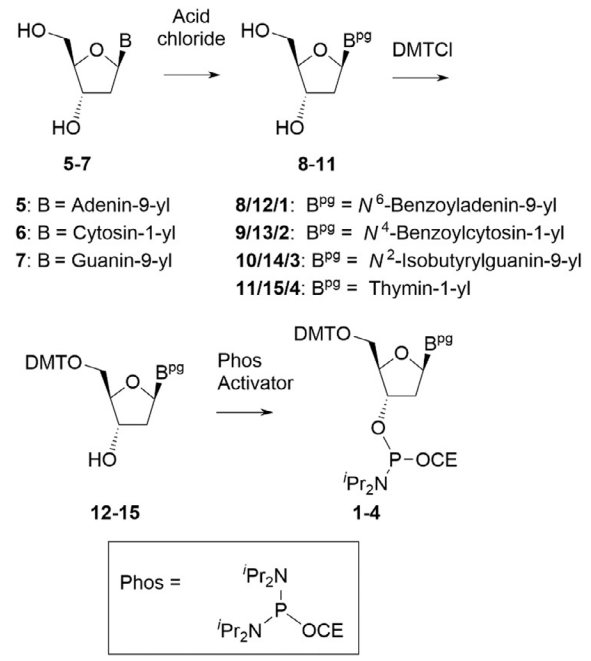
As might be expected for such established materials, the structure and physiochemical properties of deoxyamidites have been rigorously characterized. A variety of analytical techniques, for example, $^{1}H/^{13}C/^{31}P$/2D-NMR (two-dimensional nuclear magnetic resonance spectroscopy), specific rotation, and high-performance liquid chromatography with ultraviolet and mass spectrometry detection (HPLC-UV-MS) have been applied in EPOC member companies and elsewhere, providing a confidence in the robustness of their quality. These studies have led to a detailed

understanding of potential and actual impurities, as well as their fate and impact on the quality of the target oligonucleotide. Consequently, the material attributes of the deoxyamidites that may impact the API critical quality attributes (CQAs) [1] (eg, reactive critical, impurities) can be defined, and analytical methods with appropriate acceptance criteria can be validated, leading to specifications to purchase materials. This comprehensive understanding further supports acceptance of deoxyamidites as SMs, and a more detailed discussion is presented in the next section.

Deoxyamidites are free-flowing, nonhygroscopic, amorphous solids. They are derived from the corresponding nucleosides that are obtained from non-animal sources through fermentation and readily available in ton quantities in stereochemically pure form. The amidites are a mixture of the two diastereoisomers at the P atom and typically seen as double peaks both in ^{31}P NMR and high-performance liquid chromatography (HPLC). Commercially available deoxyamidites are synthesized by the following general synthesis scheme (FIGURE 4).

The 2′-deoxyribonucleosides 5–7 are acylated on the exocyclic primary amines of the nucleobases, that is, adenine, cytosine, and guanine, with desired protecting groups (step 2). Since thymine does not have an exocyclic amine, this step is not performed. Protection of the 5′-hydroxyl as the DMT ether affords the fully protected

FIGURE 4: General method used to manufacture deoxyamidites.



nucleosides (12–15, often referred to as PNS). The final step is the phosphitylation of PNS with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetra*iso*propylphosphordiamidite (often referred to as Phos reagent or P-reagent) in the presence of an activator. Preferred activators are small, weak, nonhygroscopic organic acids.† Although no systematic study has been conducted, anecdotal evidence derived from more than 20 years of experience within the EPOC partners related to deoxyamidites synthesized using two common activators (1*H*-tetrazole, DCI) indicates that activator choice does not appear to influence the amounts of reactive impurities that result in oligonucleotide drug substance impurities. For similar reasons,

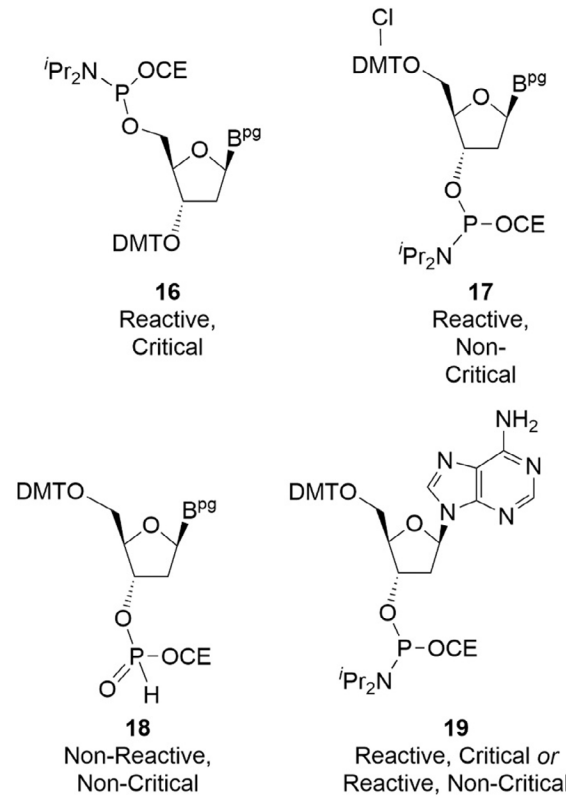
solvents used in the reaction are not considered to impact SM CQAs.

In current commercial scale processes, although no new carbon stereocenter is created from the nucleoside, the synthesis of the amidite results in an *R*/*S* stereochemical mixture at phosphorus. Crucially, the absolute configuration of the phosphorus atom of deoxyamidites does not impact the distribution of oligonucleotide diastereoisomers. This is determined by other variables during the coupling reaction in the oligonucleotide chain extension cycle; therefore, control of phosphorus stereochemistry in deoxyamidites is unimportant [13,14].

Impurities in deoxyamidites can be assigned to two broad groups based on an assessment of their reactivity during oligonucleotide coupling and the ability to purge any resulting impurity during the manufacturing process. The most important group results in impurities in the crude drug substance that are not subsequently purged. These are known as reactive, critical (or critical) impurities and generally contain both phosphoramidite functionality and an acid-labile protecting group, such that they can propagate chain elongation (FIGURE 5). It is the individual and total amounts of these critical impurities that constitute the CQAs of deoxyamidites [15].

The second group comprises deoxyamidite impurities that have no impact on the final drug substance purity and, unsurprisingly, these are commonly known as noncritical impurities. These might be such species as related nucleosides and nucleotides or residual solvents that do not react with the oligonucleotide chain during coupling. Such inert components are known as nonreactive, noncritical impurities. There is a second subset of noncritical impurities that do react with the evolving oligonucleotide chain during coupling, but do not affect product quality. This might be due to the resulting impurity in the oligonucleotide being readily purged, for example, during chromatography, or because the impurity motif is lost during

FIGURE 5: Examples of amidite impurity types.



processing and results in the target product. These are the reactive, noncritical impurities. Examples of each class are presented (TABLE 1).

The reactive, critical impurity 16 will incorporate into the oligonucleotide during the coupling reaction through a 5′-5′ internucleotide linkage. Detritylation during the subsequent deprotection step will actually release a terminal 3′-hydroxyl at the expected 5′-terminus leading to a subsequent 3′-3′ internucleotide linkage before reverting to normal progress in later cycles. This introduces a reversed nucleotide to a sequence. Due to its similarity to the parent, oligonucleotides containing 16 are not purged during downstream processing, thereby rendering it critical; 16 is controlled by purification at the PNS and amidite stages and by specification.

It is important to note that the deoxyamidite examples provided in TABLE 1 were used to illustrate the definitions of the classes of impurities, but in actual practice, there are almost no traces of critical impurities in deoxyamidites. Specifically, the levels of 16, when present at all, are very low (~0.04%) due to the high degree of selectivity between the 3′- and 5′-hydroxyls in the tritylation reaction.

TABLE 1: Origin and control of indicative critical and noncritical impurities in deoxyamidites.

Impurity type	Source	Method(s) of control
16 **Reactive, critical**	Reaction of DMT-Cl with 3′-OH rather than 5′-OH during PNS formation	PNS purification; deoxyamidite purification; deoxyamidite specification
17 **Reactive, noncritical**	Impurity in DMT-Cl reagent with one chlorine on one aryl ring	Vendor DMT-Cl specification; deoxyamidite specification
18 **Nonreactive, noncritical**	Amidite hydrolysis during phosphitylation	Control of water during PNS phosphitylation; deoxyamidite purification; deoxyamidite specification
19 **Reactive, critical or reactive, noncritical**	Bz protecting group was either never installed or was installed, but later cleaved	PNS purification; deoxyamidite purification; deoxyamidite specification

DMT, 4,4′-dimethoxytrityl; PNS, protected nucleoside.

The presence of an extra chlorine atom in reactive, noncritical impurity 17 does not materially affect the rate of subsequent detritylation, which removes this impurity motif from the chain. Oligonucleotide chains that have incorporated 17 continue to elongate into full-length parent oligonucleotide. Therefore, while impurity 17 is reactive during the synthesis, it is noncritical with respect to oligonucleotide CQAs.

The nonreactive, noncritical impurity 18 (H-phosphonate) is unreactive under normal amidite coupling conditions and therefore passes through the synthesis column and away from the oligonucleotide during amidite delivery and subsequent washing steps. While noncritical, some control over 18 is advantageous for yield consistency.

Criticality for other reactive impurities will not always be so clearly defined. Reactive impurity 19 might be present in 1 and will couple as normal, but will introduce branching impurities throughout the rest of the synthesis. During subsequent coupling cycles, one or both growing branches may fail to extend and be capped, generating a complex mixture of branched species of various lengths with as many as two DMT groups present on the 5′-termini. Depending on which coupling cycle was compromised, the resulting mixture of branched oligonucleotide impurities may be easier or harder for the chosen downstream purification method to purge. Incorporation in the first coupling cycle would result in a much larger (and generally easier to separate) oligonucleotide impurity than for the final cycle where it may just lead to parent oligonucleotide. In such cases, criticality will need to be assessed for the process chosen and the appropriate limit set for 19.

Deoxyamidites typically contain trace quantities of water and solvents (eg, acetonitrile, ethyl acetate, and toluene). These do not react with the growing support-bound oligonucleotide and are washed away or purged, as such they do not affect drug substance purity and are therefore considered noncritical. A steady evolution of deoxyamidite manufacturing means that there are almost no traces of critical impurities observed (TABLE 2).

Of 72 lots of materials manufactured by 4 vendors on multi-kg scales, 13 contained a single critical impurity in the range 0.04%–0.11% with a further 2 batches containing only 19 in the range 0.2%–0.3%. Only two batches contained >1 critical impurity, but still totaling <0.2%. Similar summary statistics on assay, purity by ^{31}P NMR, water content, and residual solvents (TABLE 3) show deoxyamidites to be of consistently high quality from multiple vendors.

Specifications for deoxyamidites focus on control of critical impurities and overall purity (TABLE 4). The need to control critical impurities will be evident from the foregoing discussion, but control of overall purity can also be important. For example, reversed-phase purification ruggedly purges failure sequences, whereas

TABLE 2: Deoxyamidite Impurity Lot History Summary

Vendor	No. of lots	Lots with critical impurities	Assay range (% a/a)	^{31}P purity range (% a/a)	Water (% w/w)	Residual solvents (% w/w)
1	31	8	94.0–99.6	96.8–100.0	0.11–0.35	0.23–2.90
2	22	5	94.4–99.7	98.8–100	0.07–0.67	0.84–2.42
3	10	1	97.0–99.5	99.3–99.9	0.10–0.28	0.22–2.36
4	9	1	94.9–100.5	97.9–100	0.11–0.23	ND–1.1
Total	72	15				

ND, not detected.

TABLE 3: Additional summary statistics for 72 lots of deoxyamidites.

Characterization	Mean	Median	SD
Assay (% w/w)	97.5	98.1	1.62
Purity by ^{31}P NMR (% a/a)	99.2	99.4	0.83
Water (% w/w)	0.19	0.15	0.108
Residual solvents (% w/w)	1.07	0.95	0.568

NMR, nuclear magnetic resonance spectroscopy.

with some oligonucleotide sequences, strong anion exchange chromatography is sensitive to quality of input materials. If the chosen process for oligonucleotide manufacture cannot completely purge coupling failures, then coupling efficiency becomes a critical process parameter and, by extension, the overall purity of the deoxyamidites becomes critical. Even if the oligonucleotide process can purge all failed sequences, it is still advantageous to control the overall deoxyamidite purity to aid process robustness (and to avoid paying for expensive noncritical impurities). When considering critical impurities, it is also necessary to consider the multiplicity of deoxyamidite incorporation due to the impurity family approach typically applied to oligonucleotide impurities [11]. The presence of a single, critical reactive amidite impurity at 0.05% w/w in each amidite during the synthesis of a 20-mer oligonucleotide could lead to a maximum of 1.0% (20×0.05) of the corresponding oligonucleotide impurity.

TABLE 4: Additional summary statistics for 72 lots of deoxyamidites.

Test	Method	Acceptance criterion
Appearance	Visual inspection	White to yellow powder
Identification	LC-UV-MS	MoIM of the sample and the reference standard agree to within an amu limit. Retention times of both main peaks of the sample and reference standard agree to within a limit
Assay	LC[a,b]	NLT 90.0% Critical impurity NMT 0.20% Any unspecified critical impurity[c] NMT 0.15% Total critical impurities
Impurity profile	LC[a,b]	NMT 0.50%
Purity	^{31}P NMR	≥95.0% a/a
Water content	KF	NMT 1.0% w/w
Residual solvents	GC	NMT 4.0% w/w

[a] LC methods may be reported as either area percent or weight percent at a specific wavelength that can vary depending upon the method.

[b] Both UV and MS detection methods have been applied.

[c] Any impurity that contains both an amidite moiety and DMT protecting group may be critical and should be investigated; all other impurities are noncritical.

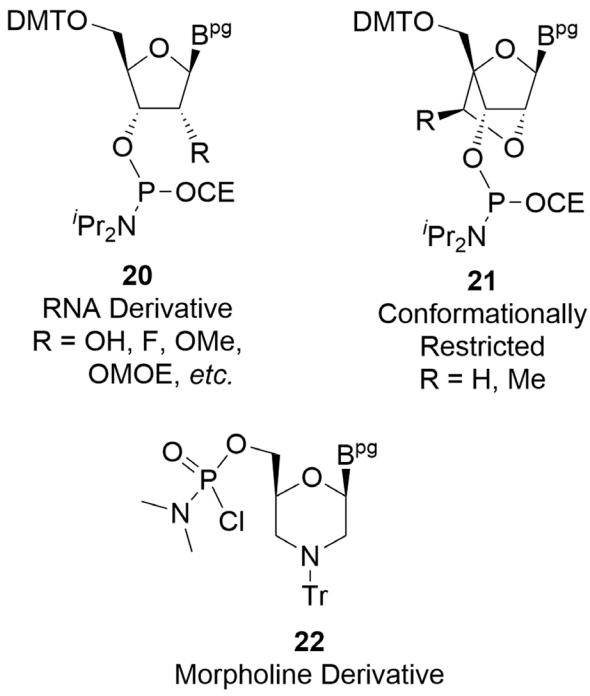
GC, gas chromatography; KF, Karl Fischer; LC-UV-MS, liquid chromatography with ultraviolet and mass spectrometry detection; MoIM, monoisotopic mass; NLT, not less than; NMT, not more than.

This is due to amplification depending upon how often the individual amidite is used in a specific sequence since the motif will be incorporated at low level during each coupling cycle. This may not be a problem if amidite impurities are qualified appropriately, but could lead to surprises if not anticipated and controlled in the deoxyamidite specification.

More complex amidites

As the oligonucleotide space has matured, a broader range of amidites has been accommodated within sequences (FIGURE 6). The more popular of these have been those derived from RNA nucleotides 20 such as 2′-F [16] and 2′-OMe [17], more elaborate versions such as the LNAs 21 [18], and other ring systems such as the morpholinos 22 that

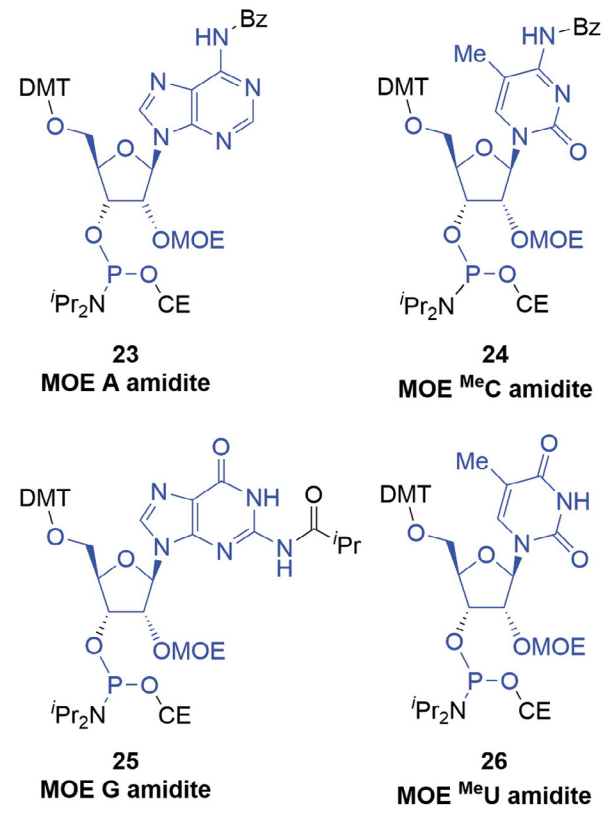
FIGURE 6: Examples of more complex amidites.



lead to the phosphomorpholino oligonucleotides (PMOs) [19], as well as many others. It should come as no surprise that the same approaches and impurity classifications identified for the deoxyamidites can be applied to these more complex amidites.

One of the most popular ribose-derived monomers used in oligonucleotide APIs are the 2′-*O*-(2-methoxyethyl)ribonucleoside amidites (MOE amidites, 23–26 R = OMOE) (FIGURE 7). MOE amidites are SMs for a number of marketed products (Kynamro, Tegsedi, Spinraza, and Waylivra) and will be used to extend the deoxyamidite argument into a more complex case.

FIGURE 7: MOE amidites (atoms marked in *blue* are incorporated into the oligonucleotide). MOE, 2′-(methoxyethoxy)ribose.



These materials are coupled in the oligonucleotide synthesis in the same manner as deoxyamidites. They are also stable solids and their raw materials (non-animal sourced nucleosides 27) are available in ton quantities. The synthesis of commercially available MOE amidites follows a general approach dependent on whether they bear purine or pyrimidine bases (FIGURE 8).

