Applications of modified oligonucleotides

Modified oligonucleotides are now being used in many applications, most notably, diagnostics and therapeutics.

Introduction

As we will see in the following sections, modifying an oligonucleotide enables the development of diagnostic tests, therapeutics, detection methods and genetic analysis tools. Whilst there is a multitude of applications such as gene synthesis, genetic profiling, biosensors, cosmetics and agriculture, the two main sectors utilising modified oligonucleotides are diagnostics and therapeutics.

Diagnostics

The diagnostics sector is the fastest growing sector in the oligonucleotide market. This is primarily driven by the advances in terms of detection methods, particularly with respect to qPCR and sequencing techniques. These improvements in turn demand the need for new and improved detection labels such as fluorophores or electrochemical labels to fine tune these techniques to give higher sensitivity and selectivity.

There is a vast array of oligonucleotide related diagnostic tests but, with the exception of one or two techniques, they fall into one of three categories: fluorescence (e.g. probe based qPCR); electrochemical (e.g. CombiMatrix microarrays); or colourimetric detection (e.g. ELISA assay), see pages 78, 91 and 89, respectively, for further information on each topic..

Therapeutics

Oligonucleotide therapeutics[29] is a broad term which actually covers a number of modes of action of similarly structured molecules. Therefore, whilst the design and construction of the oligos is often similar, there are several ways in which therapeutic effects can be induced. These include antisense, anti-miRs, aptamers,[30] DNAzymes and ribozymes, exon skipping, siRNA, transcription factor decoys (TFD) and immunostimulatory effects. Currently, the main focus in terms of oligonucleotide therapeutics is on antisense and siRNA technologies.

29 **Therapeutic Oligonucleotides**, RSC Biomolecular Sciences, Ed. J. Kurreck, 2008, ISBN 978-0-85404-116-9.
30 **Aptamers as therapeutics**, *A.D. Keefe, S.* Pai and A. Ellington, Nature Reviews Drug Discovery, *9, 537-550, 2010.*

Antisense therapy

The concept underlying antisense technology is relatively straightforward: the use of a sequence, complementary by virtue of base pair hybridisation, to a specific mRNA can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein.[31] The development of antisense oligonucleotide technologies as therapeutics agents in recent years led to the first FDA approval for the commercialisation of an antisense oligonucleotide, Vitravene (for cytomegalovirus retinitis[32]), and to numerous clinical trials of therapeutic oligonucleotides.[33]

Phosphorothioate oligonucleotides (see page 62) were the first modified oligos to be used in antisense applications (e.g. Vitravene). Their endonuclease resistance and their RNase H activity make them suitable candidates for this purpose, for both RNase H directed and steric block antisense applications.

However, where the oligonucleotide has a high degree of phosphorothiolation, non-specific binding is known to occur.[34] There is also the issue where binding efficiency to RNA is much lower than that of DNA. In spite of this, full phosphorothioate and part-phosphorothioate oligos are still used in the development of antisense oligonucleotide therapeutics.

In order to resolve these issues base, sugar and other phosphate modifications have been developed. These "second-generation" oligonucleotides are resistant to degradation by cellular nucleases (see page 71) and hybridise specifically to their target mRNA with higher affinity than the isosequential phosphodiester or phosphorothioate. However, such antisense effects result from RNase H-independent mechanisms.

In this respect, the most common oligonucleotide modification involves use of 2-O-methyl groups (see page 72). These oligonucleotides form high melting heteroduplexes with targeted mRNA[35] and induce an antisense effect by a non-RNase H-dependent mechanism[36], i.e. via a steric blocking mechanism.

