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INSIDE

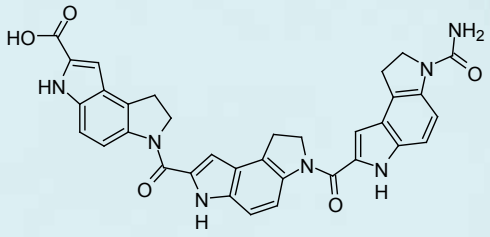
CDPI$_3$ MGB™-OLIGONUCLEOTIDE CONJUGATES AND THEIR APPLICATIONS

Author: Eugene Lukhtanov
ELITechGroup Molecular Diagnostics
21720 23rd Drive SE, Suite 150
Bothell, WA 98021

INTRODUCTION

The tripeptide of dihydropyrroloindole-carboxylate (CDPI$_3$)[1] (Figure 1) is a minor groove binding (MGB) moiety derived from the natural product CC-1065 with strong DNA binding properties. CDPI$_3$ is a crescent-shaped molecule which binds isohelically within the B-form DNA minor groove. The reversible binding is mediated via hydrophobic and van der Waals interactions between the MGB and the floor of the groove. CDPI$_3$ moiety occupies a region of duplex DNA of approximately 5 bases long (Figure 2) and binds to both A/T and G/C rich sequences with association constants of Ka ~1x10^7 M^{-1} and Ka~1x10^5 M^{-1}, respectively.

FIGURE 1: STRUCTURE OF CDPI$_3$

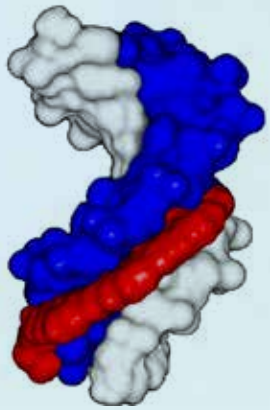


1,2-Dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate tripeptide (CDPI$_3$)

Synthetic oligonucleotides (ODNs) with covalently-attached CDPI$_3$ moieties were first introduced in 1995 by Lukhtanov et al.[3] It has since been shown that such ODNs have enhanced DNA affinity and have improved the hybridization properties of sequence-specific DNA probes. Short CDPI$_3$-oligonucleotides hybridize with single-stranded DNA to give more stable DNA duplexes than unmodified ODNs of similar length. For example, a DNA duplex formed between the CDPI$_3$-(dTp)$_8$ conjugate and poly(dA) template has a melting temperature (T_m) that is 44°C higher than the T_m of the unmodified duplex. Mismatch discrimination of short CDPI$_3$-ODNs is also enhanced in comparison to unmodified oligonucleotides. It was demonstrated that mismatches under the CDPI$_3$ binding region were more easily discriminated for a 15-mer CDPI$_3$-ODN in comparison with unmodified ODN with up to 3-fold increase in free energy difference.[4]

The tethered CDPI$_3$ moiety has a preference for A/T rich B-form DNA duplex but is capable of binding to DNA duplexes with mixed sequences as well as

FIGURE 2: DNA DUPLEX OF CDPI$_3$-ODN AND COMPLEMENT



Connolly surface representation of a decamer DNA duplex formed between a CDPI$_3$-ODN and its complement[2]. Red represents the CDPI$_3$ and the linker moieties, Blue is the CDPI$_3$-labeled strand and light gray is the complementary strand.

(Continued on Page 2)

(Continued from Front Page)

to some modified backbone nucleic acid as investigated by Kutyavin et al.[5] The A/T sequence preference has a useful practical application. It is well known that DNA duplex melting temperature is dependent on A/T and G/C content with A/T rich sequences being much less stable. $CDPI_3$ tethering significantly reduces the difference, such that probes of equal length have similar T_ms regardless of base composition.

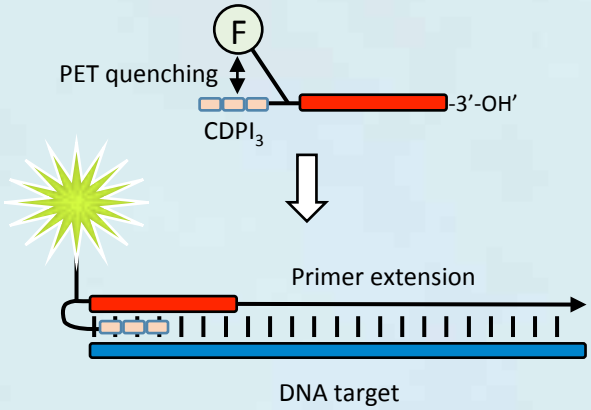
APPLICATIONS

1. Arrest of primer extension and PCR blockers

$CDPI_3$-ODN conjugates were investigated for potential use as antigene agents via the inhibition of DNA polymerase.[6] The study, which was done in context of a single-stranded DNA phage, demonstrated that T7 DNA polymerase was physically blocked when a complementary 16-mer 5'-$CDPI_3$-ODN was hybridized to a downstream site. Blockage was abolished when a single mismatch was introduced. A 16-mer with 3'-$CDPI_3$ moiety also failed to arrest primer extension. The exceptional efficiency of the primer extension arrest was attributed to DNA polymerase's inability to displace the 5' end of the duplex super-stabilized with the $CDPI_3$ group.

It has since been shown that 5'-$CDPI_3$-ODNs are able to arrest Taq DNA polymerase and therefore can be used at PCR temperatures as well. This came as a surprise since it was expected that the 5' exonuclease Taq polymerase activity would degrade such duplexes. Evidently, 5'-$CDPI_3$ labeling makes ODNs resistant to 5' exonuclease digestion. Such ODNs can be used as PCR blockers to prevent amplification of selected DNA sequences.

FIGURE 3: MECHANISM OF ACTION OF FLUOROGENIC $CDPI_3$-FLUOROPHORE-PRIMERS



2. Short and fluorogenic PCR primers

Efficient priming of PCR was demonstrated with 5'-$CDPI_3$-ODNs as short as 8-mers using modified (touch-down) PCR cycling conditions or 10-16-mers using regular PCR cycle.[7] The PCR was shown to produce specific amplification products of expected size. The reduced length primers were suggested for use for PCR amplification of viral sequences which possess a high degree of variability or techniques such as gene hunting and differential display which amplify multiple sequences using short primer pairs.

5'-$CDPI_3$-primers which also have an attached 5'-fluorophore are able to quench the dye fluorescence via the photo-induced electron transfer (PET) mechanism, depicted in Figure 3. Such primers are significantly quenched in a single strand state but become highly fluorescent when incorporated into the PCR amplicon allowing for detection of target amplification.

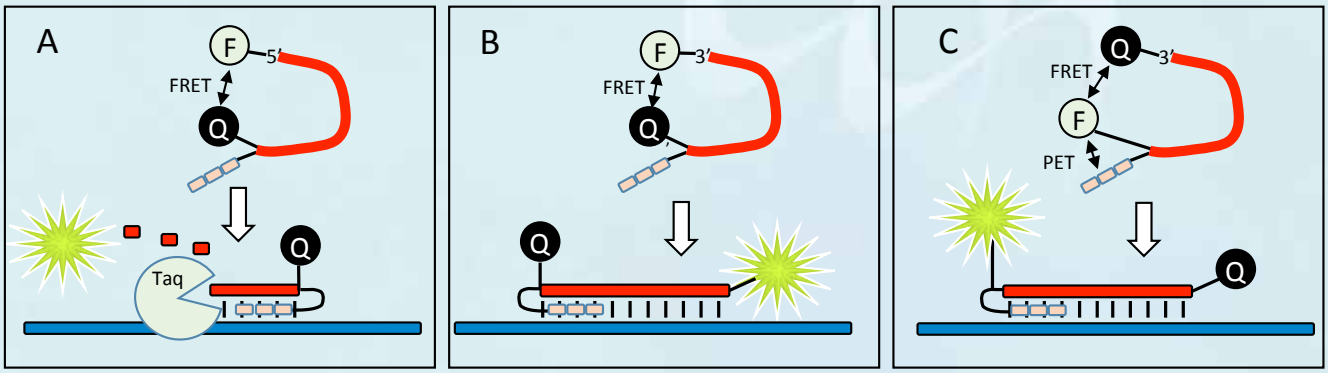
3. Real-time PCR probes

The stronger binding of $CDPI_3$-ODNs in comparison with unmodified ODNs allows for more stringent hybridization conditions to be used in DNA hybridization assays. Short $CDPI_3$-ODNs with improved mismatch discrimination are especially useful in PCR assays since they bind efficiently and specifically during the high-temperature primer extension cycle. Several types of real-time PCR probes that utilize the $CDPI_3$ moiety have been developed.

- MGB TaqMan® probes[4] have $CDPI_3$-Quencher and Fluorophore tethered to the 3' and 5' ends, respectively. Provided that specific sequence is present in the target DNA, the TaqMan probes are degraded by Taq polymerase during PCR, releasing unquenched fluorophore.

- MGB Eclipse® hybridization probes[8] have the $CDPI_3$-Quencher and Fluorophore attached at the 5' and 3' ends, respectively. They are non-degradable and their fluorescence is strongly increased upon

FIGURE 4: PUTATIVE MECHANISMS OF ACTION OF MGB TAQMAN (A), MGB ECLIPSE (B) AND MGB PLEIADES (C) PROBES



probes' hybridization to amplified targets during the annealing step.

- MGB Pleiades® probes[9] are also non-degradable hybridization probes. They have the $CDPI_3$-Fluorophore and Quencher moieties tethered to the 5' and 3' ends, respectively. These probes utilize the unique dual fluorescence quenching mechanism to significantly reduce background fluorescence and improve signal-to-background ratio.

4. miRNA Inhibitors

In a recent US patent,[10] it was disclosed that $CDPI_3$-ODNs with 2'-OMe sugar-phosphate nucleic acid backbone are highly efficient and specific miRNA inhibitors. The inhibition is more pronounced when the minor groove binder is tethered to the 5' end of an miRNA inhibitor. The mechanism of this effect is not fully understood but could be attributed to the abilities of 5'-$CDPI_3$ moiety to stabilize nucleic acid duplexes and block enzymatic activities.

$CDPI_3$-ODN-based miRNA inhibitors have also shown improved cellular uptake and promise as pharmaceuticals by modulating gene expression.[11]

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[11] D. L. Mcelligott WO2012119051 Enhanced biodistribution of oligomers

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5'-$CDPI_3$ MGB™ PHOSPHORAMIDITE AND 3'-$CDPI_3$ MGB™ CPG

As described in the preceding article by Eugene Lukhtanov of ELITechGroup Molecular Diagnostics, the tripeptide of dihydropyrroloindole-carboxylate ($CDPI_3$) (Figure 1, Front Page) is a crescent-shaped molecule which binds isohelically within the B-form DNA minor groove. The reversible binding is mediated via hydrophobic and van der Waals interactions between the minor groove binder (MGB) and the floor of the groove. $CDPI_3$ occupies a region of duplex DNA approximately 5 bases long.

