The Glen Report

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GLEN RESEARCH

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INSIDE

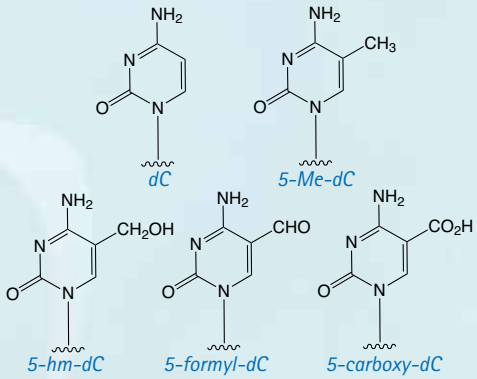
DNA METHYLATION REVISITED

Epigenetics is the study of heritable changes in gene expression and regulation that are not due to changes in the DNA sequence itself. Rather, epigenetic control primarily involves methylation of cytidine in CpG islands and the modification of histones. Aberrant methylation of DNA is associated with a variety of disorders such as Beckwith-Wiedermann, Prader-Willi, and Angelman syndromes, as well as cancers such as neuroblastoma, Wilms tumour, and osteosarcoma, among many others. A few of the chronic diseases associated with dysregulation of DNA methylation patterns include cardiovascular disease, obesity, lupus, and certain types of leukemia.

Until recently, 5-methyl-2'-deoxyCytidine (5-Me-dC) was the only known modification of DNA for epigenetic regulation. In 2009, however, Kriaucionis[1] discovered a second methylated cytidine, 5-hydroxymethyl-2'-deoxyCytidine (5-hmdC). While most prominent in neuronal tissues such as the brain and spine, 5hmdC has also been identified in a broad range of tissues such as the bladder, heart, liver and pituitary glands.[2] At present, its role in the epigenetic control of gene expression remains unclear. However, since it prevents the targeting of a number of transcriptional repressors specific for 5-Me-dC, it appears to serve a subtle regulatory role.[3] Removal of the methyl group from 5-Me-dC (demethylation) also plays an important role in cellular reprogramming, embryogenesis, autoimmune disorders and is critical in the establishment of maternal and paternal methylation patterns.

DNA demethylation occurs through active and passive mechanisms. Passive mechanisms simply involve the synthesis of DNA with the absence of methylation. Active DNA demethylation is thought to occur through a number of different mechanisms such as base excision repair (BER), nucleotide excision repair (NER), or the direct removal of the methyl group from 5-Me-dC.[4] Direct demethylation from cytidine requires the breaking of a carbon-carbon bond through an unidentified mechanism. More likely, the removal of the methyl group involves the hydroxylation of 5-Me-dC to 5-hmdC or deamination of 5-Me-dC to thymidine and subsequent BER. An alternative demethylation pathway could involve the oxidation of 5-Me-dC to 5-formyl-dC or 5-carboxy-dC with subsequent decarboxylation to cytosine, though these active demethylation intermediates were not detected using a sensitive HPLC-MS assay.[2,5]

dC ANALOGUES



We are pleased to introduce two new cytidine analogs, 5-carboxy-2'-deoxyCytidine (dC^{COO}) (1) and 5-formyl-2'-deoxyCytidine (dC^{Fo}) (2) as CE phosphoramidites (Figure 1) that complement our existing tools for epigenetics research. We hope they will aid in the development and understanding of this exciting field.

PROPERTIES OF 5-CARBOXY-2'-DEOXYCYTIDINE (5-CARBOXY-dC)

Despite the extra negative charge of the 5-carboxylic acid, the incorporation of 5-carboxy-dC into a DNA duplex has a stabilizing effect on DNA as indicated by a modest increase in the Tm (~2 °C per incorporation).[6] The 5-carboxy moiety of cytidine

(Continued on Page 2)

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projects into the major groove of a duplex, minimizing the potential negative steric effect of the modification. In comparison, the incorporation of 5-carboxy-dC into triplex forming oligos (TFO) as the third strand has a destabilizing effect, lowering the melting temperature by approximately 4-5° C. This is most likely because of the repulsion of the extra negative charge with the phosphate backbone and the crowding of the Hoogesten base pairing.

The presence of the carboxy moiety does not significantly alter the pKa of the N3 on cytidine with a pKa of 4.4 for cytidine versus a pKa of 4.0 for 5-carboxy-dC (Table 1), as determined by spectral analysis.[6] The Watson-Crick hybridization of G-C^{C00-} is essentially unaffected by the modification.

OLIGONUCLEOTIDE SYNTHESIS USING 5-CARBOXY-dC

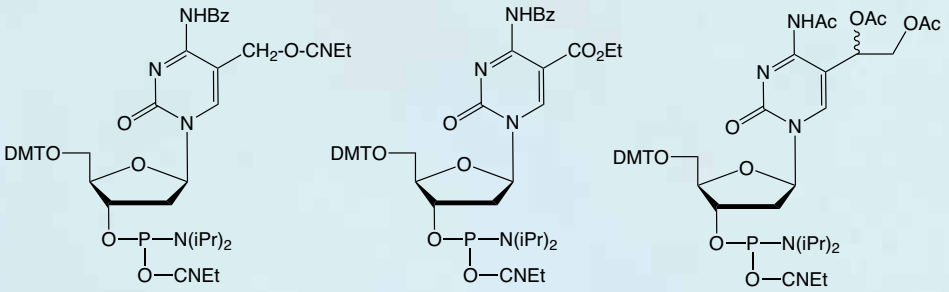
Incorporation of 5-carboxy-2'-deoxyCytidine into oligonucleotides is simple and does not require any changes to the coupling conditions. Deprotection requires the use of sodium hydroxide in methanol to prevent amide formation during the deprotection of the oligo.

PROPERTIES OF 5-FORMYL-2'-DEOXYCYTIDINE (5-FORMYL-dC)

The duplex stability of oligos containing 5-formyl-dC is comparable to oligos containing 5-Me-dC, with the Tm for 5-Me-dC being approximately 1.3° C higher per incorporation.[7] Interestingly, the misincorporation of thymidine opposite 5-formyl-dC was 3-4 times higher compared to control oligos containing dC and 5-Me-dC.[7] The higher misincorporation rate was speculated to be a result of the changes in shape and the presence of the electron-withdrawing formyl group of 5-formyl-dC in comparison to 5-Me-dC. The overall effect is that 5-formyl-dC is considered highly mutagenic and increases the rate of transition mutations when evaluated by polymerase extension.[7]

In contrast to 5-carboxy-dC, the presence of the electron-withdrawing formyl group lowers the pKa to 2.4 compared to a pKa of 4.4 for dC and 4.5 for 5-Me-dC (Table 1).The hybridization of G-dC^{Fo} is unaffected by the modification as indicated by thermal denaturation.

FIGURE 1: 5-HYDROXY-dC AND RELATED PHOSPHORAMIDITES

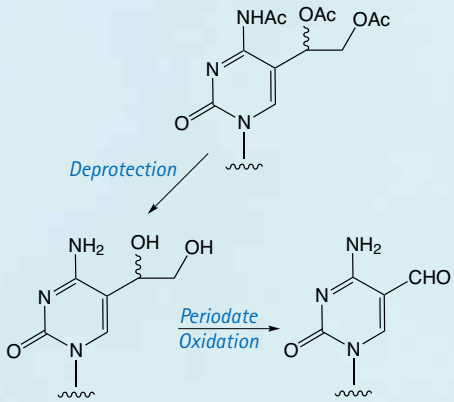


5-hydroxymethyl-dC | *5-Carboxy-dC (1)* | *5-Formyl-dC (2)*

OLIGONUCLEOTIDE SYNTHESIS USING 5-FORMYL-dC

The incorporation of 5-formyl-dC requires a 3 minute coupling time with 1H-tetrazole. Standard deprotection removes the 1,2-acetoxy protecting groups and subsequent oxidation with sodium periodate converts the 1,2-diol to formyl-dC, as shown in Figure 2.