Stable oligos have also been produced that do not possess the natural phosphate-ribose backbone. PNAs (see page 54) have an uncharged, flexible, polyamide backbone comprised of repeating N-(2-aminoethyl) glycine units to which the bases are attached. These oligomers can form very stable duplexes or triplexes with nucleic acids: single or double-strand DNA or RNA.[37] The property of high-affinity nucleic acid binding can be explained by the lack of electrostatic repulsion because of the absence of negative charges on the PNA oligomers. Because PNAs are not substrates for the RNase H or other RNases, the antisense mechanism of PNAs depends on steric hindrance. PNAs can also bind to DNA and inhibit RNA polymerase initiation and elongation,[38]

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32 *(a)* **Technology evaluation: fomivirsen. Isis Pharmaceuticals Inc/CIBA vision**, *R.M. Orr, Curr. Opin. Mol. Ther., 3, 288 –294, 2001; (b)* **Fomivirsen approved for CMV retinitis**, *B. Roehr, J. Int. Assoc. Physicians AIDS Care, 4, 14 –16, 1998.*
33 **Antisense therapeutics: is it as simple as complementary base recognition?,** *S. Agrawal and E.R. Kandimalla, Mol. Med. Today, 6, 72 –81, 2000.*
34 *(a)* **Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein**, *L. Benimetskaya, J.D. Loike, Z. Khaled,G. Loike, S.C. Silverstein, L. Cao, J. el Khoury, T.Q. Cai and C.A. Stein, Nat. Med., 3, 414 –420,1997; (b)* **Controversies in the cellular pharmacology of oligodeoxynucleotides**, *C.A. Stein, Ciba Found. Symp., 209, 79 –89, 1997; (c)* **Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix**, *M.A. Guvakova, L.A. Yakubov, I. Vlodavsky, J.L. Tonkinson and C.A. Stein, J. Biol. Chem., 270, 2620 –2627, 1995; (d) Inhibition of high affinity basic fibroblast growth factor binding by oligonucleotides, S.M. Fennewald and R.F. Rando. J. Biol. Chem., 270, 21718 –21721, 1995.*
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36 **2'-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells**, *B.F. Baker, S.S. Lot, T.P. Condon, S. Cheng-Flournoy, E.A. Lesnik, H.M. Sasmor and C.F. Bennett, J. Biol. Chem., 272, 11994 –12000, 1997.*
37 *(a)* **Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide**, *P.E. Nielsen, M. Egholm, R.H. Berg and O. Buchardt, Science, 254, 1497 –1500, 1991; (b)* **Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique**, *K.K. Jensen, H. Orum, P.E. Nielsen and B. Norden. Biochemistry, 36, 5072 –5077, 1997.*
38 **Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal**, *G. Cutrona, E.M. Carpaneto, M. Ulivi, S. Roncella, O. Landt, M. Ferrarini and L.C. Boffa, Nat. Biotechnol., 18, 300 –303, 2000; (b)* **Invasion of the CAG triplet repeats by a complementary peptide nucleic acid inhibits transcription of the androgen receptor and TATA-binding protein genes and correlates with refolding of an active nucleosome containing a unique AR gene sequence**, *L.C. Boffa, P.L. Morris, E.M. Carpaneto, M. Louissaint and V.G. Allfrey, J. Biol. Chem., 271, 13228 –13233, 1996; (c)* **Antisense and antigene properties of peptide nucleic acids**, *J.C. Hanvey, N.J. Peffer, J.E. Bisi, S.A. Thomson, R. Cadilla, J.A. Josey, D.J. Ricca, C.F. Hassman, M.A. Bonham, K.G. Au et al, Science, 258, 1481 –1485, 1992.*

as well as the binding and action of transcription factors.[39] PNAs can also bind mRNA and inhibit splicing[40] or translation initiation and elongation.[41]

Although such modifications in their own right have proved efficient in terms of antisense applications, it is the combination of these modifiers, including the use of phosphorothioate linkages, which have given the most dramatic improvements. Specificity, as well as efficacy, can be increased by using a chimeric oligonucleotide, in which the RNase H-competent segment, usually a phosphorothioate moiety, is flanked on one or both termini by a higher- affinity region of modified RNA,[42] frequently 2'-O-alkyloligoribonucleotides. This substitution not only increases the affinity of the oligonucleotide for its target but reduces the cleavage of nontargeted mRNAs by RNase H.[43]

Other examples of "second-generation" antisense oligonucleotides include phosphorodiamidate morpholino oligomers,[44] and N3'-P5' PN, which result from the replacement of the oxygen at the 3' position on ribose by an amine group.[45]

siRNA

Unlike antisense oligonucleotides, siRNA is a duplex made up of a sense (passenger) and antisense (guide) strands. In this case, the mechanism of gene silencing is more complex. First the duplex loads onto the RNA-Induced Silencing Complex (RISC) where the strands separate. The antisense strand then guides sequence specific cleavage of the target mRNA with the protein Agronaute, the latter being the catalytic component of RISC.

