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REVERSIBLE M^{6}A RNA MODIFICATION

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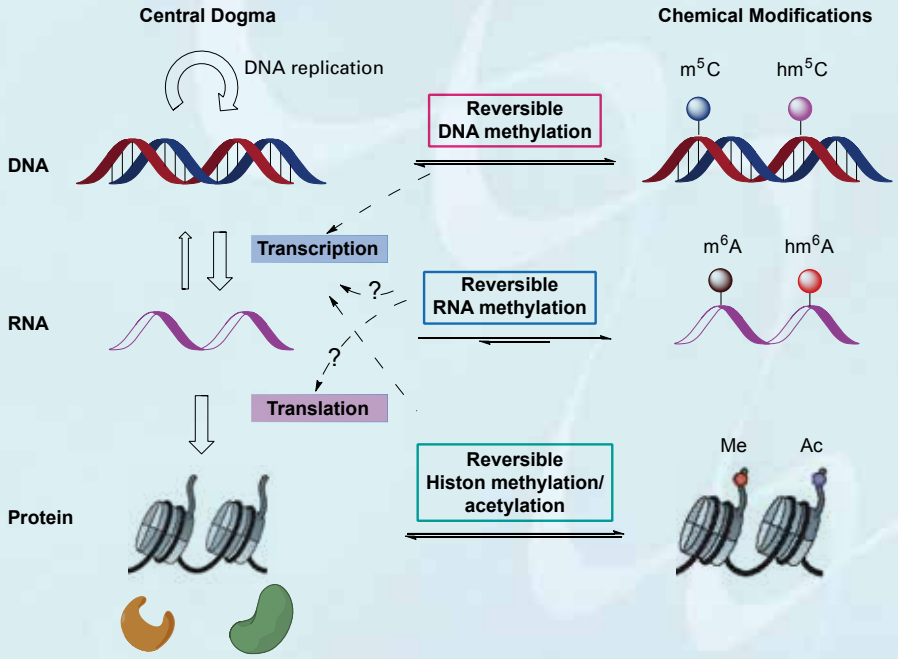
In the central dogma of molecular biology, genetic information flows from DNA to RNA and then to protein. Reversible epigenetic modifications on genomic DNA[1] and histone[2] have been known to substantially regulate gene expression (Figure 1). On the other hand, there exists more than 100 naturally occurring chemical modifications in RNA[3]; however, the functions of these RNA modifications are largely unknown. Whether some of these modifications in RNA can be reversed and could impact gene expression in the central dogma was unknown until the recent discovery of N^6-methyladenosine (m^{6}A) as the first example of reversible RNA methylation[4].

Discovered in the 1970s[5], the m^{6}A modification exists in different types of RNA including rRNA, snRNA, tRNA, mRNA, and lncRNAs etc. It is the most prevalent internal modification in mRNAs and lncRNAs in higher eukaryotes[6]. The amount of m^{6}A in isolated RNA was estimated to be 0.1–0.4% of that of adenines (that is, ~3–5 m^{6}A sites per mRNA) in mammals and the identified consensus sequence is [G/A/U][G>A]m^{6}AC[U>A>C][7]. The genome-wide distribution of m^{6}A in mammals was recently

FIGURE 1: **REVERSIBLE CHEMICAL MODIFICATIONS THAT REGULATE THE FLOW OF GENETIC INFORMATION**



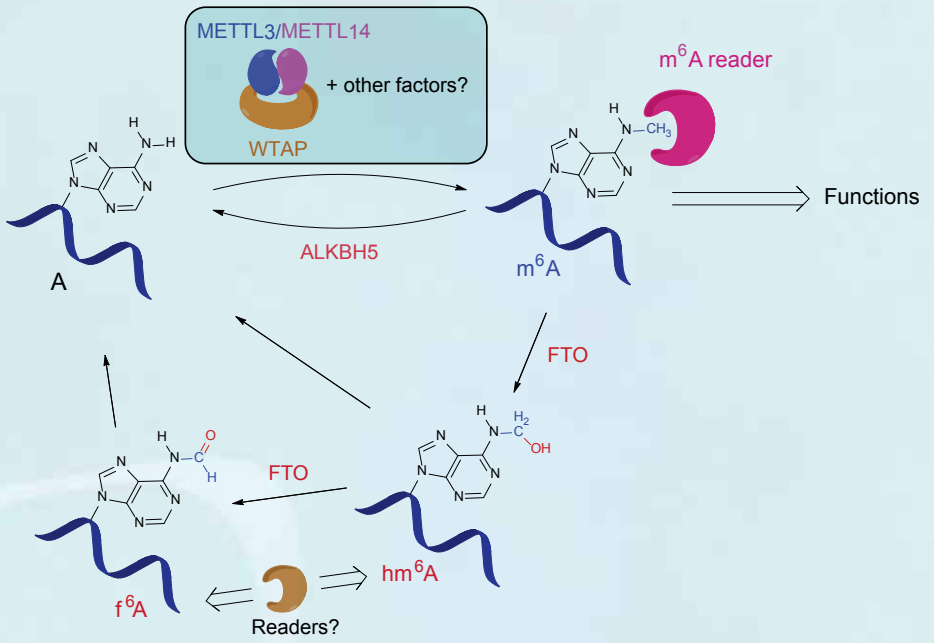
(Adapted from Y. Fu *et al.*, Nat. Rev. Genet. 2014, **15**, 293-306).

revealed using m^{6}A-seq by two laboratories[8]. The resulting maps have shown that m^{6}A is widely distributed in more than 7,000 mRNA and 300 non-coding RNA (ncRNA) transcripts in human cells and mouse brain. m^{6}A is also enriched around stop codons, in 3' untranslated regions (3'UTRs), and within internal long exons. Many m^{6}A peaks are well conserved between humans and mice, and dynamic changes of certain peaks have been observed under different stress conditions. These observations further suggest that the m^{6}A modification in mRNA and lncRNA may have significant biological functions.

In 2011, the discovery of the α-ketoglutarate-dependent dioxygenase FTO as the first RNA demethylase reignited investigations of m^{6}A biology[4a]. FTO is an intriguing protein that has been associated with human obesity. This protein efficiently demethylates m^{6}A via two unprecedented intermediates, N^6-hydroxymethyladenosine (hm^{6}A) and N^6-formyladenosine (f^{6}A), which were generated through the FTO-catalyzed oxidation of m^{6}A (Figure 2)[9]. Further experiments showed that silencing of FTO in HeLa and 293FT cells increased the total m^{6}A level in polyadenylated RNA, whereas over-expression of FTO decreased the total m^{6}A level in polyadenylated RNA. Subsequently, ALKBH5 was found to be the second demethylase that shows efficient demethylation activity towards m^{6}A in mRNA and other nuclear RNA[4b]. The reversible nature of the m^{6}A modification and the unique distribution pattern of m^{6}A in mRNA have attracted great attention from the biological community since.

It has been known that METTL3 is an active component of the m^{6}A methyltransferase complex in mammalian cells but other components are required to achieve optimal activity *in vitro*. In 2014, METTL14 was characterized to be another active component of the m^{6}A methyltransferase complex and forms a stable hetero complex with METTL3[10] (Figure 2). Biochemical characterization revealed that these two proteins form a stable complex with a stoichiometric ratio of 1:1. The combination of both methyl transferases leads to a substantially enhanced methylation activity. This heterodimer also shows a strong preference for the cognate m^{6}A consensus sequence and a modest preference for less structured

FIGURE 2: **METHYLATION OF ADENOSINE AND DEMETHYLATION OF M^{6}A IN mRNA AND OTHER NUCLEAR RNA**



(Adapted from Y. Fu *et al.*, Nat. Rev. Genet. 2014, **15**, 293-306).

RNA *in vitro*. WTAP, a protein involved in splicing, is the third critical component of the m^{6}A methyltransferase complex *in vivo*[10a,11] (Figure 2).

For the m^{6}A group to have a biological function, it needs to be recognized through "reading" by specific proteins. This process could resemble the roles of proteins that read 5-methylcytosine (5mC) in DNA, or methylated or acetylated amino acid residues of histones in order to exhibit biological functions associated with the modifications and to enable reversible tuning. Pull-down experiments using synthetic RNA probes in the presence and absence of m^{6}A identified the YTH family proteins as m^{6}A-specific binding proteins[8a,12]. One of the reader proteins, YTHDF2, preferentially recognizes m^{6}A-containing mRNA and regulates the degradation of the methylated transcripts[12] (Figure 2). A recent structural characterization revealed a well-conserved hydrophobic pocket used by the YTH family proteins to selectively recognize the methyl group of m^{6}A in RNA[13].