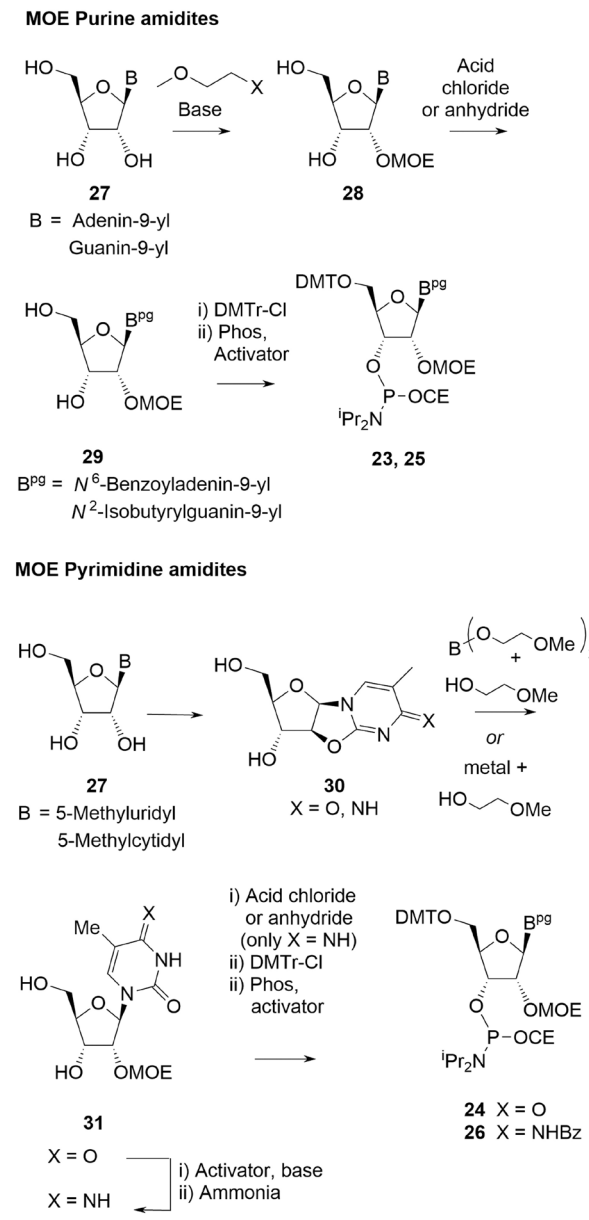
Introduction of the MOE group onto the 2′-hydroxyl of nucleoside 27 is achieved

in one of two ways. If 24 possesses a purine nucleobase (ie, adenine and guanine), direct alkylation of the nucleoside at the 2′-hydroxyl is achieved with an activated form of 2-methoxyethanol [20]. Thus, analogous to the deoxyamidites, all the ribose stereocenters of, for example, 23 and 25, are derived directly from the corresponding sugar and stereochemical integrity is maintained during conversion to the amidites. Stereoisomers of 23 or 25 should, therefore, not be treated as CQAs.

When the nucleobase in 27 is a pyrimidine (eg, 5-Me cytosine and 5-Me uracil), it is first converted to a bicyclic oxazolidine 30, inverting the stereochemistry at the 2′-position following an S_N2 mechanism [21]; 30 can only be formed as a single stereochemical isomer following neighboring group displacement of the activated 2′-OH by the nucleobase. Ring opening of 30 with 2-methoxyethanol or a nucleophilic variant also results in inversion of C2′, that is, the overall effect can only be for double inversion at C2′, thus retaining the natural configuration.

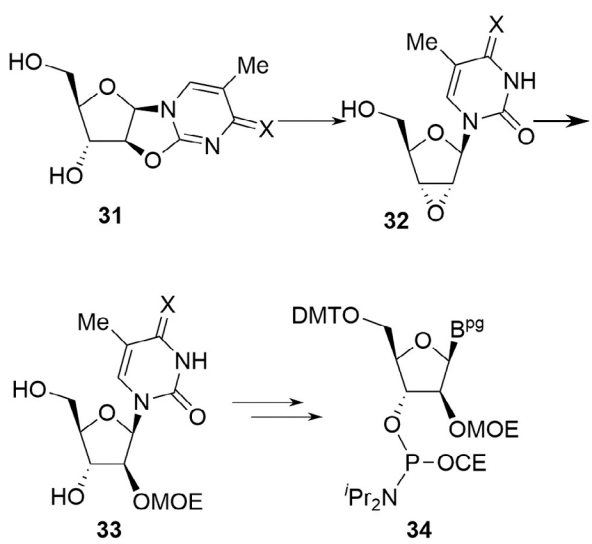
For both ring types, nucleosides 28 and 31 are converted into the corresponding MOE amidites in the same way as for deoxyamidites. The exocyclic primary amines of the nucleobases are acylated (adenine, methylcytosine, and guanine), the 5′-hydroxyl is protected as the DMT ether, and finally, the 3′-hydroxyl is phosphitylated with Phos reagent in the presence of an activator. For pyrimidines, nucleoside 31 (X = O) can also be converted into 31 (X = NH) if required.

FIGURE 8: General approaches to MOE amidites.



In the case of pyrimidine MOE amidites, there is a theoretical risk that 30 could be subject to an alternative reaction involving neighboring group attack from the 3′-hydroxyl leading to epoxide 32 (FIGURE 9). In this situation, epoxide opening of 32 by methoxyethanol would give 33 as an impurity in 31, which, on completion of the synthesis, would be expected to provide 34 as a critical, diastereomeric MOE amidite impurity. As with all such investigations, it is always important to ensure that the potential for impurity formation through reasonable reaction pathways has been considered. In this case, the isomer pathway leading to 34 has never been observed, despite extensive investigations (McPherson A, 2020, unpublished data), although it might be viewed as prudent to demonstrate specificity for 34 in the release methods, for example, 24 and 26. As a consequence of this observation and, since the integrity of all other stereocenters remains intact during conversion of 27 to 24 or 26, the stereochemical integrity of pyrimidine MOEs is not treated as a CQA.

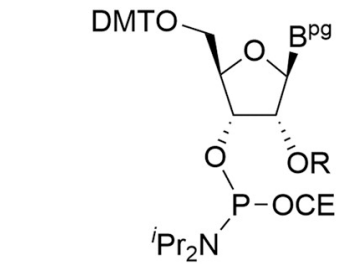
FIGURE 9: Theoretical C-2′ inversion of MOE amidite precursors.



In the same way as for deoxyamidites, MOE amidite impurities can be treated as noncritical or critical and the same conditions apply. Species that do not react (eg, solvents, phosphonates, phosphonoamidates, and phosphoramidates), either do not contain an activatable amidite group or are analogous to the reactive, noncritical deoxyamidite impurities described earlier and are readily removed during purification. Introduction of the alkyl side chain at the 2′-*O* position does introduce some 2′-*O* impurities 35 that can be incorporated into the oligonucleotide during synthesis and should be considered critical impurities (FIGURE 10). These alkylation impurities are monitored in the MOE amidites by HPLC-UV-MS and are readily controlled in the SM specifications.

As with deoxyamidites, MOE amidites carry the potential for regioisomeric impurities 36 (sometimes called inverted amidites) if tritylation occurs on the 3′-hydroxyl and phosphitylation occurs on the 5′-hydroxyl (FIGURE 11). Furthermore, an alternative 3′-alkylation of purine MOE nucleosides could occur followed by 2′-phosphitylation to form a different set of regioisomer impurities 37.

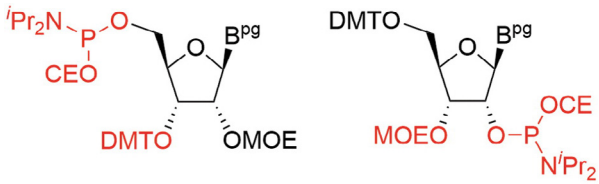
FIGURE 10: 2′-*O* impurities in MOE amidites.



35

R = Me, Et, *n*-Bu, CH_2CH_2OEt, $CH_2CH_2OCH_2CH_2OMe$

FIGURE 11: Regioisomeric MOE amidite impurities.



36 **37**

Any oligonucleotide impurity derived from 36 and 37 would be not be removed during downstream processing due to the similarity with the parent; 36 and 37 are therefore controlled by purification in the MOE amidite SM synthesis and by SM specifications.

As might be expected from the additional manipulations in their synthesis, MOE amidites typically have more critical impurities than their deoxyamidite analogs. Of 104 lots of materials manufactured on multi-kg scales by 4 vendors, critical impurities ranged from below the limit of detection (<0.04% a/a) to a high of 0.8% a/a (TABLE 5). Similar summaries on assay, purity by ^{31}P NMR, water content, and residual solvents show that MOE amidites are manufactured to a consistently high quality from multiple vendors.

Generally, MOE amidite specifications track water content and solvents similar to

TABLE 5: MOE amidite impurity range summary.

Amidite	No. of lots	Assay (% w/w)	Purity by ^{31}P NMR (% a/a)	Total critical impurities (% a/a)	Water (% w/w)	Residual solvents (% w/w)
MOE ^{Me}U	24	91–101	95–100	0.04–0.8	0.1–0.5	0.3–1.7
MOE ^{Me}C	28	95–101	97–100	0.04–0.25	0.1–0.3	0.2–2
MOE A	25	97–101	97–100	0.04–0.4	0.1–0.7	0.3–1.7
MOE G	27	92–98	96–99	0.04–0.7	0.2–0.9	0.3–3.2

MOE amidites in Table 6 have the base protection schemes depicted in Fig. 5.
MOE, 2′-(methoxyethoxy)ribose.

TABLE 6: Example impurity specifications for MOE amidites.

Test	Method	Acceptance criterion	
		Critical Impurity	NMT 0.2–0.4% a/a
		Any unspecified critical impurity[b]	NMT 0.15% a/a
Impurity profile	HPLC[a]	Total critical impurities	NMT 0.5–0.8% a/a
Purity	^{31}P NMR		NLT 95.0% a/a

[a] Both UV and MS detection methods have been applied.

[b] Any impurity containing both a amidite moiety and a DMT protecting group may be critical and should be investigated; all other impurities are noncritical.

HPLC, high-performance liquid chromatography.

those for deoxyamidites, but mainly focus on control of critical impurities and overall purity. Typical values for impurity limits used in clinical and commercial products by EPOC partners are outlined (TABLE 6) (Note: limits should be defined and be fit for each new oligonucleotide sequence and tailored to the controls for the specific manufacturing process—one size does not fit all). The specifications for individual critical impurities and the totals are somewhat higher than for the corresponding deoxyamidites. This reflects that higher levels of detectable impurities have been observed and used successfully in clinical and commercial manufacturing by EPOC partners.

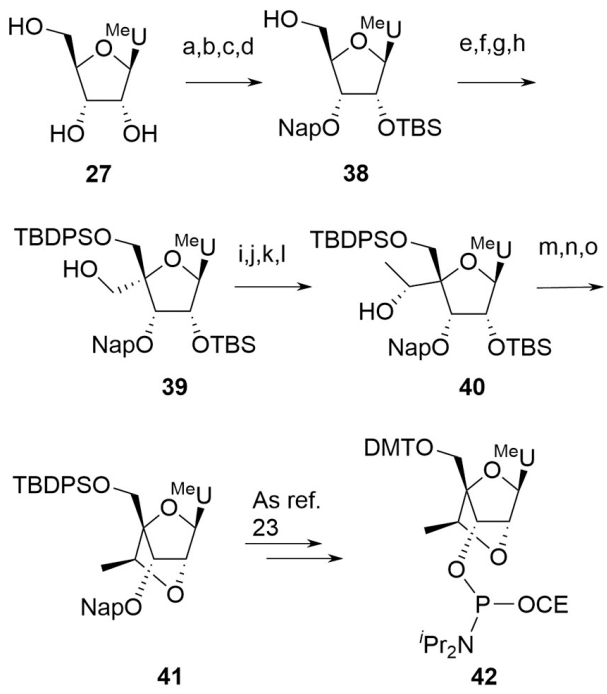
All the points raised for deoxyamidites and MOE amidites can be extended further for even more complex amidites. These are often proprietary in nature, which brings the additional complication that supply chains may not be so well established as the deoxyamidites and MOE amidites. In addition, multiple synthetic routes might be employed to deliver materials.

The LNA derivatives are typical examples of this additional complexity and, in the case of the more challenging constrained ethyl (cEt) amidites (cEts, 21 R = Me) (FIGURE 6), a number of chemical routes to these materials have been published [22–24]. A common characteristic is that all follow lengthy synthetic sequences, although the final stages are similar.

Salinas *et al.*'s approach [23] (FIGURE 12) is a linear synthesis and starts from the appropriate RNA nucleoside 26 in a similar manner to the MOE amidites. Although only demonstrated for cEt ^{Me}U 42, extension to other amidites should be possible.

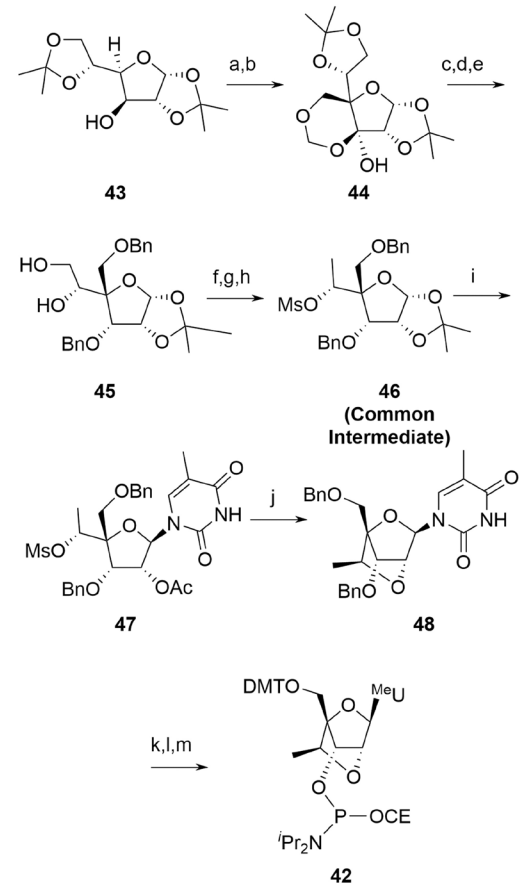
The Seth *et al.* [22] and Blade *et al.* [24] syntheses offer a more complete approach

FIGURE 12: Salinas cEt route.



Reagents. a) 2-NapCH(OMe)$_2$; b) $TiCl_4$, $NaBH_3CN$; c) TBSCl; d) TFA, AcOH; e) Dess-Martin periodinane (DMP); f) CH_2O then $NaBH_3CN$; g) TrCl; h) TBDPSCl then TFA; i) DMP; j) $AlMe_3$; k) DMP; l) $LiAlH_4$; m) MsCl; n) HCl; o) K_2CO_3

FIGURE 13: Blade cEt route.



Reagents. a) NaOCl. TEMPO, KBr, NaHCO, DCM; b) CH_2O, Et_3N, 2-MeTHF; c) $NaBH_4$, MeOH; d) BnBr, NaOH, Bu_4NHSO_4, 2-MeTHF; e) HCO_2H, AcOH, water; f) TsCl, $BnN(Et)_3Cl$, NaOH, toluene; g) $LiAlH_4$, THF; h) MsCl, DMAP, MTBE; i) AcOH, H_2SO_4, EtOAc then thymine, bis(trimethylsilyl)acetamide, TMSOTf, toluene; j) NaOH, MeOH; k) H_2, $Pd(OH)_2/C$, EtOH; l) DMTCl m) Phos, DCl

to delivering a range of cEt amidites. In both cases, a linear sequence provides a common intermediate such as 46 (FIGURE 13), which supports a divergent approach to the required amidites; 46 contains the key skeletal elements present in the final amidite, although not in the final, structural presentation. Given acceptable molecular properties, 46 also represents a convenient storage point if flexibility is required, for example, to support a broad development portfolio. The Salinas synthesis is identical to that of Seth's from 39 onward. A similar divergent approach is applied toward the LNAs (21, R = H) [25,26].

The nature of the route (divergent vs. linear) is important since critical reactive impurities would generally be expected to possess comparable kinetics to the required phosphoramidites during coupling. The linear

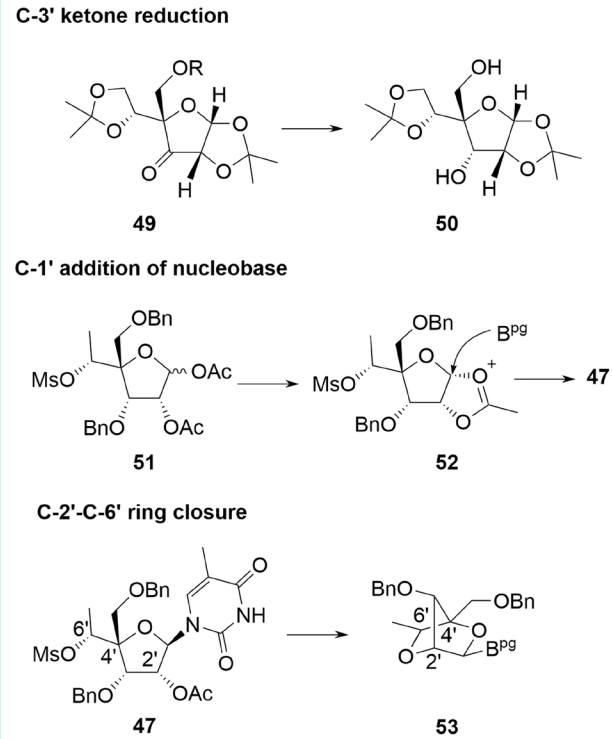
approach reflects a more traditional situation for SMs where provision of each cEt amidite can be viewed as an independent activity with impurity identification and purging only relevant to that specific SM.

In the case of the divergent approaches, the situation is somewhat more complex. Any inherent impurity not purged before isolation of 46 could, in principle, lead to analogous impurities in all cEt amidites generated from that batch, and in turn result in higher levels of the corresponding oligonucleotide impurity. Impurities generated downstream from 46 will be discrete to the individual cEt amidites, although the chemistry across the divergent stages is quite similar and therefore one might see common issues to various extents.

In practice, the linear approach is not pursued at scale and cEt amidites are produced by one or other of the divergent syntheses. As with deoxyamidites and MOE amidites, the precursor di(acetone) glucose is chiral, naturally derived, and well characterized with unambiguous stereochemistry. A key difference in the case of the cEt amidites is that reaction occurs at four of the five original stereocenters with overall inversion at each, potentially resulting in a more complex chemistry to follow for the SM. Confidence in stereochemical integrity can be provided by approaches such as X-ray structural elucidation or NMR correlation studies following key transformations. Some general mechanistic observations can also be applied, however, which further mitigate the potential impact of this apparent complexity:

- The stereochemistry at C-3′ is inverted from that originally in di(acetone) glucose as a result of oxidation and later reduction. This is a common reaction sequence on protected glucose to invert C-3′ and, hence, obtain less common sugars [27]. Steric crowding ensures that delivery of hydride during reduction of 49 (FIGURE 14) occurs from the convex face of the [3.3.0] ring system, resulting in the desired 3′-(*S*) configuration in 50.

FIGURE 14: Stereochemical control in cEt amidites.



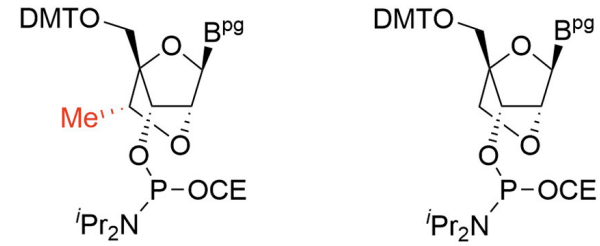
- The base is added through a Vorbrüggen reaction [28,29], whereby an equilibrating mixture of activated C-1′ acetate isomers 51 is internally displaced by participation of the C-2′ acetate, giving acetoxy-bridged intermediate 52 (FIGURE 14). This is a well-documented process and is followed by opening of 52 at the activated C-1′ by the nucleobase leading to 47. This again occurs from the convex face of a [3.3.0] bicyclic system resulting in the desired 1′-(*R*) configuration.
- Hydrolysis of the C-2′ acetate in 47 induces nucleophilic attack of the oxyanion on to the C-6′ mesylate to provide the cEt bicyclo [2.2.1] framework 53 (FIGURE 14). This mandates the relationship between C-2′ and C-4′ since both the 2′-OH and the mesylate-bearing C-4′ side chain must be on the same face to successfully react. Since C-2′ retains the natural configuration found in glucose, this provides additional confidence for the stereochemistry at both C-2′ and C-4′. Also, in this step, S_N2 displacement of the C-6′ mesylate sets the stereochemistry at C-6′ by inversion of the natural glucose stereochemistry.