FIGURE 2: FORMYL-dC FORMATION



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2. D. Globisch, *et al., PLoS One,* 2010, **5**, e15367.
3. S.G. Jin, X. Wu, A.X. Li, and G.P. Pfeifer, *Nucleic Acids Res.,* 2011.
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6. M. Sumino, A. Ohkubo, H. Taguchi, K. Seio, and M. Sekine, *Bioorganic & Medicinal Chemistry Letters,* 2008, **18**, 274-277.
7. N. Karino, Y. Ueno, and A. Matsuda, *Nucleic Acids Res.,* 2001, **29**, 2456-2463.

TABLE 1: PKa VALUES OF CYTIDINE AND ANALOGS[7]

Nucleoside	*pKa*
dC	4.4
5-methyl-dC	4.5
5-carboxy-dC	4.0
5-formyl-dC	2.4

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5-Me-dC-CE Phosphoramidite	10-1060-90	100 µmole	50.00
	10-1060-02	0.25g	120.00
5-Hydroxymethyl-dC-CE Phosphoramidite	10-1062-95	50 µmole	335.00
	10-1062-90	100 µmole	650.00
	10-1062-02	0.25g	1675.00
5-Carboxy-dC-CE Phosphoramidite	10-1066-95	50 µmole	230.00
	10-1066-90	100 µmole	450.00
	10-1066-02	0.25g	1200.00
5-Formyl-dC-CE Phosphoramidite	10-1514-95	50 µmole	610.00
	10-1514-90	100 µmole	1200.00
	10-1514-02	0.25g	3225.00

NEW PRODUCTS PREVENT BRANCHING AT SECONDARY AMINES USING DCI ACTIVATOR

The Glen Research catalog of products includes several where a secondary amine remains unprotected. However, all of our work with these products indicated that branching during synthesis due to the coupling of phosphoramidites at the secondary amine occurred at a very low level. Indeed, efficient manufacture of the phosphoramidites would not be possible if significant phosphitylation occurred at the secondary amino position. All of that work was carried out using 1H-tetrazole as activator. During subsequent work using 4,5-dicyanoimidazole (DCI) as activator, we noticed in some routine experiments that branching at the secondary amino positions was very significant.

Our main causes for concern were two minor base phosphoramidites, N6-Me-dA (10-1003) (1) and N4-Et-dC (10-1068)[1] (2), as well as unprotected biotin phosphoramidites. For example, Figure 2 shows the differences between two simple oligos containing N4-Et-dC when using 1H-tetrazole or DCI as activator. Similarly, Figure 3 illustrates the coupling of N4-Et-dC to a universal support using 1H-tetrazole or DCI. From both experiments, coupling with 1H-tetrazole leads to a trace of branching, while DCI leads to around 15% branching.

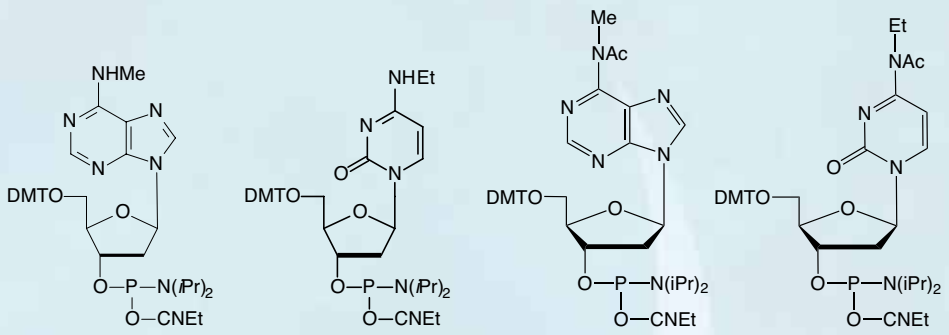
Our first priority was to address the situation with these two popular minor base analogues. In collaboration with Berry and Associates, the acetyl protected monomers (3) and (4) were prepared. Acetyl protection was chosen since it would block branching reactions while being compatible with all deprotection strategies from UltraMild to UltraFast.

Oligonucleotides synthesized using monomers (3) and (4) indeed proved to be compatible with all popular deprotection strategies. When the acetyl protected monomers were compared with the unprotected monomers using DCI as activator, branching was reduced from 15% to zero.

We are happy to add the acetyl protected monomers to our catalog but we will also continue to maintain supply of the unprotected monomers for optimal use with 1H-tetrazole or other members of the tetrazole family of activators.

Increased levels of branching are also observed when unprotected biotin products are used along with DCI as activator. In this case, branching can be avoided by using tetrazole activators. Alternatively, we have a line of new serinol-based biotin products that are already protected with the t-butylbenzoyl group. Biotin-dT is similarly protected, although the protecting group in this case was originally chosen to confer better solubility to the product. 5'-Biotin phosphoramidite is already protected with a DMT group. All of our biotin supports are aggressively capped as acetates to include the biotin N1 position so minimal branching should be observed from our supports.

FIGURE 1: PHOSPHORAMIDITE STRUCTURES



(1) N6-Me-dA (2) N4-Et-dC (3) N6-Ac-N6-Me-dA (4) N4-Ac-N4-Et-dC

FIGURE 2: IEX HPLC OF OLIGOS USING TETRAZOLE OR DCI AS ACTIVATOR

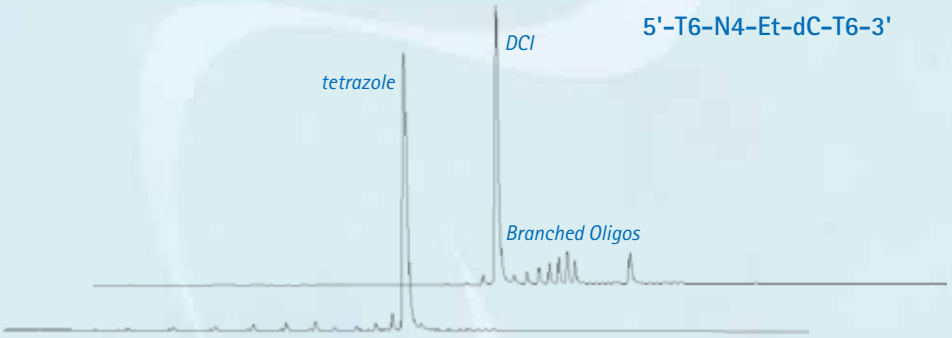
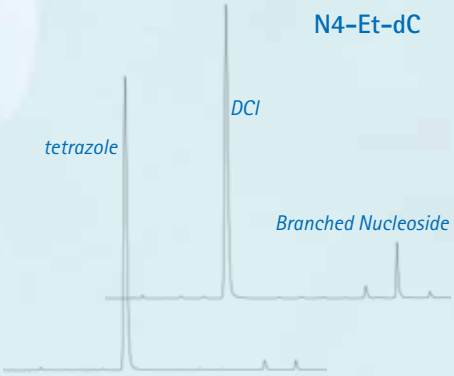


FIGURE 3: RP HPLC OF N4-Et-DC PREPARED ON US III USING TETRAZOLE OR DCI AS ACTIVATOR



REFERENCE:

1. H.K. Nguyen, P. Auffray, U. Asseline, D. Dupret, and N.T. Thuong, *Nucleic Acids Res,* 1997, **25**, 3059-65.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
N6-Ac-N6-Me-dA-CE Phosphoramidite	10-1503-90	100 µmole	162.50
	10-1503-02	0.25g	495.00
N4-Ac-N4-Et-dC-CE Phosphoramidite	10-1513-95	50 µmole	125.00
	10-1513-90	100 µmole	225.00
	10-1513-02	0.25g	675.00

NEW PRODUCTS – CLICK CHEMISTRY UPDATE

Through our collaboration with baseclick GmbH, Glen Research has been introducing a selection of products and reagents for use with click chemistry. In this update to an earlier Glen Report article,[1] we cover the additional nucleoside products that we have available, add an interesting azide product, and detail a procedure suitable for click chemistry on a 1 µmole synthesis scale.