DNA probes with conjugated MGB groups form extremely stable duplexes with single-stranded DNA targets, allowing shorter probes to be used for hybridization based assays. In comparison with unmodified DNA, MGB probes have higher melting temperature (T_m) and increased specificity, especially when a mismatch is in the MGB region of the duplex. MGB probes, therefore, can be significantly shorter than traditional probes, providing better sequence discrimination and flexibility to accommodate more targets.

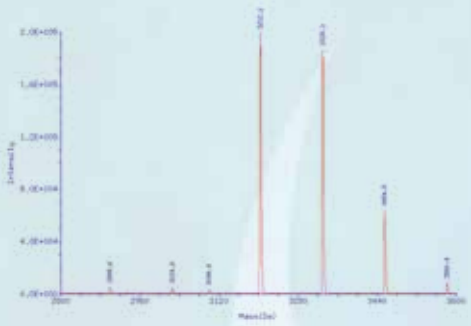
The simplest approach to MGB probe design is to use an MGB support, add a quencher molecule as the first addition and complete the synthesis with a 5'-fluorophore. Alternatively, a fluorophore support could be used with the 5' terminus containing a quencher molecule followed by a final MGB addition at the 5' terminus.

USE OF $CDPI_3$ MGB

The iodine oxidation step during DNA synthesis cycles has the potential to damage minor bases and modifiers. So it was no surprise when it was found that the indole residues of $CDPI_3$ MGB CPG are susceptible to iodination when standard 0.02 M Iodine oxidizer is used during synthesis. (This is only observed in the $CDPI_3$ MGB CPG which lacks the ethoxycarbonyl protecting groups on the nitrogens of the indole rings of the 5'-$CDPI_3$ MGB phosphoramidite.)

Figure 5 on Page 4 shows chromatograms 1) and 2) of the sequence 5'-T8-$CDPI_3$ MGB-3' deprotected in 30% ammonium hydroxide for 2 hours at room temperature. The first oligo was synthesized using non-iodine oxidation with 0.5 M CSO and a 3 minute oxidation time while the second used 0.02 M iodine oxidizer. However, as shown in the third chromatogram, the iodination is reversible when the oligo is deprotected for 17 hr at 55 °C in EtOH/NH4OH 1:3 (v/v).

FIGURE 6: ESI MS OF OLIGO 2, 5'-T8-CDPI$_3$ MGB-3'



Oligo 2 of Figure 5 prepared with 0.02M iodine oxidation and deprotected with ammonium hydroxide 2h/RT

To determine how CDPI$_3$ MGB fared with a more "real-world" oligo, 19mer oligonucleotides of the sequence,

5'-GCC TAA CTT CTG GAG ATG T- 3'

were synthesized with either a 3' or 5' CDPI$_3$ MGB. The CDPI$_3$ MGB phosphoramidite was found to be hydrophobic enough that it required 10% THF in ACN to go completely into solution at a 0.1 M concentration and required a 3 minute coupling time.

CDPI$_3$ MGB Phosphoramidite

Diluent: 10% THF in ACN
Coupling time: 3 minutes
Oxidation: 0.02 M Iodine in THF/pyridine/water
Deprotection: EtOH/NH4OH 1:3 (v/v) 17 hr at 55 °C
Purification: GlenPak™ purified

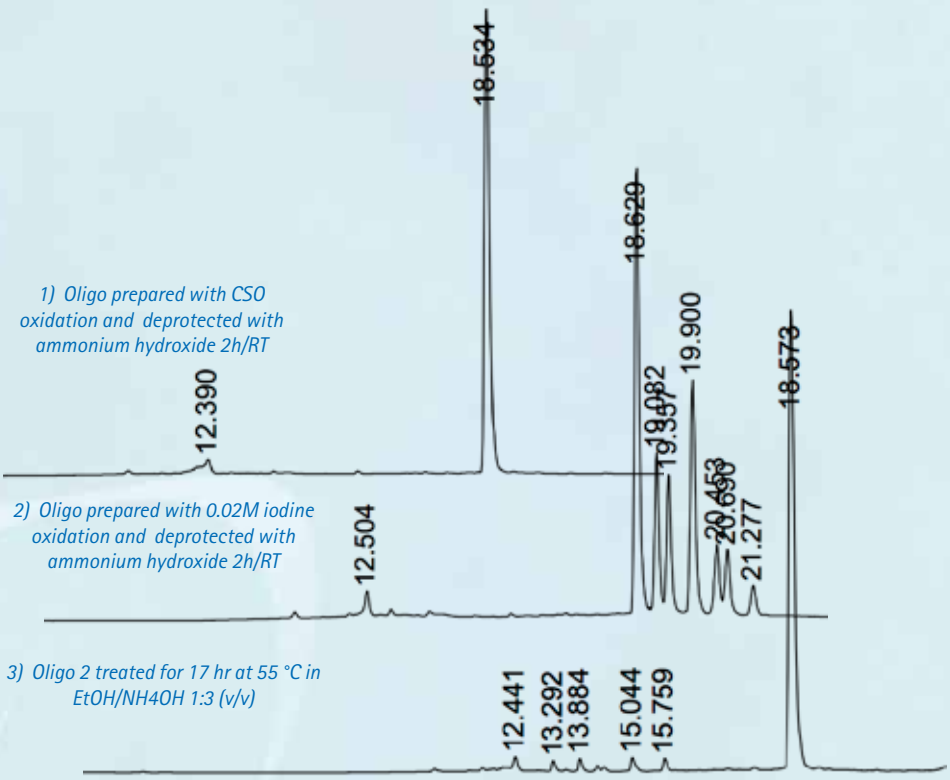
Shown in Figure 7 is the chromatogram for the 5'-CDPI$_3$ MGB probe after deprotection in EtOH/NH4OH 1:3 (v/v) 17 hr at 55 °C and GlenPak™ purification.

With the CDPI$_3$ MGB CPG, the optimum results are obtained if UltraMild monomers and Cap A are used during synthesis along with 0.5 M CSO oxidizer. However, the use of standard monomers with iodine oxidation followed by deprotection with EtOH/NH4OH 1:3 (v/v) for 17 hr at 55 °C will give acceptable results.

CDPI$_3$ MGB CPG

Coupling time: regular with UltraMild monomers and Cap A used during synthesis
Oxidation: 0.5 M CSO in ACN (3 minute oxidation time)
Deprotection: 30% ammonium hydroxide 2 hr at room temperature
Purification: GlenPak™ purified

FIGURE 5: CHROMATOGRAMS OF 5'-T8-CDPI$_3$ MGB-3'



Chromatogram 1 shows the oligo prepared using CSO oxidation. Chromatogram 2 shows the result of iodine oxidation with the various permutations of 0, 1, 2 or 3 iodines coupled to the indoles of the CDPI$_3$ MGB - as determined by ESI MS (Figure 6) - most likely at the 3 position of the indoles as described by Boger and Sakya *J. Org Chem.* 1992, **57**, 1277-1284. Chromatogram 3 shows the oligo of Chromatogram 2 after deprotection with ethanolic ammonium hydroxide to reverse the iodination reactions.

FIGURE 7: CHROMATOGRAMS OF 5'-CDPI$_3$ MGB AND 3'-CDPI$_3$ MGB PROBES

Also shown in Figure 7 is the chromatogram of 5'-GCC TAA CTT CTG GAG ATG T-CDPI$_3$ MGB-3' after deprotection in 30% NH4OH for 2 hours at room temperature and GlenPak™ purification. Given the hydrophobic nature of CDPI$_3$ MGB, HPLC purification is preferred as the short failures containing the CDPI$_3$ MGB are not efficiently removed by GlenPak™ purification.

This oligo was used in a melting study comparing the T_m of the CDPI$_3$ MGB-labeled probe against that of a control probe lacking the CDPI$_3$ MGB. The probes were annealed to both matched (G-C) and mismatched (G-A) targets. As seen in the plot in Figure 8, a single incorporation of the CDPI$_3$ MGB gave rise to an almost 12 °C increase in the melting temperature of the matched CDPI$_3$ MGB probe compared to the unlabeled control. In addition, the selectivity - that is the ΔTm (match - mismatch) - remained practically unchanged between the unlabeled control (8.1 °C) and the CDPI$_3$ MGB-labeled oligo (7.0 °C) so the target specificity remains high despite the large increase in duplex stability.

	Tm (°C)
MGB Probe : target C (match)	72.0
Control G : target C (match)	60.1
MGB Probe : target A (mismatch)	65.0
Control G : target A (mismatch)	52.0
ΔTm (MGB Probe - control):	+11.9 °C
ΔTm (match - mismatch)	
MGB Probe:	-7.0 °C
Control G:	-8.1 °C

We are pleased to add the 3'-CDPI$_3$ MGB CPG and the 5'-CDPI$_3$ MGB phosphoramidite to our repertoire of tools for increasing duplex stability, for use in PCR, DNA arrays, and for antisense applications. The structures are shown in Figure 9.

Starting on Page 13 of this newsletter, we review the sequence modifiers that might be useful in the design of MGB probes containing a fluorophore, quencher, etc., attached to the 5 position of dU.