Just like antisense oligonucleotides, it is important to build in nuclease resistance to the therapeutic. Similarly, high binding efficiency between the antisense strand and the target mRNA is highly desirable. As a consequence, the aforementioned modifiers developed to improve antisense technology are equally applicable to siRNA. Typical modifications are combinations of 2'- or sugar modified nucleosides such as 2'-OMe, 2'-F, LNA and phosphorothioate. The first FDA-approved siRNA drug; Patisiran for the treatment of hATTR is an siRNA oligo developed by Alnylam.

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Aptamers

Nucleic acid 'aptamers' are single stranded (ss) oligonucleotides (DNA or RNA), which fold into well-defined three-dimensional structures, forming shapes with complementary interactions with a desired target (e.g. small molecules, proteins, cells and even whole organisms).[46,47] As a consequence of this 'lock and key' binding mechanism, they generally have very high affinity (nano- and pico-molar dissociation constants) and specificity (>1000-fold) equivalent to that of antibodies (Figure 2). Importantly they offer several advantages over antibodies. Firstly, from a synthetic perspective, aptamers can be cost-effectively produced on a large scale and site-specifically modified by chemical methods.[48,49]

In addition, they are intrinsically stable to heat and, unlike antibodies, can undergo multiple heat denaturation steps and still refold. Secondly, for therapeutic applications, they penetrate tissue faster due to their smaller size (8–25 kDa aptamers versus ~150 kDa of antibodies)[47] and generate lower toxicity and immunogenicity as they are not normally recognised by the host immune system.

Aptamers are generally derived from a random library of 10^{58} – 10^{61} ssDNA or ssRNA molecules through an *in vitro* selection technique called SELEX (systematic evolution of ligands by exponential enrichment), developed in the 1990s.[51,52] The process is depicted in Figure 3. ssDNA libraries are often prepared by the strand separation of double-stranded PCR products.[53] In contrast, ssRNA aptamer libraries are prepared by *in vitro* transcription of double stranded (ds) DNA using recombinant T7 RNA polymerase[51] SELEX involves the incubation of random oligonucleotide libraries with the target molecule, separation of bound from unbound nucleic acids, elution of the bound nucleic acids from the target and amplification of enriched population to use as the 'new' starting libraries in the next round of selection. Subsequently, cloning and sequence analysis are carried out. Conventional SELEX carried out manually can take up to several weeks to be completed. Several types of *in vitro* selection methods have been studied including nitrocellulose membrane filtration-based SELEX,[7,54,55] affinity chromatography[56,57,58] and magnetic bead-based SELEX,[59,60,61] capillary electrophoresis-based SELEX,[62,63,64] microfluidic-based SELEX,[65,66] cell-SELEX[67,68,69] and other less common techniques.[70,71] Recently developed technologies have shown a significant improvement in SELEXbased discovery of aptamers.[72]

From a clinical point of view there have been a small number of important advances. An anti- VEGF aptamer, (Pegaptanib or Macugen) has been approved for the treatment of neovascular (wet) age-related degeneration disease (AMD).[73,74] In addition, numerous aptamers are now at preclinical or clinical trial stages for gene

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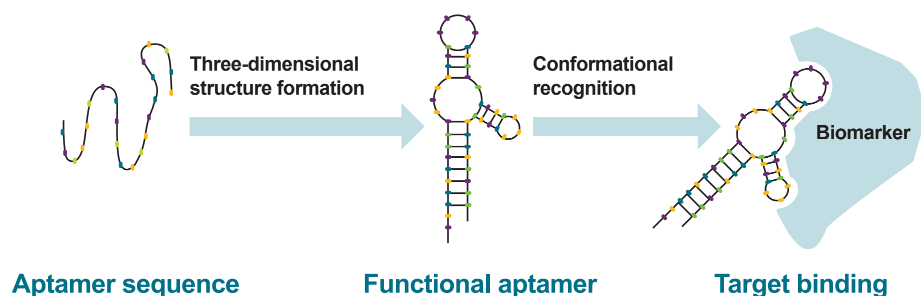