ALKYNE NUCLEOSIDE ANALOGUES

Since the launch of our collaboration with baseclick, we have had available for sale C8-Alkyne-dT-CE Phosphoramidite (1) and C8-TIPS-Alkyne-dC-CE Phosphoramidite (2) for copper(1)-catalyzed azide alkyne cycloaddition (CuAAC) click reactions.[2,3] We are now happy to add C8-TMS-Alkyne-dC-CE Phosphoramidite (3), C8-TMS-Alkyne-dT-CE Phosphoramidite (4), and C8-Alkyne-dC-CE Phosphoramidite (5). C8-TIPS-Alkyne-dT-CE Phosphoramidite (6) makes up the complete family of pyrimidine derivatives for click, click-click, and click-click-click applications.[4,5] This set of reagents, therefore, allows sequential and specific reactions of alkynes on column, after oligo deprotection, and after removal of the TIPS protecting group.

COUMARIN AZIDE - PRO-FLUORESCENT CLICK LABEL

With our selection of azides, our goal is to expand the library of products available for oligonucleotide labelling rather than to duplicate products in our existing catalog. We are specifically interested in labels that are incompatible with DNA synthesis or deprotection, or labels where click chemistry offers a clear advantage over amine-NHS ester conjugation.

7-Hydroxycoumarin, also known as umbelliferone, is a highly fluorescent, pH-sensitive fluorophore that emits in the blue region of the spectrum. However, its fluorescence is strongly quenched if the hydroxyl is alkylated or phosphorylated, making it useful in high-throughput screening for phosphatases and lipases. Interestingly, it was found that the 3-azido derivative (7) is also highly quenched but, upon reaction with an alkyne in the presence of copper to form

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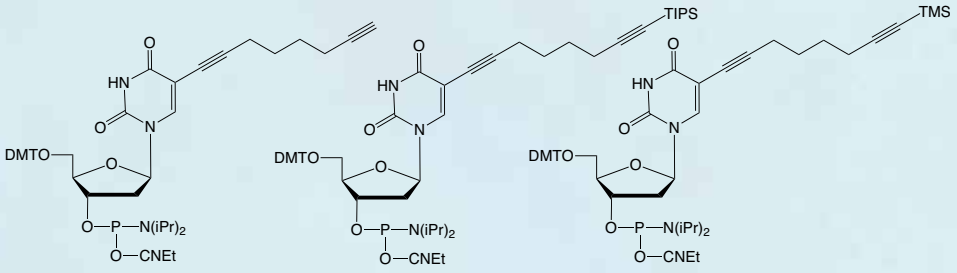
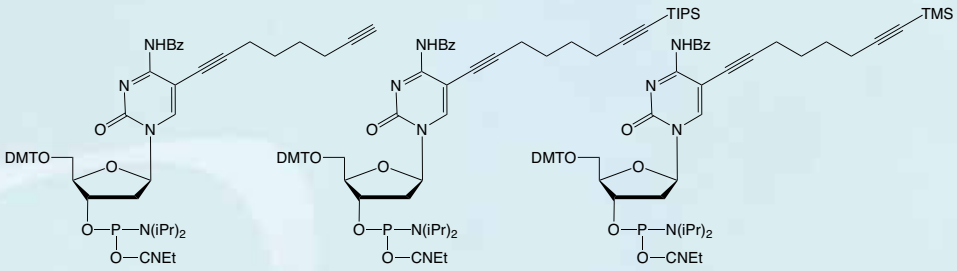
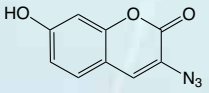
FIGURE 1: C8-ALKYNE NUCLEOSIDE PHOSPHORAMIDITES



(1) C8-Alkyne-dT *(6) C8 -TIPS-Alkyne-dT* *(4) C8 -TMS-Alkyne-dT*

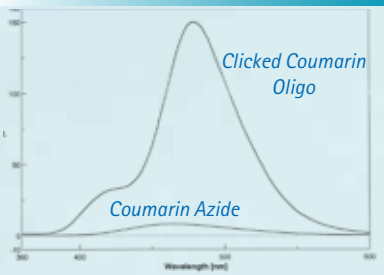


(5) C8-Alkyne-dC *(2) C8 -TIPS-Alkyne-dC* *(3) C8 -TMS-Alkyne-dC*



(7) Coumarin azide

FIGURE 2: FLUORESCENCE SPECTRA



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
C8-Alkyne-dT-CE Phosphoramidite	10-1540-95	50 µmole	165.00
	10-1540-90	100 µmole	315.00
	10-1540-02	0.25g	900.00
C8-TIPS-Alkyne-dC-CE Phosphoramidite	10-1541-95	50 µmole	295.00
	10-1541-90	100 µmole	575.00
	10-1541-02	0.25g	1275.00
C8-TMS-Alkyne-dC-CE Phosphoramidite	10-1542-95	50 µmole	270.00
	10-1542-90	100 µmole	525.00
	10-1542-02	0.25g	1275.00
C8-Alkyne-dC-CE Phosphoramidite	10-1543-95	50 µmole	225.00
	10-1543-90	100 µmole	435.00
	10-1543-02	0.25g	1125.00
C8-TIPS-Alkyne-dT-CE Phosphoramidite	10-1544-95	50 µmole	inquire
	10-1544-90	100 µmole	
	10-1544-02	0.25g	
C8-TMS-Alkyne-dT-CE Phosphoramidite	10-1545-95	50 µmole	205.00
	10-1545-90	100 µmole	395.00
	10-1545-02	0.25g	1050.00
Coumarin Azide	50-2004-92	25 µmole	115.00
	50-2004-90	100 µmole	350.00

NEW PRODUCT – ULTRAFAST PHOTO CROSS-LINKER

In an accompanying review on our web site (http://www.glenresearch.com//Technical/Crosslink.pdf) of cross-linking strategies over the last 15 years, the potential of 3-cyanovinylcarbazole nucleoside (^{CNV}K) for photo-induced cross-linking was clearly apparent.[1] When ^{CNV}K is incorporated into an oligonucleotide, very rapid cross-linking to the complementary strand can be induced at one wavelength and rapid reversal of the cross-link is possible at a second wavelength. Neither wavelength has the potential to cause significant DNA damage.

The nucleoside analogue, ^{CNV}K, can be readily converted into a CE phosphoramidite (1) and its use in oligonucleotide synthesis is straightforward. The modified oligo can also be simply deprotected by a variety of popular techniques.

As shown in Figure 2, irradiation of a duplex containing a single incorporation of ^{CNV}K at 366nm led to 100% cross-linking to thymine base in 1 second, although complete cross-linking to cytosine takes 25 seconds.[1] A 30 second irradiation time should cover all situations. In addition, it was demonstrated that the purine bases were unreactive to cross-linking, allowing differentiation between pyrimidines and purines at the target site. The authors also determined the effect of sequence contexts around the ^{CNV}K site and demonstrated that the identity of bases on either side of the cross-linking site has little effect on the reaction. Once cross-linked, the UV melting temperature of the duplex was raised by around 30 °C relative to the duplex before irradiation.

Complete reversal of the cross-link takes place at 312nm in 3 minutes. This facile reversal reaction is, therefore, accomplished with no damage to normal DNA.

In a later publication, as shown in Figure 3, a further application of this cross-linking technique was investigated.[2] When ^{CNV}K was cross-linked with a dC residue in duplex DNA, heating at 90°C for 3.5 hours led to deamination of the cytosine base to form uracil in the complementary strand. Reversal of the cross-link at 312nm led to a DNA strand in which dC had been converted to dU. The authors showed that this transformation is specific for the dC residue opposite the ^{CNV}K and any further adjacent dC residues are unaffected. Similarly, the authors have shown that ^{CNV}K can be cross-linked to an adjacent RNA strand.[3]

We are happy to introduce ^{CNV}K-CE phosphoramidite. We expect applications for ^{CNV}K in research in general interstrand cross-linking studies, gene expression, and DNA and RNA mutation.