Over and above the previously described concerns for deoxyamidites and MOE amidites, the major novel challenges for cEt amidites arise from the following:

- Control of stereochemistry for the pendant 6′-Me on the 2′-4′ bridging group
- 6′-Me-deletion impurity (*M-14*, 55).

For all approaches, there is potential for low levels of the 6′-(*R*) diastereomer 54 to be formed (FIGURE 15). Dependent on the synthesis employed, this is either a consequence of incomplete stereochemical inversion during an oxidation/reduction sequence (39→40) or through activation of the secondary alcohol rather than the primary during an epoxide formation (45→46); 54 can be readily identified at the point of formation, but reacts in subsequent steps in a similar manner to the parent 6′-(*S*) isomer and the resulting impurities can be tracked through the synthesis.

FIGURE 15: Critical cEt amidite impurities.



Impurity 55 is a feature of both the Seth and Salinas approaches arising from incomplete reaction during methyl addition to an aldehyde (39→40). Such an impurity is to be expected and

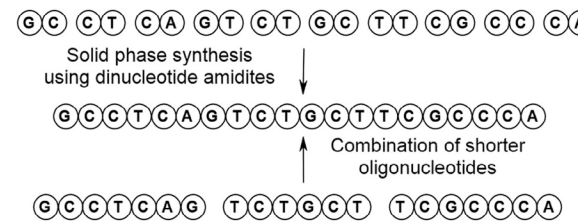
the requirement for a demonstrable understanding of its fate should be anticipated. The Blade approach avoids this methylation step completely and a paper assessment had ruled out this impurity motif. Surprisingly, 55 was observed as a byproduct, proposed to result from an unanticipated bond cleavage mechanism. This observation further emphasizes the need for vigilance during SM synthesis as with the previous situation relating to the anticipated, but unobserved 2′-diastereomer for MOE pyrimidine amidites 34. The importance of a detailed understanding of generation and fate of impurities rather than taking an assumed position cannot be overemphasized. Once such an impurity is observed and identified, actions can be taken to proactively purge if deemed appropriate to reach desired quality levels (eg, through reactive chemistry or recrystallization of an intermediate). The impact of purging on these impurities is presented (TABLE 7).

TABLE 7: Purging of impurities 54 And 55 during constrained ethyl manufacture.

Stage	*54* (% a/a)	*55* (% a/a)
Initial formation	0.24–0.30	0.7–1.0
26	0.05–0.12	0.5
cEt amidite	ND	0.1–0.2

cEt, constrained ethyl.

FIGURE 16: Critical cEt amidite impurities.



Alternative synthetic approaches to oligonucleotides

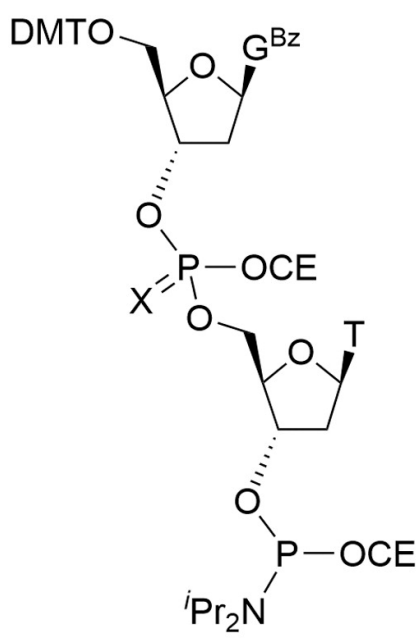
In comparison with small molecule synthesis where convergent approaches are viewed as desirable, oligonucleotide synthesis has largely remained as a linear (and lengthy) exercise. In an effort to introduce convergency, alternative approaches have been considered such as the use of dinucleotide amidites [12,30–33] or the combination of shorter oligonucleotides as demonstrated using the templated ligation approach exemplified by Crameri *et al.* (FIGURE 16) [34].

Such approaches are usually described as blockmers as exemplified by the deoxy GT blockmer amidite 56 (FIGURE 17). Similar criteria to that described previously should be applied when designating SMs for these alternative approaches. A number of obvious additional challenges can be identified due to the existence of the internucleotide phosphorus linker. The first is associated with the coupling cycle required to deliver 56. This results in a series

of impurities more typically associated with finished oligonucleotides, for example, coupling failures, overcoupling, P = O (for X = S). Thus the set of reactive, critical impurities may be more significant than for the corresponding monomer deoxyamidites.

The presence of the second phosphorous functional group compounds this issue. The amidite group has already been identified previously as bringing a mixture of diastereomers. Aside from the special case of a stereo-specific phosphorothioate, the blockmer introduces a second, variable, chiral element leading to all discrete species being present as 4 diastereomers. In the case of a phosphate linkage, this complexity does not extend through to the API where the phosphate is achiral.

FIGURE 17: Representative blockmer deoxy amidite.



56
X= : [P(III)]
O, S [P(V)]

This additional stereochemistry has a dual impact since signal/noise is reduced and the number of potentially observable components increased, both by a factor of approximately two over standard monomer amidites. The consequence is that blockmers present greater technical challenges in identifying/quantifying impurities, thus increasing complexity during the development of an appropriate control strategy for these molecules. A blockmer approach will also require a larger number of potential SMs across a portfolio of projects rather than the limited number of monomer amidites generally employed to manufacture oligonucleotides.

No marketed oligonucleotides currently apply such convergent approaches, but as the pressure to supply ever larger quantities of oligonucleotides to meet growing patient demands continues to build, scalable alternatives to the current solid-supported manufacturing process may entertain these types of routes. The Q&A for ICH Q11 advises that convergent syntheses are acceptable and in answer to question 3, "ICH Q11 general principles apply to the selection of starting materials for linear or convergent syntheses [1]. The ICH Q11 general principles should be applied independently

to each branch of a convergent synthesis, unless the point of convergence of the branches occurs upstream of an appropriate starting material." In addition, although a blockmer contains multiple nucleotide subunits, they may be considered SMs as highlighted in answer to question 2, "ICH Q7 states that an 'API starting material' is a raw material, intermediate, or an API that is used in the production of an API. When a chemical, including one that is also an API, is proposed to be a SM, all ICH Q11 general principles still need to be considered." More optimistically, it should be noted in the conceptually similar field of peptides that, at least one EPOC member company has reported successful justification of 2-mer peptides as SMs for the construction of larger peptides. In that case, several agencies accepted a specification for dipeptides that comprised comprehensive identity (composition and sequence), purity and impurity profile, chiral purity, water (Karl Fischer [KF]) or limit of detection (LoD), and residual solvents (A. Charaf, 2019, personal communication).

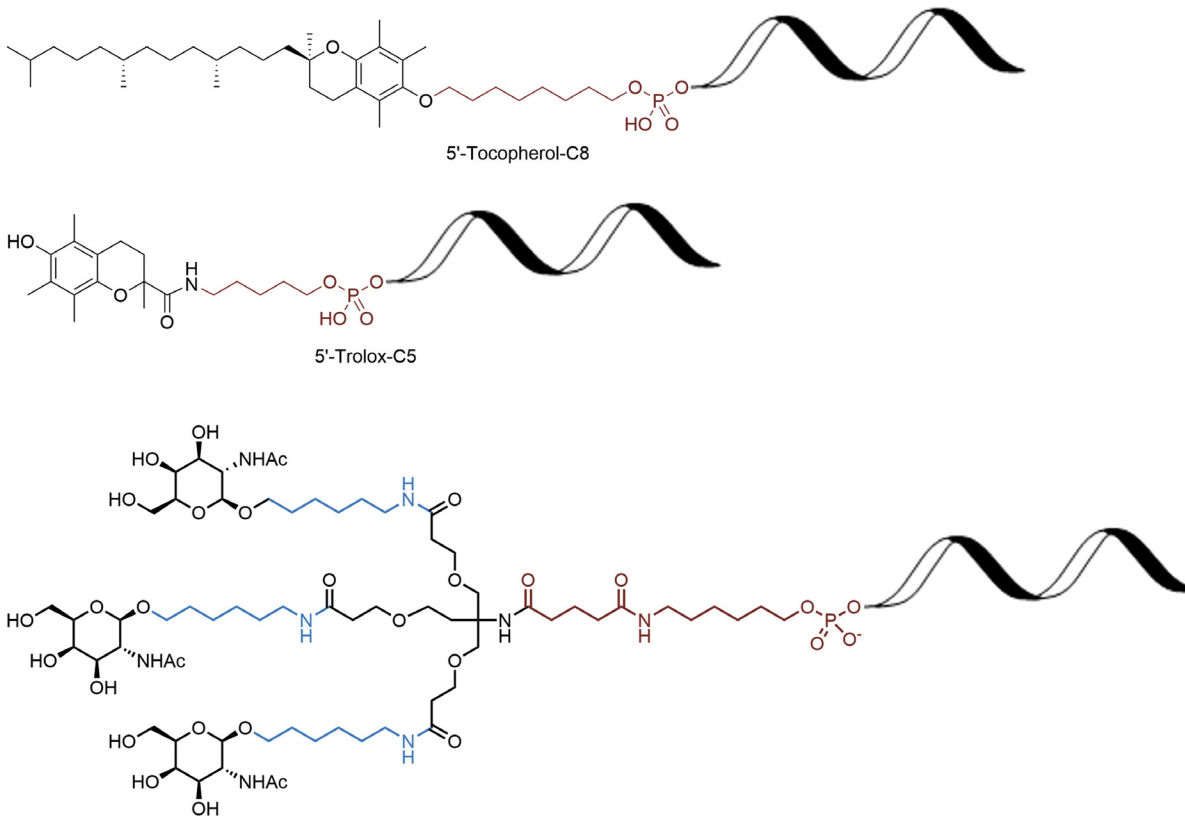
Conjugating agents

Due to the cost of oligonucleotides, efforts to reduce patient dosage have become more important. Besides modified nucleotide chemistries, the use of conjugates has become more common to improve pharmacokinetics and distribution and facilitate cellular uptake mainly for antisense oligonucleotide (ASO) and small interfering RNA (siRNAs). The most common modifications are conjugate agents that are small molecules attached to the oligonucleotide (FIGURE 18) such as cholesterol [35], tocopherol [36], anisamide [37], folic acid [38], peptides [39], anandamide [40], *N*-acetyl-d-galactosamine (GalNAc) [41], and poly(ethyleneglycol) ethers (PEGs) [42]. These are generally attached to the oligonucleotide through a cleavable tether often referred to as a linker (highlighted in red, FIGURES 18 and 19). There is further activity, whereby large molecules such as antibodies [43] and aptamers [44] are applied, although this is out of scope for this discussion.

Most of these modifications can occur at either the 3′ or 5′ end of the oligonucleotide, although other modifications are also possible such as an internucleotide phosphonate [45].

N-acetyl-d-galactosamine (GalNAc) conjugation has become increasingly popular for the targeted delivery of chemically modified oligonucleotides to hepatocytes through binding to ASGR (asialoglycoprotein receptor) [46,47]. Although the chemical modification can take several forms, they retain a common feature, in that, several GalNAc moieties (typically 3—the triantennary structure) are connected to an oligonucleotide through a linker for optimal binding. Beyond this, a variety of differences in the nature of the spacer (eg, alkyl, ethylene glycol) and the point of attachment (eg, tris and lysine-lysine) to the oligonucleotide have been applied. These are schematically summarized (FIGURE 19) for the most widely used approaches [48,49].

FIGURE 18: Selected small molecule conjugates.

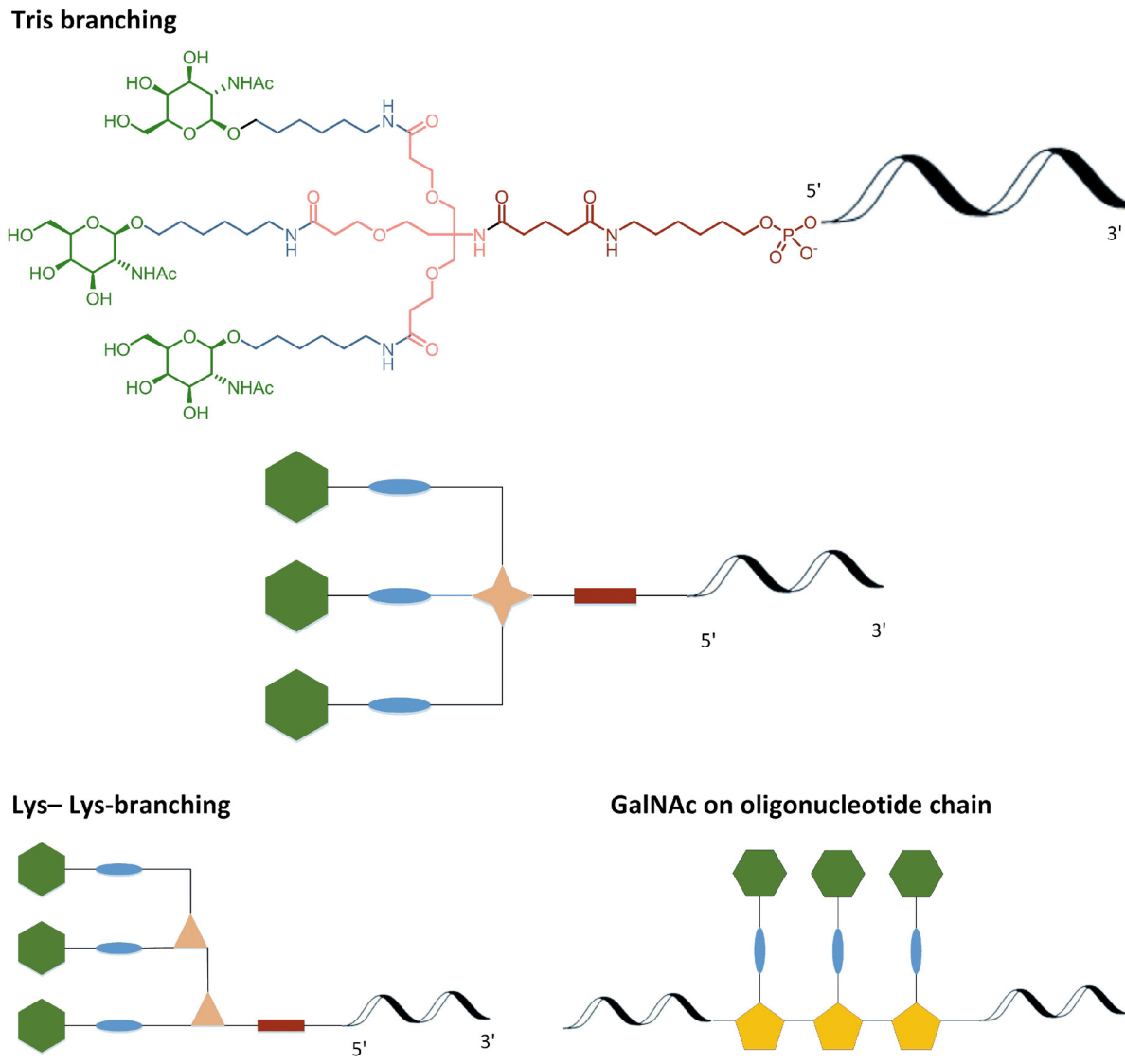


Attachment of GalNAc at the 5′-oligonucleotide terminus serves as a useful example of the treatment of conjugate fragments. The conjugation can be done after the solid-phase synthesis or starting with the oligonucleotide construct loaded on the solid support (FIGURE 20) [**50**].

As an example of the approach, this publication will focus on post-oligonucleotide synthesis conjugation, that is, "5′-GalNAc," specifically bis-lysine cluster 58. "GalNAc on oligonucleotide" and "3′-GalNAc" [41] will not be discussed, although the principles set out are equally applicable.

For 5′-GalNAc, completion of the oligonucleotide fragment synthesis is typically followed by the solid-phase coupling of a spacer amidite (such as 57) and, finally, by the solution-phase coupling of the fully assembled GalNAc cluster, for example, 58. Compared to naked oligonucleotides, GalNAc-conjugated oligonucleotides therefore contain two additional significant structural elements of the API. In a similar way to the (deoxy)ribose

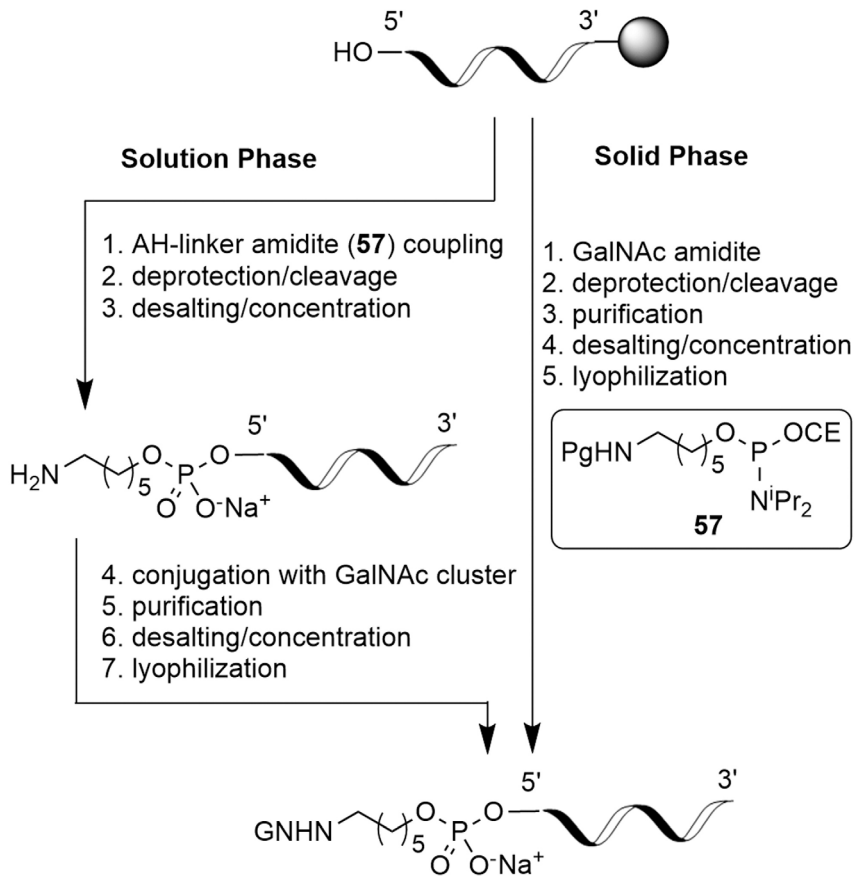
FIGURE 19: Selection of GalNAc attachment motifs. GalNAc, N-acetylgalactosamine.



amidites, this fulfills the most fundamental criterion for an SM.