The discoveries and characterization of the m^{6}A writers, erasers and readers, together with the parallel development of high-throughput assays that profile this methylation on a transcriptome-wide scale, set the stage and provided tools for functional investigations that aim to identify the mechanisms by which m^{6}A is translated into biological outcomes.

The synthesis of m^{6}A phosphoramidite and its incorporation into RNA have helped to discover the reader and eraser proteins and will find more applications in studies on how m^{6}A affects RNA structures, recruits binding proteins, and interacts with writers and erasers. It may also find applications on developing inhibitors to interfere with the m^{6}A writing, erasing, and reading processes as potential drug candidates.

The synthesis of the first version of m^{6}A phosphoramidite (I) without protecting the MeNH- group has been reported[14] (Figure 3). This version of m^{6}A phosphoramidite couples as efficiently as unmodified phosphoramidites when tetrazole is used as the activator. After deprotection, HPLC analysis showed the full-length RNA to be the major product. It is possible that the unprotected MeNH- group may also be subject to coupling to give a branched RNA byproduct, especially when DCI is used as the activator.

To completely eliminate the possible coupling of MeNH- to form an undesired byproduct, Glen Research has developed a new version of m^{6}A phosphoramidite with MeNH- protected by the PhOAc group (II). This protecting group can be readily removed under normal base treatment

during deprotection. The availability of this new m^{6}A phosphoramidite will significantly facilitate investigations of m^{6}A and its related biological functions.

References:

1 (a) M. M. Suzuki, *et al.*, Nat. Rev. Genet., 2008, **9**, 465–476. (b) R. M. Kohli, *et al.*, Nature, 2013, **502**, 472–479. (c) P. A. Jones, Nat. Rev. Genet., 2012, **13**, 484–492. (d) M. R., Branco, *et al.*, Nat. Rev. Genet. 2012, **13**, 7–13. (e) N. Bhutani, *et al.*, Cell, 2011, **146**, 866–872.

2 (a) B. D. Strahl, *et al.*, Nature, 2000, **403**, 41–45. (b) Y. Shi, Nat. Rev. Genet., 2007, **8**, 829–833. (c) R. J. Klose, *et al.*, Nat. Rev. Genet., 2006, **7**, 715–727. (d) A. Bird, Science. 2001, **294**, 2113–2115.

3 M. A. Machnicka, *et al.*, Nucleic Acids Res. 2013, **41**, D262–D267.

4 (a) G. Jia, *et al.*, Nat. Chem. Biol. 2011, **7**, 885–887. (b) G. Zheng, *et al.*, Mol. Cell, 2013, **49**, 18–29.

5 (a) R. P. Perry, *et al.*, Cell, 1974, **1**, 37–42. (b) R. Desrosiers, *et al.*, Proc. Natl. Acad. Sci. USA, 1974, **71**, 3971–3975.

6 C. M. Wei, *et al.*, Cell, 1975, **4**, 379–386.

7 (a) P. Narayan, *et al.*, Science, 1988, **242**, 1159–1162. (b) T. Csepany, J. Biol. Chem., 1990, **265**, 20117–20122. (c) P. Narayan, *et al.*, Nucleic Acids Res., 1994, **22**, 419–426.

8 (a) D. Dominissini, *et al.*, Nature, 2012, **485**, 201-208. (b) K. D. Meyer, *et al.*, Cell, 2012, **149**, 1635–1646.

9 Y. Fu *et al.*, Nature Commun., 2013, **4**, 1798.

10 (a) J. Liu, *et al.*, Nature Chem. Biol., 2014, **10**, 93-95. (b) Y. Wang, *et al.*, Nat. Cell Biol., 2014, **16**, 191–198.

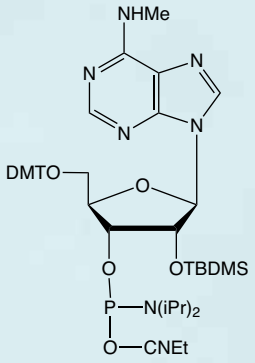
11 X. L. Ping, *et al.*, Cell Res. 2014, **24**, 177–89.

12 X. Wang *et al.*, Nature, 2014, **505**, 117–20.

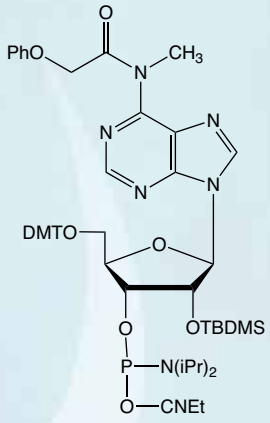
13 C. Xu, *et al.*, Nat. Chem. Biol. 2014, **10**, 927–9.

14 Q. Dai *et al.*, Nucleic Acids Res., 2007, **35**, 6322–6329.

***FIGURE 3:* STRUCTURES OF M^{6}A PHOSPHORAMIDITES**



m^{6}A phosphoramidite (I)



Protected m^{6}A phosphoramidite (II)

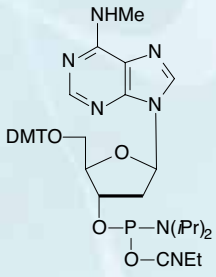
NEW PRODUCT - N6-Me-A - REVERSIBLE M^{6}A RNA MODIFICATION

Since 2009, Glen Research has been active in providing monomers that may be implicated in the mechanisms of DNA methylation and demethylation. We were intrigued to see that a similar scenario may be playing out in the field of reversible RNA methylation and we were delighted when Chuan He and his colleague Qing Dai offered to review the field with specific emphasis on their own work at the University of Chicago.

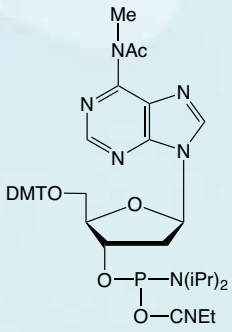
As they note in the accompanying article, it has been shown that the unprotected secondary amine of our original N6-Me-dA monomer (1) could support branching during oligonucleotide synthesis. In an article in The Glen Report 23.1 in 2011, we showed that although branching was minimal using tetrazole as activator, it became very significant (~15%) using DCI as activator. Consequently, we now offer the acetyl protected version of N6-Me-dA (2), which eliminates this branching reaction.

It was our decision to offer the N6-Me-A RNA monomer with a phenoxyacetyl protecting group to minimize potential branching. We have shown the phenoxyacetyl protected N6-Me-A (3) to be completely compatible with all popular RNA synthesis and deprotection methods, from UltraMild to the most popular procedure using AMA for deprotection.

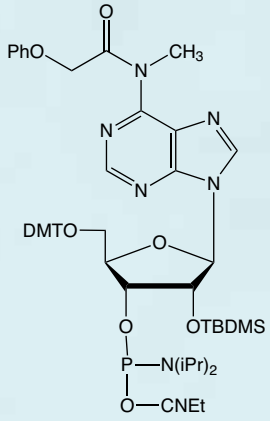
***FIGURE 1:* STRUCTURES OF GLEN RESEARCH N6-Me-dA AND N6-Me-A PHOSPHORAMIDITES**



N6-Me-dA (1)



N6-Ac-N6-Me-dA (2)



N6-Me-A (3)

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
N6-Me-A-CE Phosphoramidite	10-3005-95	50 µmole	285.00
	10-3005-90	100 µmole	550.00
	10-3005-02	0.25g	1295.00

NEW PRODUCT - 5'-CARBOXY-MODIFIER C5

Conjugation reactions in organic chemistry are generally fairly straightforward with a nucleophile reacting with an electrophile to form the conjugate. In the field of oligonucleotide synthesis, it has proven to be expedient to include the nucleophile in the oligonucleotide for post synthesis conjugation with a suitable electrophile. There is a catalog of nucleophilic modifiers available to fit virtually any circumstances.