REFERENCES:

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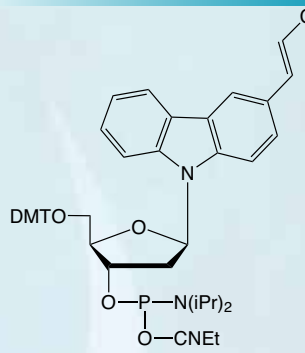
2. K. Fujimoto, K. Konishi-Hiratsuka, T. Sakamoto, and Y. Yoshimura, *ChemBioChem,* 2010, **11**, 1661-4.
3. Y. Yoshimura, T. Ohtake, H. Okada, and K. Fujimoto, *ChemBioChem,* 2009, **10**, 1473-6.

FIGURE 1: **STRUCTURE OF CROSS-LINKER**



(1) ^{CNV}K-CE-Phosphoramidite

FIGURE 2: **SCHEMATIC DIAGRAM OF PHOTO CROSS-LINKING WITH C OR T**

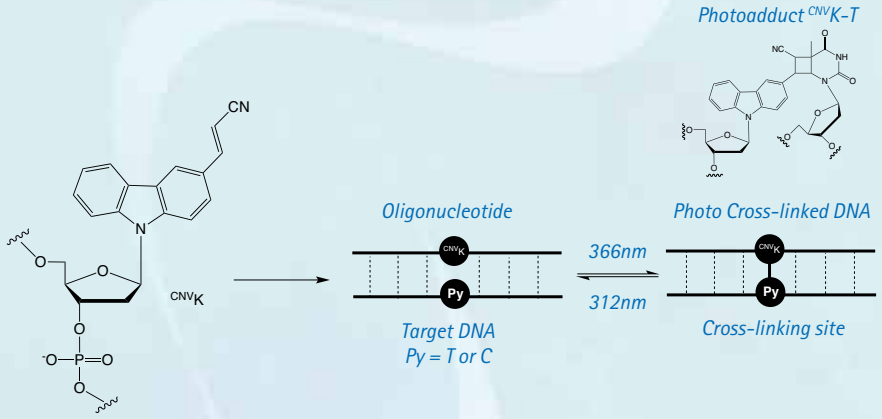
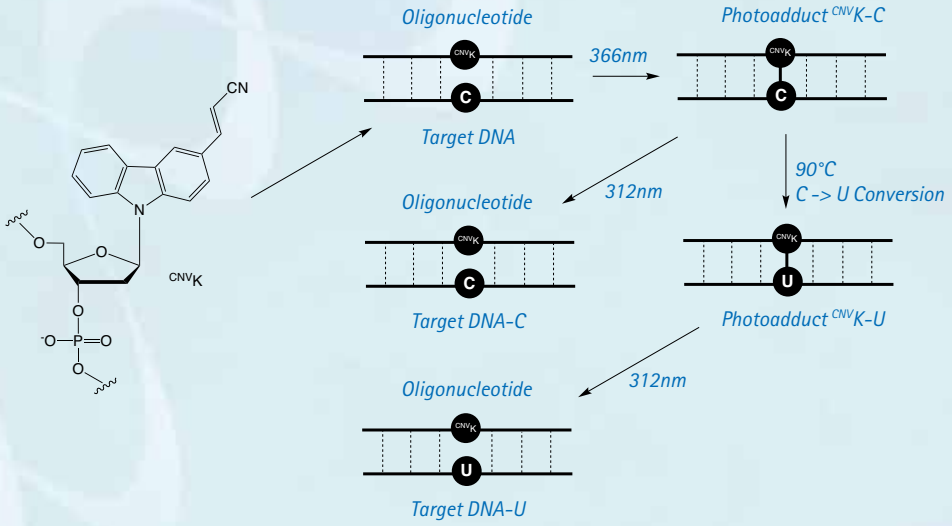


FIGURE 3: **SCHEMATIC DIAGRAM OF PHOTO CROSS-LINKING WITH C AND CONVERSION TO U**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
3-Cyanovinylcarbazole Phosphoramidite (^{CNV}K)	10-4960-95	50 µmole	200.00
	10-4960-90	100 µmole	390.00
	10-4960-02	0.25g	1125.00

PRODUCT UPDATE - UNNATURAL BASE PAIRS

For the last several years, Glen Research has collaborated with TagCyx Biotechnologies on the commercial development of an unnatural base pair that relies on hydrophobic interaction rather than the normal hydrogen bonding of the standard A – T and C – G base pairs. In this article, we will briefly outline the strategies for using the existing product line. We will also introduce a new highly fluorescent nucleoside analogue and suggest some applications for its use in this unnatural base pair environment.

Figure 1 shows all of the products that are currently commercially available for this technology, including dDss, the new fluorescent analogue. Although the array of products may be complicated at first glance, the set breaks down simply as they appear in the TagCyx name as y and x:

	y	x
DNA		
	dDs	dPa
	dDss	dPa
	ds	dPa
RNA		
	sTP	BiotinPaTP

Potential applications make use of the varying physical properties of these nucleoside analogues.

DNA RESEARCH

dDs is a weakly fluorescent and stable nucleoside analogue that can be readily incorporated into oligonucleotides for studies where dPa is the complementary "base", as shown in Figure 2.[1]

ds is a fluorescent analogue which also pairs with dPa. Pac-ds CE Phosphoramidite has a phenoxyacetyl (Pac) protecting group so is best used with Pac anhydride as the capping reagent. The fluorescence of ds is very sensitive to its environment and this attribute makes ds very useful in structural analysis.[2]

As shown in Figure 3, dDss is strongly fluorescent and is useful as a fluorescent tag for DNA detection. dDss also forms a base pair with dPa.[3]