FIGURE 8: **MELTING STUDY USING THE 3'-CDPI$_3$ MGB-LABELED PROBE**

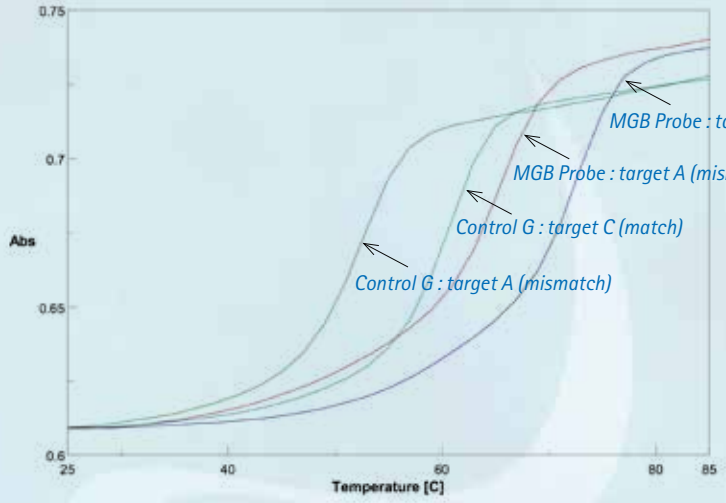
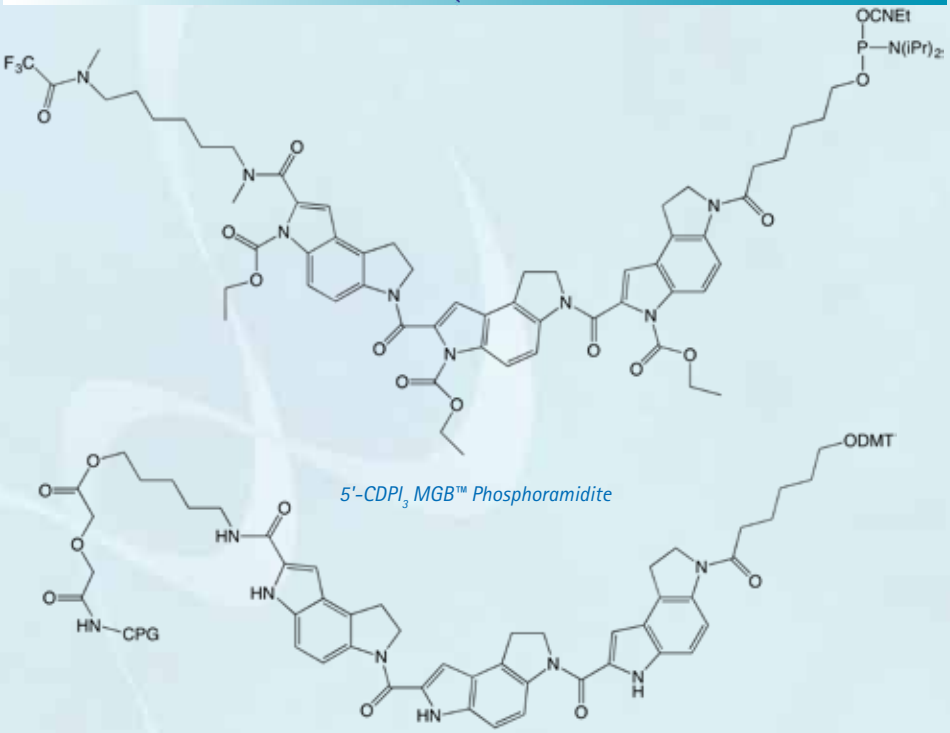


FIGURE 9: **STRUCTURES OF CDPI$_3$ MGB PHOSPHORAMIDITE AND CPG**



5'-CDPI$_3$ MGB™ Phosphoramidite

CDPI$_3$ MGB™ CPG

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-CDPI$_3$ MGB™ Phosphoramidite	10-5924-95	50 µmole	705.00
	10-5924-90	100 µmole	1390.00
	10-5924-02	0.25g	2600.00
CDPI$_3$ MGB™ CPG	20-5924-01	0.1g	215.00
	20-5924-10	1.0g	1800.00
1 µmole columns	20-5924-41	Pack of 4	325.00
0.2 µmole columns	20-5924-42	Pack of 4	165.00
10 µmole column (ABI)	20-5924-13	Pack of 1	925.00
15 µmole column (Expedite)	20-5924-14	Pack of 1	1395.00

STUDYING DNA REPAIR PATHWAYS USING SITE-SPECIFICALLY MODIFIED OLIGONUCLEOTIDES

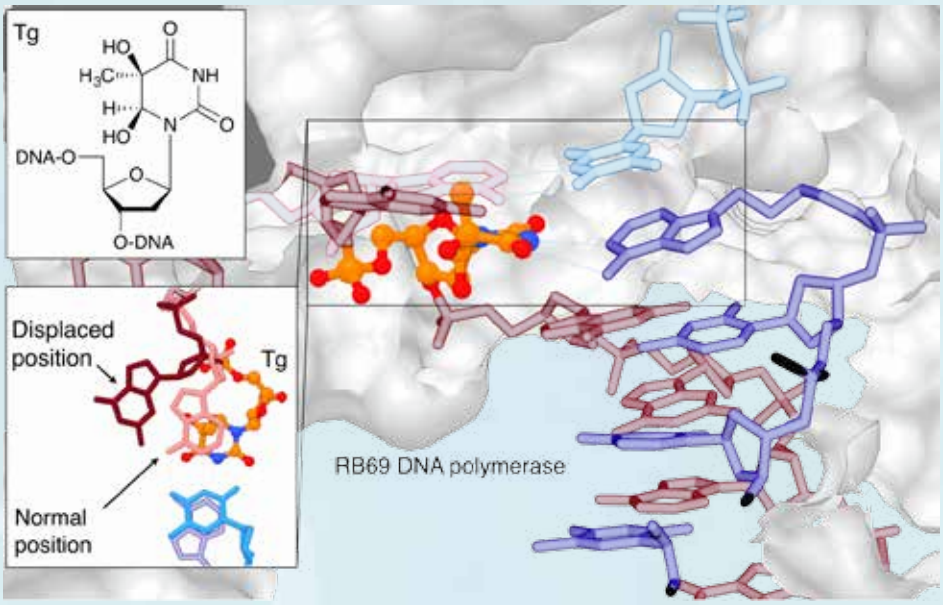
Young K. Cheun & Orlando D. Schärer
Center for Genomic Integrity, Institute for Basic Science, Ulsan National Institute of Science and Technology, Ulsan 44919, South Korea
young.cheun@ibs.re.kr
orlando.scharer@ibs.re.kr

1. INTRODUCTION

DNA is an inherently unstable molecule that can react with both endogenous (water, reactive oxygen species) and exogenous agents (UV light, environmental mutagens, antitumor agents).[1] Errors during replication of damaged sites in DNA leads to mutations in genes and eventually cancer. More recently, it has become clear that persistent DNA lesions are associated with many additional pathologies including premature aging and neurodegeneration.[2] Fortunately, a variety of pathways exist to repair DNA to counteract this threat. Mutations in DNA repair genes lead to debilitating inherited disorders and contribute to disease predisposition.

Conversely, DNA repair pathways have also become much sought-after targets for antitumor therapy for two main reasons.[3] First, a number of antitumor agents such as cisplatin or temozolomide cause cytotoxicity by inflicting DNA damage to tumor cells and the damage is processed by DNA repair enzymes similarly to endogenous DNA damage. The inhibition of DNA repair enzymes in tumors can therefore lead to improved therapeutic outcomes. Second, the vast majority of tumors have a defect in one of the DNA repair pathways. This allows them to acquire the number of mutations needed to develop into full-blown tumors. Such DNA repair defects provide specific vulnerabilities that can be exploited by targeting a second pathway that acts on the same type of lesion through the principle of synthetic lethality. This is the principle behind the clinically approved PARP inhibitor olaparib (trade name Lynparza), which was approved for the treatment of BRCA1/2-deficient cancers in 2015 and is on the way to become a blockbuster drug. Many start-up and established drug companies have programs for discovering the next wave of DNA repair inhibitors for use in oncology.

FIGURE 1: **THYMINE GLYCOL (Tg) BLOCKING REPLICATION**



Thymine glycol (Tg) blocks the replication by displacing the base adjacent to the lesion so that it can no longer base pair during replication. Tg is shown in orange/atom color, the G adjacent to it in salmon (normal position) or burgundy (displaced position) The images were created using PDB ID 2DY4 and 1IG9 and UCSF Chimera. (See https://www.cgl.ucsf.edu/chimera/docs/licensing.html)

These developments were in no small part enabled by the detailed studies of the molecular mechanisms of DNA repair pathways, which in turn were made possible by using site-specifically modified oligonucleotides. We will discuss some examples of how the availability of phosphoramidites to synthesize oligonucleotides with DNA lesions has contributed to the field, using primarily structural studies for illustration.

2. DNA POLYMERASES AND GLYCOSYLASES INTERACTING WITH THYMINE GLYCOL

Thymidine Glycol CE Phosphoramidite[4]

Thymine glycol (Tg, 5,6-dihydro-5,6-dihydroxythymine) is the most common oxidation product of thymine. It can be produced endogenously by aerobic metabolism or exogenously by chemical oxidants and ionizing radiation. The oxidation of the double bond in thymine leads to loss of aromaticity and base stacking, but does not affect the Watson-Crick base pairing properties. Tg is therefore not considered to be mutagenic, but it is a strong block to replication, making it a highly cytotoxic lesion.[5]Replication is blocked at the extension step immediately past the Tg rather than the insertion step opposite Tg. To explain this observation, Aller *et al.*[6] took a snapshot of the reaction of a replicative DNA polymerase with a Tg-containing oligonucleotide by X-ray crystallography. The structure offers insights into how Tg blocks DNA polymerase from extending beyond the lesion site (**Figure 1**). The loss of planarity of the pyrimidine ring places the methyl group into an axial position and induces steric hindrance forcing the 5' templating guanine out of the polymerase active site,[6] where it is stabilized in its misplaced position by the two vicinal diols of the Tg.[6] This guanosine is therefore unable to pair with dCTP, leading to a block of the polymerase reaction.

Tg is primarily removed by base excision repair (BER) pathway. In BER, DNA glycosylases recognize specific lesions and cleave the glycosidic bond of the damaged base. The Neil family of DNA glycosylases cleave oxidized pyrimidines including Tg.[7] To gain information into this recognition process, Imamura *et al.*[8] used a Tg-containing oligonucleotide and a viral ortholog of NEIL1 glycosylase in their X-ray crystallographic studies. In the electron

density map, the Tg lesion is flipped out of the helix to be positioned in the active site of the glycosylase (**Figure 2**).[8] This nucleotide flipping mechanism is conserved for most DNA glycosylases, allowing these enzymes to probe the modification outside the duplex, while gaining access to the glycosidic bond, which is normally hidden in the stack of the DNA duplex. Interestingly, NEIL1 makes few direct hydrogen bond interactions between the lesion and the amino acid residues in thymine glycol recognition site,[8] Instead, the propensity of Tg to assume an extrahelical conformation in DNA is a driving force for recognition by the Neil proteins.

