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INSIDE

METHYLENE BLUE PHOSPHORAMIDITE FOR DNA LABELLING

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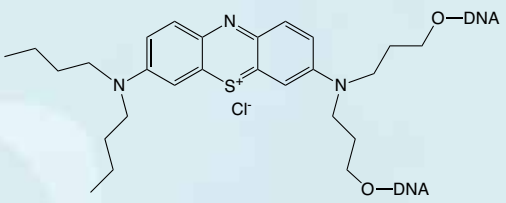
Dimethylaminophenothiazin-5-ium chloride, better known as Methylene Blue (MB), has many properties in chemistry and biology. For decades, this compound has been used for many applications, including redox indicator,[1] photosensitizer,[2-8] dye for cellular staining procedures,[9] antiseptic[2,10] and in medicine against Alzheimer's disease.[11]

For the detection of biological analytes, MB was used by many groups as a redox reporter bound to DNA probes.[12-15] For instance, several studies were conducted by K. Plaxco *et al.* in the design of DNA, protein, antibody and drug biosensors.[16-20] MB provided efficient electron transfer, good sensitivity and stability in such detection systems, and proved better than ferrocene.[21]

The position of MB along the DNA probe seems to be critical for sensor performance. For instance, internally labelled probes have shown better signal suppression after target addition than end-labelled probes.[13] In this context, Barton's group studied the electrochemical response of MB bound to dsDNA with a flexible linker and demonstrated both direct reduction at the electrode and DNA-mediated reduction dependent on the π-stacking of MB.[22,23]

Intercalation properties of MB with DNA have been widely investigated but only with free MB molecules in solution.[24-28] DNA structures with an intercalated MB still have to be considered, and novel binding chemistries have to be explored. However, only a post-synthesis functionalization of DNA with an NHS-ester derivative has been used to incorporate MB into oligonucleotides.[22,29,30] This method is limited by the requirement for amino functions on the oligonucleotide chain. Although the introduction of amino groups is relatively straightforward with

METHYLENE BLUE IN OLIGO



the appropriate phosphoramidites, the procedure requires an additional deprotection step followed by an additional coupling/purification procedure to incorporate the MB. This final step could be tedious due to the trend of MB to interact with DNA *via* electrostatic and π-stacking interactions with the phosphate backbone and nucleic bases (in particular with guanine residues), respectively, thus decreasing the efficiency of covalent incorporation.[31,32]

METHYLENE BLUE PHOSPHORAMIDITE

Our approach is to synthesize MB derivatives allowing covalent introduction into the desired position of an oligonucleotide sequence without supplementary manipulation. Phosphoramidite chemistry seems the most straightforward way to achieve this purpose. While most fluorescent dyes (fluorescein, rhodamine, cyanine, etc.) are now available as phosphoramidites, there is a lack of redox markers which can be incorporated during oligonucleotide solid-phase synthesis. For example, we can reference our work with ferrocene phosphoramidite that was shown to be efficient to incorporate numerous redox markers into sequences.[33,34]

Since many dyes are unstable under strong basic conditions, labelled DNA may have to be deprotected under mild basic conditions to ensure

(Continued on Next Page)

their integrity. It is worth noting that MB is not stable in these mild basic conditions and the molecule needs to be chemically modified to improve its stability.

We report the first synthesis of an MB phosphoramidite and its use for labelling synthetic DNA. First, a series of symmetrical MB analogues were synthesized and stability tests were performed in DNA synthesis and under DNA deprotection conditions. Second, after selection of the most stable molecule, unsymmetrical derivatives were obtained and modified as phosphoramidites.[35]

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NEW PRODUCT - METHYLENE BLUE C3 PHOSPHORAMIDITE

Glen Research is delighted to offer Methylene Blue C3 Phosphoramidite, (1) in Figure 1, under license from the University of Lyon. In consultation with Carole Chaix, we chose to prepare this propyl version for optimal performance and ease of synthesis.

Methylene Blue C3 Phosphoramidite is used with a coupling time of 3 minutes. *However, UltraMild monomers and capping must be used to allow deprotection with 0.05M potassium carbonate in methanol (Catalog No. 60-4600-30).*

The UV/Visible spectrum of an MB labelled oligo is shown in Figure 2.

We would like to thank Christian Chapelle of the Technology Transfer Office of the University of Lyon for his help in setting up a license agreement. Also, thanks are due to Carole Chaix for her help and encouragement in making the commercial availability of Methylene Blue C3 Phosphoramidite possible.