Based on ICH Q11, "enough of the drug substance manufacturing process should be described in the application. …" In the case of 5′-GalNAc conjugates, the 5′-GalNAc cluster is typically introduced in the final synthetic step offering fewer transformations than would be considered acceptable in the realm of traditional small molecules. However, the extensive downstream processing, including chromatographic purification, desalting by ultrafiltration/diafiltration, and isolation,

FIGURE 20: 5′-GalNAc conjugation options.



may compensate for the reduced number of chemical transformations.

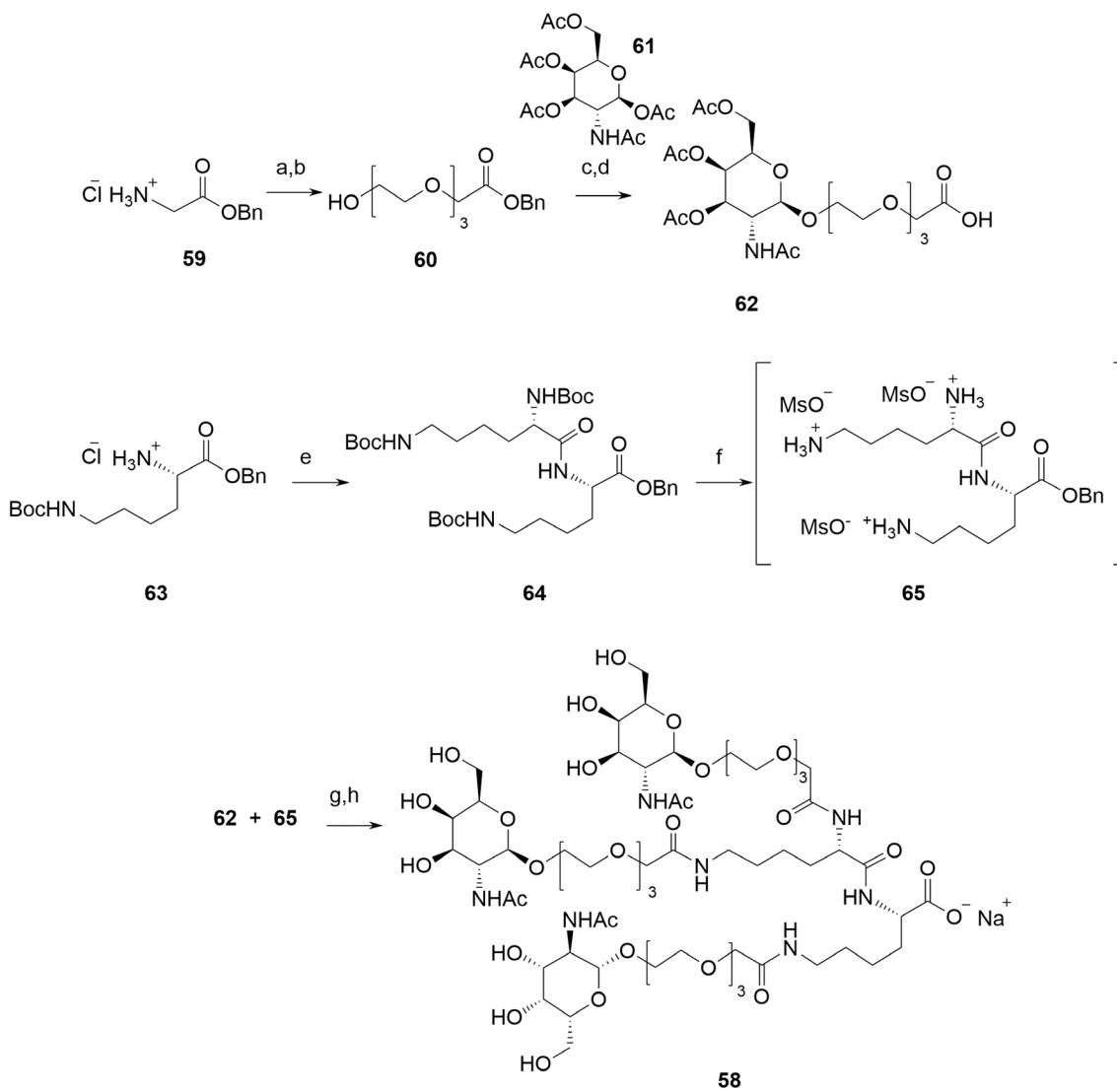
The linker used in 5′-GalNAc conjugates is typically a protected 6-aminohexyl phosphoramidite (6-AH) such as 57. This is of a similar size to conventional small molecule SMs and can therefore be treated as such; it will not be discussed in any further detail. GalNAc cluster 58 is a typical example of its type and features a bis-lysine moiety to allow for sufficient branching and a triethyleneglycol spacer to enable additional separation of GalNAc from oligonucleotide (FIGURE 21).

The convergent synthesis commences with benzyl glycinate 59, which is converted to triethyleneglycol spacer 60 in a diazotization-promoted substitution [51]. Glycosidation with *N*-acetyl-d-galactosamine tetraacetate 61 mediated by TMSOTf provides β-anomer 62. The *bis*-lysine coupling partner 65 is readily prepared from

Boc-protected l-lysine benzyl ester 63, which is coupled to bis-Boc-protected l-lysine under standard conditions (T3P, DIPEA) to give 64 followed by amine deprotection under acidic conditions to give 65.

Crude 65 is used without further purification and coupled with 62 using T3P to give the protected GalNAc cluster, which is taken to the global deprotection step (aq. NaOH and MeOH) without further

FIGURE 21: Synthesis of GalNAc Cluster 58.



Reagents. a) $NaNO_2$, H_2O, toluene; b) Triethylene glycol, $BF_3.OEt_2$, CH_2Cl_2; d) Pd/C, H_2, THF; e) Boc-Lys(Boc)-OH, T3P, $^{i}PrNEt_2$, THF; f) MsOH, MeOH; g) T3P, THF, $^{i}PrNEt_2$, EtOAc, MeCN; h) NaOH, MeOH

purification. GalNAc cluster 58 is purified using preparative HPLC.

58 is an isolated, well-characterized, and stable amorphous material manufactured using a reliable and robust process. Its precursors are naturally occurring amino acid or sugar building blocks, which are readily available in ton scale in enantiomerically pure form. To date, the synthesis was carried out on multi-kg scale with appropriate analytical controls using standard techniques (HPLC).

It is important to point out preparative HPLC purification of 58 should not be considered a specific "unit operation" in the sense laid out in ICH Q11 Q&A. The choice of this technique is motivated rather by the lack of crystallinity of 58 than by failure of other purification techniques to provide material of sufficient quality.

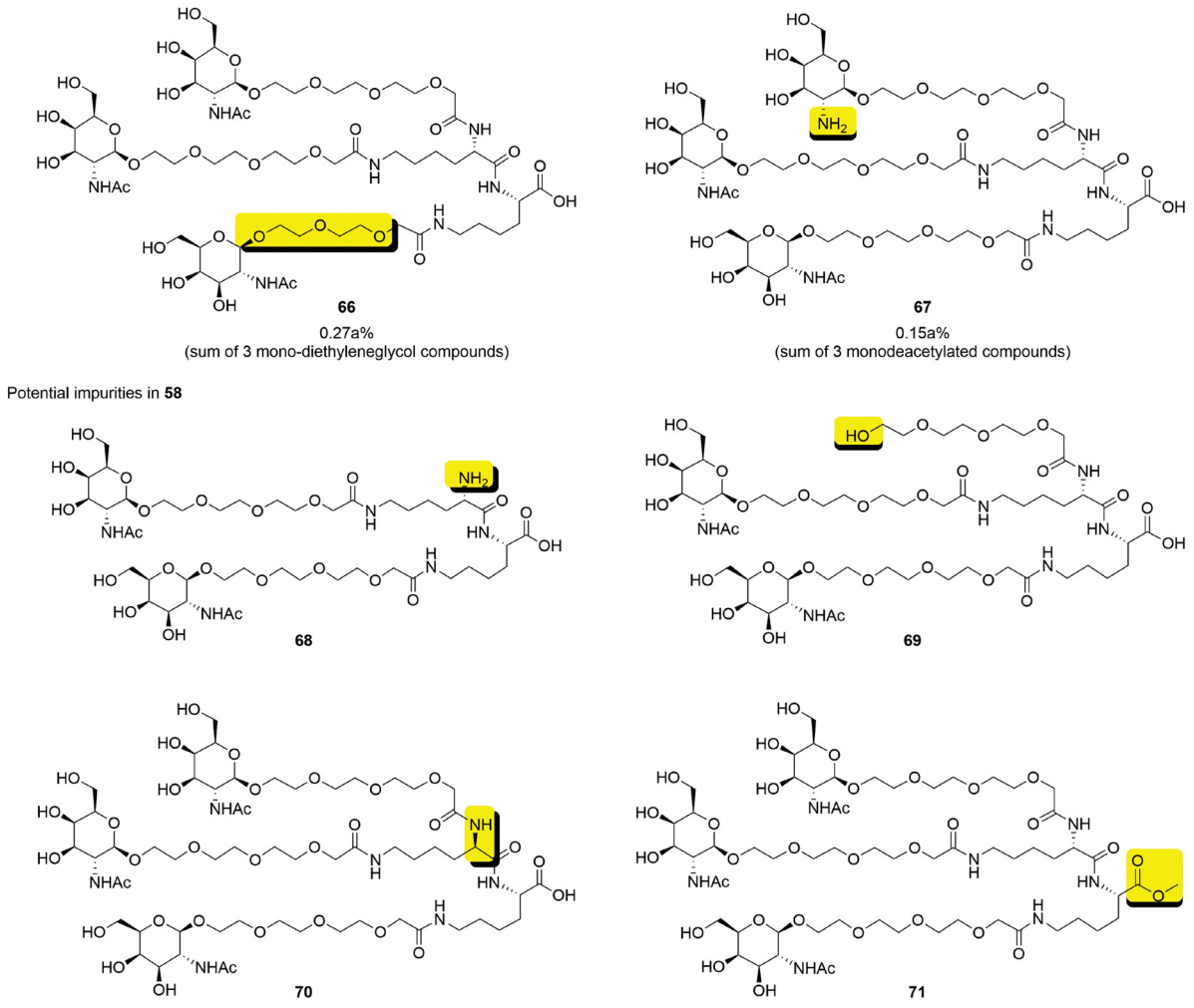
Even at a relatively early stage of development, a number of potential and actual impurities in 58 have been identified (FIGURE 22). Potential process-related impurities will be continuously evaluated during further development. Any new unknown impurities detected above the reporting limit of the analytical method in future batches will be characterized, and their fate will be investigated in the subsequent processing steps, if needed.

The only impurities present in 58 at a level >0.10% area are critical impurities 66 (0.27% area[‡]) and 67 (0.15% area[§]). An impact of these two impurities on a drug substance (DS) CQA can be excluded based on their specification level in 58 and hence, no additional fate-of-impurity data are required. Impurities 68 and 69 are controlled by the process and their levels are below the reporting limit in 58. It is advisable to ensure that analytical methods can assess the configuration of stereocenters, which are synthetically derived and/or prone to epimerization (eg, anomeric center of galactosamine or α-position of lysine amino acids in 70). The nonreactive, noncritical impurity 71 has been shown to be depleted in the downstream purification step. In the context of ICH Q11, "impact on DS quality" is defined as level above the identification threshold. Since the amount present in GalNAc is significantly lower than the ID threshold in the API, an impact can be excluded.

The specification of 58 is based on current knowledge and will be revised as additional batch history data and/or process development data become available before the manufacture of the commercial drug substance batches.

Given the breadth of different GalNAc clusters used across the industry and the above-mentioned lack of regulatory guidance for oligonucleotides, a general recommendation regarding their acceptability as SM is difficult. Sponsors are encouraged to evaluate their GalNAc cluster using a science-based approach founded in the principles set forth in ICH Q11 and provide data demonstrating safety

FIGURE 22: Impurities in GalNAc cluster 58 (impurity motif highlighted).



to patients.** The holistic approach outlined in ICH Q11 and associated Q&A provide a good framework for this assessment. Understanding of how the impurity profile of the SM affects the drug substance quality is necessary and should be supported by a sound specification for release testing of GalNAc. The application of GalNAc conjugates in a commercial setting is a very immature area; therefore, the advice presented illustrates scientific concepts that EPOC member companies feel are relevant to support GalNAc and related structures.

Solid support

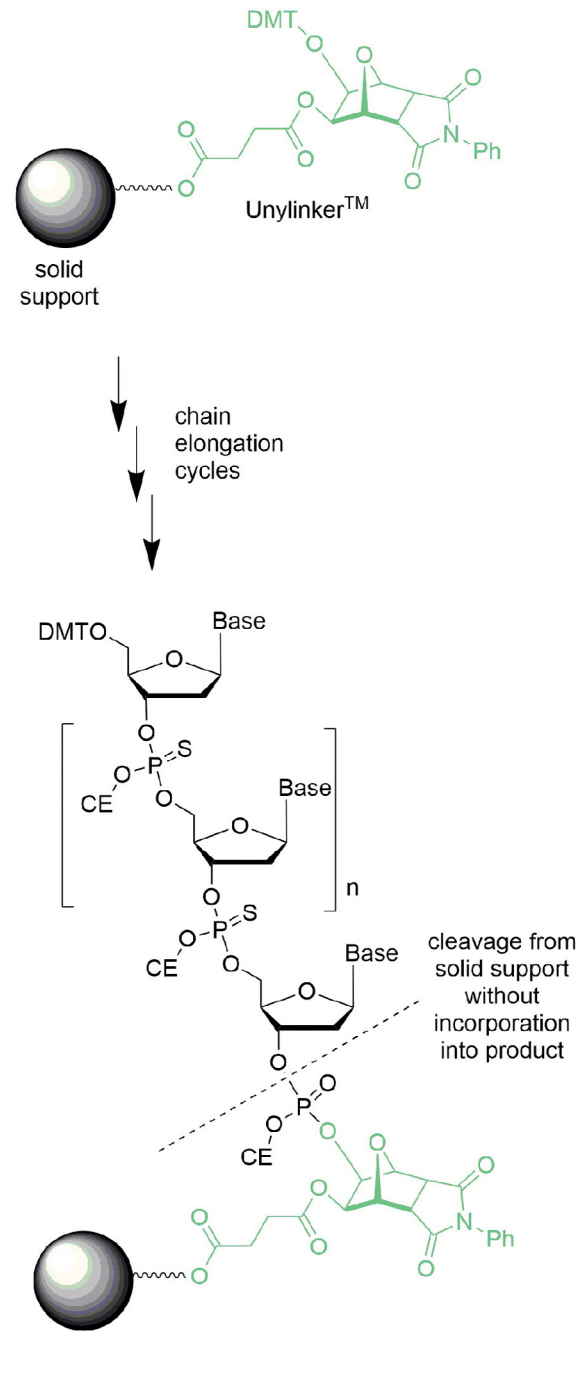
One important element in most oligonucleotide syntheses is the synthesis support (eg, NittoPhaseHL® and †† Primer Support 5G®‡‡) and their derivatives functionalized with an appropriate linker

(eg, UnyLinker®§§ and succinate). Typically, the first manufacturing step begins after deprotection of the commercial support (eg, NittoPhase UnyLinker HL350®) followed by coupling of the first nucleotide. Since the synthesis resin functioned as a stationary phase (or has even been described as a 3′-protecting group) for the automated synthesis, it contributed no material to the oligonucleotide at the end of the process and would be described as a noncontributory raw material (FIGURE 23).

As with other noncontributory raw materials and reagents, the resin still exerts an effect on the synthesis and robust performance during oligonucleotide synthesis under GMP requires monitoring of appropriate attributes of the solid support. Sample preparation is key to examine impurities derived from solid supports and varies with the support [**54**], a few typical attributes are listed (TABLE 8).

Although amidite coupling is generally very high yielding, there can be situations where the first coupling can be more challenging (eg, due to added steric hindrance/ lower reactivity at the secondary alcohol of UnyLinker). Since oligonucleotide manufacturing is quite expensive, knowledge of the yield for the first coupling may be advantageous, but is not readily ascertained during manufacture because of the inability to sample the packed column. One approach to circumvent this difficulty is to apply preloaded supports where the first nucleotide or even 3′-GalNAc is already coupled to the resin. Such resins are available

FIGURE 23: Relationship of NittoPhase UnyLinker® and oligonucleotide.



from most suppliers and loadings can be assayed, therefore increasing confidence for the subsequent manufacture.

In these cases, the input support contains structural elements of the final oligonucleotide and the noncontributory status cannot be applied. As a result, sponsors may seek to justify the preloaded support as an SM. It should be anticipated that loaded solid supports would require more extensive characterization than unmodified supports. For instance, performance characteristics of the support may involve derivatization and/or wet chemical techniques to examine loading capacity or impurities derived from the element that is bound to the loaded support (TABLE 9). There are complexities involved in the validation and routine use of these types of tests; so their necessity should be informed by a well-developed risk assessment that examines the capability of the assays and their ability to control for critical properties of the loaded solid support.

TABLE 8: Standard quality attributes generally monitored for solid supports.

Test	Method	Acceptance criterion
Appearance	Visual examination	Mixture of powder and aggregates
Identification	FTIR by ATR or NIR	Sample spectrum conforms to standard spectrum
DMT loading	Spectrophotometry	Measured in µmol/g
Impurities	HPLC	NMT 0.10% a/a

ATR, Attenuated total reflection spectroscopy; FTIR, Fourier transform infrared spectroscopy; NIR, near-infrared spectroscopy.

TABLE 9: Modified solid support control and characterization.

Physical characteristics	Loading capacity	Quality attributes (cleavage may be required)
Particle size (microscopy)	Use test	Identity of loaded support (IR) or loaded entity (HPLC)
Swell volume (eg, in MeCN)	Use test	Related substances (HPLC) Water (KF) Residual solvents (GC)
Bulk density	Testing for stoichiometry	Loss on drying

IR, infrared.

CONCLUSIONS

In this white paper, we provide guidance on the application of risk-based strategies, founded on the collective experience of member companies of the European Pharma Oligonucleotide Consortium. The purpose is to enable a more uniform approach to the justification of various general classes of oligonucleotide SMs.

As we describe, oligonucleotides are explicitly out of scope with respect to ICH Q11. EPOC member companies have, however, sought to apply the ethos and principles of ICH Q11 in their development activities, but in conjunction with awareness of the practices and knowledge of oligonucleotide processing. We recognize not only that step count in its traditional sense is not a helpful construct for SM justification during oligonucleotide processing but also that chromatographic purification and other downstream operations should serve to provide some mitigation. We describe how a detailed understanding of the synthetic steps in both SM and oligonucleotide processing can support a regulatory SM proposal addressing the importance and fate of impurities and providing confidence for patient safety.

We introduced our position using deoxyamidites 1–4 as the simplest and most common of the SMs used in oligonucleotide API manufacture. These clearly satisfy the criteria for SMs, in line with the guiding principles of ICH Q11:

Impurity classes in these materials are well characterized and well understood
The criticality of the various impurity classes toward impact on API quality are well understood (reactive, critical; reactive, noncritical; and nonreactive, noncritical)
A high level of control is achievable (enabling very stringent purity-focused specifications)

For the related ribonucleoside amidite building blocks, such as the MOE amidites 23–26 and cEt amidites such as 42, similar arguments can be applied. Although these materials are of greater synthetic complexity and can contain larger numbers of impurities than deoxyamidites, the control strategies employed and acceptance criteria in specifications are comparable and driven by the same risk-based principles. Analogously, with the appropriate understanding and control of impurities, amidite dimers or blockmers such as 56 would follow the ICH Q7 and Q11 SM principles as part of convergent oligonucleotide syntheses.