3. RECOGNITION OF 8-OXOGUANINE

8-oxo-dG-CE Phosphoramidite[9]

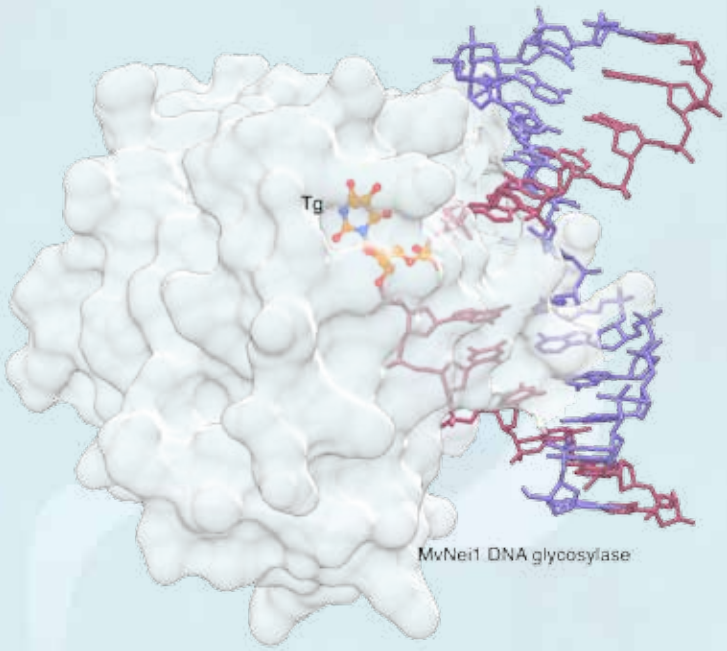
8-oxoguanine (8-oxoG) is the prototypical lesion caused by oxidative stress. Extensive studies of the recognition of 8-oxoG by the DNA glycosylase hOGG1 have not only revealed how this lesion is recognized in the flipped-out state, but also at various stages leading up to the ultimate recognition complex. Early structural studies showed early on that hOGG1 also recognizes DNA lesions by flipping out the 8-oxoG nucleotide into the active site pocket.[10] Dramatic insights into the how hOGG1 differentiates binding to 8-oxoG over non-damaged DNA were made possible through tethering the cysteine-engineered protein to the N4 cytosine of the DNA via a disulfide linkage using convertible nucleotide technology.[11] These studies showed that G and 8-oxoG occupy distinct sites on hOGG1 (**Figure 3**). These studies provided the basis for a full description of the recognition process of hOGG1 by a combination of structural, computational and biochemical studies.

4. CYCLOBUTANE PYRIMIDINE DIMER (CPD) – THE MOST IMPORTANT EXOGENOUS DNA ADDUCT

Cis-syn Thymine Dimer phosphoramidite[12]

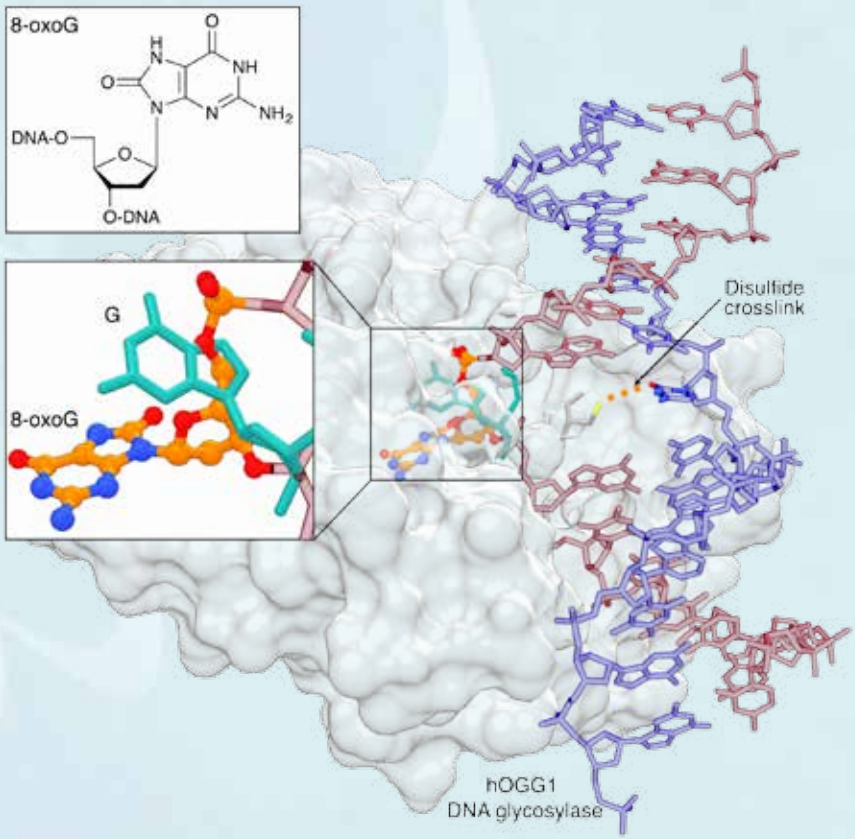
Cyclobutane pyrimidine dimers (CPDs) are perhaps the most important and well-known environmental DNA adducts. Under ultraviolet light (UV) exposure, two adjacent pyrimidines can undergo photochemical

FIGURE 2: MvNei1 FORCING THYMINE GLYCOL (TG) OUT OF THE HELIX



MvNei1, a viral ortholog of human DNA glycosylase NEIL1, can recognize the Tg lesion and force it to flip out of the helix. The flipped nucleotide is shown in orange/atom color in the base recognition site. The image was created using PDB ID 3VK8 and UCSF Chimera.

FIGURE 3: hOGG1 DISCRIMINATING 8-oxoG FROM G



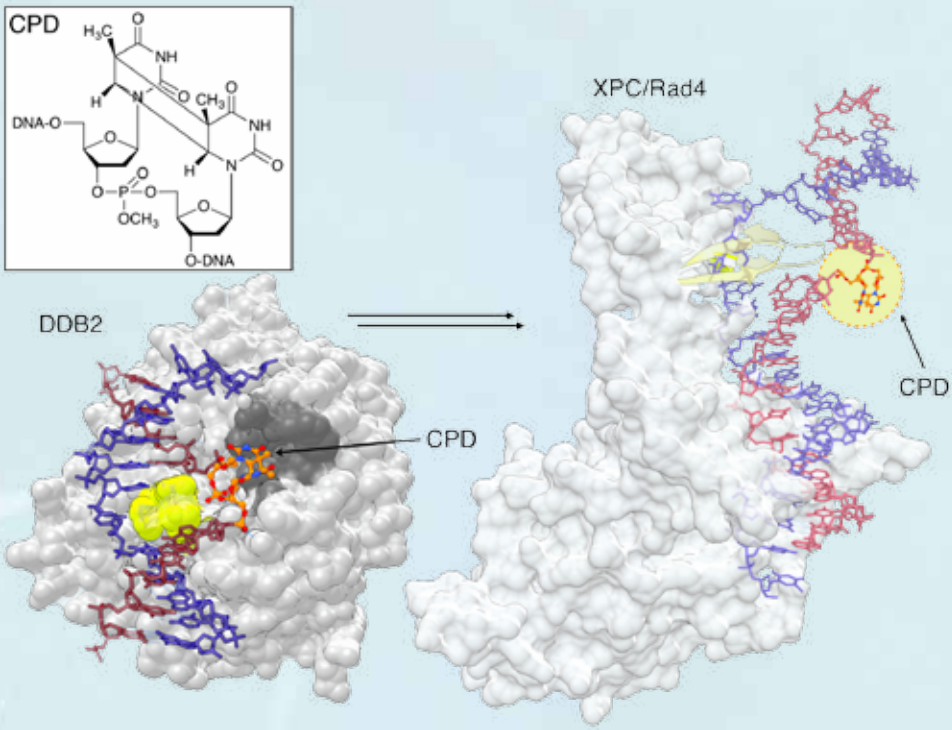
Human 8-oxoguanine DNA glycosylase I (hOGG1) can discriminate 8-oxoG from G before it is fully flipped out from the helix. The native (teal) and damaged (orange/atom color) bases are shown occupying distinct binding sites. The image was created by using PDB ID 1YQR and 1YQK and UCSF Chimera.

crosslinking reactions to form CPD (approx. 75%) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs, approx. 25%).[13] Like most bulky lesions, both are primarily repaired by nucleotide excision repair (NER). NER is a multistep process and includes two proteins that have affinity for bulky DNA adducts. XPC-RAD23B is a general damage sensor that recognizes lesions that thermodynamically destabilize the DNA duplex. UV-DDB is a more specialized protein complex that recognizes lesions in the context of chromatin. While UV-DDB is not required for the repair of all NER substrates, it is essential for NER of CPDs, because they induce very little helical distortion into a DNA duplex.[14]

The CPD phosphoramidite has allowed researchers to design an oligonucleotide with a CPD lesion at a specific position, playing a crucial role in elucidating the CPD repair mechanism by NER. The X-ray structure of UV-DDB–CPD-containing DNA by Fischer *et al.*[15] shows how the binding pocket of DDB2 subunit is optimized for the recognition of CPD lesions. It has a shallow binding pocket on the surface that can accommodate CPD and a wedge that helps extrude the CPD from the duplex (**Figure 4**). DDB2 is part of a ubiquitin ligase complex and its binding to DNA lesions triggers ubiquitination of DDB2 and XPC, facilitating binding XPC to damaged sites.

Min *et al.*[16] designed a "flipped-out" CPD lesion as a substrate for Rad4-Rad23, the yeast ortholog of XPC-RAD23B, to illustrate the binding mode of the protein. Rad4 has two main DNA binding domains. The first one anchors the protein on the unmodified duplex DNA, and the other uses two beta-hairpins to encircle the unpaired bases on the non-damaged strand opposite the CPD lesion (**Figure 4**).[16] A more recent structure of Rad4 used convertible nucleotides to tether XPC to undamaged DNA, and showed that the binding mode is identical for damaged and undamaged DNA.[17] Together with spectroscopic and thermodynamic studies, the authors suggested that the binding affinity of Rad4 with DNA correlates with the propensity of a lesion to lead to an "open" DNA structure, explaining the observed preference of NER to repair lesions that destabilize the DNA duplex. [17]

***FIGURE 4:* DDB2 RECOGNIZING THE CPD LESION**



DDB2 is highly specialized for recognizing CPD lesion, which only mildly distort duplex. DDB2 has a shallow binding pocket (dark gray) to accommodate the CPD and a wedge (lime) to move it out of the duplex. Rad4/XPC binds to undamaged nucleotides opposite the lesion through two ß-hairpins (yellow) and does not contact the CPD (orange/atom color) directly. The CPD is not visible in the electron density map of the structure, but was modeled here for visual illustration. The image was created by using PDB ID 4A08 (left) and 2QSG (right) and UCSF Chimera.