Methylene Blue C3 Phosphoramidite is covered by PCT application number WO2013128099 and is sold under license from the University of Lyon.

FIGURE 1: METHYLENE BLUE C3 PHOSPHORAMIDITE

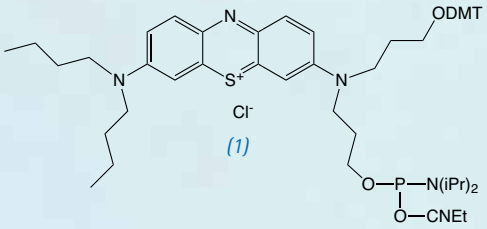
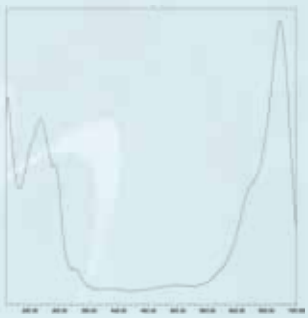


FIGURE 2: UV/VIS SPECTRUM OF MB OLIGO



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
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

NEW PRODUCT - 5'-DICHLORO-DIMETHOXY-FLUORESCEIN PHOSPHORAMIDITE

5'-Dichloro-dimethoxy-fluorescein is more commonly known as JOE™ to those familiar with the dye sets used in Applied Biosystems DNA PRISM sequencers - e.g., Dye Set 20 and 32, which contain FAM/JOE/TAMRA/ROX and FAM/JOE/NED/ROX, respectively.

As a dye, 5'-dichloro-dimethoxy-fluorescein gained popularity because its emission is nicely resolved from both FAM and TAMRA, falling exactly between them. This allows multiplex detection without too much signal bleed through into other channels, making it extremely useful in automated DNA sequencing. In addition, because of the electron-withdrawing groups on the xanthene ring, the 5'-dichloro-dimethoxy-fluorescein dye is less prone to quenching due to protonation. As such, its fluorescence is much less pH sensitive than its popular cousin, fluorescein. Even at pH 5, our in-house testing indicates 5'-dichloro-dimethoxy-fluorescein's signal dropped by only 30%.

A high extinction coefficient of 75,000 L/mol.cm, a quantum yield of fluorescence of 0.58, and excellent stability to standard deprotection conditions in ammonium hydroxide are the reasons why 5'-dichloro-dimethoxy-fluorescein has been a popular addition to our ever-expanding selection of fluorophore phosphoramidites.

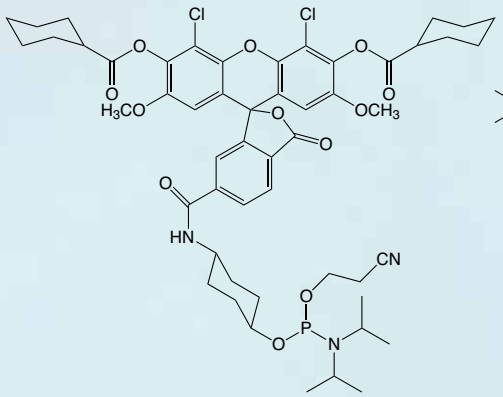
This product, (1) in Figure 1, has been available from Glen Research since 2008. Beginning in 2012, due to its increasing popularity, we have been evaluating options for scale up of the current product. Due to several factors, we have concluded that synthesis of this product on a larger scale is not feasible and we have decided to replace it with the optimized product described below.

5'-DICHLORO-DIMETHOXY-FLUORESCEIN II

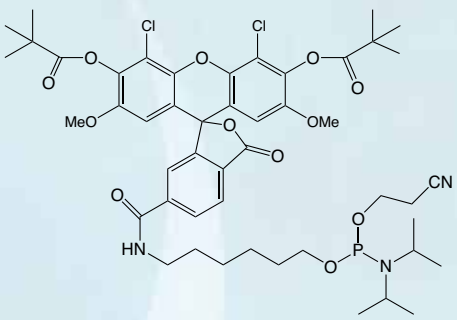
This improved version of 5'-dichloro-dimethoxy-fluorescein phosphoramidite (2) is shown in Figure 1. This second generation version uses the identical chromophore for 5'-dichloro-dimethoxy-fluorescein but more standard pivaloyl protecting groups are used to protect the fluorophore during synthesis. The spacer used is also the more standard C6 spacer.