Nucleophile	Target Electrophile
Amine	*Activated Carboxylate*
Thiol	*Maleimide*
Aminooxy	*Aldehyde*

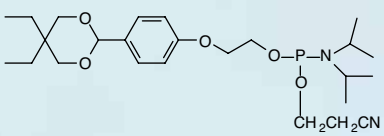
However, the electrophilic modifiers are a little more complicated. Although oligonucleotides are fairly easily modified with electrophilic aldehyde groups, which can readily be conjugated with hydrazide, hydrazine and aminooxy groups, our maleimide modifier requires some delicate chemistry to generate a maleimide-modified oligo in preparation for reaction with a thiol. And, in this article, we will focus on carboxy modification of oligonucleotides as we introduce a new 5'-Carboxy-Modifier.

The simplest approach to carboxy modification of oligos is to include the carboxylate NHS ester in the phosphoramidite to form the protected and activated carboxylate *in situ*. Both of our NHS ester carboxy-modifiers (5'-Carboxy-Modifier C10 and NHS-Carboxy-dT) use this approach. While the oligo is still fully protected and attached to the support, it can be reacted with amines on the synthesis column. The reaction is fast and specific while any precious excess amine can be recovered. A downside to the approach of simple amine conjugation on column is that the reacted amino species must be able to survive the conditions of cleavage and deprotection.

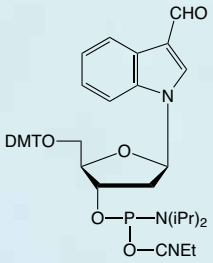
If the amine chosen for conjugation is not stable to the conditions of cleavage and deprotection, the oligonucleotide has first to be cleaved and deprotected with sodium hydroxide to generate the carboxylate sodium salt. Deprotection with sodium hydroxide is a standard technique which is not very popular since the solution can not be simply evaporated like ammonium hydroxide or AMA. If ammonium hydroxide or AMA were to be used instead, the carboxylate NHS ester would be substantially converted to unreactive amide. Our Carboxy-dT also falls into this category in that the methyl ester protecting the carboxylate has to be hydrolyzed with sodium hydroxide.

An alternative approach to 5'-carboxy-modification was described[1] a few years ago in which the carboxylate is protected with a 2-chlorotrityl group. This protecting group is simply removed using the standard deblock cycle to generate a free carboxyl group on an otherwise fully protected oligonucleotide. Using a standard peptide

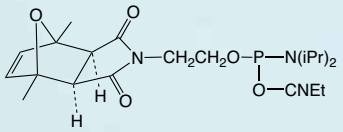
FIGURE 1: STRUCTURES OF GLEN RESEARCH ELECTROPHILIC PHOSPHORAMIDITES



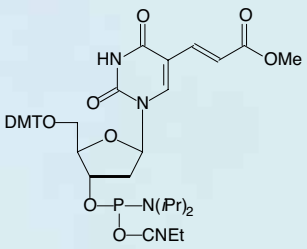
5'-Aldehyde-Modifier C2



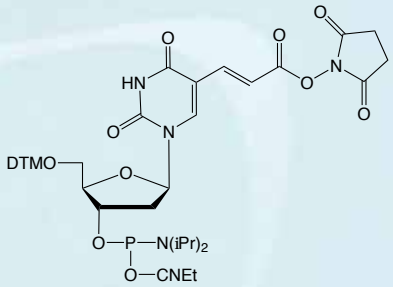
Formylindole



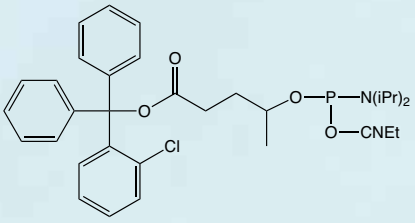
5'-Maleimide-Modifier



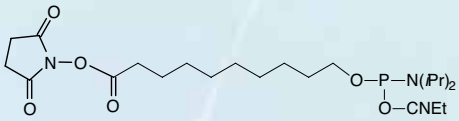
Carboxy-dT



NHS-Carboxy-dT



5'-Carboxy-Modifier C5



5'-Carboxy-Modifier C10

TABLE 1: COMPARISON OF 5'-CARBOXY-MODIFIERS

NHS Ester Protected	2-Chlorotrityl Protected
Preactivated for On Column conjugation with simple amines	*Requires trityl group to be removed and further activation*
On Column conjugate must be stable to deprotection conditions	*On Column conjugate must be stable to deprotection conditions*
Isolation of free carboxylate requires deprotection with sodium hydroxide	*Isolation of free carboxylate can be done using ammonium hydroxide, AMA or sodium hydroxide*
	No need to remove 2-chlorotrityl group prior to deprotection
	Useful for conjugation with amino acids and small peptides for library formation

coupling reaction, the carboxyl group can be reacted with amines or amino acids to form conjugates. The procedure, shown in Figure 2, is also fully compatible with the formation of oligonucleotide peptide conjugates or libraries.

Alternatively if a 5′ free carboxylate is desired, the oligonucleotide can be cleaved and deprotected trityl-on *or* trityl-off using ammonium hydroxide or AMA with no amide formation, as shown in Figure 3.

We have found that the optimal coupling time for this product is 3 minutes and the chlorotrityl group can be removed using the standard deblock procedure on the synthesizer. It should be noted that the chlorotrityl group is a different color from the regular DMT group so trityl monitors may not register the release properly.

It should also be noted that the 2-chlorotrityl group is removed during oligo deprotection, as shown in Figure 3, and is incompatible with RP purification techniques.

We are happy to introduce 5′-Carboxy-Modifer C5 to extend our range of electrophilic modifiers.

Reference:

1. A.V. Kachalova, *et al.*, *Helv Chim Acta*, 2002, **85**, 2409-2416.

PROCEDURE FOR ON-COLUMN CONJUGATION

1. Synthesize the 5′-carboxylate-modified oligo trityl-off and retain on the support.
2. Pre-activate the carboxylic acid by treating the support-bound oligo with HATU (100 equivalents) and HOBT (100 equivalents) in dry DMF (100µL).
3. Warm the reaction to 35°C and shake support for 35 minutes.
4. After activation of the acid is complete, add triethylamine (100 equivalents) and the amine (100 equivalents).
5. Warm the conjugation mixture to 35°C and shake the support for 1 hour.
6. The unbound amine can easily be removed from the solid support by washing successively with DMF (2 x 100µL), ethanol (2 x 200µL), and distilled water (2x 200µL).
7. The conjugate can then be deprotected and removed from the solid support using ammonium hydroxide or AMA using conditions appropriate for deprotection of the nucleobases.
8. The conjugate is now ready for purification.

FIGURE 2: **ON-COLUMN CONJUGATION OF AMINE, AMINO-ACID OR PEPTIDE**

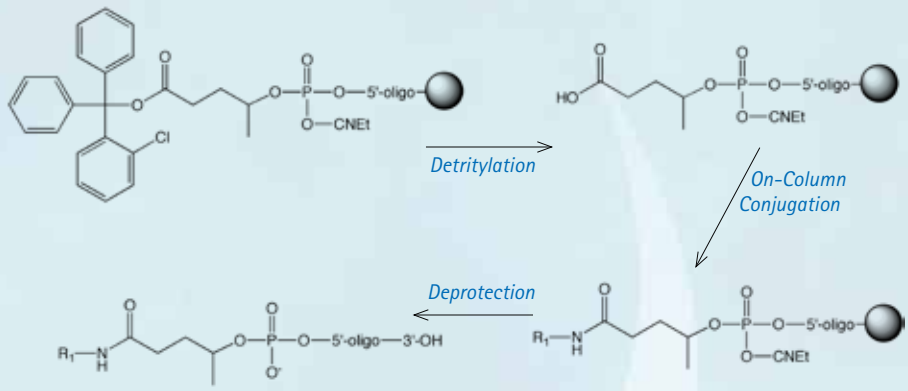
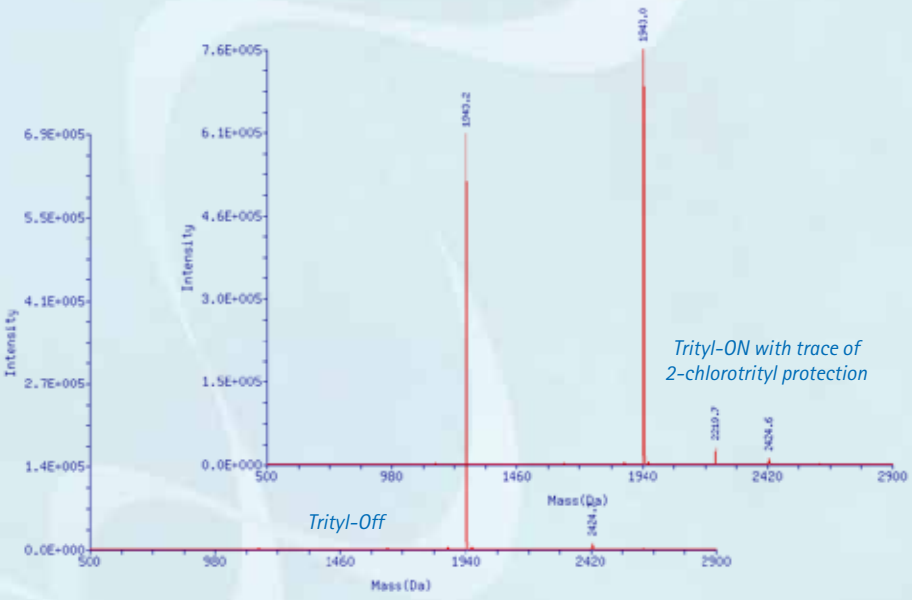