Figure 2. Schematic presentation of aptamer conformational interaction with its target to form an aptamer-target complex.[22]

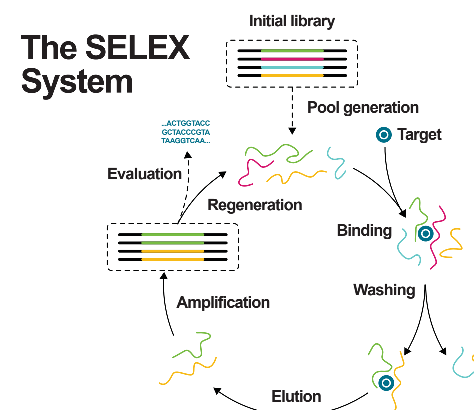

Figure 3. Overview of a typical SELEX procedure

therapy,[75,76,77,78] immunotherapy,[79,80] cancer therapy[81,82,83] and as molecular imaging agents.[84,85,86]

For therapeutic applications, a major limitation of natural nucleic acid-based aptamers is their poor stability in biological media. They are susceptible to nuclease degradation and are sensitive to the composition of the local environment (e.g. acidic or basic media, metal ions).[70,87] To address the former point, the 2'-position of the ribose sugar is often functionalised with fluoro (-F), amino (-NH2), O-alkyl (e.g. -OMe) or thiol (-SH) groups (Figure 4).[70,88,89,90,91]

Recently, Tolle et al. reported a 'click-SELEX' procedure that greatly enhances the structural diversity of aptamers by introducing bulky modifications during post-PCR step by click chemistry (CuAAC reaction).[92] This eliminates problems caused by the limitations of DNA polymerases in incorporating modified dNTPs that must otherwise be overcome during the PCR amplification steps in SELEX.[93] Furthermore, Kimoto et al. discovered DNA aptamers that contain two hydrophobic artificial (unnatural) nucleotides in addition to four natural bases, enhance aptamer affinity and target specificity.[94]

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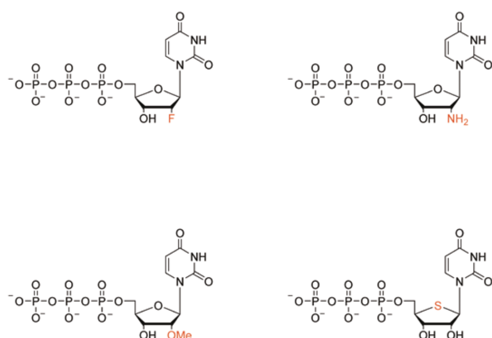

Figure 4 Chemical structures of sugar modified nucleotides used in the aptamer generation resulting in increased nuclease resistance : 2'-fluorouridine-5'-triphosphate, 2'-aminouridine-5'-triphosphate, 2'-methoxyuridine-5'-triphosphate and 4'-thiouridine-5'-triphosphate, These are currently not available from Biosearch Technologies as stock items. However, contact our Customer Service for a custom order (terms apply).

CRISPR/Cas gene editing

The ability to make targeted changes to genes within living organisms has undergone a revolution in recent years, largely due to the rapid development of CRISPR/Cas technology. This technology has its origins in a bacterial immune defence mechanism that is defined by its use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) nucleases to recognise and destroy foreign DNA from invading viruses.[95]

Several types of CRISPR/Cas nucleases have been identified, of which Cas9 and Cas12a (also known as Cpf1) are the most commonly used. Both nucleases require an RNA molecule, referred to as guide RNA, to specifically target a genetic locus. Together, the guide RNA (gRNA) and nuclease target and cut both strands of double-stranded DNA. When this is performed *in vivo*, the DNA is repaired by an error-prone mechanism that can result in a genetic change. As an RNA molecule, gRNAs are susceptible to hydrolysis and this liability can hinder utilisation of CRISPR/Cas systems in environments where cellular nucleases are present.