Having implemented these changes, we feel confident that we are in a much better position now to support future growth of 5'-dichloro-dimethoxy-fluorescein with much better synthesis scale and consequent pricing profile.

FIGURE 1: STRUCTURES OF JOE PHOSPHORAMIDITES



(1) 5'-Dichloro-dimethoxy-fluorescein Phosphoramidite



(2) 5'-Dichloro-dimethoxy-fluorescein Phosphoramidite II

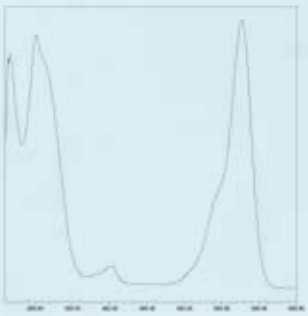
USE OF 5'-DICHLORO-DIMETHOXY-FLUORESCEIN II

Diluent: Anhydrous Acetonitrile
Coupling: 6 minute coupling time recommended.
Deprotection: Use Ammonium Hydroxide and deprotect as required by nucleobases. If AMA is used, a small amount of a non-fluorescent impurity will be formed. To eliminate this impurity, first deprotect with ammonium hydroxide for 30 minutes at room temperature, add an equal volume of 40% methylamine and then complete the deprotection as required by the nucleobases - e.g., 10 minutes at 65°C or 2 hours at room temperature for standard bases.
Storage: Freezer storage, -10 to -30°C, dry
Stability in Solution: 1-2 days

It is important to note that the spectral properties of the 5'-Dichloro-dimethoxy-fluorescein fluorophore are unchanged from the original to the new phosphoramidite versions of JOE. Also, the protecting groups and spacer used for the second generation product are actually standard for all of our fluorescein-based products.

SPECTRAL CHARACTERISTICS

The UV/visible spctrum of a simple T6 oligo labelled with JOE II is shown below:



E260	12,000 L/mol.cm
Abs. Max.	525 nm
Emax	75,000 L/mol.cm
Emission Max.	548 nm
QY	0.58

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-Dichloro-dimethoxy-Fluorescein Phosphoramidite II (JOE)	10-5906-95	50 µmole	105.00
	10-5906-90	100 µmole	198.00
	10-5906-02	0.25g	495.00

NEW PRODUCTS - TEMPO SPIN LABELS FOR CLICK CHEMISTRY AND 5-ETHYNYL-dU

Spin labels are molecules containing sites with unpaired electrons, usually stable nitroxide free radicals, and generate a paramagnetic center that can be probed using magnetic resonance spectroscopy. Electron Paramagnetic Resonance (EPR) spectroscopy is a sensitive technique for the characterization of materials and has been used to determine the molecular environment of spin labels[1,2], including the location, orientation and motion of spin labels within biomolecules. Most naturally occurring biomolecules do not contain unpaired electrons. Consequently, spin labels localized within biomolecules can act as bioorthogonal chemical reporters for studying the structure and dynamics of biomolecules using EPR spectroscopy.

Recently, the spin label 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) has been used to determine RNA structure using paramagnetic relaxation enhancement (PRE).[3]

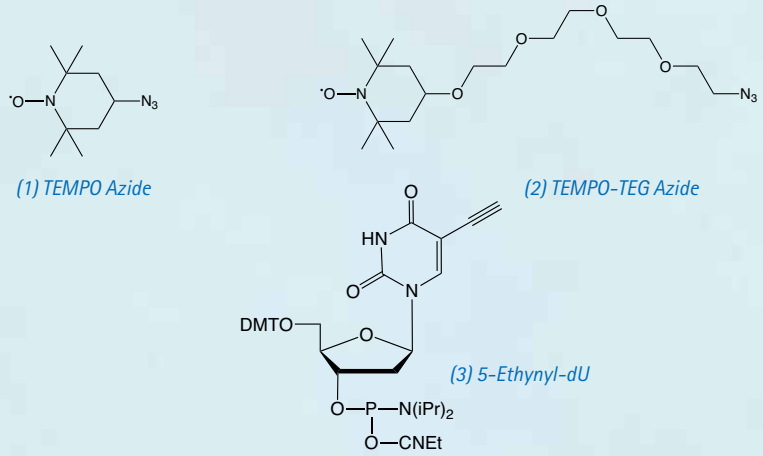
Spin labels can be easily incorporated into biomolecules by covalent attachment using Click Chemistry. The wide variety of alkyne modifications already available from Glen Research offers a diverse set of options for site-specific conjugation of spin labels to oligonucleotides.