RNA RESEARCH

Using template DNA containing dDs, RNA transcription using BiotinPaTP leads

FIGURE 1: **UNNATURAL BASE PAIR PHOSPHORAMIDITES AND TRIPHOSPHATES**

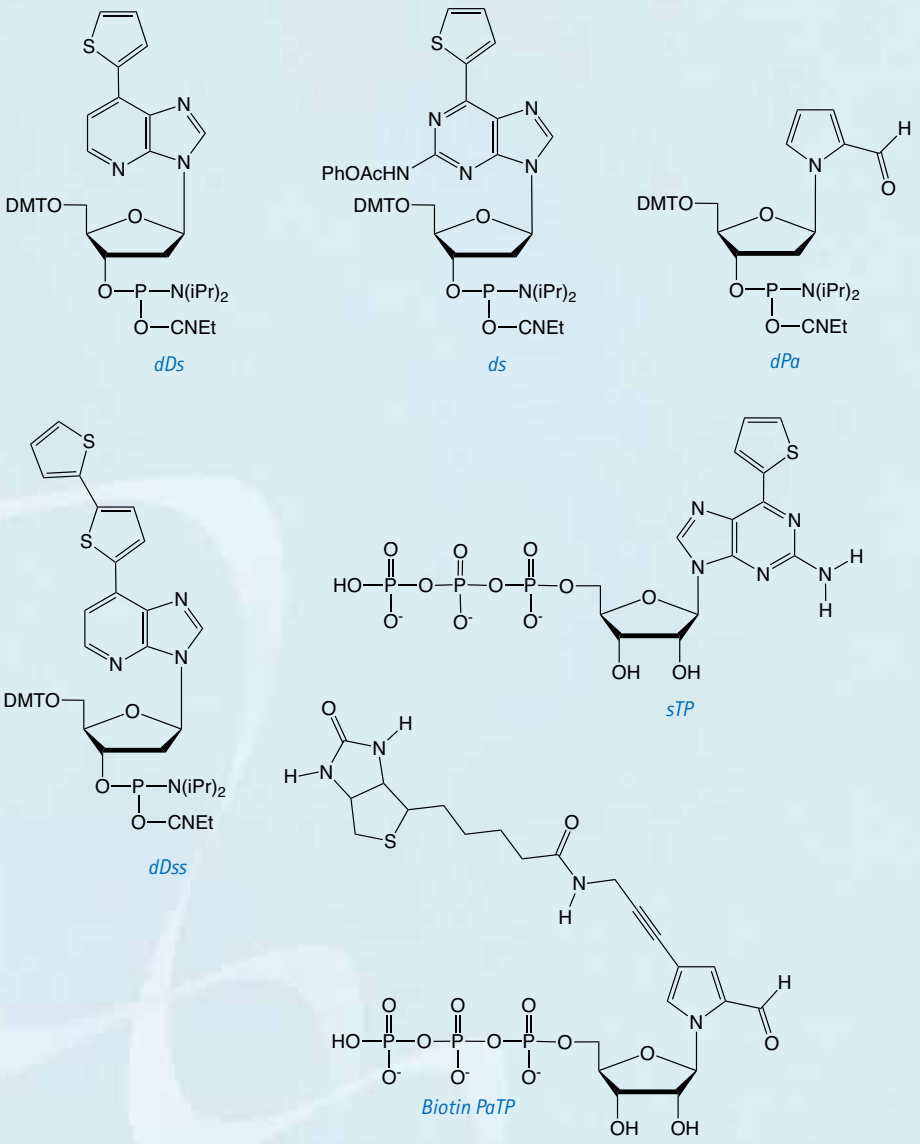
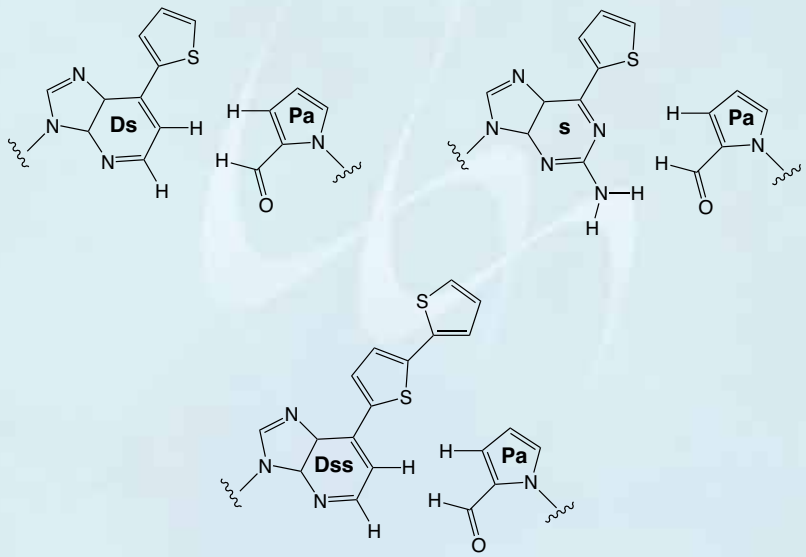


FIGURE 2: **UNNATURAL BASE PAIRING DUE TO HYDROPHOBIC INTERACTION**



to RNA biotinylated at sites complementary to dDs. The presence of biotin can be used to capture and immobilize the transcribed RNA.[4]

Similarly, a DNA template containing dPa can be used along with sTP to generate transcribed RNA containing s at defined locations. Since the fluorescence of s is sensitive to its environment, as illustrated in Figure 4, partial structural analysis of the transcribed RNA can again be developed.[5]

PROPERTIES OF dDss

dDss-CE Phosphoramidite is incorporated into oligonucleotides by standard procedures. The modified oligonucleotides can be deprotected by any of the popular procedures.[3] dDss has an absorption maximum at 380nm with an extinction coefficient of 31,000 L/mole. With an emission maximum for dDss at 470nm, dabcyl would be a good quencher.

The future for this unnatural base pair family is promising and we expect to see further refinements in the base pairs for specific applications in the future.[6]

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INTELLECTUAL PROPERTY

FIGURE 3: **COMPARISON OF FLUORESCENCE PROPERTIES OF Ds, s AND Dss**

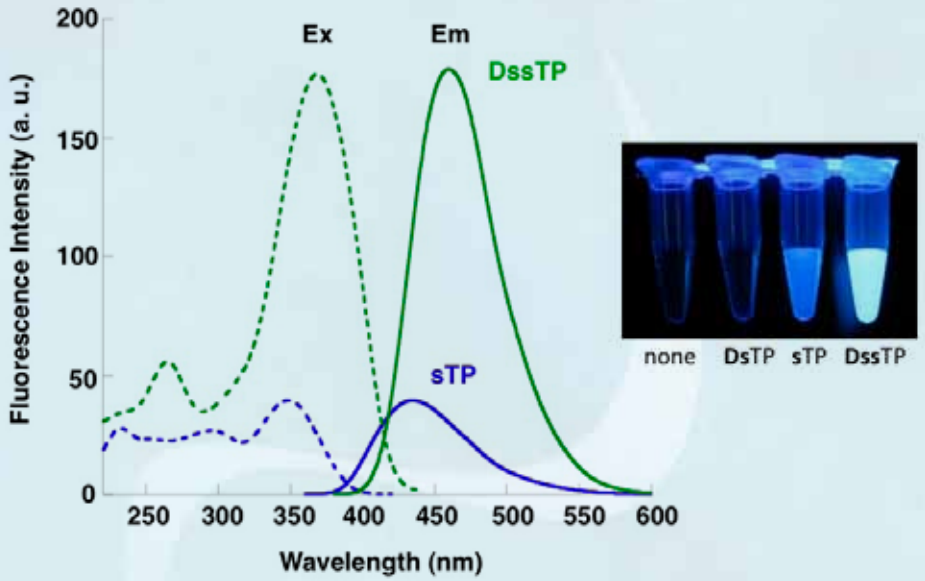
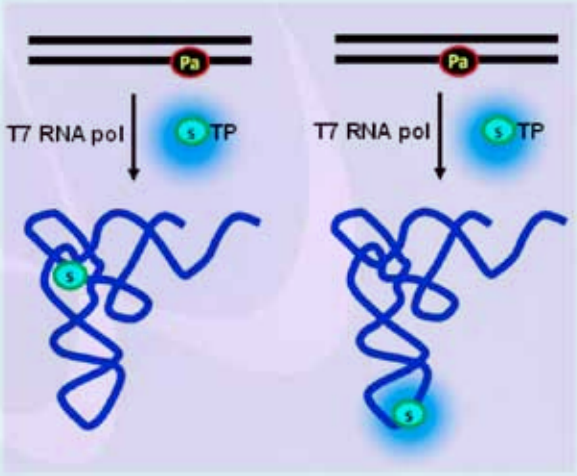


FIGURE 4: **FLUORESCENCE OF S IN RNA TRANSCRIPTS VARIES WITH ENVIRONMENT**



We thank Professor Hirao for permission to use the illustrations in Figures 3 and 4.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
dDs-CE Phosphoramidite	10-1521-90	100 µmole	145.00
	10-1521-02	0.25g	420.00
Pac-ds-CE Phosphoramidite	10-1522-90	100 µmole	170.00
	10-1522-02	0.25g	420.00
dPa-CE Phosphoramidite	10-1523-90	100 µmole	130.00
	10-1523-02	0.25g	420.00
dDss-CE Phosphoramidite	10-1524-95	50 µmole	130.00
	10-1524-90	100 µmole	250.00
	10-1524-02	0.25g	675.00
sTP 10mM	81-3522-02	25 µL	350.00
Biotin PaTP 10mM	81-3525-02	25 µL	450.00

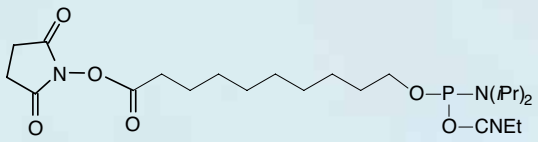
Conjugating reporter molecules to DNA has been an important functionalizing technique for use in many diagnostic, therapeutic and research applications. One of the most popular conjugation methods is the reaction of an amino group with an activated carboxylic acid to form an amide linkage.