Chemical modifications that protect gRNAs from degradation have been shown to improve gene editing by CRISPR/CAS systems.[96,97,98,99] These modifications include the addition of phosphorothioate bonds in the backbone between the first and last three nucleotides of gRNA, as well as modifications to the 2'-hydroxyl, such as replacement with 2'-O-methyl or 2'-fluoro groups. While these modifications protect gRNAs from cellular RNA exonuclease activity, they can only be placed in certain locations within the gRNA sequence without affecting Cas activity.

Fluorescently labelled gRNAs have been used in various applications. Fluorescent guides enable monitoring of Cas-gRNA delivery into cells following transfection. Additionally, cells that have received a fluorescent gRNA can be sorted by fluorescent-activated cell sorting (FACS), which allows for enrichment of cells containing Cas-gRNA complexes and therefore improves the likelihood of obtaining edited cells. Inactive Cas nucleases complexed with gRNA can be used to tag loci without cleaving them. In an example of this approach, a gRNA was engineered to include an annealing site for a molecular beacon, which is a structured oligonucleotide appended with a fluorophore and quencher.[100] CRISPR/Cas has also been used in synthetic biology applications. One such approach uses fluorescent guides to study CRISPR/Cas synthetic DNA circuits.[101]

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Modifications for epigenetics

The study of DNA damage resulting from metabolic processes and environmental factors, along with their associated repair mechanisms has led to a better understanding of the occurrence of genetic mutations, neurodegenerative diseases, cancers and the aging process.

Ordering epigenetic reagents

At Biosearch Technologies, we offer a range of modifiers for this purpose. In particular we have amidites of 5-hydroxy-dC (**LK2543**), 5-hydroxy-dU (**LK2541**), 5-hydroxymethyl-dU (**LK2542**), 5-hydroxymethyl-dC (**LK2544**), 5-carboxy-dC (**LK2545**), 5-formyl-dC (**LK2546/ BA0367**), 5-hydroxymethyl-dC II (**LK2547/ BA0371**) and 5-formyl-dC III (**LK2548**) for use in the study of oxidative damage and repair, methylation and epigenetics.

Oxidised pyrimidines such as 5-hydroxy dU and 5-hydroxy dC are derived from dC via oxidative metabolic processes, UV or ionising radiation to form 5-HO-dC which spontaneously undergoes deamination to form 5-HO-dU (see Figure 5).

Although there are repair mechanisms to convert 5-HO-pyrimidines back to dC,[102] the fact that they are observed in cellular DNA at consistent levels suggests that these repair mechanisms are inefficient,[103] at least in certain cell types. Oligonucleotides modified with **LK2541** or **LK2543** are useful in understanding such processes.

The presence of either 5-HO-dU or 5-HO-dC can both lead to mutations resulting from their ability to mismatch with A and A/C respectively hence where the repair mechanism fails, such mutations can be permanently incorporated into the resulting gene.

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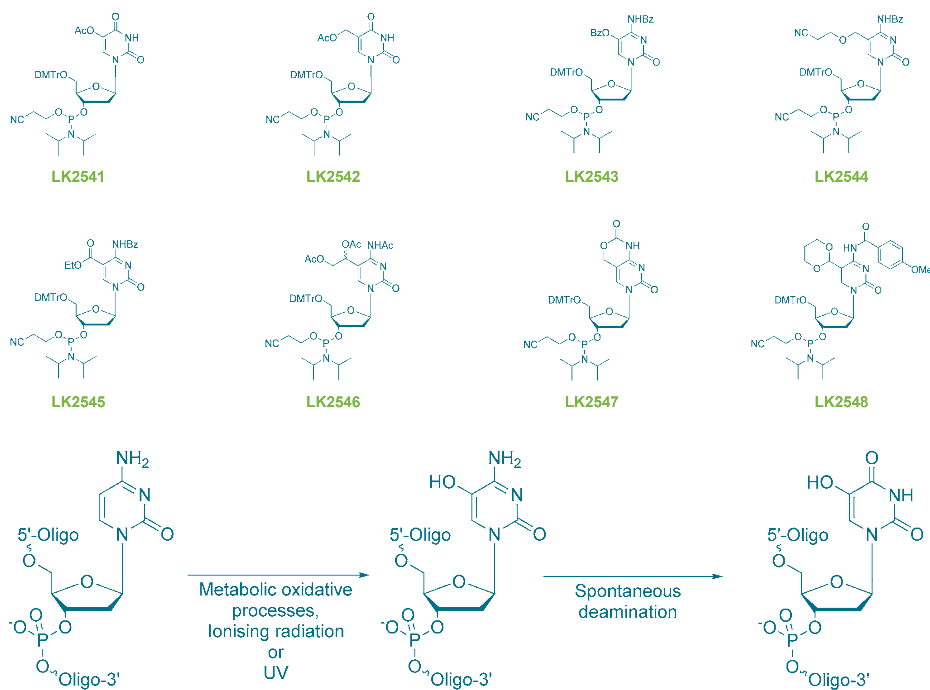