To exemplify oligonucleotide conjugates, a 5′-GalNAc case study of a linker-modified oligonucleotide was used to illustrate two further SM types to consider—the linker amidite 57 and the GalNAc cluster 58. The GalNAc clusters were identified as a larger challenge; consequently, a general recommendation of their acceptability as SM is less straightforward; however, we recommend that sponsors apply the same risk- and science-based approaches defined in ICH Q11 to assess their own particular situation.

This article also gives consideration to the solid supports themselves. For the standard resins and the linkers used for attachment of the initial nucleotide unit to the resin, these are clearly defined as noncontributory raw materials, and the guidance around key attributes associated with use of these noncontributory raw materials is reiterated. In specific cases where a sponsor may choose to employ a functionalized solid support where a significant contributory element of the API is already present on the support, it is possible that further justification as an SM may be required, on a case-by-case basis, with a focus on the characterization and performance characteristics of the functionalized support.

In general, through the cross-industry examples provided in this article, we outline a clear and uniform approach to the designation of SM status for the amidite building blocks utilized in oligonucleotide API manufacture, firmly rooted in the principles of ICH Q11. We have also provided insight and guidance on the designation of more complicated SMs such as those required for oligonucleotide conjugates, as well as setting out a clear position relating to the solid supports required for synthesis. We believe that publication of these arguments will be of great value in enabling both sponsors and regulatory bodies to achieve greater clarity and harmony in justifying the status of SMs for use in cGMP oligonucleotide manufacture.

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AUTHOR DISCLOSURE STATEMENT

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A.F. and M.M. are employed by F. Hoffmann-La Roche Ltd.; T.W. is employed by Sanofi; and S.R. and G.H. are employed by Thermo Fisher Scientific. No competing financial interests exist.

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† Discussions with deoxyamidite vendors indicate that typical activators applied include 4,5-dicyanoimidazole (DCI), 1*H*-tetrazole, 5-(ethylthio)-1*H*-tetrazole (ETT), 5-(benzylthio)-1*H*-tetrazole (BTT), 5-(3,5-bis(trifluoromethylphenyl)-1*H*-tetrazole), 5-(4-nitrophenyl)-1*H*-tetrazole, benzimidazolium triflate, and *N*-methylimidazolium triflate. Activators must possess an aqueous pKa value comparable to acetic acid [$pKa_{(aq)} = 4.5$] and produce a conjugate base that is both a good nucleophile and leaving group. The choice of activator used to phosphitylate the nucleoside precursor does not appear to impact amidite CQAs.

‡ Following oligonucleotide impurity conventions, this level accounts for three different impurities, which have a single diethylene glycol unit in one of the three spacer units.

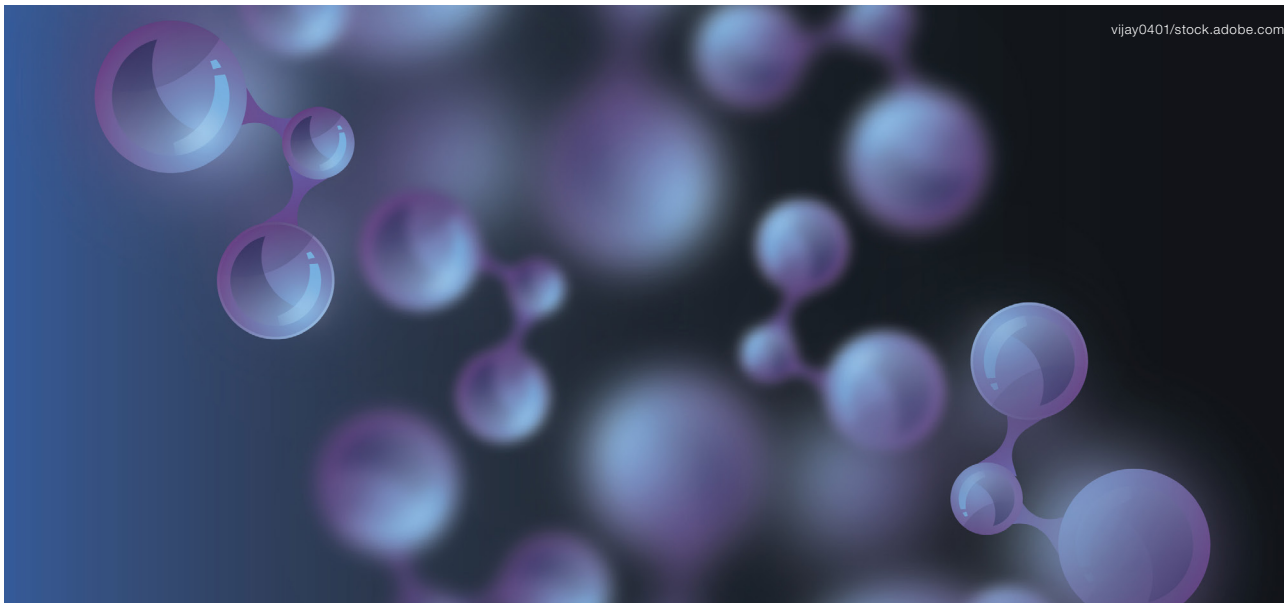
§ This level accounts for three different impurities where any one of the three acetyl groups was cleaved.

** It is important to understand that the GalNAc cluster is a targeting moiety, which is cleaved from the active compound during metabolism and does not modulate the target. Toxicological coverage of the GalNAc cluster (including related impurities) is achieved through data generated with the API in animals and humans [52,53].

†† NittoPhase is a trademark of Nitto Denko Corporation.

‡‡ Primer 5G is a trademark of General Electric companies.

§§ UnyLinker is a trademark of Ionis Pharmaceuticals, Inc.



Therapeutic Oligo Quality: Profiling and Controlling for Raw Material Impurities

By Indra Pal*, Grant Fernstrum, Chandrashekar Gudise and Gary Held

Minimizing and controlling upstream single critical impurities can help reduce stringency of oligo purification and increase overall yields.

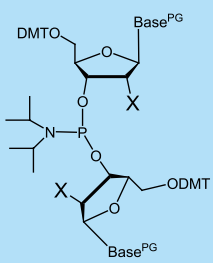
Introduction

As a growing number of therapeutic oligonucleotide compounds continue to be introduced into the clinical pipeline, and advancing into larger, late phase clinical trials, an increasingly stringent demand is placed upon the phosphoramidite supply chain.

With global raw material suppliers scaling up production to meet market demands, heightened concern surrounds the increased potential for generating novel, as well as previously identified, impurities. The impurities found within the material supply chain can directly impact the quality of phosphoramidite synthesis, and thus potentially affect the quality of a therapeutic oligo.

Therefore, an increased focus has been placed upon controlling incoming raw materials, understanding the impact to phosphoramidite chemistry, including impurity profile, and subsequent effects to oligo purity. Thermo Fisher Scientific Milwaukee continues to undertake a comprehensive approach to supply chain management through partnerships with raw material suppliers, as well as customers, to define raw material specifications, including control of impurity levels to satisfy the dynamic quality requirements.

Recently, the Process Development team performed a deep investigation into the quality of an integral raw material, 4-4'-Dimethoxytrityl Chloride (***I***, DMTr-Cl). The team capably identified the role and potential deleterious impact of two potential impurities, 4-Acetoxy-4'-methoxytrityl Chloride (***ii***, AMTr-Cl) and 4-Hydroxy-4'-methoxytrityl Chloride (***iii***, HMTr-Cl), in the synthesized phosphoramidite (illustrated in **Synthetic Schemes**).



The impurities found within the material supply chain can directly impact the quality of phosphoramidite synthesis, and thus potentially affect the quality of a therapeutic oligo.

Discussion

AMTr-Chloride in DMTr-Chloride generates non-critical impurity B. HMTr-Chloride generates the critical impurities C, D, E and F. Impurities A, B, C, D and F have been synthesized and further characterized by ^{31}P NMR, HPLC and LC-MS.

All the critical impurities will produce deletion sequences during oligo synthesis. The number and percentage of total impurities in an oligonucleotide due to these critical impurities in phosphoramidites will depend on the sequence length. For example, a 0.1% critical impurity in the phosphoramidite may generate up to 2.0% of impurities in a 20mer oligo.

Conclusions

Thermo Fisher Scientific collaborates with our suppliers and therapeutic oligo manufacturing and developmental partners to offer phosphoramidites that reflect the high standards for which our TheraPure Phosphoramidtes have been known since 2002.

Synthetic Schemes: 5'-DMT-N^4-Bz-dC- Phosphoramidite (***A***); 5'-AMT-N^4-Bz-dC- Phosphoramidite (***B***); 5'-HMT-N^4-Bz-dC-di Phosphoramidite (***C***); HMT Phosphoramidite (***D***); HMT-di Phosphoramidite (***E***); and N^4-Bz-dC-3',5'-di Phosphoramidite (***F***).

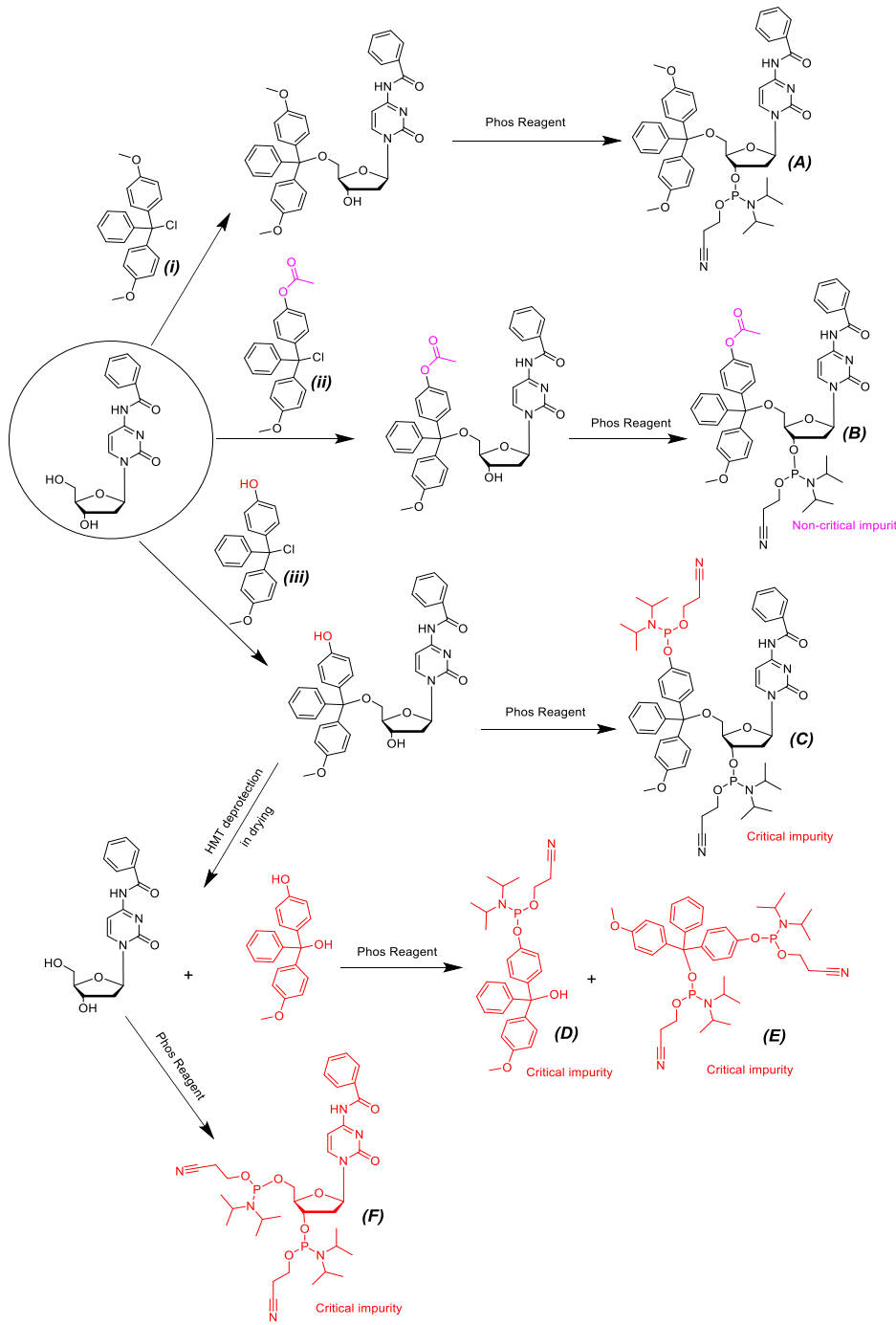


FIGURE 1: ^{31}P NMR Chemical shift comparison of 5'-DMT-N^4-Bz-dC-Phosphoramidite (***A***) and HMT Phosphoramidite (***D***).

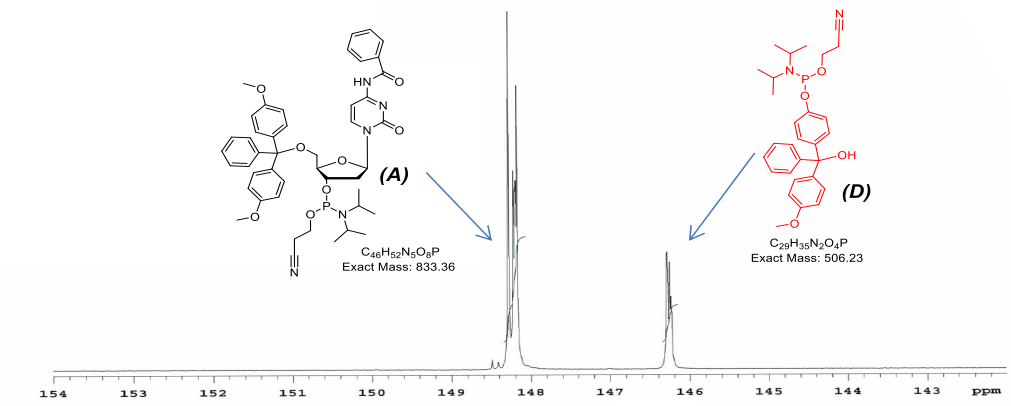


FIGURE 2: ^{31}P NMR Chemical shift comparison of 5'-AMT-N^4-Bz-dC-Phosphoramidite (***B***) and 5'-HMT-N^4-Bz-dC-di Phosphoramidite (***C***).

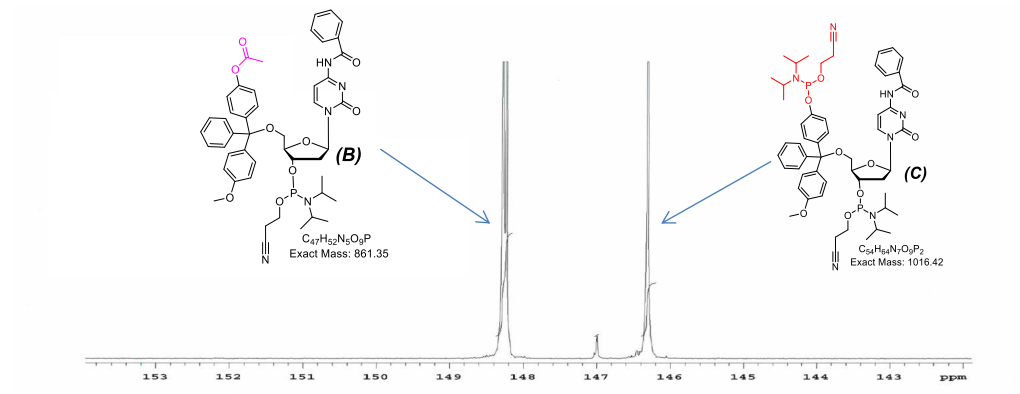


FIGURE 3: ^{31}P NMR Chemical shift of N^4-Bz-dC-3',5' Phosphoramidite (***F***).

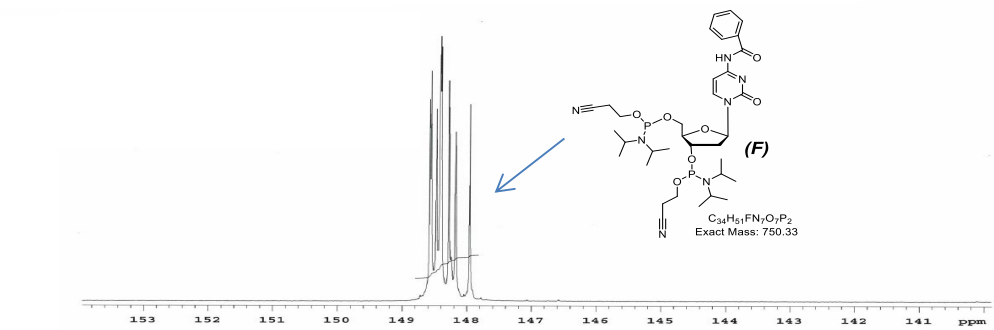
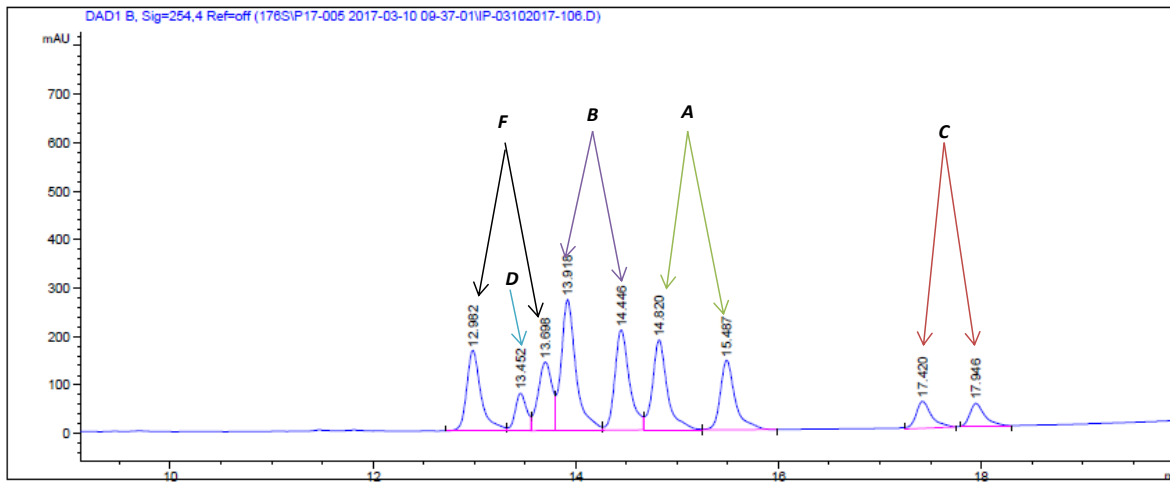


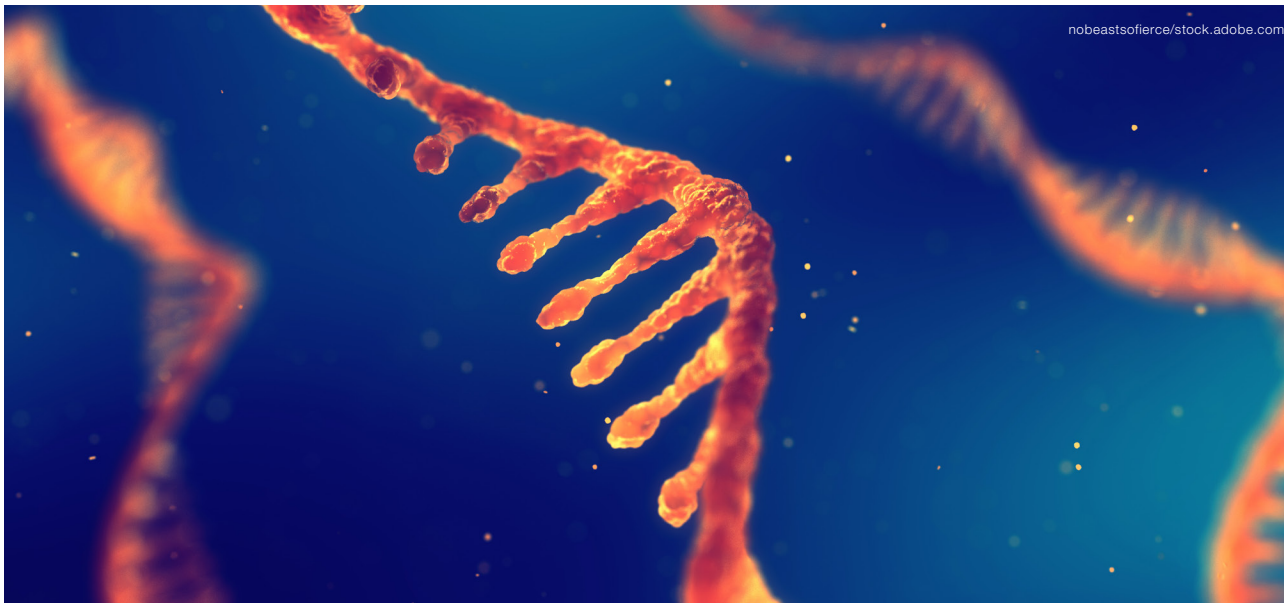
FIGURE 4: HPLC comparisons of 5'-DMT-N^4-Bz-dC-Phosphoramidite (***A***), 5'-AMT-N^4-Bz-dC-Phosphoramidite (***B***), 5'-HMT-N^4-Bz-dC-di Phosphoramidite (***C***), HMT Phosphoramidite (***D***) and N^4-Bz-dC-3',5'-di Phosphoramidite (***F***)



Minimizing and controlling upstream single critical impurities as demonstrated in DMT-Cl, can help reduce stringency of oligo purification and increase overall yields.