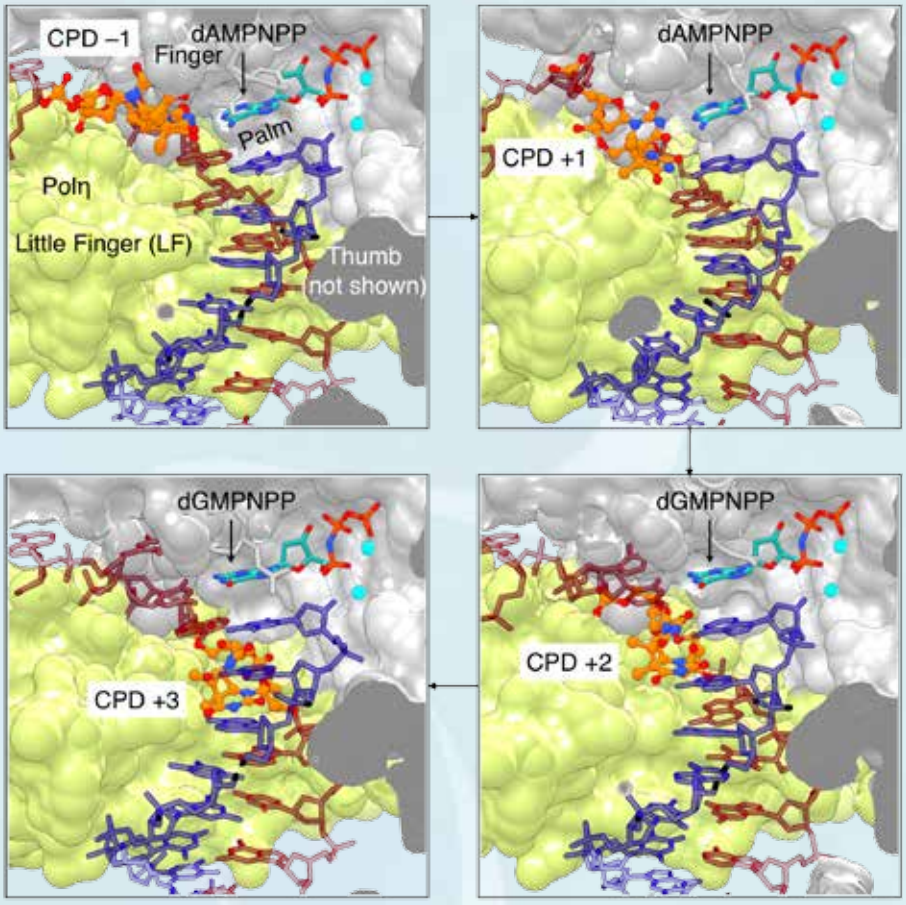
If a CPD lesion evades NER surveillance and the cell proceeds to the S phase, the bulky CPD can block replication. When a polymerase stalls at a lesion, a translesion synthesis (TLS) polymerase is recruited to bypass the lesion.[18] TLS polymerases have larger and more open active site cavities, allowing the bypass of DNA lesions, often at the expense of accuracy, in order to prevent the replication stalling. CPD-containing templates were used to solve X-ray structures of CPD lesion bound to Polη, showing how Polη can accurately bypass CPDs to prevent UV-induced mutations (Figure 5). The large active site of Polη can accommodate two bases of the CPD lesion in active site and use them as templating bases. Biertümpfel *et al.*[19] compared the X-ray structures of Polη in which the CPD lesion is located in either +1, +2, or +3 position after bypass. The authors found that the little finger (LF) domain, responsible for binding the template-primer, is very rigid and acts as a "molecular splint" that straightens the CPD-containing DNA to maintain the shape of natural B-form, facilitating the correct insertions up to 3 base pairs after the CPD.[19] Consistent with biochemical studies, these structures suggested the CPD lesion is likely to experience a steric clash with the Polη after three bases[19], explaining the dissociation of Polη three bases after the CPD bypass.[20]

5. CONCLUSIONS AND OUTLOOK

In summary, phosphoramidites allowing for the generation of oligonucleotides containing site-specific lesions have been vital components for studying the mechanism of DNA repair. For many research projects, obtaining the lesion-containing phosphoramidites is a limiting factor. New DNA lesions are still being discovered[21] and the study of their biological consequences will require their site-specific incorporation into oligonucleotides. In

addition to some of the commercially available phosphoramidites discussed here, various oxidative DNA lesions, bulky DNA adducts formed by polyaromatic hydrocarbons or aromatic amines or the second major UV adduct – 6-4PPs are much sought-after lesions in studies of DNA repair enzymes. More complex lesions such as DNA interstrand crosslinks, which covalently link two strands of DNA and are formed by many antitumor agents[22] or DNA-protein crosslinks[23] have captured a lot of recent attention due to their dramatic effects on DNA replication. The increased availability of phosphoramidites for the synthesis of lesion-containing oligonucleotides should facilitate many future discoveries in the broad area of DNA damage and repair.

FIGURE 5: HUMAN Polη BYPASSING CPD LESIONS



Human Polη is highly efficient at bypassing CPD lesions, as it can accommodate CPDs (orange/atom color) in its large active site and force the CPD-containing template into a "regular" B-DNA shape by using its little finger (LF) domain (lime) as a molecular splint. With the help of this splint, Polη can extend the primer 3 nucleotides past the lesion. The images were created using PDB ID 3MR3, 3SI8, 3MR5, and 3MR6 (clockwise from top left) and UCSF Chimera.

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
Thymidine Glycol CE Phosphoramidite	10-1096-95	50 µmole	180.00
	10-1096-90	100 µmole	360.00
	10-1096-02	0.25g	975.00
8-Oxo-dG-CE Phosphoramidite	10-1028-95	50 µmole	177.50
	10-1028-90	100 µmole	355.00
	10-1028-02	0.25g	975.00
Cis-syn Thymine Dimer Phosphoramidite	11-1330-95	50 µmole	2100.00
	11-1330-90	100 µmole	4200.00
	11-1330-02	0.25g	10200.00

N-ACETYLGALACTOSAMINE (GalNAc) OLIGONUCLEOTIDE CONJUGATES

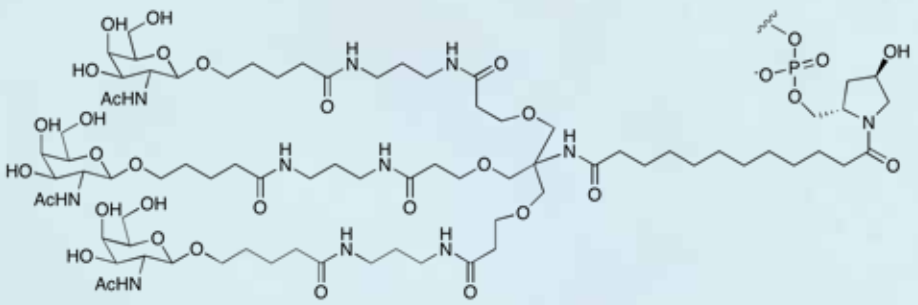
Oligonucleotides are predominantly hydrophilic species and require help in permeating cell membranes. One strategy to improve cellular uptake of therapeutic oligonucleotides is to conjugate them with non-toxic, lipophilic molecules. Glen Research offers products for cholesteryl, α-tocopheryl and stearyl labelling of oligonucleotides and this strategy has proved to be useful for delivering therapeutic oligonucleotides to a broad distribution of targets.

A more directed approach to the delivery of therapeutic oligonucleotides specifically to the liver has been to target the asialoglycoprotein receptor (ASGPR) using a suitable glycoconjugate. Indeed, ASGPR is the ideal target for delivery of therapeutic oligonucleotides to the liver since it combines tissue specificity, high expression levels and rapid internalization and turnover. The use of oligonucleotide glycoconjugates has led to significant advances in therapeutic delivery as evidenced by the work of Alnylam Pharmaceuticals which has developed multivalent N-acetylgalactosamine (GalNAc) conjugated siRNAs that bind at nanomolar levels to ASGPR.[1] A similar strategy has been applied at Ionis Pharmaceuticals directed at the development of antisense oligonucleotide therapeutics.[2]

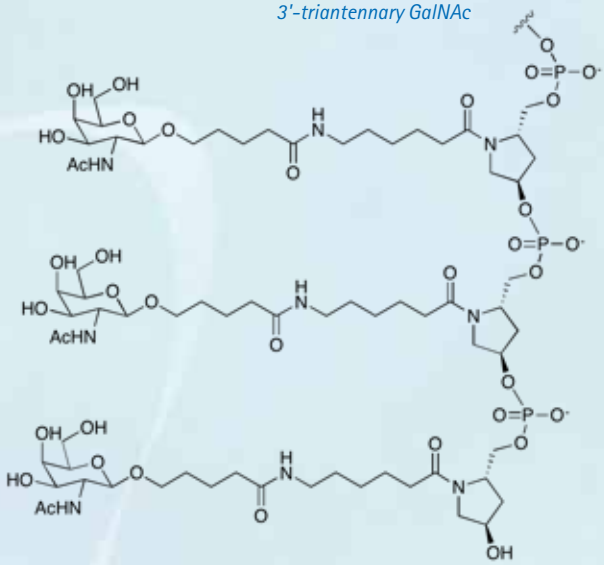
The GalNAc ligand originally used by Alnylam is shown in Figure 1. This so-called triantennary ligand would seem to lend itself to formation by post synthesis conjugation to the 3' terminus but a complex trivalent GalNAc support would also be perfectly applicable, if challenging to produce. However, an alternative approach[3] using a monovalent GalNAc support with two additions of a monovalent GalNAc phosphoramidite was also described and yielded a structure shown in Figure 1. This (1+1+1) trivalent GalNAc structure led to GalNAc modified siRNA oligos with potency equal to the equivalent siRNA with the triantennary GalNAc ligand both *in vitro* and *in vivo*.

Researchers at Ionis have developed antisense oligonucleotides containing the GalNAc cluster. In their case, they were able to show[2] that moving the triantennary GalNAc ligand to the 5' terminus led to improved potency *in vitro* and *in vivo*. As

FIGURE 1: **STRUCTURES OF 3'-GalNAc MODIFIED OLIGONUCLEOTIDES**

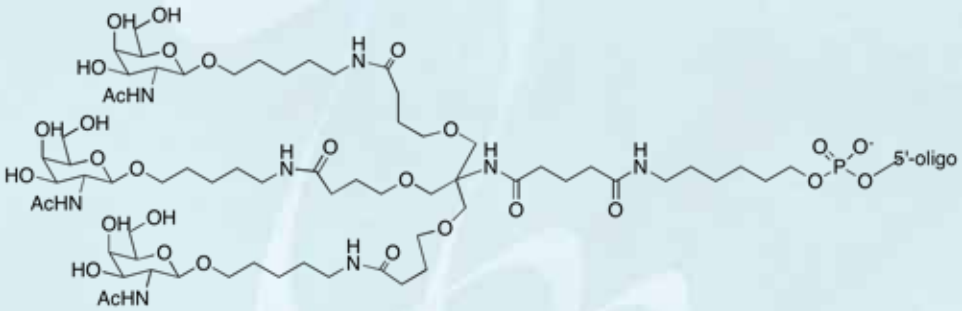


3'-triantennary GalNAc



3'-(1+1+1) trivalent GalNAc

FIGURE 2: **STRUCTURE OF 5'-GalNAc MODIFIED OLIGONUCLEOTIDES**



5'-triantennary GalNAc

may be expected, such a large complex ligand lends itself to solution phase chemistry to produce GalNAc modified antisense oligos. However, a solid phase synthetic approach was also described, and compared to the solution phase approach.[4] The structure of the 5'-GalNAc triantennary ligand is shown in Figure 2.