FIGURE 3: **ESI MS DATA FOR OLIGOS DEPROTECTED WITH AMA - TRITYL-ON VERSUS TRITYL-OFF**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5′-Aldehyde-Modifier C2 Phosphoramidite	10-1933-90	100 µmole	85.00
	10-1933-02	0.25g	325.00
Formylindole CE Phosphoramidite	10-1934-90	100 µmole	85.00
	10-1934-02	0.25g	325.00
5′-Maleimide-Modifier Phosphoramidite	10-1938-90	100 µmole	70.00
	10-1938-02	0.25g	335.00
Carboxy-dT	10-1035-90	100 µmole	180.00
	10-1035-02	0.25g	360.00
NHS-Carboxy-dT	10-1535-90	100 µmole	210.00
	10-1535-02	0.25g	550.00
5′-Carboxy-Modifier C10	10-1935-90	100 µmole	50.00
	10-1935-02	0.25g	200.00
5′-Carboxy-Modifier C5	10-1945-90	100 µmole	95.00
	10-1945-02	0.25g	330.00

DNA-MEDIATED CHARGE TRANSPORT: A NOVEL PROPERTY OF DNA WITH DIVERSE APPLICATIONS

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California Institute of Technology
Pasadena, CA 91125

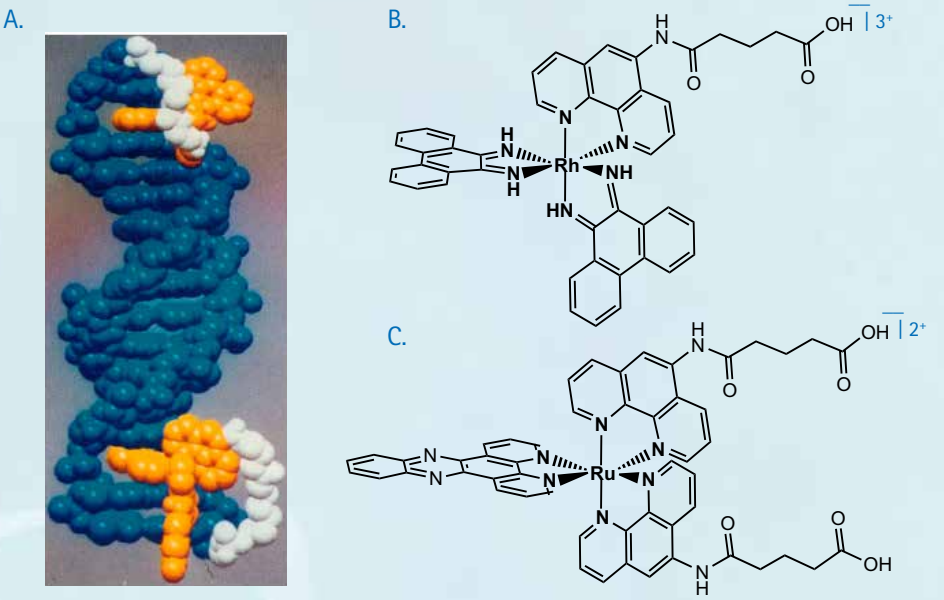
DNA is traditionally thought of as the cell's library of genetic information. However, work in the Barton lab over the last three decades has brought to light a new side of DNA, revealing the ability of this molecule to facilitate the transport of charge over long molecular distances. This fascinating property of DNA has been investigated under biologically relevant conditions using a variety of redox probes.

One of the first studies to examine DNA-mediated charge transport utilized covalently bound metallointercalators to explore long range quenching of luminescence by DNA-mediated electron transfer. $[Ru(phen')_2(dppz)]^{2+}$ and $[Rh(phi)_2(phen')]^{3+}$ were covalently tethered to opposite ends of a 15-mer DNA duplex[1]. Here covalent tethering made electron transfer through the π-stacked base pairs the only pathway fully able to explain the observed luminescence quenching. This experiment really provided the first indication that long range electron transfer could proceed through the DNA duplex.

While the above studies were able to establish the occurrence of DNA-mediated charge transport in excited state systems, a different method was required to investigate these processes in the ground state. Electrochemical techniques provided the ideal means of carrying out this aim. In these experiments, an organic redox probe was tethered to one end of a DNA strand which was then annealed to an alkanethiol-modified complement. The thiol linker facilitated the formation of self-assembled DNA monolayers on a gold electrode, allowing direct interrogation of electron transfer between the surface and the probe.

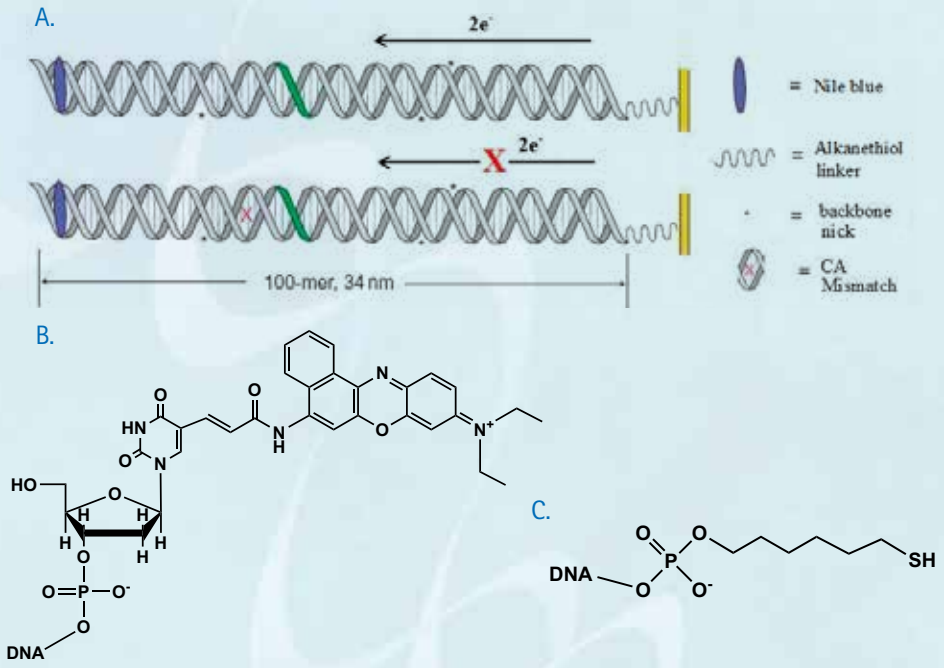
Using cyclic voltammetry with a Nile Blue reporter covalently bound and well coupled electronically to the DNA, a key set of experiments compared electron transfer rates between a 100-mer and a 17-mer DNA duplex[2]. In fact, ground state electron transfer could proceed through the 100-mer duplex to reduce the covalently bound Nile Blue. Moreover, electron transfer rates were virtually indistinguishable, showing almost no attenuation over 100 base pairs (a distance of 34 nm). Remarkably, the presence of multiple nicks in the phosphate backbone had no effect on this process, while the incorporation of a single mismatched base pair significantly attenuated the signal, revealing that long-range charge transfer through DNA is dependent on base-pair stacking alone. Interestingly, the attenuation associated with the presence of the single base mismatch was

FIGURE 1: DNA BINDING BY INTERCALATING METAL COMPLEXES



Fluorescent $[Ru(phen')_2(dppz)]^{2+}$ and $[Rh(phi)_2(phen')]^{3+}$ quencher covalently tethered to a 15-mer DNA duplex.
A) Intercalated metal complexes, with Rh (III) at top and Ru (II) at bottom.
B) Free $[Rh(phi)_2(phen')]^{3+}$. C) Free $[Ru(phen')_2(dppz)]^{2+}$.
C.J. Murphy, M.R. Arkin, Y. Jenkins, N.D. Ghatlia, S.H. Bossmann, N.J. Turro, and J.K. Barton
Science (1993) 262 p. 1025.