Our continuous commitment to the pursuit of deeper control, analytical refinement and quantitation of the phosphoramidite supply chain will maintain Thermo Fisher Scientific as an industry leader and a sustainable partner for the continued growth and safety of oligotherapeutic medicines.

Acknowledgement: We thank Amal Audi, Syed Raza, Rick Matusik, Roger Knight, Amy Pasley, and Michael Singer for their support.



Identification and Quantitation of Oligonucleotides, Impurities, and Degradation Products

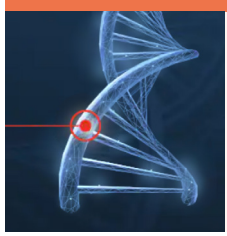
By Haichuan Liu[1], Kevin Guo[2], Jennifer Sutton[1], Keeley Murphy[1], Min Du[2]

The ddMS^2 approach enables confident identification, mapping, and relative quantitation of oligonucleotides and their impurities in a single experiment.

Introduction

Oligonucleotides are synthesized, polymeric sequences of nucleotides (RNA, DNA, and their analogs) that are increasingly being developed as direct therapeutic agents against a wide range of disease conditions. They have attracted increasing attention from the biopharmaceutical industry due to the successes of applying this new modality for the treatment of rare diseases as well as their potential in treating common diseases and even coronavirus disease-2019 (COVID-19).[1-3] Therapeutic oligonucleotides produced by chemical synthesis carry various types of product-related impurities, including deletion sequences ('shortmers'), addition sequences ('longmers'), and the modified full-length species.[4-7]

The n–x shortmers, the most common impurities present in synthetic oligonucleotides, are formed due to failed base coupling at the 5' end followed by incomplete capping, which may also result in n–1 impurities with different single deletions.[4] The longmers are mostly the n+1 or n+2 species, while the modified impurities correspond to the full-length product with modifications on its nucleobases or phosphorothioate linkages.[4] Degradation of synthetic oligonucleotides may introduce additional species in the products. Although chemically modified oligonucleotides are normally quite stable, the rate of degradation may be affected by their sequences and the presence of different stressors.[4-6]



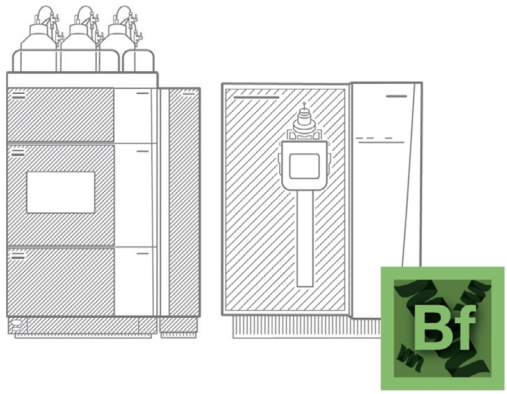
Advanced analytical tools are indispensable for the characterization of various oligonucleotide impurities and degradation products, some of which are present at a very low level. One popular method for oligonucleotide analysis is ion-pair reversed-phase liquid chromatography coupled with mass spectrometry (IP-RP LC-MS).[4-6] The MS1-based LC-MS method offers intact mass confirmation for oligonucleotides and their common impurities, however, it does not provide base-by-base sequence information and localization of modifications. Additionally, it is challenging to apply the MS1-based method for the identification of impurities with modifications and degradation products. By comparison, an HRAM based ddMS^2 method allows confident identification and mapping of unmodified and modified oligonucleotides, as demonstrated in our recent application note.[8] The introduction of powerful Oligonucleotide Analysis tools in Thermo Scientific™ BioPharma Finder™ 4.0 software enables fast processing and annotation of ddMS^2 data, as well as a comparative analysis of multiple raw files simultaneously.

In this application note, the capability of ddMS^2 was extended to the characterization of impurities and degradation products of a synthetic oligonucleotide using a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system, a Thermo Scientific™ DNAPac™ RP column, and BioPharma Finder software. The optimization of ddMS^2 was performed using a strategy described previously.[8] The optimal ddMS^2 method was then applied to identification, mapping, and relative quantitation of oligonucleotide impurities in samples purified with different methods or treated under different stress conditions. The power of HRAM and ddMS^2 for structural characterization of high MW impurities will also be highlighted.

Experimental

Equipment

- Thermo Scientific Orbitrap Exploris 240 mass spectrometer (P/N BRE725535)
- Thermo Scientific Vanquish Horizon UHPLC system (P/N 5400.0105)



Software

- Thermo Scientific BioPharma Finder 4.0 software (P/N OPTON-30988)

Columns

- Thermo Scientific DNAPac RP column (4 µm, 2.1 × 50 mm, P/N 088924)

Vials and closures

- Thermo Scientific™ 11 mm Autosampler Snap-It Caps (P/N C4011-50B)
- Thermo Scientific™ 11 mm Plastic Crimp/Snap Top Autosampler Vials (P/N C4011-13)
- Eppendorf™ DNA LoBind Microcentrifuge Tubes (P/N 022431005)

Solvents

- Thermo Scientific™ UHPLC-MS Water (P/N W81)
- Thermo Scientific™ UHPLC-MS Methanol (P/N A456-1)
- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), >99.0%, TCI Chemicals (P/N H0424)
- *N*,*N*-Diisopropylethylamine (DIPEA), >99.0%, TCI Chemicals (P/N D1599)
- Solvent A: 2% (~190 mM) HFIP and 0.1% (~5.7 mM) DIPEA in water (pH 7.8)
- Solvent B: 0.075% (~7.1 mM) HFIP and 0.0375% (~2.1 mM) DIPEA in methanol

Oligonucleotide samples

A lyophilized DNA 21mer (CAG TCG ATT GTA CTG TAC TTA) was purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA) in the following two forms by choosing different purification methods available from IDT.

A. 21mer purified with standard desalting, and
B. 21mer purified using HPLC

No further purification was performed in house prior to LC-MS analysis. The 21mer sample described in this application note, unless otherwise mentioned as HPLC purified, is referred to as Sample A.

A stock solution of 1 mg/mL was prepared for two 21mer samples of different purity. This solution was diluted to 100 µg/mL before LC-MS analysis.

Forced degradation

In a heat stress study, the 21mer (1 mg/mL) was heated at 80 °C for 1, 2, 4, 6, and 24 hours. The samples were let to cool to room

temperature and then diluted to 100 µg/mL for LC-MS analysis.

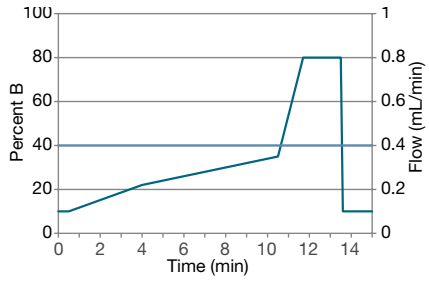
The oxidative stress was performed by incubating the 21mer (1 mg/mL) with 5% H_2O_2 at room temperature for 1, 2, 4, 6, and 24 hours. The samples were diluted to 100 µg/mL before LC-MS analysis.

Ion-pair reversed-phase liquid chromatography

Oligonucleotide separations were performed with a DNAPac RP column using a Vanquish Horizon UHPLC system. The autosampler was held at 5 °C while the column was maintained at 60 °C with the column oven Thermostatting Mode set to Still Air. The solvents were prepared in the original UHPLC solvent bottles to minimize salt contamination. The LC gradient used in this study is shown in FIGURE 1.

FIGURE 1: LC gradient for oligonucleotide separation at a flow rate of 0.4 mL/min

Time (min)	%B
0	10
0.5	10
4	22
10.5	35
11.7	80
13.5	80
13.6	10
15	10



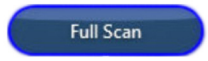
Mass spectrometry

The Orbitrap Exploris 240 mass spectrometer was operated with Thermo Scientific™ Xcalibur™ 4.4 software and controlled by Orbitrap Exploris Series 2.0 instrument control software. Instrument calibration was performed using Thermo Scientific™ Pierce™ FlexMix™ calibration solution. Data acquisition was performed in negative ion mode. The ddMS^2 methods were based on templates provided with the Orbitrap Exploris instrument control software (ICSW). TABLES 1 and 2 list the global and scan parameters of the ddMS^2 methods, respectively.

TABLE 1: Ion source properties and global method settings of ddMS^2 methods

Ion source properties	Value
Ion source type	H-ESI
Spray voltage	Static
Negative ion (V)	2,500
Sheath gas (Arb)	40
Aux gas (Arb)	20
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	300
Method settings	**Value**
Application mode	Peptide
Method duration (min)	15
Expected LC peak width (s)	6
Advanced peak determination	Checked
Default charge state	1
Internal mass calibration	Off

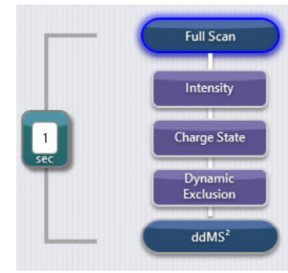
TABLE 2: Full Scan only and Full Scan/ ddMS^2 method settings

Full scan only	
Full scan	**Value**
	
Orbitrap resolution	120,000
Scan range (*m/z*)	550–2,000
RF lens (%)	70
AGC target	Custom
Normalized AGC target (%)	100
Max. injection time mode	Custom
Max. injection time (ms)	100
Microscans	1
Data type	Profile
Polarity	Negative

Oligonucleotide analysis in BioPharma Finder software

The details about ddMS^2 method optimization and data analysis using BioPharma Finder software were described in an application note published recently.[8] Briefly, the identification and mapping of 21mer and its impurities were performed in the Oligonucleotide Analysis workflow within BioPharma Finder software. The main identification parameters include: Use MS/MS = Use All MS/MS, Mass Accuracy = 5 ppm, and minimum confidence = 0.80. The task of "Find All Ions in the Run" under Component Detection was chosen for most of the data analysis. The summed peak area of all charge states of 21mer was obtained by choosing the task of "Find All Masses in the Run".

TABLE 2: Full Scan only and Full Scan/ ddMS^2 method settings (continued)

Full Scan/ddMS^2	
Full scan	**Value**
Orbitrap resolution	60,000
Scan range (*m/z*)	550–2,000
RF lens (%)	70
AGC target	Custom
Normalized AGC target (%)	100
Max. injection time mode	Custom
Max. injection time (ms)	100
Microscans	1
Data type	Profile
Polarity	Negative
Intensity	**Value**
Intensity threshold	5.0e3
Charge state	**Value**
Include charge state(s)	1–20
Dynamic exclusion	**Value**
Dynamic exclusion mode	Auto
	
ddMS^2	**Value**
Isolation window (*m/z*)	2
Isolation offset	Off
Collison energy mode	Stepped
Collision energy type	Normalized
HCD collision energies (%)	18,20,22[i]
Orbitrap resolution	30,000
Scan range mode	Auto
AGC target	Standard
Max. injection time mode	Custom
Max. injection time (ms)	200
Microscans	1
Data type	Profile
Data dependent mode	Cycle time
Time between master scans (s)	1

[i] Stepped NCE was varied from 10-12-14 to 20-22-24 with an incremental value of 1. A stepped NCE of 18-20-22 was used here for impurity identification, mapping, and relative quantitation in one experiment, as described in detail below.

Accurate masses of high MW impurities were obtained by performing the deconvolution of MS1 data using the Intact Mass Analysis workflow within BioPharma Finder software. The Xtract (Isotopically Resolved) deconvolution algorithm was used together with the Sliding Windows algorithm for generating source spectra in the retention time (RT) range above the full-length product (7.5–9.5 min). The output mass and charge state ranges were set 5,000 to 20,000 and 3 to 30, respectively. The sequence matching mass tolerance was set to 5 ppm.

BioPharma Finder software provides relative quantitation results of impurities in the modification results table under the Modification Summary section. The components used for % abundance calculation can be modified in the components table. The % abundance of the impurities checked in the modification result table are visualized in a modification plot. Some of the plots in this application note were generated in a spreadsheet using the export function from the modification results table.

Nomenclature of impurities

In this application note, the conventional "n-x" nomenclature is applied to deletion sequences produced from incomplete base coupling at the 5' end. In BioPharma Finder software, the impurities are assigned as "x-y" where x and y represent the position numbers of the first and last bases, respectively. The "x-y" species may carry -OH or -PO_4H at 5' end. For example, "A2-A21 [A2+Dephosphorylation]" assigned in the BioPharma Finder result stands for the n-1 impurity of 21mer with -OH at 5' end, while "A2-A21 [Nonspecific]" corresponds to that with 5'-PO_4H. Unless otherwise mentioned, the "n-x" impurities in this application are referred to as those with 5'-OH.

Results and discussion

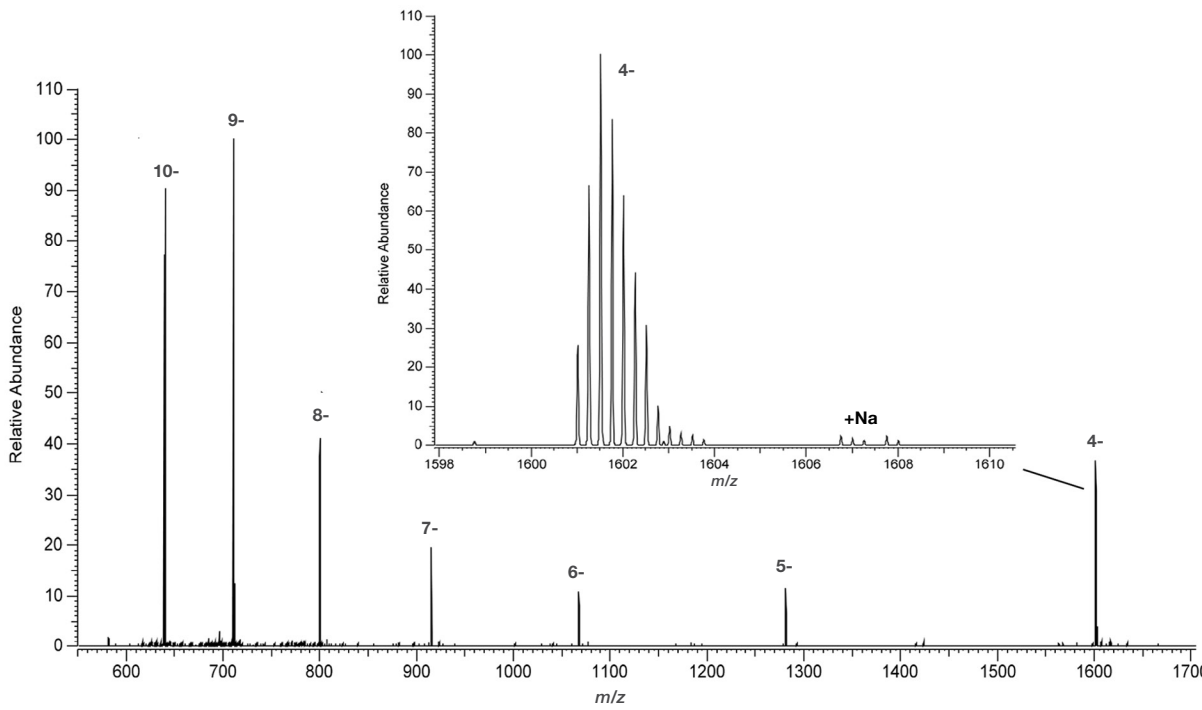
HRAM mass spectrometry of 21mer

The Vanquish Horizon UHPLC system coupled with a DNAPac RP column provides robust and reproducible separation of oligonucleotides and impurities. FIGURE 2 displays a representative mass spectrum of 21mer. A resolution setting of 120,000 at *m/z* 200 provided baseline separation of the 21mer isotopes, thereby allowing accurate mass measurement of this intact oligonucleotide (~1 ppm). The inset in FIGURE 2 highlights the low salt adduct (< 2%) under the experimental conditions employed in this study. To minimize the level of salt adducts, it is recommended to carefully choose the ion-paring reagents and LC solvents.

Optimization of the stepped NCE for 21mer and impurities

The optimal NCE varies depending on the oligonucleotide sequence, size, modification, and charge state, and hence needs to be carefully determined. As described previously,[8]

FIGURE 2: Mass spectrum of 21mer acquired at a resolution setting of 120,000 (at m/z 200). The inset shows the isotopic envelope of the 4- and its sodium adduct present at a very low level (< 2%).