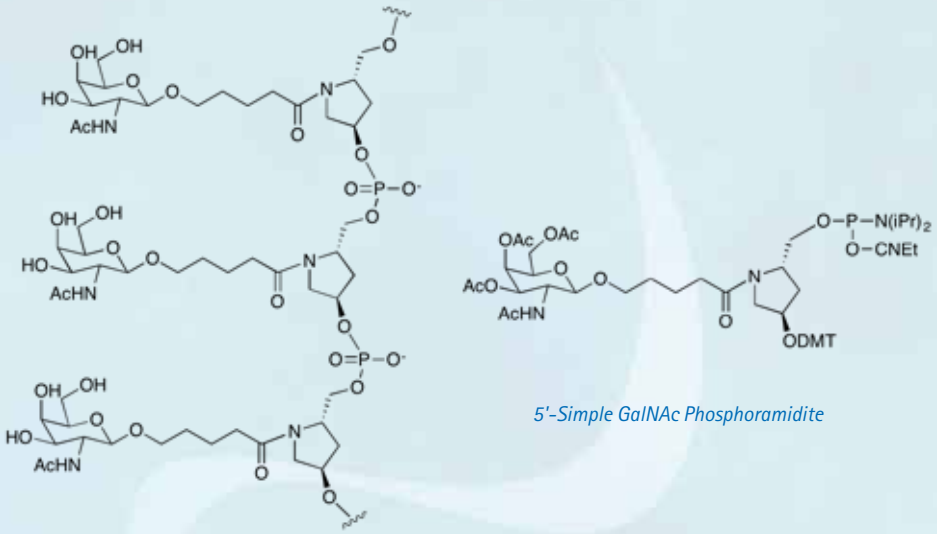
A further report on antisense oligonucleotides demonstrated[5] the effectiveness of modifying at the 5' terminus using monovalent GalNAc ligands. Up to five GalNAc monomers[3] were added in a serial manner (Figure 3) and it was shown that activity of the antisense oligonucleotides improved as the number of GalNAc units increased. The authors also showed that phosphodiester linkages between the GalNAc units were preferable to phosphorothioate linkages in their testing.[5]

Glen Research is delighted to introduce a GalNAc modification strategy using a monomeric GalNAc support and the equivalent GalNAc phosphoramidite, as shown in Figure 4.

The innovative linker in these products is a specialty of AM Chemicals LLC. At the core of the linker is a piperidine ring to which a 1,3-diol structure is attached. As we have noted before, 1,3-diol structures do not suffer the same level of elimination as 1,2-diol structures following synthesis and deprotection. Incorporating the piperidine ring in the structure also ensures that a chiral center is not formed when attaching a trityl group and a phosphoramidite to the two hydroxyl groups. In acyclic structures, the chiral center formed leads to diastereomers once an oligonucleotide is attached, which can lead to two product peaks of varying ratio in RP HPLC analysis. The piperidine structure removes this potential confusion of product peaks.

For this line of products, a trimethoxytrityl (TMT) group has been chosen instead of the more typical 4,4'-dimethoxytrityl (DMT) protecting group. In this structure, a DMT group is released more slowly than is typically seen on a 5'-hydroxyl of a nucleoside. The use of the more labile TMT groups ensures a rate of release equivalent to the regular DMT release. For those wishing to analyze the release of the TMT cation

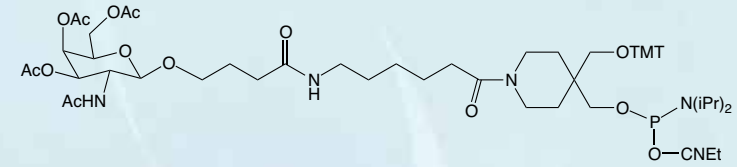
FIGURE 3: STRUCTURE OF SIMPLE MONOMER AND GalNAc MODIFIED OLIGONUCLEOTIDES



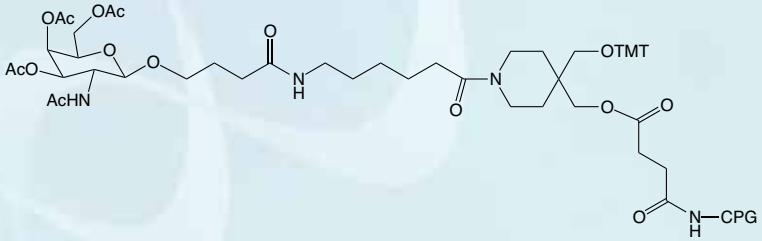
5'-(1+1+1) trivalent Simple GalNAc

5'-Simple GalNAc Phosphoramidite

FIGURE 4: STRUCTURES OF GalNAc C3 LINKER PHOSPHORAMIDITE AND CPG

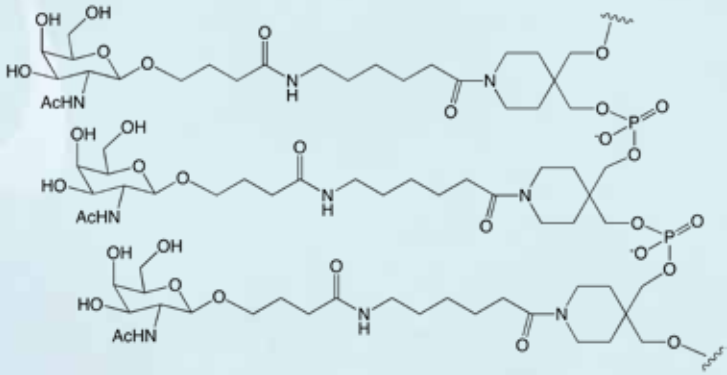


5'-C3 Linker GalNAc Phosphoramidite



C3 Linker GalNAc CPG

FIGURE 5: STRUCTURE OF GalNAc C3 LINKER MODIFIED OLIGONUCLEOTIDE



3' or 5'-(1+1+1) trivalent C3 Linker GalNAc

spectrophotometrically, the extinction coefficient at 482nm is 78,300 L/mol.cm.

Our experimental work has shown that these products are fully compatible with regular oligonucleotide synthesis and deprotection. A coupling time of 12 minutes is recommended for the phosphoramidite, while the support does not require any changes of cycle.

Oligonucleotides containing the GalNAc group can be deprotected using standard procedures during which the acetyl protecting groups on the GalNAc group are removed. The following conditions have been tested and shown to be acceptable:

Reagent	*Conditions*
Ammonium hydroxide	65°C/2h
	55°C/17h*
	RT/2h

* This is not optimal since low, but probably acceptable, levels of degradation were observed.

Ammonium hydroxide/40% Methylamine(AMA)	65°C/10 min.
Potassium Carbonate in Methanol	RT/17h

The chromatogram in Figure 6 demonstrates the high purity of 5'-GalNAc C3-T_6 synthesized using a 12 minute coupling time. For the ESI spectrum (Figure 6, Inset), the target mass for the GalNAc C3-T6 is 2372.80 Da (Obs: 2372.7). Note: the higher molecular weight species with a mass of 2538.2 Da is the HFIPA adduct.

As shown in Figure 7, we have demonstrated that 5'-GalNAc C3 phosphoramidite can be used to prepare oligonucleotides with three consecutive GalNAc additions at the 5' terminus.

Glen Research is delighted to be able to offer these GalNAc C3 products under an agreement with AM Chemicals LLC. We thank Andrei Guzaev for helpful discussions while preparing this article to introduce these products for sale.

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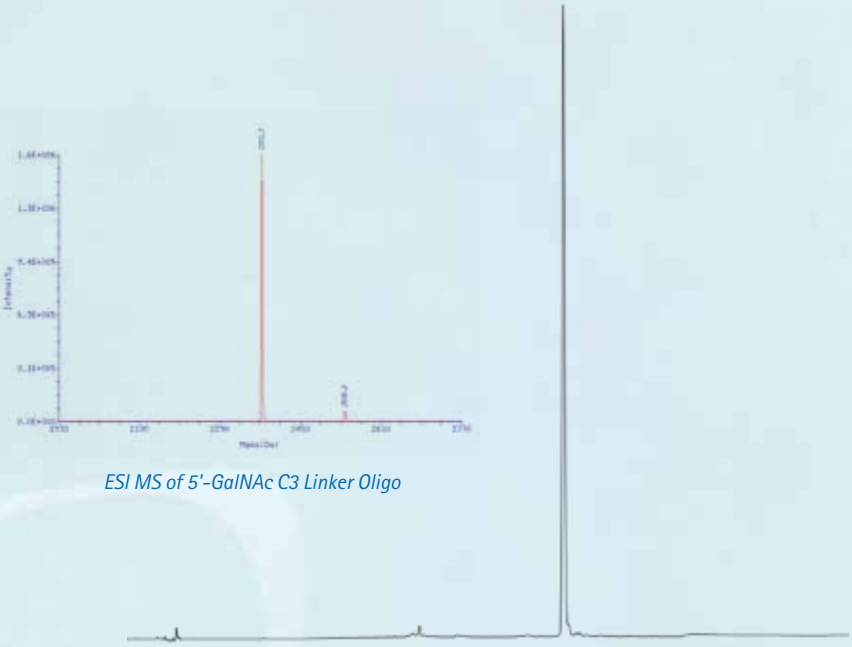
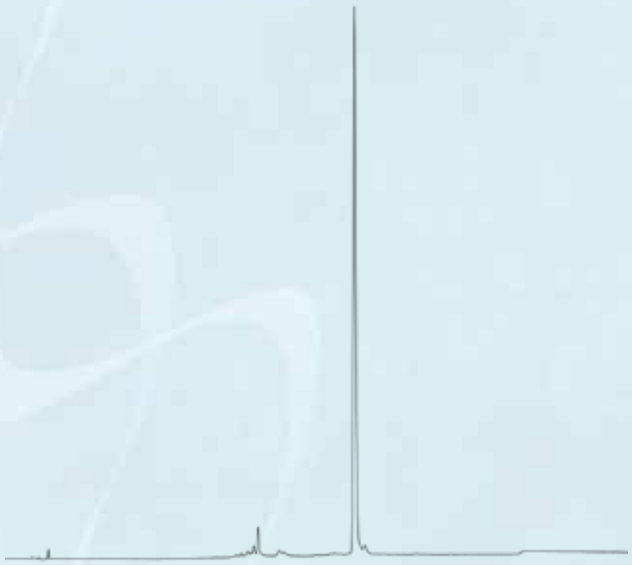
FIGURE 6: **RP HPLC OF 5'-GalNAc C3 LINKER OLIGO**



ESI MS of 5'-GalNAc C3 Linker Oligo

FIGURE 7: **RP HPLC OF A 5'-(1+1+1) TRIVALENT GalNAc C3 MODIFIED OLIGONUCLEOTIDE**



ORDERING INFORMATION

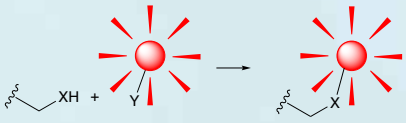
Item	*Catalog No.*	*Pack*	*Price($)*
5'-GalNAc C3 Phosphoramidite	10-1974-95	50 µmole	137.50
	10-1974-90	100 µmole	255.00
	10-1974-02	0.25g	500.00
GalNAc C3 CPG	20-2974-01	0.1g	40.00
	20-2974-10	1.0g	320.00
1 µmole columns	20-2974-41	Pack of 4	100.00
0.2 µmole columns	20-2974-42	Pack of 4	60.00
10 µmole column (ABI)	20-2974-13	Pack of 1	180.00
15 µmole column (Expedite)	20-2974-14	Pack of 1	280.00

SEQUENCE MODIFICATION USING GLEN RESEARCH'S 5-MODIFIED dU FAMILY

Glen Research's first nucleoside for sequence modification, Amino-Modifier C6 dT (10-1039), was introduced in October 1989 and has led to a family of analogues that are useful for a variety of purposes. Although these are truly dU analogues, they behave as dT in primers and probes. By using the 5 position of the dU base (1), the attached tags project into the major groove of double stranded DNA where they are readily available for detection. Even extremely large molecules, like the enzyme horseradish peroxidase, can be attached at that position with minimal perturbation of normal hybridization.