FIGURE 2: DNA ELECTROCHEMISTRY WITH TETHERED NILE BLUE



Charged transport through a 100-mer. A) Nile blue-modified 100-mer DNA duplex illustrating mismatch discrimination. B) Nile blue modifier. C) Thiol linker.
J.D. Slinker, N.B. Muren, and J.K. Barton
Nat. Chem. (2011) 3 p. 228.

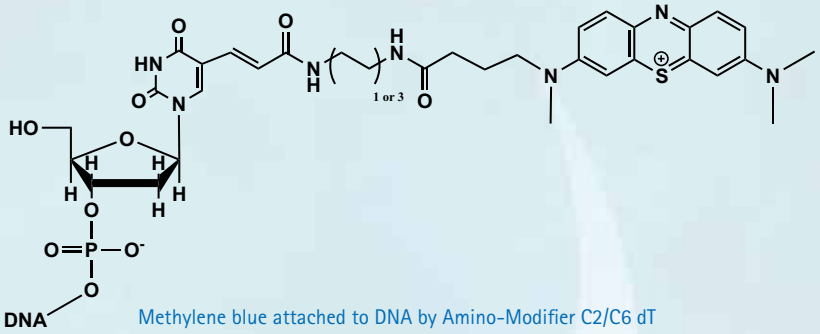
equivalent for the 100-mer and for the 17-mer. Finally, the probe signal could be removed by reaction with a blunt end restriction enzyme, showing that surface-bound DNA exists in a biologically accessible conformation and paving the way for further applications of electrochemistry with DNA.

While the work with Nile Blue made great progress in establishing the general mechanisms of DNA charge transport, the short tether length of Nile Blue does not permit intercalation into the base stack, resulting in relatively small signals. This issue was resolved by the use of methylene blue covalently bound to amino-modified DNA. Covalent methylene blue exhibits the same desirable electron transfer properties as Nile Blue, but the flexible linker allows the probe to directly intercalate into the base stack, improving charge transfer signal size. An additional degree of versatility with this probe can be achieved by varying the surface density of the DNA monolayer on gold, favoring DNA-mediated (high density) or surface-mediated (low density) processes[3], thus making covalent methylene blue suitable for a wide range of diagnostic applications.

Building on this body of earlier work, DNA-mediated charge transport has seen widespread applicability in areas ranging from analyte detection to biochemistry. Organic probes have been particularly useful in these pursuits, as demonstrated by the recent use of covalent methylene blue as a reporter of human Dnmt1 methyltranferase activity[4] (known to be aberrant in several cancer types). Tethered metal complexes have been applied in novel ways in recent work, as exemplified by a study which used photoexcited $[Ru(phen)(dppz)(bpy')]^{2+}$ to oxidize guanines in DNA (a process requiring potentials too high to observe on gold electrodes) with or without a bacterial ferritin, allowing the capability of this protein to prevent oxidative DNA damage in bacterial pathogens to be assessed[5].

As the examples above have shown, the applications of DNA-mediated charge transport have expanded dramatically in recent years, a trend which shows no signs of slowing. The addition of methylene blue and ferrocene phosphoramidites to the Glen Research catalogue at this time is thus particularly exciting, and will no doubt contribute significantly to the continued advancement of this field.

FIGURE 3: COVALENTLY TETHERED METHYLENE BLUE



Methylene blue attached to DNA by Amino-Modifier C2/C6 dT

References:

(1) C.J. Murphy, M.R. Arkin, Y. Jenkins, N.D. Ghatlia, S.H. Bossman, N.J. Turro, and J.K. Barton. Long-Range Photoinduced Electron Transfer Through a DNA Helix. *Science* 1993, **262**(5136), 1025-1029

(2) J.D. Slinker, N.B. Muren, S.E. Renfrew, and J.K. Barton. DNA charge transport over 34 nm. *Nat. Chem.* 2011, **3**(3), 228-233

(3) C.G. Pheeney and J.K. Barton. DNA Electrochemistry with Tethered Methylene Blue. *Langmuir* 2012, **28**, 7063-7070

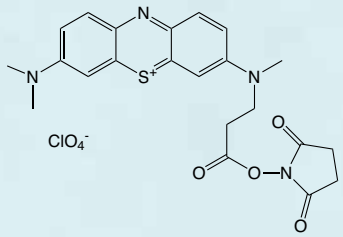
(4) N.B. Muren and J.K. Barton. Electrochemical Assay for the Signal-On Detection of Human DNA Methyltransferase Activity. *J. Amer. Chem. Soc.* 2013, **135**, 16632-16640

(5) A.R. Arnold and J.K. Barton. DNA Protection by the Bacterial Ferritin Dps via DNA Charge Transport. *J. Amer. Chem. Soc.* 2013, **135**, 15726-15729

NEW PRODUCT - METHYLENE BLUE NHS ESTER

We are delighted to be able to publish this review article from Jackie Barton and Phil Bartels from Caltech. Glen Research is pleased to have been a supplier of products to the Barton group and several others at Caltech for many years. Some of the products used in this research are shown below. We are also introducing Methylene Blue NHS Ester (1) to supplement the phosphoramidite that we recently added to our catalog.

FIGURE 1: METHYLENE BLUE NHS ESTER



(1) Methylene Blue NHS Ester

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
Amino-Modifier C2 dT	10-1037-90	100 µmole	180.00
	10-1037-02	0.25g	360.00
	10-1037-05	0.5g	720.00
Amino-Modifier C6 dT	10-1039-90	100 µmole	180.00
	10-1039-02	0.25g	360.00
	10-1039-05	0.5g	720.00
3'-Thiol-Modifier C3 S-S CPG	20-2933-01	0.1g	85.00
	20-2933-10	1.0g	600.00
1 µmole columns	20-2933-41	Pack of 4	125.00
0.2 µmole columns	20-2933-42	Pack of 4	75.00
Ferrocene-dT-CE Phosphoramidite	10-1576-95	50 µmole	170.00
	10-1576-90	100 µmole	330.00
	10-1576-02	0.25g	670.00
Methylene Blue C3 Phosphoramidite	10-5960-95	50 µmole	315.00
	10-5960-90	100 µmole	610.00
	10-5960-02	0.25g	1500.00
Methylene Blue NHS Ester (Dissolve 5.4mg in 60µL of DMSO)	50-1960-23	5.4mg	540.00

TECHNICAL BRIEF - WHICH 3'-AMINO-MODIFIER?

In previous Glen Reports, we have presented Technical Briefs covering: 'Which 5'-Amino-Modifier?'; and 'Chemical Phosphorylation, Considering The Options'. Therefore, we think it would be timely to present this article on 'Which 3'-Amino-Modifier?'

We offer two types of 3'-amino-modifier – the first consists of a pair of branched chain linkers where the amine is protected with the ubiquitous fluorenylmethoxycarbonyl (Fmoc) protecting group; and the second uses a straight chain linker to the amine connecting to the support through a phthaloyl (PT) amide group.

FMOC-PROTECTED AMINO SUPPORTS

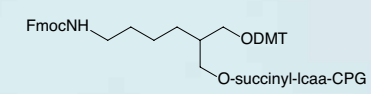
Our selection of Fmoc-protected 3'-Amino-Modifiers is shown in Figure 1. Both supports are based on a 1,3-diol backbone with a 6 atom linker to the Fmoc-protected amino group. We prefer the 1,3-diol type linkage to the support since it is very much less likely to eliminate on deprotection than the alternative 1,2-diol linkage. The mechanism of this elimination reaction is detailed in Figure 2. In contrast, the 1,3-diol does not have the same tendency to form the cyclic phosphate intermediate that leads to elimination of the linker to 3'-OH.

A consequence of the branch in the linker to accommodate attachment to both the support and the DMT group is a chiral center at the branch point. Once an oligo is synthesized, cleaved and deprotected, the chiral branch point leads to a pair of diastereomers, which can be separated chromatographically. However, the diastereomers are normally only observed in short oligos.