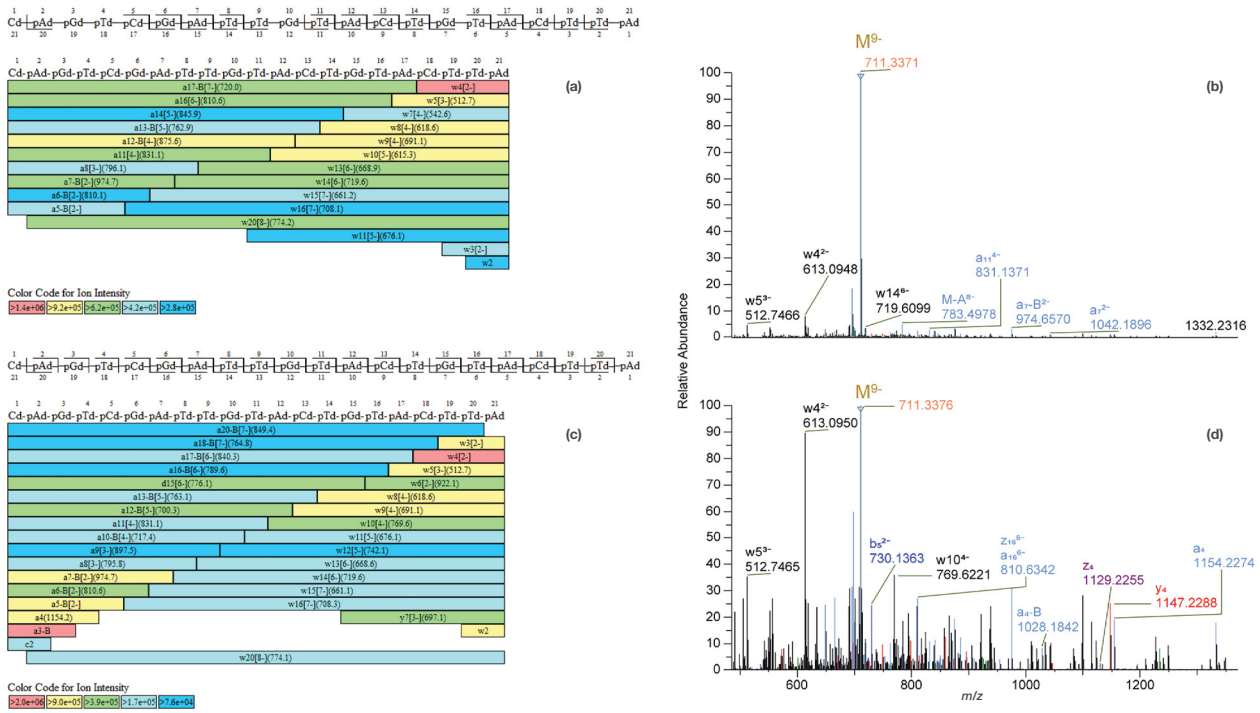


the ability to compare multiple raw files in BioPharma Finder software led to a quick survey and determination of the optimal stepped NCE value or range for an oligo. The same strategy for optimizing stepped NCE was applied to 21mer in this study.

FIGURE 3 displays fragment cover maps and annotated MS^2 spectra of the 9- of 21mer acquired using two different stepped NCEs. While a stepped NCE of 10-12-14 provided insufficient fragmentation and hence incomplete coverage of 21mer (FIGURE 3A AND 3B), a stepped NCE of 14-16-18 gave 100% coverage for this oligo.

The average structural resolution (ASR) is a score that evaluates the completeness of fragment coverage of an oligonucleotide. An ASR of 1.0 indicates a full fragment coverage of an oligonucleotide sequence. The larger the ASR is than 1.0, the less complete the fragment coverage map is. FIGURE 4 shows ASR scores measured for eleven ddMS^2 raw files of 21mer (9-) and a selected impurity (n-7, 3-) acquired at stepped NCEs ranging from 10-12-14 to 20-22-24. While a wide range of stepped NCEs (11-13-15 to 20-22-24) provided complete or nearly complete fragment coverage for the 9- of 21mer (ASR = 1.0-1.2; FIGURE 4A), higher stepped

FIGURE 3: Fragment coverage maps and annotated MS2 spectra of the 9- of 21mer acquired at stepped NCEs of 10-12-14 (a-b) and 14-16-18 (c-d)



NCEs (NCE ≥ 18-20-22) worked better for the 3- of the n-7 species (ASR = 1.3-1.5; FIGURE 4B). A stepped NCE of 18-20-22 was determined to give good coverage for 21mer and its impurities; hence it was used for studies described in the following sections. However, if large impurities (e.g., n-1) are of interest, it is recommended to use stepped NCEs optimal for the full-length product, which is between 12-14-16 and 16-18-20 for the 21mer studied here (FIGURE 4A).

Identification, mapping, and relative quantitation of impurities of 21mer purified using a standard desalting vs. an HPLC method

The ddMS^2 method not only allows quick confirmation of oligonucleotide sequence and localization of modifications (if any), but it can also confidently identify very low abundant impurities that are not discernible in a chromatogram. FIGURES 5A and 5B display total ion chromatograms (TICs) of two 21mer samples (desalting and HPLC) with shaded colors highlighting the impurities identified in each sample. The inset of FIGURE 5A shows selected ion chromatogram (SIC) (top) and zoomed TIC of n-16 present at a very low level (~0.015%, NL: 6.9E+4). However, this impurity was confidently identified by its high-quality MS^2 spectrum (FIGURE 5C) and a complete fragment coverage map (FIGURE 5D).

FIGURE 4: Comparative analysis of raw files acquired using 11 different stepped NCE settings for (a) 21mer (9-) and (b) n-7 (3-).

Shaded in green, yellow, and red are ASRs of 1.0-1.2, 1.3-1.5, and >1.5, respectively.

(a) 21mer, 9-

No.	Condition	MS Area	Delta (ppm)	Confidence Score	Average Structural Resolution	ID Type
1	NCE101214	1.10E+08	-1.36	100.0	1.4	MS2
2	NCE111315	1.08E+08	-1.28	100.0	1.2	MS2
3	NCE121416	9.97E+07	-1.06	100.0	1.0	MS2
4	NCE131517	1.01E+08	-1.13	100.0	1.0	MS2
5	NCE141618	1.34E+08	-1.06	100.0	1.0	MS2
6	NCE151719	1.40E+08	-0.52	100.0	1.0	MS2
7	NCE161820	1.38E+08	-0.52	100.0	1.0	MS2
8	NCE171921	1.39E+08	-0.60	100.0	1.1	MS2
9	NCE182022	1.34E+08	-0.52	100.0	1.2	MS2
10	NCE192123	1.41E+08	-0.83	100.0	1.1	MS2
11	NCE202224	1.32E+08	-0.60	100.0	1.2	MS2

(b) n-7, 3-

No.	Condition	MS Area	Delta (ppm)	Confidence Score	Average Structural Resolution	ID Type
1	NCE101214	1.13E+05	0.63	100.0	2.0	MS2
2	NCE111315	1.11E+05	-0.18	100.0	2.0	MS2
3	NCE121416	1.12E+05	0.63	100.0	2.0	MS2
4	NCE131517	1.10E+05	0.97	100.0	2.0	MS2
5	NCE141618	1.14E+05	1.20	100.0	1.8	MS2
6	NCE151719	1.14E+05	0.63	100.0	1.8	MS2
7	NCE161820	1.14E+05	1.09	100.0	2.0	MS2
8	NCE171921	1.15E+05	0.40	100.0	1.6	MS2
9	NCE182022	1.14E+05	1.32	100.0	1.4	MS2
10	NCE192123	1.15E+05	0.97	100.0	1.4	MS2
11	NCE202224	1.13E+05	0.74	100.0	1.3	MS2

FIGURE 5: Figure 5. TICs of 21mer purified using (a) standard desalting vs. (b) HPLC method with the shaded representation of oligonucleotide and impurities identified.

The inset in (a) displays the zoom-in of TIC (bottom) and SIC (top) of n-16, a very low abundant (~0.015%) impurity in 21mer. (c) MS2 spectrum and (d) fragment coverage map of n-16 confirming the identity of this low-level impurity. Note: Not all impurities identified are shown in Figures 5a and 5b.

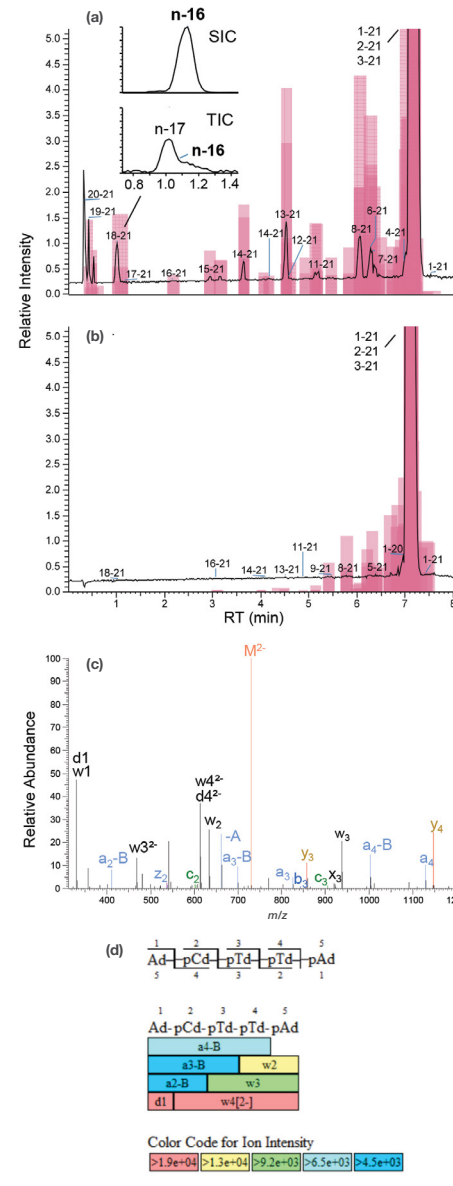
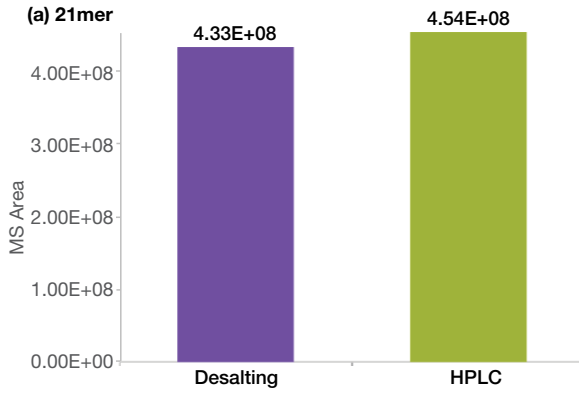
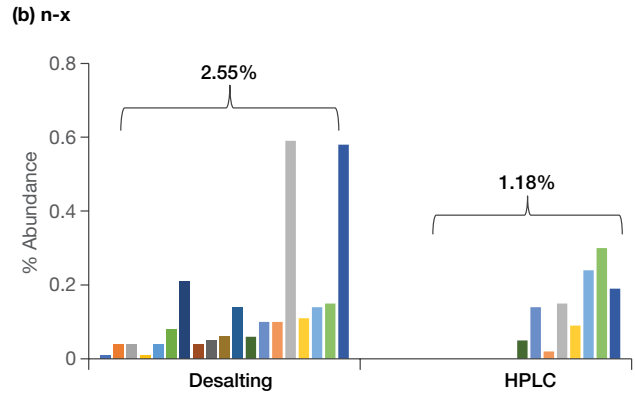


FIGURE 6 compares the MS area of 21mer and % abundances of its n-x impurities in two samples purified with the desalting or HPLC method. While the total peak area of the full-length product (21mer) remained nearly the same in two samples (FIGURE 6A), a drastic decrease in % abundance of n-x impurities (from 2.55% to 1.18%), particularly the shorter ones, was measured in the HPLC-purified sample as compared to the desalting

FIGURE 6: (a) Total MS area of all detected charge states (4- to 11-) of 21mer in two samples purified with the desalting or HPLC method.
(b) Modification plots and (c) % abundance table of n-x impurities of 21mer in the desalting vs. HPLC samples.

This table was created using the spreadsheet export functionality in BioPharma Finder software.





(c) n-x

Impurity	Desalting	HPLC	Impurity	Desalting	HPLC
n-1	0.58	0.19	n-11	0.05	0
n-2	0.15	0.30	n-12	0.04	0
n-3	0.14	0.24	n-13	0.21	0
n-4	0.11	0.09	n-14	0.08	0
n-5	0.59	0.15	n-15	0.04	0
n-6	0.10	0.02	n-16	0.01	0
n-7	0.10	0.14	n-17	0.04	0
n-8	0.06	0.05	n-18	0.04	0
n-9	0.14	0	n-19	0.01	0
n-10	0.06	0	Total	2.55	1.18

sample (FIGURE 6B and 6C), as anticipated. A similar trend was observed for n-x impurities with phosphate at 5' end (data not shown). These results showcase the ease of using BioPharma Finder software for relative quantitation of oligonucleotide impurities and quick assessment of sample purity.

Forced degradations of 21mer

Degradation of DNA can occur at high rates *in vivo* or under physiological conditions.[6] To understand degradation pathways of synthetic oligonucleotides, the effects of acid, base, oxidative, thermal, and photolytic stresses on these molecules have been

extensively studied.[4-6] In this work, thermal and oxidative stresses on 21mer were investigated.

FIGURE 7 illustrates that heating 21mer for an extended period (up to 24 hours) resulted in significant degradation of this oligonucleotide. A drastic decrease in the abundance of 21mer was observed between 2 to 4 hours of heat stress. At 6 hours, abundant degradation products in various lengths were detected. Extending the heat stress to 24 hours led to nearly complete degradation of 21mer and larger impurities.

The ddMS^2 results of heat stress samples revealed different behaviors for different impurities (FIGURE 8). While the % abundance of n-1 gradually decreased, the ratio of n-5 increased at 1 h and then steadily declined (FIGURE 8A). By contrast, a significant increase in % abundance of small impurities (e.g., n-18) was seen at 24 h (FIGURE 8A). However, the main changes upon heat degradation

FIGURE 7: (a) TICs and (b) SIC of the 21mer samples heated at 80 °C for various time points. (c) Total MS area of all charge states of 21mer in the control and heat stress samples.

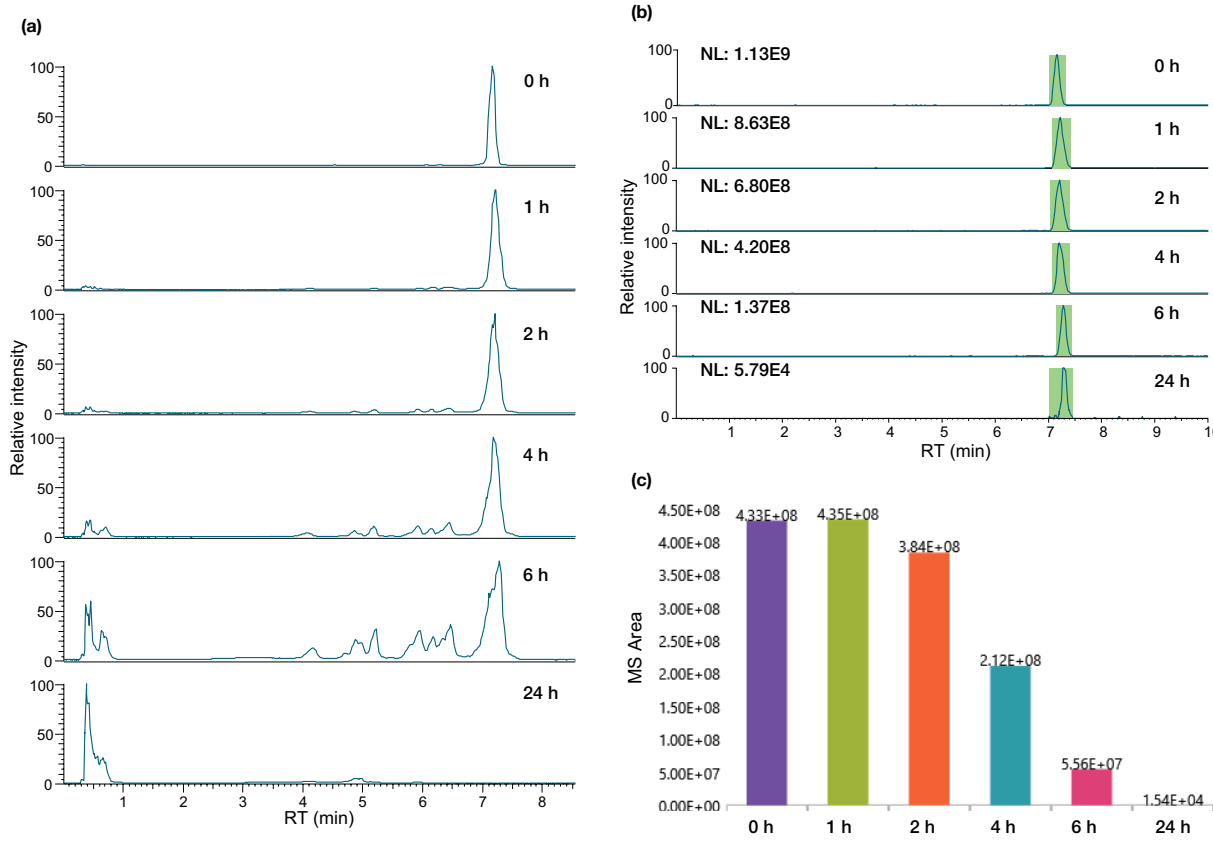
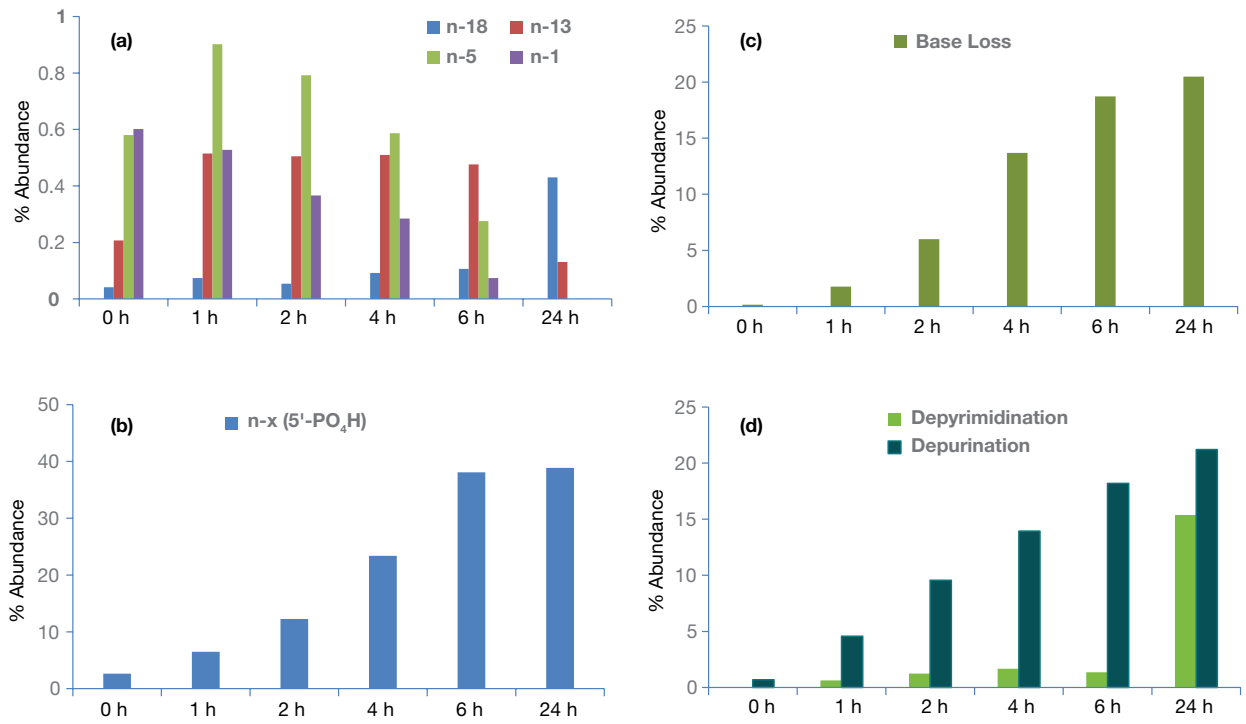