In this article, we will cover familial subsets designed for modification, fluorescent labelling, quenchers and click chemistry.

MODIFICATION



Amino-Modifier C6 dT (10-1039) has a long spacer designed to project the tag beyond the hybridization limit, whereas closely related Amino-Modifier C2 dT (10-1037) allows the tag to interact with adjacent DNA strands. In both cases, the primary amino group is revealed on deprotection, ready for post synthesis conjugation to a suitable tag, e.g., an N-hydroxysuccinimide (NHS) ester. In both cases, the amino group is protected as a trifluoroacetate (TFA).

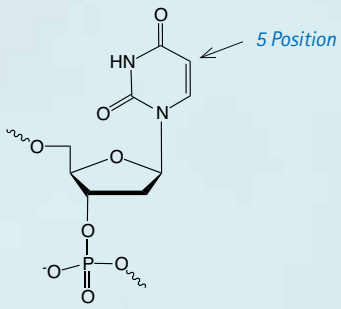
We have never found conditions which allow the TFA group to be removed from an amino-modifier while the oligonucleotide remains attached to the support. We are able to solve this problem by using a 9-fluorenylmethoxycarbonyl (Fmoc) protecting group. The Fmoc group can be removed on the synthesis column to allow on-column reaction with a variety of activated esters. Fmoc Amino-Modifier C6 dT (10-1536) is our dT analogue for this purpose.

Carboxy-dT (10-1035) is hydrolyzed during deprotection with 0.4M methanolic sodium hydroxide (methanol:water 4:1) for 17 hours at room temperature and can be coupled directly to a molecule containing a primary amino group by a standard peptide coupling or via the intermediate NHS ester.

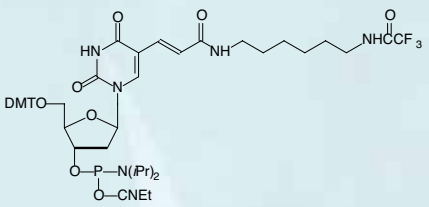
With the introduction of NHS-Carboxy-dT (10-1535), it is possible to label one or multiple sites within an oligonucleotide with an amino tag while still on the support. This opens up the possibility to label any number of different dyes or molecules within an oligonucleotide when the phosphoramidite is unavailable. Doing so is straightforward and may be done manually off the synthesizer or even in a fully-automated manner on the DNA synthesizer. (http://www.glenresearch.com/GlenReports/GR23-16.html)

Following many requests to allow a thiol to be created rather than an amino group, we introduced S-Bz-Thiol-Modifier C6-dT (10-1538) to join the ranks of thiol-modifiers for oligonucleotide synthesis. Thiol-Modifier C6-dT can be added as usual at the desired locations within a sequence.

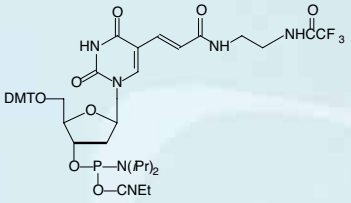
FIGURE 1: **STRUCTURES OF SEQUENCE MODIFIERS**



(1) 5 Position of dU



10-1039



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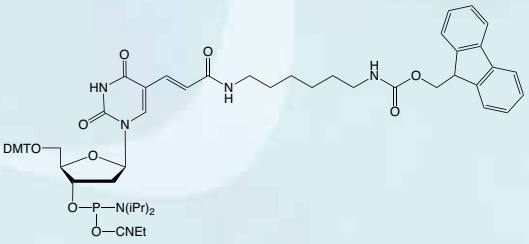

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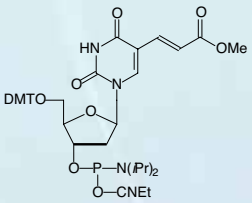
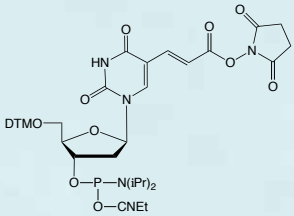
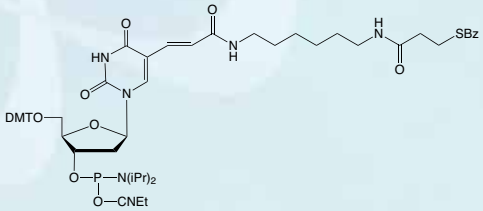


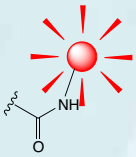
10-1035



10-1535



10-1538



Of course, the ability to amino-modify oligos with Amino-Modifier C6 dT rapidly led to requests to have the most popular labels preinstalled. Biotin and fluorescein are the two most popular labels, so Biotin-dT (10-1038) and Fluorescein-dT (10-1056) were introduced.

The earliest version of FRET probes used tetramethylrhodamine (TAMRA) as an internal quencher of the 5'-fluorophore. This was relatively inconveniently achieved using an oligo modified internally at a dT site with Amino-Modifier C6 dT and labelling post synthesis with TAMRA-NHS Ester. The introduction of TAMRA-dT (10-1057) allowed direct synthesis of such FRET probes.

Molecular beacon probes and other hairpin probes have come to rely on the fluorescence quenching properties of the dabcyl molecule.

A standard molecular beacon has a stem loop structure with a fluorophore like fluorescein at the 5'-terminus along with the quencher, usually a dabcyl group, at the 3'-terminus.

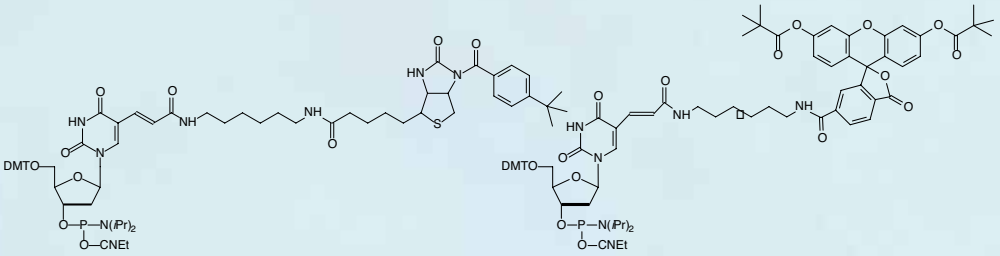
However, in other hairpin probes, the fluorophore is again at the 5'-terminus but the dabcyl group is located within the sequence. The structure of the oligo should allow it to form a hairpin in such a way that the fluorophore is spatially adjacent to the quencher in the hairpin stem. The oligos can then be used as PCR primers. The fluorescence intensity of the amplified product correlates with the amount of incorporated primer since the hairpin no longer exists in the double-stranded amplified product. Dabcyl-dT (10-1058) can be used for direct incorporation into the quencher site.

SIMA (HEX) is a more stable analogue of HEX which, unlike HEX, can be deprotected with AMA and SIMA (HEX)-dT(10-5945) is useful for sequence labelling.

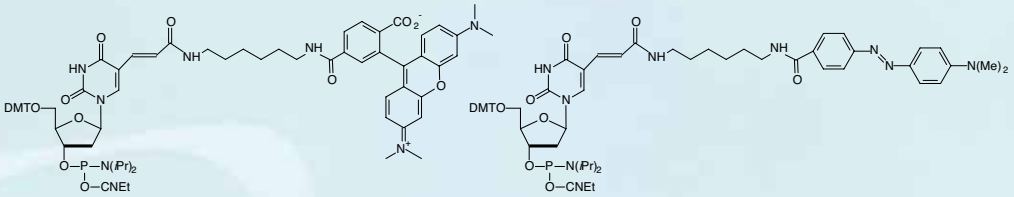
EDTA-C2-dT (10-1059) contains the triethyl ester of EDTA which allows sequence-specific cleavage of single- and double-stranded DNA and RNA. The cleavage reaction is only initiated once Fe(II) and dithiothreitol are added and so is readily controlled. Cleavage and deprotection of EDTA-C2-dT should be carried out with sodium hydroxide in aqueous methanol (0.4M NaOH in methanol/water 4:1) overnight at room temperature.

With an excellent stability profile, ferrocene has always attracted considerable interest for DNA labelling to generate probes for electrochemical detection. Ferrocene-dT (10-1576) is easily added to oligonucleotides with no disruption of regular hybridization behavior.