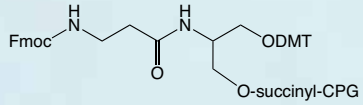
One of the major issues we have observed with Fmoc protection over the years is that the group can be replaced with acetyl during capping of the bulk support in the production process. This acetyl group of the protected amine impurity is not removed during regular oligonucleotide deprotection and so that percentage of available amine for further reaction is lost.

Interestingly, the percentage of the acetyl capped impurity is generally formed in a significantly higher amount in the production of 500Å CPG than 1000Å CPG. The average

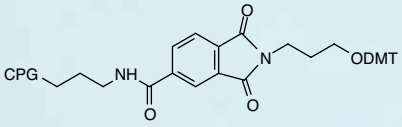
FIGURE 1: **STRUCTURES OF 3'-AMINO-MODIFIERS**



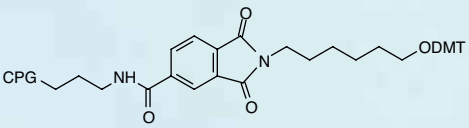
3'-Amino-Modifier C7 CPG



3'-Amino-Modifier Serinol CPG



3'-PT-Amino-Modifier C3 CPG



3'-PT-Amino-Modifier C6 CPG

FIGURE 2: **MECHANISM OF ELIMINATION OF 3'-AMINO-MODIFIERS BASED ON 1,2-DIOL LINKERS**

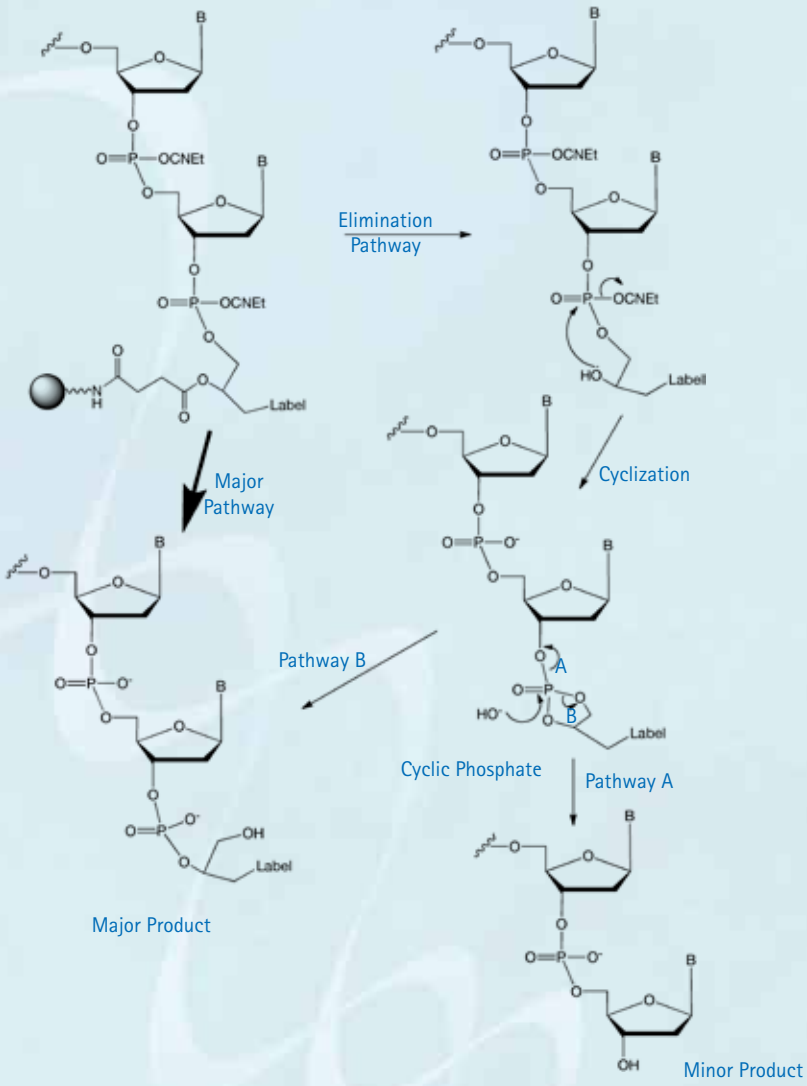


TABLE 1: **DEPROTECTION CONDITIONS FOR 3'-PT-AMINO-MODIFIERS**

Deprotection Reagent	Deprotection Conditions	Time
Ammonium Hydroxide	*Room Temperature*	*48 hours*
Ammonium Hydroxide	*55°C*	*17 hours*
AMA	*Room Temperature*	*2 hours*
AMA	*65°C*	*10 minutes*
0.4M NaOH in Methanol/Water (4:1)	*Room Temperature*	*17 hours*
0.05M Potassium carbonate in methanol		*Not Compatible*

content of the acetyl capped impurity in the 500Å batches released over the last two years was 3.7%. In the case of the 1000Å CPG version, the average content was 1.6%.

The last two years of batches of 500Å and 1000Å CPG also revealed that the average loading of 500Å batches (20-2957) was 43.9 µmoles/g with a range of 38-49 µmoles/g. The dropoff point (where the synthesis begins to falter due to steric hindrance) has averaged 60-mer in length. The average loading of the equivalent 1000Å batches (20-2958) was 43.4 µmoles/g with a range of 36-48 µmoles/g. The average dropoff point was >100-mer in length.

The 500Å CPG version is a historical anomaly in our catalog in that we use 1000Å CPG for all of our other modifiers. The data provided in this article clearly show that the 1000Å CPG product is superior in quality, and consequently in performance, to the 500Å equivalent. Effective January 1, 2015, this support is being discontinued and we will routinely stock only the 1000Å version.

3'-PT-AMINO-MODIFIERS

We offer two linker lengths in our 3'-PT-Amino-Modifiers, C3 and C6, and the structures are shown in Figure 1. In these supports, the amino group that is destined to be the 3'-amino-modification is incorporated into a phthaloyl (PT) group and is fully protected throughout the synthesis procedure. The amino group is then fully hydrolyzed from the phthaloyl moiety under conditions shown in Table 1. Although cleavage in ammonium hydroxide is fairly slow, it should be noted that standard cleavage/deprotection with AMA at 65°C for 10 minutes is sufficient for complete hydrolysis to the primary amine. There are no side reactions and only pure 3'-alkylamine is released into solution. In addition, there is no chiral center in the linker so no diastereomers with the potential to confuse future purification steps are formed on deprotection.

CONCLUSION

Our 3'-Amino-Modifiers are popular products but how do you choose which is appropriate for your application? Table 2 contains a comparison of these two types of 3'-Amino-Modifiers and demonstrates the pros and cons of both.

TABLE 2: COMPARISON OF 3'-AMINO-MODIFIERS

Fmoc-Protected Amino Supports

Uses

- Blocking the 3' terminus from exonuclease digestion
- Labelling with active esters

Pros

- Fmoc group can be specifically removed before or after oligo synthesis prior to cleavage and deprotection to allow conjugation while the oligo is fully protected
- Compatible with all deprotection schemes from UltraFast to UltraMIId

Cons

- Diastereomers formed on deprotection
- Acetyl capped impurity formed during manufacture
- Amine prone to cyanoethylation

3'-PT-Amino-Modifiers

Uses

- Blocking the 3' terminus from exonuclease digestion
- Labelling with active esters

Pros

- With AMA deprotection, this is the most straightforward approach to producing a 3'-amino-modified oligo for subsequent labelling
- A simple straight chain alkyl linker connects the amino group to the 3'-terminus
- 100% of the 3'-amino group is available for conjugation

Cons

- Slow cleavage from support with ammonium hydroxide
- Incompatible with UltraMIId deprotection
- Oligo not released from support until PT group cleaved