N-hydroxysuccinimyl (NHS) Esters are activated carboxylic acids that react preferentially with primary amines and can be used in aqueous and organic solvents. For the conjugation of oligonucleotides with NHS Esters, the most common approach is to include a primary amine in the oligonucleotide sequence using an amino-modifier attached to a nucleoside, or to one of the termini, or to an internal non-nucleosidic linker. After the synthesis is complete, the oligo is deprotected and cleaved from the support. The amine-labelled oligo is then reacted with an NHS Ester to form the oligonucleotide conjugate. An excess of the NHS Ester is required to complete the reaction in a short amount of time and the excess NHS Ester as well as hydrolysis by-products are removed by desalting or HPLC purification.

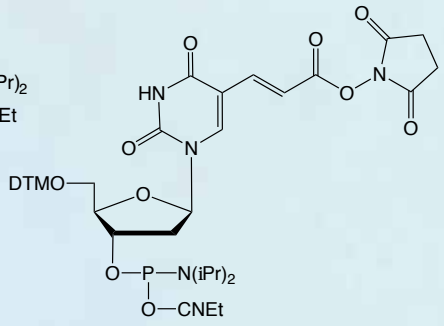
Several years, ago we introduced Carboxy-Modifier C10 (10-1935) (1) and later NHS-Carboxy-dT (10-1535) (2) which use an alternative strategy for labelling oligonucleotides. These products place the NHS Ester in the protected oligo on its solid support instead of on the label. A major benefit of this approach is to allow the incorporation of dyes and other reporter molecules that are not available as phosphoramidites. This approach also greatly simplifies purification as the excess reagents are conveniently washed away while the oligo is still on the support. There is also the advantage that excess reporter molecule can be recovered from the reaction solution. In this article, we focus primarily on labelling within the sequence using NHS-Carboxy-dT.

Earlier. we described a simple protocol where the NHS-Carboxy-dT was incorporated using conventional phosphoramidite chemistry, the synthesis was paused to conjugate the label, and then the synthesis completed. A caveat in this protocol (and Scheme 1) is that the label needs to be compatible with one of the methods for cleaving and deprotecting oligos and must not support branching.

FIGURE 1: STRUCTURES OF CARBOXY-MODIFIER C10 AND NHS-CARBOXY-DT



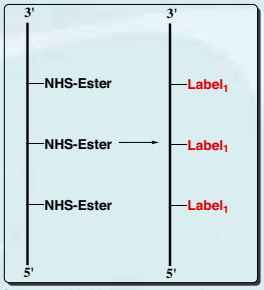
(1) Carboxy-Modifier C10



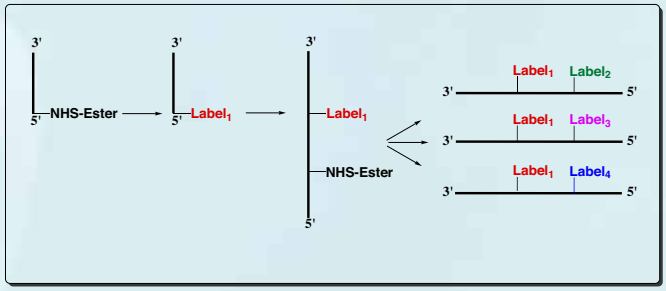
(2) NHS-Carboxy-dT

FIGURE 2: LABELLING STRATEGIES

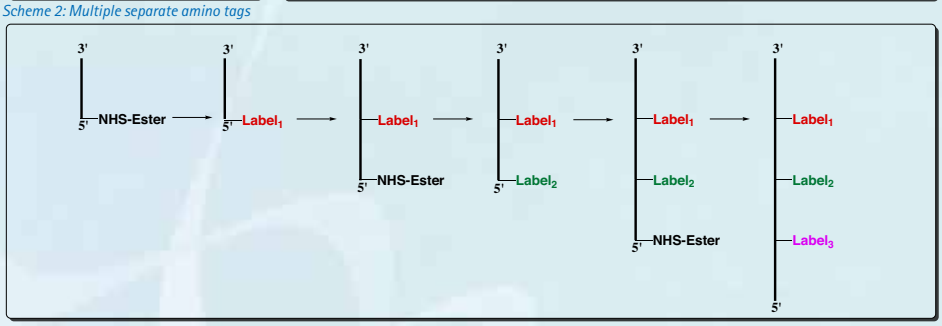
Scheme 1: Multiple identical amino tags



Scheme 3: Multiple separate amino tags, combinatorial approach



Scheme 2: Multiple separate amino tags



PROCEDURES

Different solvents have been used including DMSO, Acetonitrile, and Dichloromethane. The choice of solvent depends on the properties of the label.

On-Column Labelling Reaction (1.0 µmole scale)

1. Dissolve amino-modified label (5-10 equivalents) in 1 mL anhydrous solvent.
2. Add 0.5 µL of diisopropylethylamine (DIEA) to the solution.
3. Using two syringes to attach to the column, conjugate label solution for 2 hours at RT.
4. Rinse with 2 x 1 mL each of DMSO and acetonitrile.
5. Complete synthesis DMT-Off or DMT-ON.
6. Purify by desalting or DMT-ON purification procedure.

Post-synthesis On-Column Labelling

1. Transfer 0.2 µmole of support to a clean dry vial.
2. Dissolve amino-modified label (5-10 equivalents) in 1 mL anhydrous solvent.
3. Add 0.5 µL of DIEA to the solution.
4. Transfer 1 mL of amine solution to support.
5. Heat in water bath for four hours at 37 °C, agitating every hour.
6. Decant and wash with 1 mL each of DMSO, 10% diethylamine (DEA) in acetonitrile, methanol, and acetonitrile.
7. Cleave and deprotect using a method compatible with label.
8. Purify by desalting or DMT-ON purification procedure.

We originally investigated the possibility of synthesizing an oligo that includes one or several NHS-Carboxy-dT residues to completion and then performing the conjugation. Our initial results were acceptable but marginal due to considerable hydrolysis of the NHS Ester residues prior to conjugation. However, we have since discovered that the choice of oxidizer makes a profound difference in the stability of the NHS Ester during synthesis. When we revisited the synthesis conditions, we found that two oxidizers are quite effective for use with NHS-Carboxy-dT with no undesired hydrolysis of the ester - 0.02M Iodine in THF/Water/Pyridine (40-4330 or 40-4132) and 0.5M CSO in Acetonitrile (40-4632).

With our finding that NHS-Carboxy-dT is stable to repetitive oxidative cycles, several new options become available for oligos containing multiple modifications.

1. One or several NHS-Carboxy-dT residues can now be labelled efficiently with the same amino tag on the support after the oligo synthesis, with no need to pause the synthesis for each conjugation (Figure 2, Scheme 1).
2. Several NHS-Carboxy-dT residues can be labelled with separate amino tags by pausing the synthesis and carrying out each conjugation reaction separately (Figure 2, Scheme 2).
3. NHS-carboxy-dT could be used in a combinatorial approach for synthesizing families of labelled oligos. In this case, a single oligo synthesis could yield many oligos with different labels (Figure 2, Scheme 3).
4. The procedure is compatible with automated protocols.

To illustrate these processes, we synthesized oligos that had two or three incorporations of NHS-Carboxy-dT.

Scheme 1: The first oligo support with three incorporations of NHS-Carboxy-dT was split into two portions. One aliquot, the control, was treated with 0.4M NaOH in methanol to form carboxy-dT (Figure 3a). The second aliquot was conjugated with Dansyl Cadaverine to form the triply dansyl labelled oligo once conventional deprotection was completed (Figure 3b).

Scheme 2: For the oligo with two incorporations of NHS-Carboxy-dT, the synthesis was paused to label the first NHS-Carboxy-dT residue with Dansyl Cadaverine. The excess reagents were washed away and the synthesis completed, including the second incorporation of NHS-Carboxy-dT. After synthesis but prior to deprotection or cleavage, the oligo was labelled with Dabsylamine to form the Dansyl-Dabsyl labelled oligo. The results are compelling demonstrating that the oligo was virtually quantitatively double labelled with Dansyl Cadaverine and Dabsylamine (Figure 4).