As shown in Figure 1, we offer two new nitroxide spin labels, TEMPO Azide (1) and TEMPO-TEG Azide (2), compatible with Click Chemistry for site directed spin labelling (SDSL). TEMPO Azide (1) is most suited for conjugation to an alkyne group rigidly attached to the biomolecule in question. To facilitate this situation in oligonucleotides, we are also introducing 5-Ethynyl-dU (3), for convenient click conjugation with TEMPO Azide to generate a spin label rigidly attached to one of the oligonucleotide bases, as shown in Figure 2. We anticipate that TEMPO-TEG Azide (2), with its long flexible linker, will be more suited to interrogating the general environment of the biomolecule.

We have used these spin labels with 5-ethynyl-dU, our Copper-Free DBCO click labels, our Oligo Click Kits, and using copper sulfate mediated Click Chemistry.

In one experiment, we prepared a mixed base 20mer oligonucleotide with a 5'-alkyne group and conjugated with TEMPO Azide using the copper sulfate procedure detailed on the following page. The resulting RP HPLC traces are shown in Figure 3. The conjugation using this procedure was virtually quantitative in four hours at room temperature.

FIGURE 1: STRUCTURES OF TEMPO AZIDES AND 5'-ETHYNYL-dU



In a second experiment, a mixed base 20mer oligonucleotide, modified at the 5' terminus with DBCO-TEG, was prepared. This oligonucleotide was conjugated with TEMPO-TEG Azide using the procedure detailed on the following page. The resulting RP HPLC traces are shown in Figure 4. Again the conjugation was essentially quantitative in one hour at room temperature.

In a third experiment to illustrate the ability of the 5-ethynyl group to click efficiently, a mixed base 20mer oligonucleotide containing a 5-Ethynyl-dU residue was conjugated with 6-FAM Azide using the copper sulfate procedure detailed on the following page. The resulting RP HPLC traces are shown in Figure 5. The conjugation efficiency using this procedure was virtually quantitative in two hours at room temperature.

FIGURE 2: TEMPO-5-ETHYNYL-dU CONJUGATE

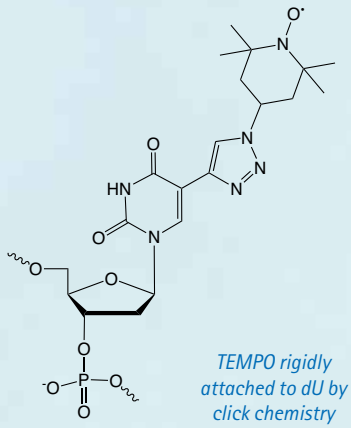
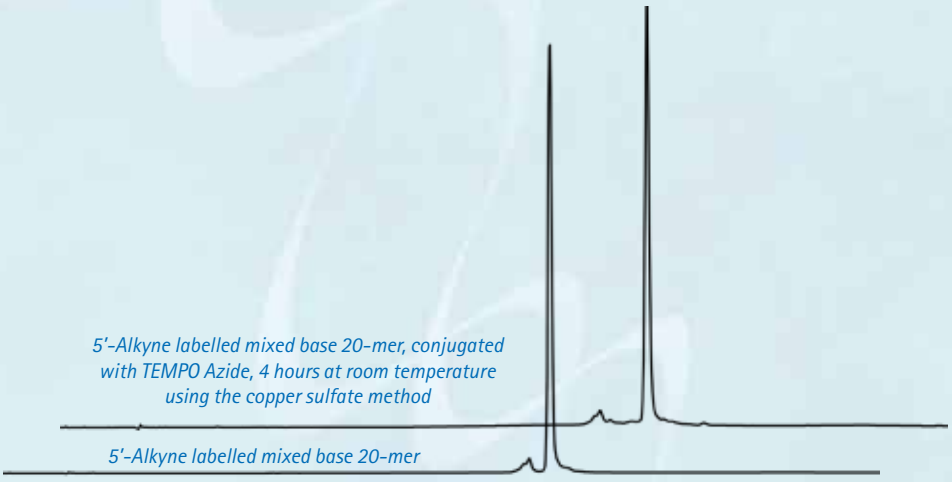


FIGURE 3: RP HPLC ANALYSIS - CONJUGATION OF 5'-ALKYNE OLIGO WITH TEMPO AZIDE



5-Ethynyl-dU-CE Phosphoramidite is used in oligo synthesis with normal coupling and synthesis cycles. However, 5-Ethynyl-dU is very susceptible to base catalyzed hydration during deprotection and we have found two options for successful use as follows:

Oligos containing 5-Ethynyl-dU can be deprotected using ammonium hydroxide at room temperature. So regular protected

monomers can be used with oligos containing iBu-dG being deprotected in 24 hours and those containing dmf-dG overnight.