FIGURE 8: % Abundances of (a) selected n-x (5'-OH) impurities and total % abundances of (b) n-x (5'-PO4H) impurities, (c) base loss products, and (d) products associated with deprimidination and depurination in the control (0 h) and heat stress (1–24 h) samples



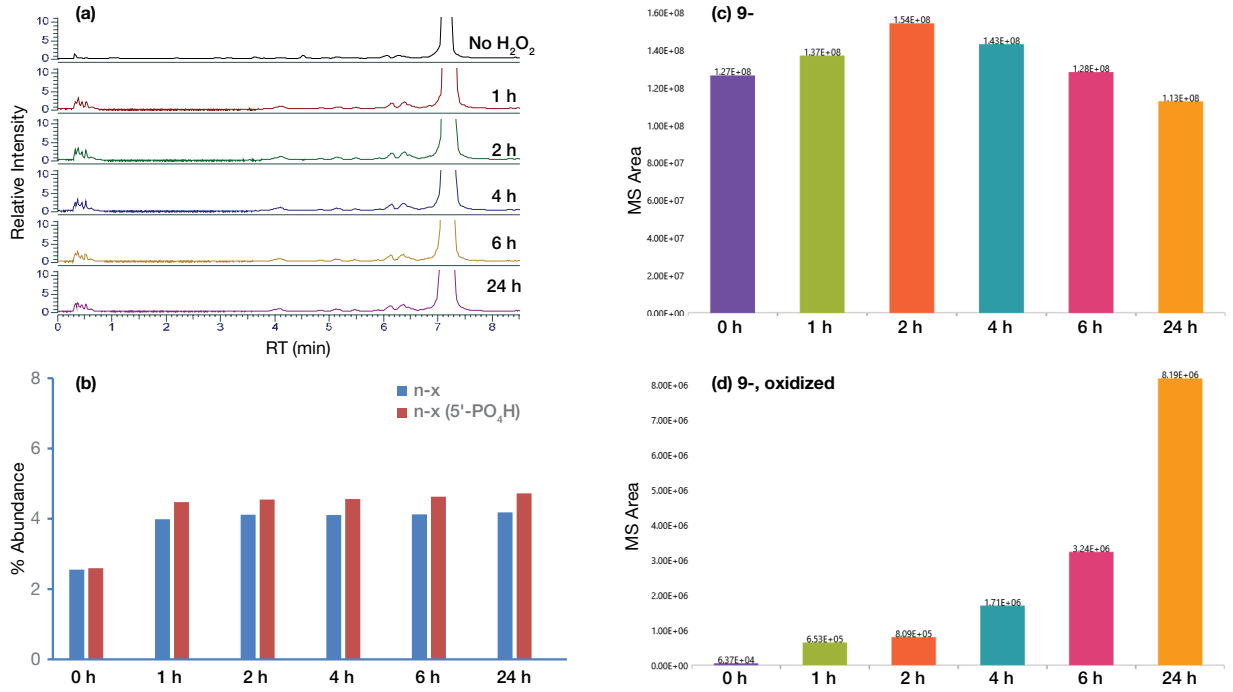
of 21mer were significant increases in % abundances of n-x impurities with 5'-PO_4H (FIGURE 8B), base loss products (FIGURE 8C), and the impurities associated with depyrimidination or depurination (FIGURE 8D).

In contrast to heat stress, oxidative stress did not result in a significant change in TICs of 21mer (FIGURE 9A). The level of n-x impurities increased slightly upon H_2O_2 treatment but then remained stable over time (FIGURE 9B). Similarly, the MS area of 21mer (FIGURE 9C) did not show a significant change (FIGURE 9C). However, the relative abundance of oxidized 21mer increased dramatically in the 24 h course of oxidative stress, as anticipated. Taken together, the results of forced degradation showcased the power of using ddMS^2 for identification and relative quantitation of impurities in a single experiment as well as for gaining insight into the degradation pathways of oligonucleotides.

High MW impurities of 21mer

In the 21mer sample purified with the desalting method, a series of high MW impurities were detected at RTs later than that of the full-length product. The main

FIGURE 9: (a) TICs of the 21mer control (no H2O2 treatment) and 21mer samples treated by 5% H2O2 for 1 h, 2 h, 4 h, 6 h, and 24 h; (b) % Abundances of n-x impurities and MS area of (c) non-oxidized and (d) oxidized 9- in the control and oxidative stress samples



high MW species was eluted out at ~9 min (FIGURE 10A). Interestingly, these MW species were completely absent in the HPLC sample (FIGURE 10B), showing the effectiveness of HPLC for the removal of these species. The chromatographic peak at RT ~ 9 min corresponds to two major charge state envelopes, one of which is displayed in FIGURE 10C. The HRAM capability of the Orbitrap Exploris 240 mass spectrometer allowed accurate mass measurement of these high MW impurities. The deconvolution of MS1 data using the Sliding Windows algorithm in the Intact Mass Analysis workflow within BioPharma Finder software revealed a series of peaks whose MWs match perfectly with the total masses of a 21mer plus its n-x impurities, i.e., "M + (n-x)" (FIGURE 10D). The mass errors measured for the eight largest impurities were all less than 1 ppm (TABLE 3). It should be mentioned that similar high MW species have been observed for oligonucleotides with A at 3' end.[9] In that study, a branched structure, where the 3'-OH of n-x is linked to the exocyclic amino group of the 3'-A of 21mer, was proposed for these high MW impurities. However, no MS^2 data of high MW impurities was acquired.

FIGURE 10: Zoom-ins of TICs of 21mer purified with (a) desalting or (b) HPLC method showing high MW species eluting at ~9 min; (c) mass spectrum of a high MW species with inset showing the isotopically resolved envelope of its 17-charge state; (d) deconvolution spectrum of high MW species assigned as "21mer + (n-x)" with x = 1-19

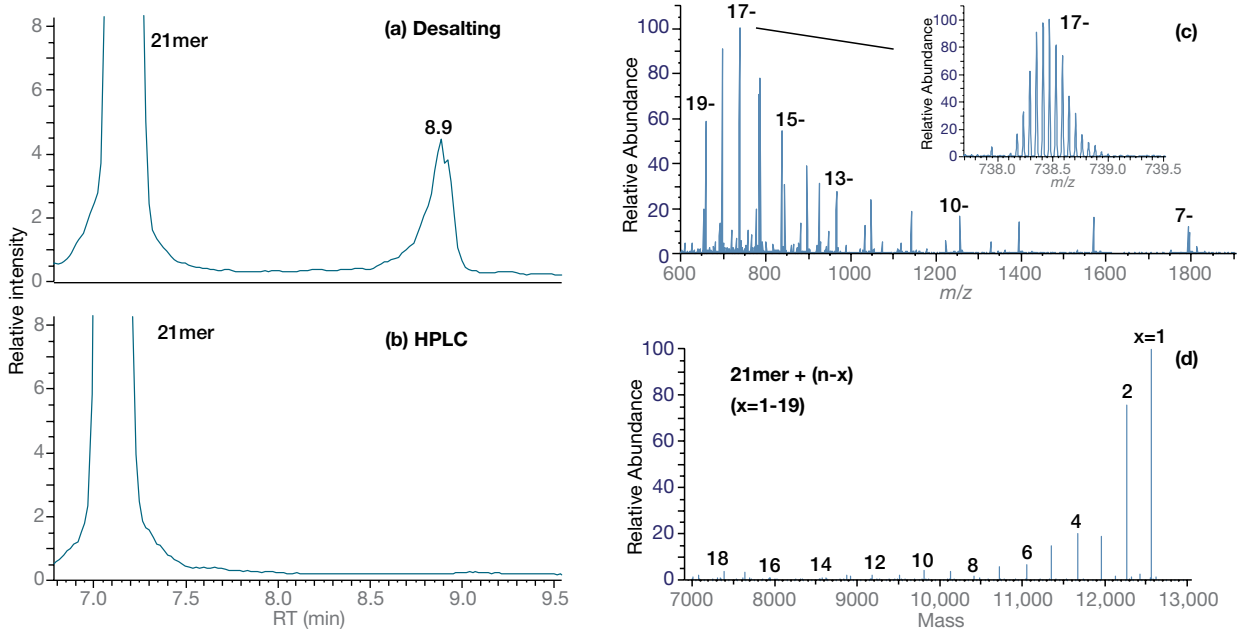
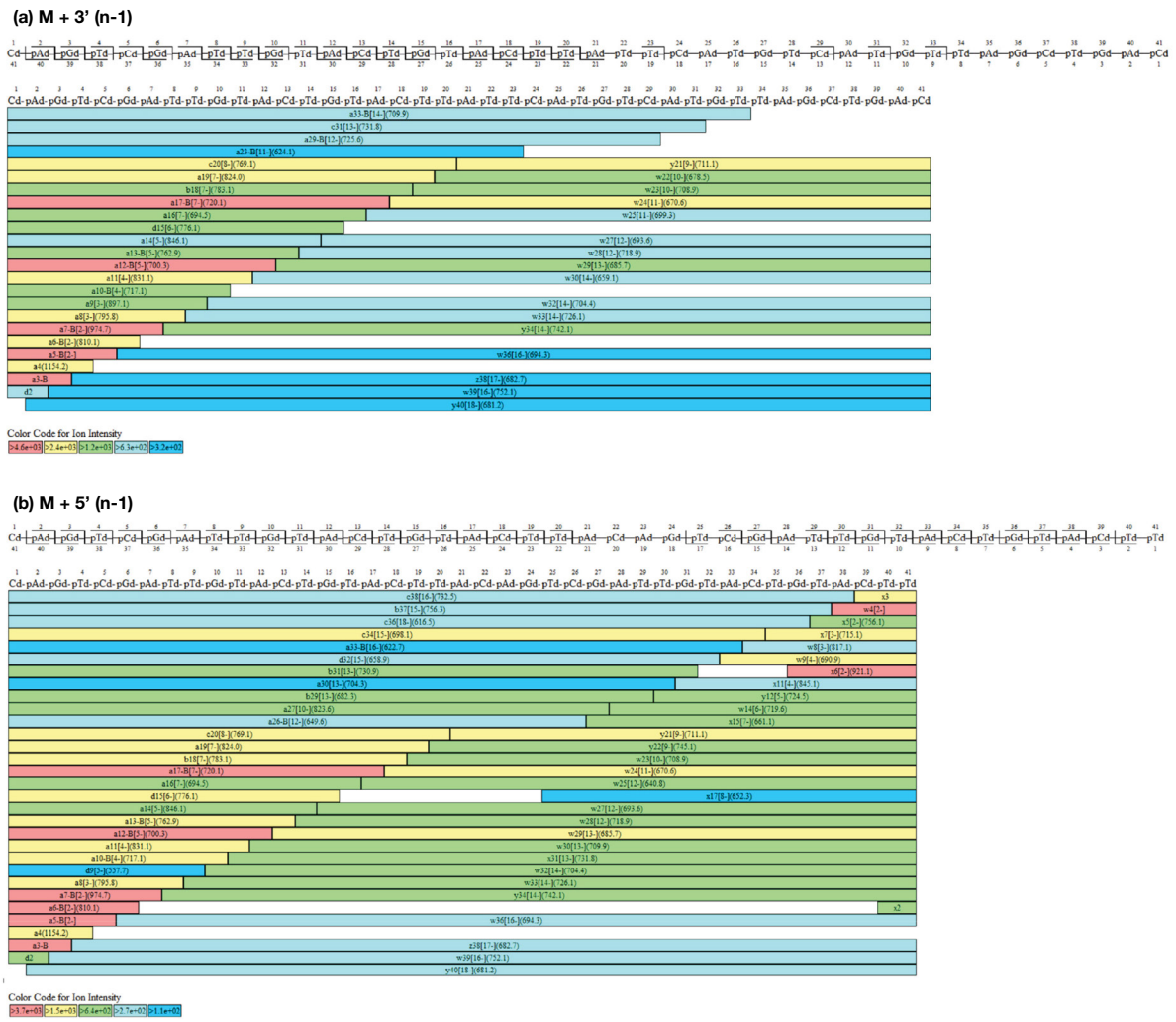


TABLE 3: Mass errors for selected high MW species. M = 21mer

Species	Exp. MW	Theo. MW	Error (ppm)
M + n-1	12261.033	12261.044	0.9
M + n-2	11956.994	11956.998	0.4
M + n-3	11667.947	11667.952	0.4
M + n-4	11354.898	11354.894	0.3
M + n-5	11050.853	11050.848	0.4
M + n-6	10721.802	10721.796	0.6
M + n-7	10417.755	10417.750	0.5
M + n-8	10128.712	10128.703	0.9

In this study, ddMS^2 was employed to facilitate structural elucidation of these high MW impurities. A stepped NCE of 11-13-15 was used to acquire ddMS^2 data of high MW impurities. FIGURE 11 displays fragment coverage maps of the "M + (n-1)" impurity obtained by searching its ddMS^2 data against a 41 nt sequence with 3'- (FIGURE 11A) or 5'-end (FIGURE 11B) of n-1 placed to the 3' end of 21mer. Interestingly, mapping the former sequence did not identify any MS^2 fragments (e.g., w_1–w_{20}) covering the 3' end of the 41 nt sequence (FIGURE 11A). By contrast, a series of *w* and *x* fragments

FIGURE 11: Fragment coverage maps of (a) "M + 3' (n-1)" and (b) "M + 5' (n-1)", in which the 3' and 5' ends of n-1 are placed to the 3' end of M (21mer), respectively, to the sequences for oligonucleotide mapping in BioPharma Finder software



were identified using the 41 nt sequence where the 5' end of n-1 was placed to the 3' end of 21mer (FIGURE 11B). Similar ddMS^2 results were also observed for other "M + (n-x)" impurities (data not shown). The two chains (21mer and n-x) likely form a similar branched structure described before[9]. However, our ddMS^2 data indicate the linkage occurring on the 5' side of n-x instead of its 3' end, as proposed in the previous study[9]. These results show that ddMS^2 can provide additional insight into the structure of high MW impurities that may not be obtained by MS1 alone. Further structural elucidation of high MW impurities will be the subject of future study.

Conclusion

In summary, the ddMS^2 approach described in this application note enables confident identification, mapping, and relative quantitation of oligonucleotides and their impurities in a single experiment. The high confident identification can be obtained for very low abundant impurities that are not discernible at the chromatographic level. The three case studies presented here demonstrate the ability of ddMS^2 for quick assessment of oligonucleotide purity, comparison of different purification methods, and in-depth understanding of forced degradations of oligonucleotides. Additionally, the capabilities of HRAM and ddMS^2 offered by the Orbitrap Exploris 240 mass spectrometer provide insight into the structure of high MW impurities, which cannot be obtained using the MS1 method alone. The new Oligonucleotide Analysis workflow introduced in BioPharma Finder software provides a streamlined process from sequence creation and oligonucleotide mapping to relative quantitation of impurities and result reviewing. An array of tools for comparative analysis offered by BioPharma Finder software allow easy optimization of ddMS^2 and comparisons of data from different studies, including impurity analysis and time-course degradation.

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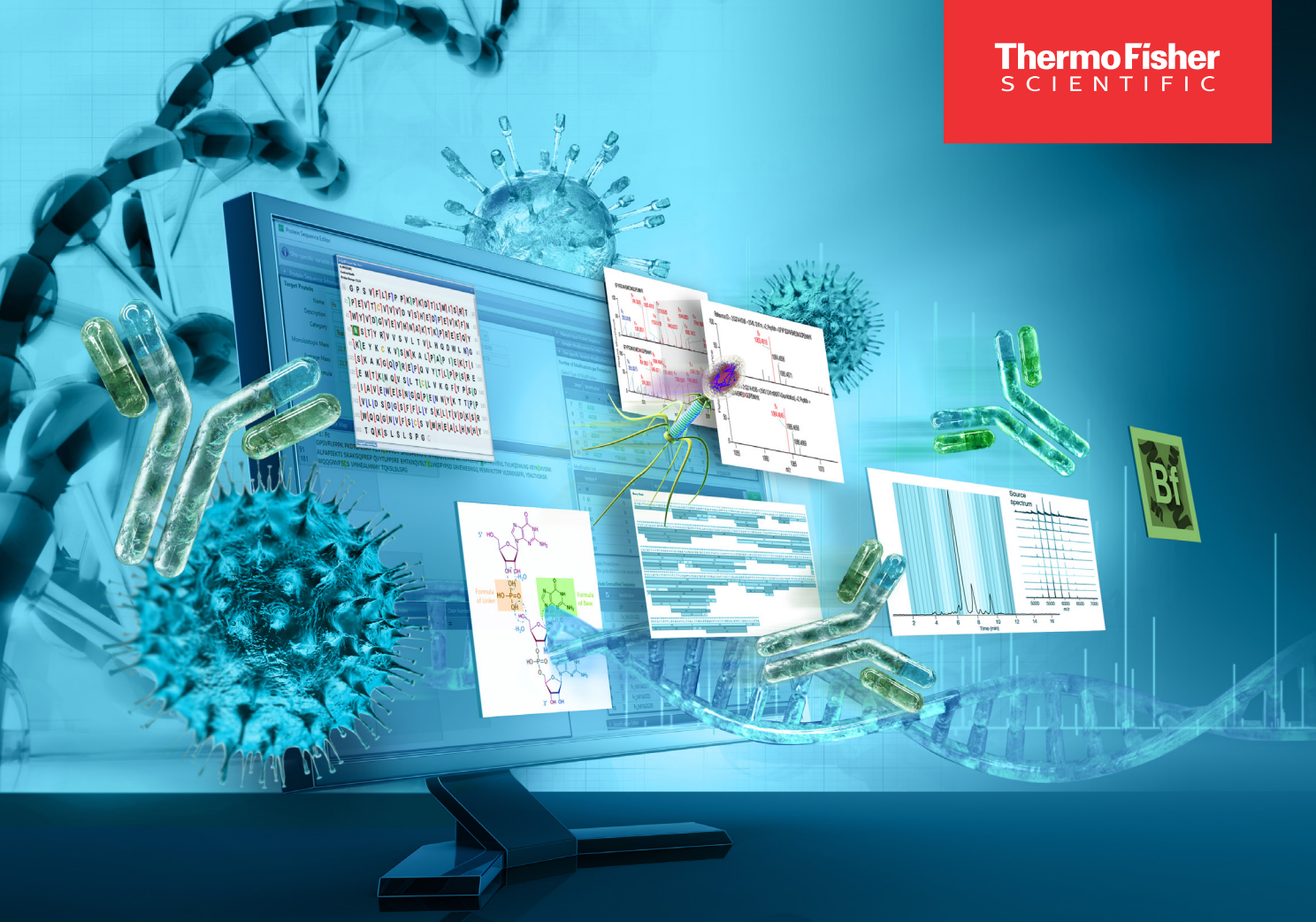

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