We conclude that on-column labelling of oligonucleotides containing a carboxylate NHS ester is a highly efficient procedure that is a preferred alternative to post synthesis labelling in aqueous solution. Not every reporter molecule can be added using this technique since the conjugated oligonucleotide still has to be deprotected and the labels must survive this treatment. However, in conjunction with UltraMild DNA synthesis and suitable deprotection strategies, NHS-Carboxy phosphoramidite labelling offers a simple and direct approach to synthesizing oligo conjugates that are not compatible with standard deprotection conditions or unavailable as phosphoramidites.

FIGURE 3: **RP HPLC OF OLIGOS WITH THREE INCORPORATIONS OF NHS-CARBOXY-dT**

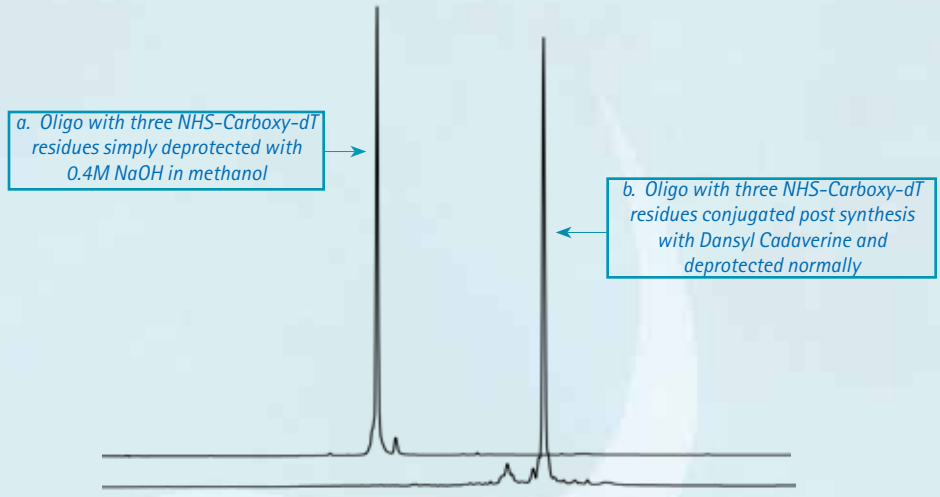
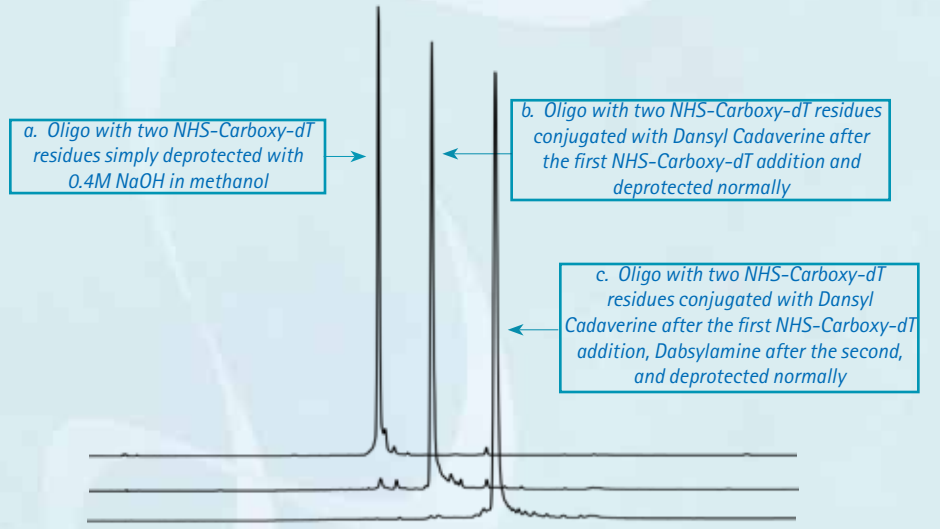


FIGURE 4: **RP HPLC OF OLIGOS WITH TWO INCORPORATIONS OF NHS-CARBOXY-dT**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
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

As new versions of products are introduced at Glen Research, the older versions may be discontinued or they may have specific attributes that warrant their continuing home in our catalog. In the case of chemical phosphorylation reagents, we have not had the need to discontinue any of our old products that have served us and our customers so well. However, when faced with so many options, customers sometimes need a concise summary of the relative strengths and weaknesses of these products, and we will endeavor to provide that in this Technical Brief.

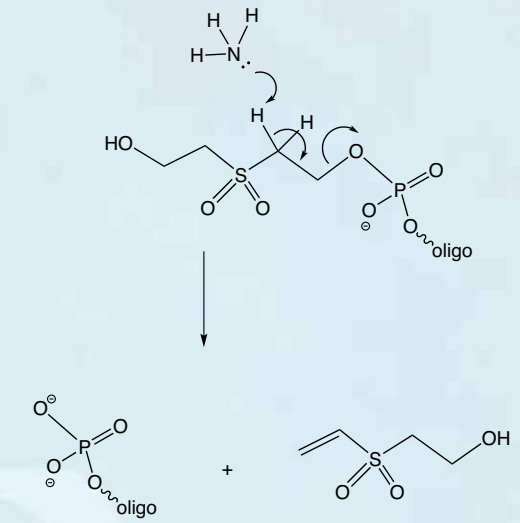
10-1900, 20-2900: Chemical Phosphorylation Reagent and Support

This is our old standby as a chemical phosphorylation reagent (CPR).[1] The phosphate is generated by the ß-elimination reaction of a diethylsulfonyl group, as shown in Figure 1. It is an inexpensive and versatile reagent that can be used for 5'-phosphorylation, as well as generating a 3' phosphate if added to a nucleoside support that will be sacrificed 3' of the insertion. This can be quite useful if a 2000Å support is required for a very long oligo synthesis but a 3' phosphate is required. However, a significant drawback of the 10-1900 is that it is not compatible with 5' DMT-ON purification. In addition, the conditions required to drive the elimination reaction to completion are not the most gentle – in ammonium hydroxide, 17 hours at room temperature or 4 hours at 55 °C. Finally, it is not compatible with standard deprotection conditions in AMA.

10-1901: Chemical Phosphorylation Reagent II

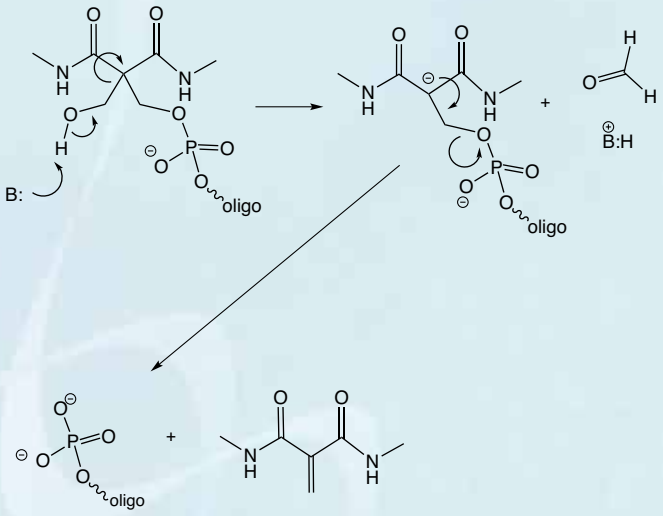
This improved CPR has one very large advantage over the earlier version – it is compatible with DMT-ON purification.[2] Figure 2 shows the mechanism of the elimination reaction, with Solid Chemical Phosphorylation Reagent II (10-1902) shown as an example. The first step in the elimination reaction is the abstraction of a proton from the hydroxyl, which occurs under very mild conditions, generating formaldehyde. The rest of the elimination reaction occurs quickly, affording the 5'-phosphate. However, when the DMT group is still protecting the hydroxyl, the elimination reaction is completely inhibited, allowing DMT-ON

***FIGURE 1:* MECHANISM FOR ß-ELIMINATION OF CPR**



3'- or 5'-Phosphate

***FIGURE 2:* MECHANISM FOR ELIMINATION OF CPR II**



3'- or 5'-Phosphate

***TABLE:* RECOMMENDATIONS FOR USE OF CHEMICAL PHOSPHORYLATION REAGENTS**

	DMT-ON Purification	Standard AMA Deprotection	UltraMild Deprotection	DEA Pretreatment	RNA
10-1900	✘	✘	✘	✔	✔-
10-1901	✔	✔	✔	✔	✔*
10-1902	✔	✔	✔	✔	✔*
20-2900	✔	✘	✘	✘	✔-
20-2903	✔	✔	✔	✔	✔

*Oligo synthesized DMT-Off
(✔-) Indicates an acceptable but not preferred method.