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
3'-Amino-Modifier C7 CPG 500 (Discontinued. Replacement 20-2958 below)	20-2957		
3'-Amino-Modifier C7 CPG 1000	20-2958-01	0.1g	95.00
	20-2958-10	1.0g	675.00
1 µmole columns	20-2958-41	Pack of 4	140.00
0.2 µmole columns	20-2958-42	Pack of 4	85.00
10 µmole column (ABI)	20-2958-13	Pack of 1	250.00
15 µmole column (Expedite)	20-2958-14	Pack of 1	375.00
3'-Amino-Modifier Serinol CPG	20-2997-01	0.1g	95.00
	20-2997-10	1.0g	675.00
0.2 µmole columns	20-2997-42	Pack of 4	85.00
1 µmole columns	20-2997-41	Pack of 4	140.00
10 µmole column (ABI)	20-2997-13	Pack of 1	250.00
15 µmole column (Expedite)	20-2997-14	Pack of 1	375.00
3'-PT-Amino-Modifier C3 CPG	20-2954-01	0.1g	95.00
	20-2954-10	1.0g	675.00
1 µmole columns	20-2954-41	Pack of 4	140.00
0.2 µmole columns	20-2954-42	Pack of 4	85.00
10 µmole column (ABI)	20-2954-13	Pack of 1	250.00
15 µmole column (Expedite)	20-2954-14	Pack of 1	375.00
3'-PT-Amino-Modifier C6 CPG	20-2956-01	0.1g	95.00
	20-2956-10	1.0g	675.00
1 µmole columns	20-2956-41	Pack of 4	140.00
0.2 µmole columns	20-2956-42	Pack of 4	85.00
10 µmole column (ABI)	20-2956-13	Pack of 1	250.00
15 µmole column (Expedite)	20-2956-14	Pack of 1	375.00
3'-PT-Amino-Modifier C6 PS	26-2956-01	0.1g	125.00
	26-2956-10	1.0g	1025.00
200 nmole columns (AB 3900)	26-2956-52	Pack of 10	220.00
40 nmole columns (AB 3900)	26-2956-55	Pack of 10	220.00

POSSIBLE CROSSLINK

As our regular readers know, Glen Research has a long term interest in oligonucleotide interstrand crosslinking. During the enforced absence of cnvK, we continue to evaluate new methods that may allow a practical approach to crosslinking.

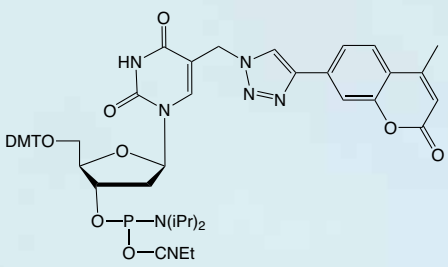
A recent publication[1] from Professor Peng and his group at the University of Wisconsin, Milwaukee, along with researchers from the University of California, Riverside, caught our attention. Using a synthetic approach involving click chemistry, a coumarin containing thymidine analogue, (1) in Figure 1, was prepared and incorporated into oligonucleotides using standard phosphoramidite chemistry. Interstrand crosslinking was achieved by irradiation using UV light at 350nm. The authors demonstrated that crosslinking occurs preferentially with dT on the opposite strand but also occurs at lower levels with dC and dA. The photo-induced crosslink with pyrimidines can be reversed with UV light at 254nm but is irreversible with purines.

While coumarin is not fluorescent (as we have noted in the past in relation to our coumarin azide) the triazole product of the click reaction is fluorescent. Although the authors describe a slightly different coumarin structure, the coumarin-thymidine analogue is fluorescent until a crosslink is formed, which shows that the conjugation system of the coumarin has been disrupted. This behavior indicates that the crosslinking occurs via a [2+2] cycloaddition with a double bond on the base opposite. The proposed crosslink with thymidine is shown in Figure 2.

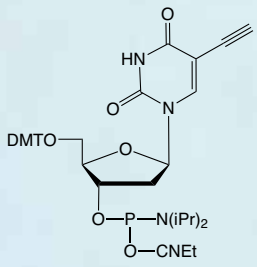
The authors also demonstrated that the interstrand crosslink was photoswitchable over six cycles of radiation at 350nm and 254nm. This may be the first observation of photoswitching of interstrand oligonucleotide crosslinks induced by a modified pyrimidine analogue.

This elegant work piqued our interest since we offer 5-ethynyl-dU, (2) in Figure 1, as a potential synthon for preparing possible crosslinking intermediates following a click reaction. As shown in Figure 3, the click reaction with our coumarin azide, (3) in Figure 1, forms an intermediate which also may be interesting for potential crosslinking. However, the location of the double bond

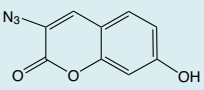
FIGURE 1: STRUCTURES OF CROSSLINKING PRODUCTS



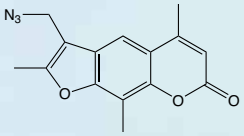
(1) Coumarin-Thymidine Analogue



(2) 5-Ethynyl-dU

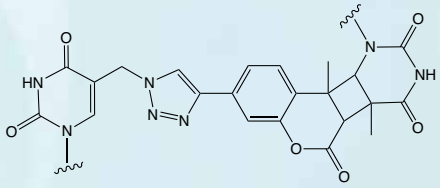


(3) Coumarin Azide



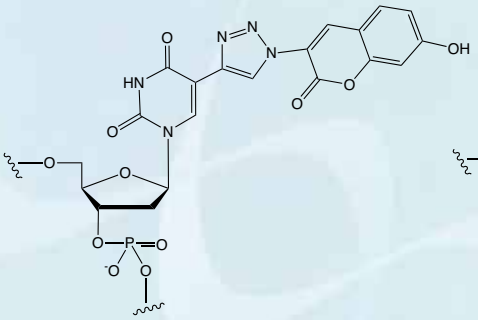
(4) Psoralen Azide

FIGURE 2: CROSSLINK BETWEEN COUMARIN ANALOGUE AND AN ADJACENT THYMIDINE

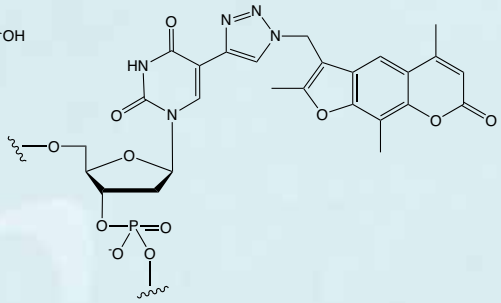


Coumarin-Thymidine Analogue Crosslinked with Thymidine

FIGURE 3: CLICK CONJUGATES BETWEEN ETHYNYL-dU AND COUMARIN AND PSORALEN AZIDES

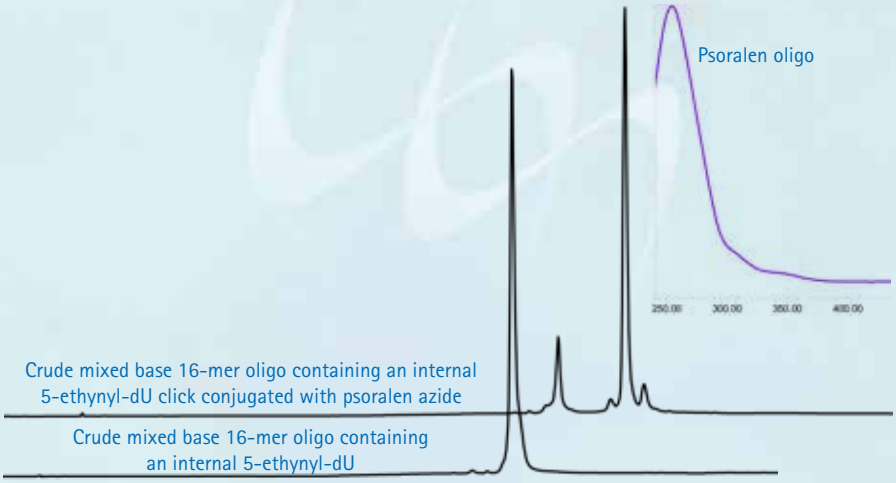


Click Product Between 5-Ethynyl-dU and Coumarin Azide



Click Product Between 5-Ethynyl-dU and Psoralen Azide

FIGURE 4: RP HPLC ANALYSIS - UNPURIFIED OLIGO CONTAINING POTENTIAL PSORALEN CROSSLINKER



which would be a partner in the [2+2] cycloaddition is close to the triazole ring and that may not be optimal in comparison to Professor Peng's structure.

A second option may be psoralen azide, (4) in Figure 1. The click reaction of psoralen azide with ethynyl-dU forms a potentially more interesting intermediate, as shown in Figure 3. In this case, the well known crosslinking behavior of psoralen with an adjacent thymidine residue may be indicated.