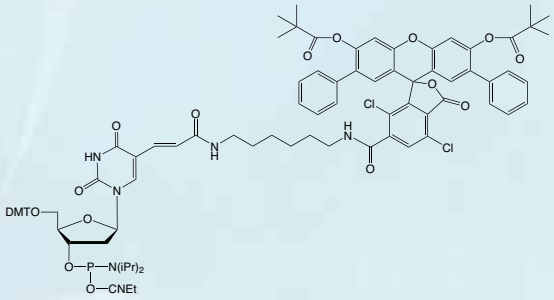
FIGURE 2: **STRUCTURES OF SEQUENCE LABELS**



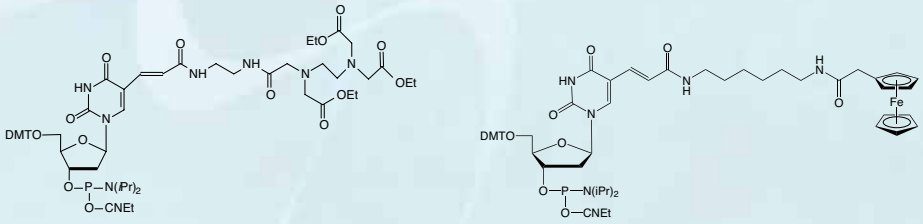
10-1038 10-1056



10-1057 10-1058

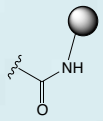


10-5945



10-1059 10-1576

QUENCHERS



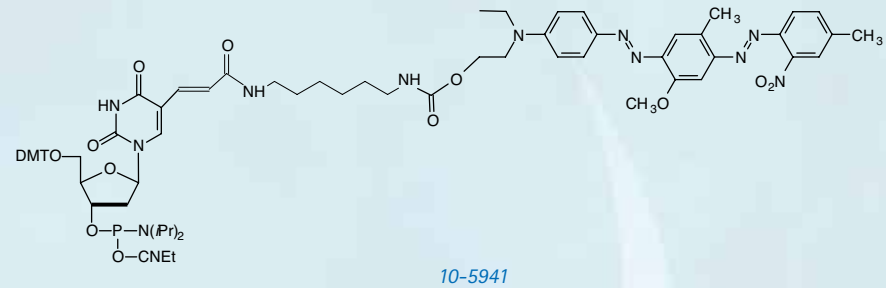
TAMRA and Dabcyl have both been used as quenchers of fluorescence by FRET or static quenching. However, other non fluorescent quenchers have become very popular over time. These include Black Hole Quenchers (BHQ), introduced by Biosearch Technologies, and Blackberry Quencher (BBQ), introduced by Berry & Associates.

Two BHQ dyes are available from Glen Research as dT analogues, BHQ-1-dT (10-5941) and BHQ-2-dT (10-5942). These two BHQ dyes are suitable for quenching fluorescence in the range 450 - 650nm.

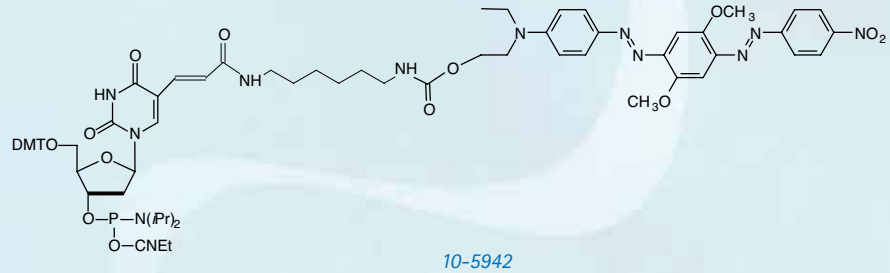
BBQ-650®-dT (10-5944) has an absorption maximum of around 650nm and is capable of quenching fluorescence in the range 550 - 750nm.

Figure 4 shows the wavelengths of fluorescence emission and quenching of various products available from Glen Research.

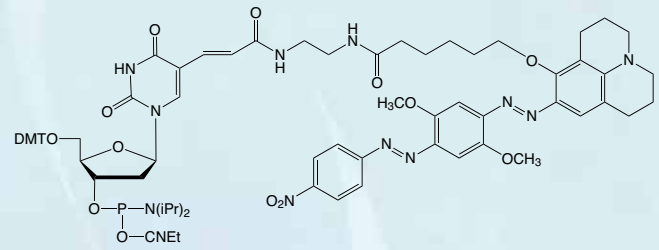
FIGURE 3: STRUCTURES OF QUENCHER MODIFIERS



10-5941

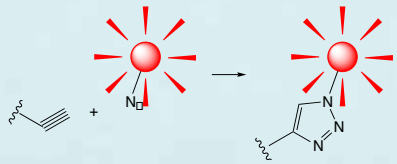


10-5942



10-5944

CLICK CHEMISTRY

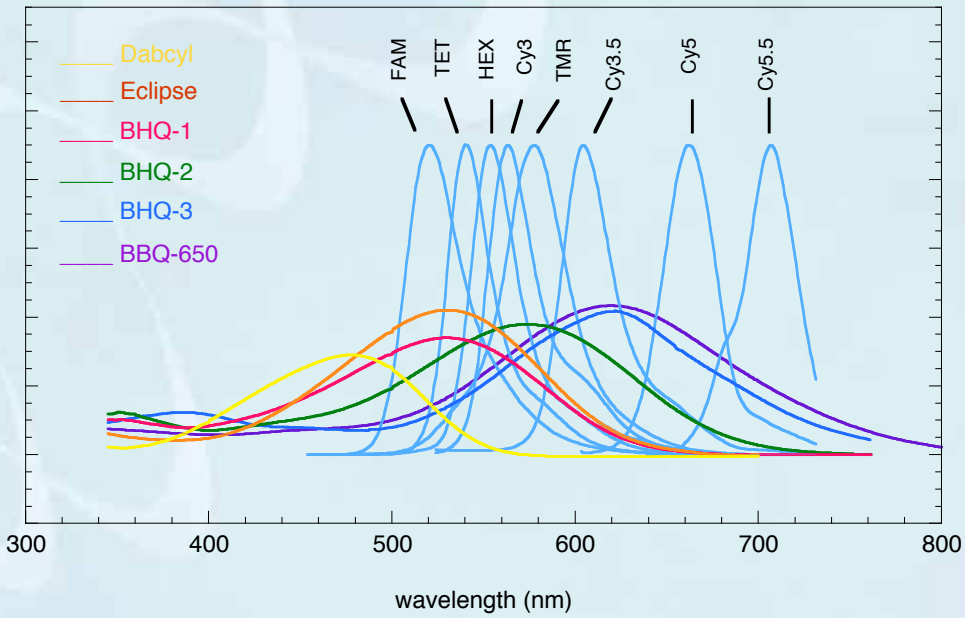


The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction between azides and alkynes to form 1,2,3-triazoles was found to be exquisitely regioselective and efficient under very mild conditions. Although copper ions are known to damage DNA, typically yielding strand breaks, the use of copper stabilizing ligands in the reaction has allowed the CuAAC reaction to be used to functionalize alkyne-modified DNA nucleobases with extremely high efficiency. This strategy allows the use of the vast library of azide tags to be used in oligonucleotide labelling.

To realize efficient click-chemistry labelling of alkyne modified oligonucleotides, our dT analogues use a 5-(octa-1,7-diynyl) side chain. Oligonucleotides bearing a single nucleosidic alkyne group can be prepared using a C8-Alkyne-dT (10-1540). Using a combination of C8-Alkyne-dT, C8-TIPS-Alkyne-dT (10-1544) and C8-TMS-Alkyne (10-1545), it is possible to label oligonucleotides with different azide tags in up to three separate click reactions.

5-Ethynyl-dU (10-1554) offers

FIGURE 4: DYE FLUORESCENCE AND QUENCHER WAVELENGTH



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22825 DAVIS DRIVE
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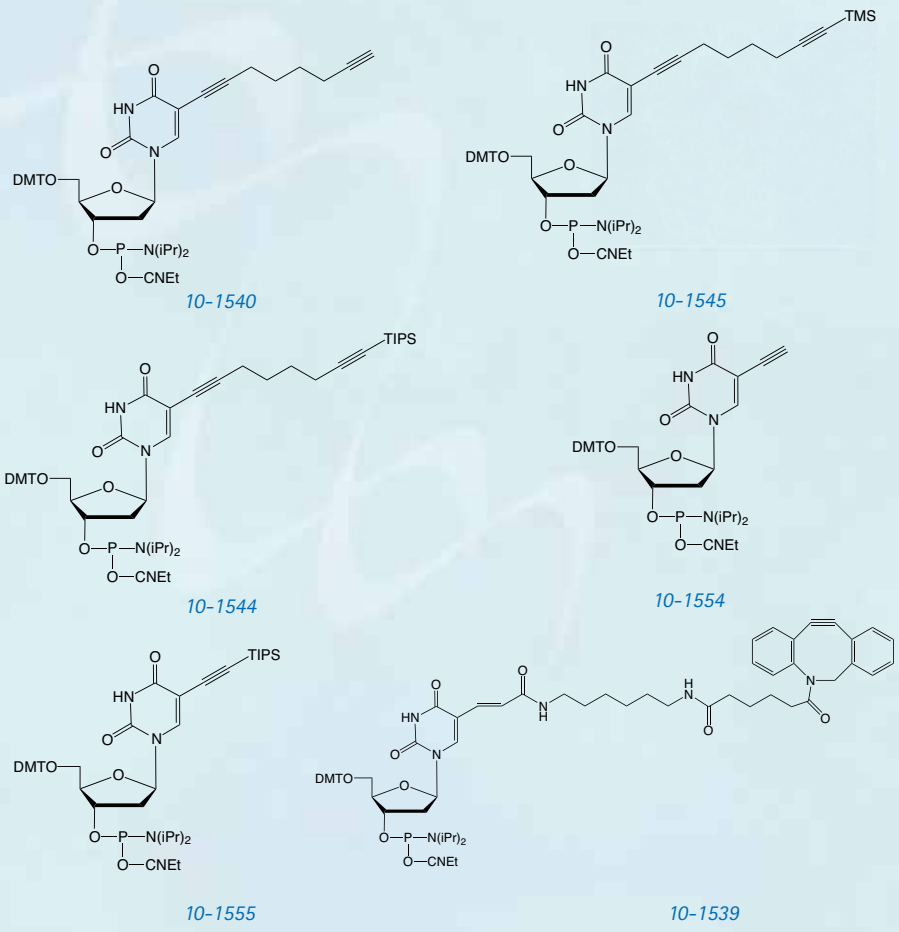
20164

convenient click conjugation with an azide to generate a label rigidly attached to one of the oligonucleotide bases. Mild deprotection conditions are necessary when using 5-Ethynyl-dU to prevent a hydration side reaction, which lowers the available alkyne level for the subsequent click reaction. TIPS-5-Ethynyl-dU (10-1555) contains an alkyne protected with a triisopropylsilyl (TIPS) group, which prevents acid or base catalyzed hydration during oligonucleotide synthesis and deprotection. A quick treatment with TBAF post synthesis removes the TIPS protecting group.

These click chemistry dT analogues are patent protected and available from Glen Research in collaboration with baseclick GmbH.

The dibenzocyclooctyl (DBCO) group exhibits the following desirable properties for use in oligonucleotide synthesis: simple to use; stable in solution on the synthesizer; stable to ammonium hydroxide and AMA; and excellent click performance in 17 hours or less at room temperature. DBCO-dT (10-1539) can be used for inserting a DBCO group at any position within the oligonucleotide and is then available for copper free click coupling with an azide.

FIGURE 5: **STRUCTURES OF CLICK CHEMISTRY SEQUENCE MODIFIERS**



10-1540 10-1545 10-1544 10-1554 10-1555 10-1539