Figure 5 Formation of 5-HO-dC and 5-HO-dU from dC.

5-Hydroxymethyl-dU (5-hmdU, **LK2542**) is also a result of oxidative process or ionizing radiation but in this case dT is modified.[104] It is also possible that 5-hmdU is formed by deamination of 5-hmdC but Müller and Carell recently showed that this does not contribute to the steady state levels of hmdU in mouse embryonic stem cells, but that dT is a substrate for ten eleven translocation enzymes (Tet) leading to the formation of 5-hmdU.[105] Hence, **LK2542** is an important reagent for the study of both oxidative processes and epigenetics.

Epigenetics is the study of heritable silencing of genes where there is no change to the coding sequence. Interest in this area has grown significantly over the past few years particularly looking at changes induced and sustained by non-coding RNA gene silencing, histone modification and DNA methylation of cytidine in CpG islands.[106] Phosphoramidites **LK2544** - **LK2548** are applicable to the latter.

Once incorporated into an oligonucleotide, these modifiers represent the various products in the biochemical pathway of the modification of dC (see Figure 6).

In DNA, cytidine is methylated by a DNA methyl transferase catalysed reaction with S-adenosylmethionine to form 5-mdC. This is oxidised by Tet enzymes to 5-hydroxymethyl-dC which is further oxidised to 5-formyl-dC, which in turn is further oxidised to 5-carboxy-dC. Both 5-carboxy-dC and 5-formyl-dC can be converted back to dC via thymidine DNA glycosylase mediated base excision repair.[107]

Until now our range of products in this area of research has been limited to 5-methyl-dC (**LK2017** [N-Bz] and **LK2529** [N-Ac]) therefore the addition of these modifiers to our catalogue provides our customers working in this area the tools required to progress our understanding of these important pathways.

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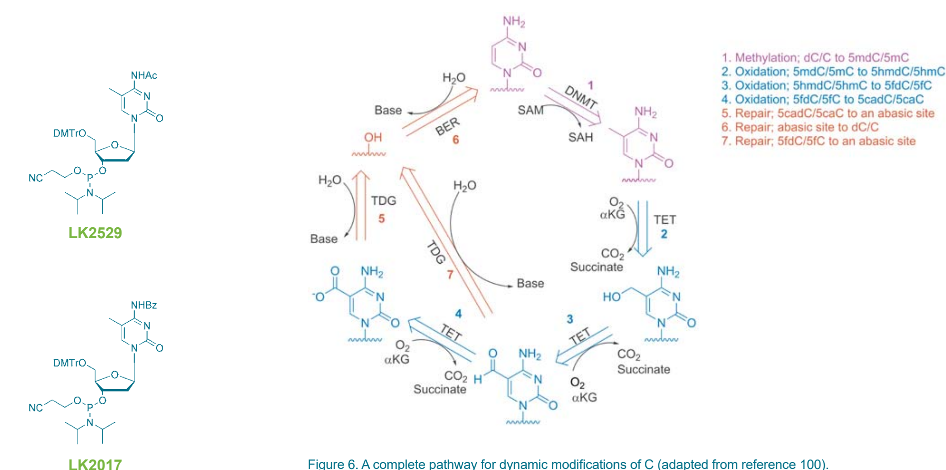

Figure 6. A complete pathway for dynamic modifications of C (adapted from reference 100).