At this point, we simply demonstrate that both coumarin and psoralen azides form triazole intermediates with 5-ethynyl-dU, as shown in the chromatograms in Figure 4. We also introduce psoralen azide to our growing selection of azido tags.

POTENTIAL AZIDE SUPPORT

One of our customers inquired whether it would be possible to prepare an azide solid support for oligonucleotide synthesis for later reaction to an alkyne containing tag. A reference from the Morvan group[2] certainly indicates that an azide solid support can indeed survive the conditions of oligonucleotide synthesis and can be used to click to a suitable alkyne containing product. So we set out to determine if an azide support could be simply prepared using our existing products. Our strategy is outlined in Figure 5.

We used our 3'-Amino-Modifier C7 CPG (20-2958) and removed the Fmoc protecting group with 20% piperidine in DMF. It was then straightforward to conjugate the free alkylamine on the support to our azidobutyrate NHS ester to generate the desired azide support.

After regular oligonucleotide synthesis and deprotection, the purified oligonucleotide was subjected to a click reaction with a dabsyl alkyne to confirm the presence of an active azide at the 3' terminus. The results, shown in Figure 6, confirm that the dabsyl dye was click conjugated and that the azide support was successfully prepared.

References:
1. M.M. Haque, H.B. Sun, S. Liu, Y.S. Wang, and X.H. Peng, *Angewandte Chemie-International Edition*, 2014, **53**, 7001-7005.
2. G. Pourceau, A. Meyer, J.J. Vasseur, and F. Morvan, *J Org Chem*, 2009, **74**, 6837-6842.

FIGURE 5: **SYNTHESIS AND CONJUGATION OF A 3'-AZIDO SUPPORT**

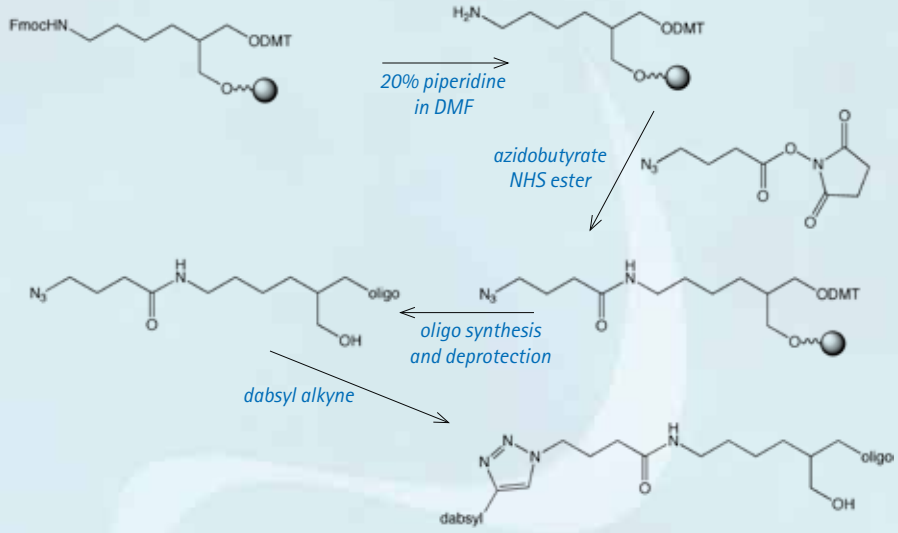
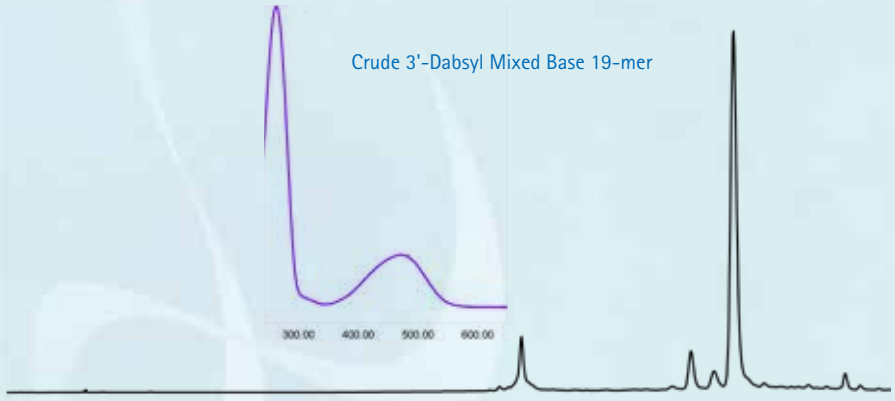
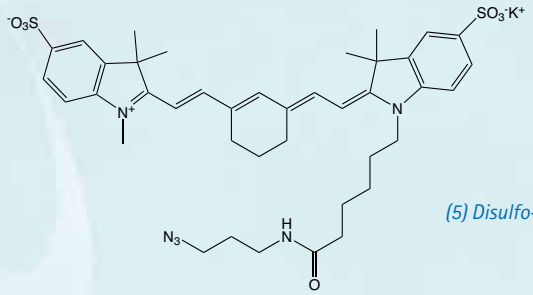


FIGURE 6: **RP HPLC ANALYSIS - CLICK CONJUGATION OF 3'-AZIDE SUPPORT WITH A DABSYL ALKYNE**



NEW PRODUCT - DISULFO-CYANINE 7 AZIDE

FIGURE 7: **STRUCTURE OF DISULFO-CYANINE 7 AZIDE**



(5) Disulfo-Cyanine 7 Azide

Near infrared fluorescent dyes (NIR) are fluorophores that absorb and emit light in the near infrared range between 600-1000nm. The usable range within biological systems is between 650nm and 900nm since hemoglobin absorbs below 650nm and water absorbs above 900nm. For NIR imaging, the ideal range is between 750nm-900nm, as biological tissues may also auto-fluoresce, contributing to high background that reduces sensitivity.

Several dyes are available for use within this range and a common cyanine dye, indocyan green (ICG), is an FDA approved fluorescent dye for bioimaging. As a fluorescent indicator, ICG has been used

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20164

for bioimaging in a number of applications including optometry, cancer screening, lymphatic visualization and whole body animal imaging.

There are several advantages of using NIR for bioimaging including the absence of ionizing radiation, low background fluorescence from biological tissues, capability for real-time monitoring, and deep tissue penetration. Cyanine dyes are particularly attractive as NIR dyes as they are very bright with high extinction coefficients and quantum yields.

Glen Research offers several cyanine-based phosphoramidites that may be useful in NIR imaging including Cyanine 5 with emission at 662nm and Cyanine 5.5 with emission at 707nm. These cyanine phosphoramidites lack the sulfo-groups that help prevent aggregation and promote solubility in aqueous solutions. To better address applications in NIR imaging, Glen Research is offering a water soluble Disulfo-Cyanine 7 azide, (5) in Figure 7, that can be easily conjugated to DNA and RNA through standard click chemistry. This long wavelength dye offers the benefits of improved solubility, reduced aggregation, and improved stability in the near-infrared spectrum along with the convenience of click chemistry.

Disulfo-Cyanine 7 azide has an excitation maximum of 750nm and an emission maximum of 773 nm. Typical click reactions are performed in aqueous solution in 15-60 minutes using our water-soluble THPTA click ligand. We foresee the use of this dye in applications requiring real-time imaging and tracking of NIR-labelled oligonucleotides.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5-Ethynyl-dU-CE Phosphoramidite	10-1554-95	50 µmole	130.00
	10-1554-90	100 µmole	245.00
	10-1554-02	0.25g	775.00
Coumarin Azide	50-2004-92	25 µmole	115.00
	50-2004-90	100 µmole	350.00
Psoralen Azide	50-2009-92	25 µmole	115.00
	50-2009-90	100 µmole	350.00
3'-Amino-Modifier C7 CPG 1000	20-2958-01	0.1g	95.00
	20-2958-10	1.0g	675.00
1 µmole columns	20-2958-41	Pack of 4	140.00
0.2 µmole columns	20-2958-42	Pack of 4	85.00
10 µmole column (ABI)	20-2958-13	Pack of 1	250.00
15 µmole column (Expedite)	20-2958-14	Pack of 1	375.00
Azidobutyrate NHS Ester	50-1904-23	2.3mg	60.00
(Dissolve 2.3mg in 60µL of DMSO)	50-1904-24	23mg	300.00
Disulfo-Cyanine 7 Azide	50-2010-92	25 µmole	325.00
	50-2010-90	100 µmole	975.00