Technical Bulletins:
http://www.glenresearch.com//Technical/TB_CPR.pdf
http://www.glenresearch.com//Technical/TB_CPR_II.pdf

purification. Upon elution of the purified oligo, treatment for 15 minutes at room temperature in dilute ammonia affords the 5'-phosphorylated oligo.

This facile elimination reaction has an additional advantage – when an oligo is synthesized DMT-Off using the Chemical Phosphorylation Reagent II, even the most mild conditions are sufficient to generate the 5'-phosphate. This is very useful when working with RNA or sensitive dyes where deprotection conditions should be as mild as possible.

One additional consideration when using this product is that the synthesis cycle should be modified to remove the capping step after coupling of the phosphoramidite to maintain phosphorylation efficiency.

10-1902, 20-2903: Solid Chemical Phosphorylation Reagent II and 3'-CPR II

As the name implies, this phosphoramidite is essentially the solid version of 10-1901. However, its use does not require any changes to the synthesis cycle since it is stable to capping and, like 10-1901, it is compatible with DMT-ON purification. In addition, because it is a solid, unlike 10-1900 and 10-1901, it is possible to provide larger pack sizes. As with 10-1901, it allows a method for gentle phosphorylation of RNA if the synthesis is performed DMT-Off.

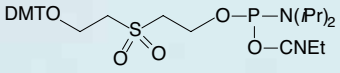
20-2903 versus 20-2900

A common post-synthetic practice performed prior to the cleavage and deprotection step is to treat the support with a solution of 10% diethylamine (DEA) in acetonitrile. This does not cleave the oligo from the support. Rather, it removes the acrylonitrile that is produced from the ß-elimination of the cyanoethyl protecting groups on the phosphodiester backbone. By doing so, alkylation of the N3 position of Thymidine by acrylonitrile is avoided. (An unexplained +53 Da peak in mass spectral analysis of oligos is likely due to alkylation by acrylonitrile.). Given the utility of this procedure, we decided to introduce (in May 2009) Solid CPR II CPG. As with both CPR II and Solid CPR II, the elimination reaction that yields the phosphate does not occur unless there is a free hydroxyl, as shown in Figure 2.

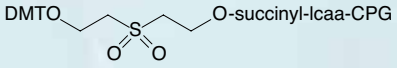
With Solid CPR II CPG, the hydroxyl is not released until the ammonolysis of the succinate linkage has occurred, which means it is perfectly compatible with post-synthesis DEA treatment.

The structures of all of our products for chemical phosphorylation are shown in Figure 3.

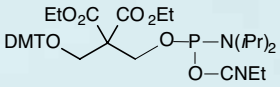
FIGURE 3: STRUCTURES OF CHEMICAL PHOSPHORYLATION REAGENTS



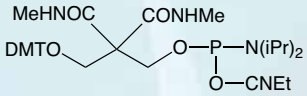
Chemical Phosphorylation Reagent



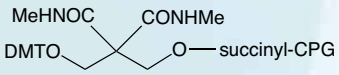
3'-Phosphate CPG



Chemical Phosphorylation Reagent II



Solid Chemical Phosphorylation Reagent II



3'-CPR II CPG

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2a. A. Guzaev, H. Salo, A. Azhayev, and H. Lonnberg, Tetrahedron, 1995, 51, 9375-9384.

2b. Chemical Phosphorylation Reagent II is covered by US Patent No.: 5,959,090 and European Patent: EP0816368.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Chemical Phosphorylation Reagent	10-1900-90	100 µmole	50.00
	10-1900-02	0.25g	160.00
3'-Phosphate CPG	20-2900-01	0.1g	70.00
	20-2900-10	1.0g	480.00
1 µmole columns	20-2900-41	Pack of 4	100.00
0.2 µmole columns	20-2900-42	Pack of 4	60.00
10 µmole column (ABI)	20-2900-13	Pack of 1	180.00
15 µmole column (Expedite)	20-2900-14	Pack of 1	280.00
3'-Phosphate PS			
200 nmole columns (AB 3900)	26-2900-52	Pack of 10	150.00
40 nmole columns (AB 3900)	26-2900-55	Pack of 10	150.00
3'-Phosphate CPG	25-2900-01	0.1g	85.00
(High Load)	25-2900-10	1.0g	600.00
2.5 µmole columns	25-2900-46	Pack of 4	120.00
Chemical Phosphorylation Reagent II	10-1901-90	100 µmole	60.00
(CPR II)	10-1901-02	0.25g	200.00
Solid Chemical Phosphorylation Reagent II	10-1902-90	100 µmole	60.00
(Solid CPR II)	10-1902-02	0.25g	200.00
3'-CPR II CPG	20-2903-01	0.1g	70.00
	20-2903-10	1.0g	480.00
1 µmole columns	20-2903-41	Pack of 4	100.00
0.2 µmole columns	20-2903-42	Pack of 4	60.00
10 µmole column (ABI)	20-2903-13	Pack of 1	180.00
15 µmole column (Expedite)	20-2903-14	Pack of 1	280.00

GLEN RESEARCH

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(Continued from Page 4)

the triazole, the fluorescence is restored.[2] The clicked coumarin emits at a lambda max of 480 nm and absorbs at 358 nm. Also, the fluorescence of the clicked coumarin is approximately 15 times higher than that of the coumarin azide (Figure 2, Page 4). This opens up the possibility to monitor a Copper-catalyzed Click reaction in real time and the detection of DNA in cells without requiring washing steps to remove the unreacted Coumarin Azide.[6]

IMPROVED CLICK REACTION PROTOCOL

The CuAAC reaction between azides and alkynes has become a very popular method for conjugation. This is because of the mild conditions employed and its orthogonal nature, which allows specific conjugations to be performed in the presence of practically any other functional groups.

One issue that we have had with this versatile reaction is the work-up after the click conjugation has been performed. The TBTA copper chelator is not water soluble nor is the CuBr. While this is not an issue with small-scale reactions, difficulties arise with large-scale reactions (~1 µmole or more). On a larger scale, simple dilution with water followed by desalting has not given us consistent results. We therefore have been working on a more robust procedure that is compatible with large-scale click reactions.

We found that the clicked oligo could conveniently be ethanol precipitated directly from the Click Reaction solution. In addition, we found that degassing the solvents prior to the Click Reaction prevented precipitation from occurring during the conjugation. This is easily accomplished by applying low vacuum to the solvents for just 2-3 minutes with gentle agitation.

Protocol for 800 nmol Reaction:

- Dissolve 12.7 mg TBTA in 240 µL DMSO/t-butanol 3:1 (v/v)
- Degas under vacuum to remove oxygen and blanket under argon.
- Transfer to 1.7 mg CuBr in vial blanketed with argon. Vortex to dissolve.
- Dissolve 800 nmole oligo in 80 µL water. Degas under vacuum and blanket under argon.
- Transfer to 2 equivalents of azide blanketed under argon. Vortex to dissolve.
- Transfer oligo/azide solution to TBTA/CuBr solution. Let react ~1 hr at 45 °C.

Protocol for Ethanol Precipitation:

- Add to solution 10 µL 3 M Sodium Acetate
- Add 1 mL Ethanol, mix well or vortex.
- Chill at – 20 °C for >20 minutes.
- Centrifuge at 15,000 rpm to pellet oligo.
- Rinse with EtOH and dry pellet under vacuum.

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