Alternatively, UltraMild conditions with deprotection using ammonium hydroxide at room temperature for 2 hours or anhydrous 0.05M potassium carbonate in methanol for 4 hours at room temperature can be used.

Note that AMA even at room temperature and ammonium hydroxide at elevated temperatures cause significant degradation of the 5-ethynyl group.

We hope that the addition of these new spin labels, along with 5-Ethynyl-dU, will prove valuable to the growing field of EPR spectroscopy.

CLICK CHEMISTRY USING THE COPPER SULFATE PROCEDURE

Prepare the following solutions:

1. DMSO-tBuOH (3:1)
2. 0.1M Copper Sulfate in water
3. 0.2M TBTA in DMSO/tBuOH
4. 0.1M Sodium Ascorbate (prepare fresh)
5. 20mM Azide in DMSO/tBuOH
6. Oligo Solution 50nmoles in 50µL
7. 5'-XTT ATT CTT GTT ATT CTT GTT – 3'
8. 10-1908, 5'-hexynyl phosphoramidite, 100µmol.

We prepared these solutions in 2mL HPLC vials with septum caps. The vials with loosened caps were sparged for 5-10 minutes using a fine needle to slowly bubble argon into the solutions.

Copper Catalyzed Click Chemistry, Copper Sulfate method

1. Mix the TBTA/Copper Sulfate (1:1), 100µL each, degas.
2. To the vial containing the oligo solution (50µL), add 50 equivalents Azide (125µL).
3. Add 25 equivalents of the Copper sulfate/ TBTA solution (25µL).
4. Vortex. Add additional DMSO/tBuOH if a precipitate forms.
5. Using a syringe with a fine needle, add 40 equivalents of 0.1M Sodium Ascorbate (20µL).
6. Degas solution briefly and agitate at room temperature for 2-4 hours.

Ethanol precipitation

1. Transfer to 1.5mL centrifuge tube.
2. Add 20µL 3M Sodium Acetate and vortex.
3. Add 520µL cold ethanol, or the amount to reach 70% ethanol, and vortex briefly.
4. Chill for 30 minutes (-20°C to -70°C).
5. Centrifuge for 10 minutes at 13,500rpm.
6. Decant, wash twice with cold ethanol and dry.
7. For downstream processes that may be sensitive to trace amounts of copper, we recommend a Glen GelPak desalting, HPLC or other suitable method.

FIGURE 4: **RP HPLC ANALYSIS - CONJUGATION OF 5'-DBCO OLIGO WITH TEMPO-TEG AZIDE**

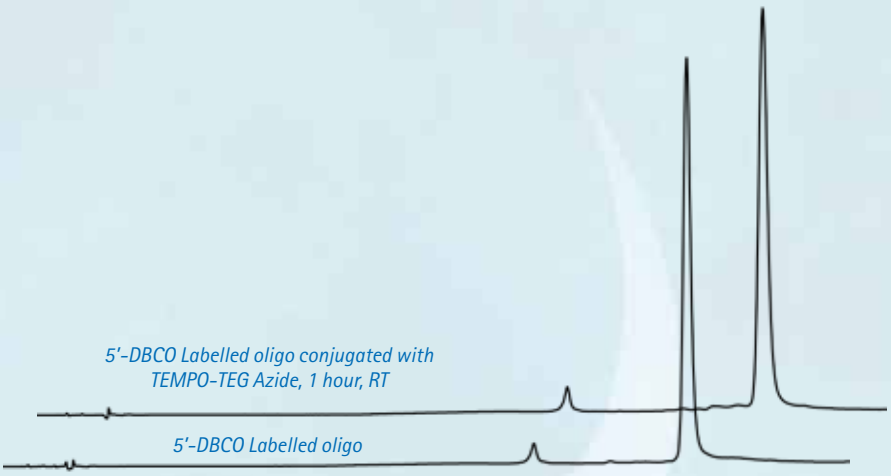
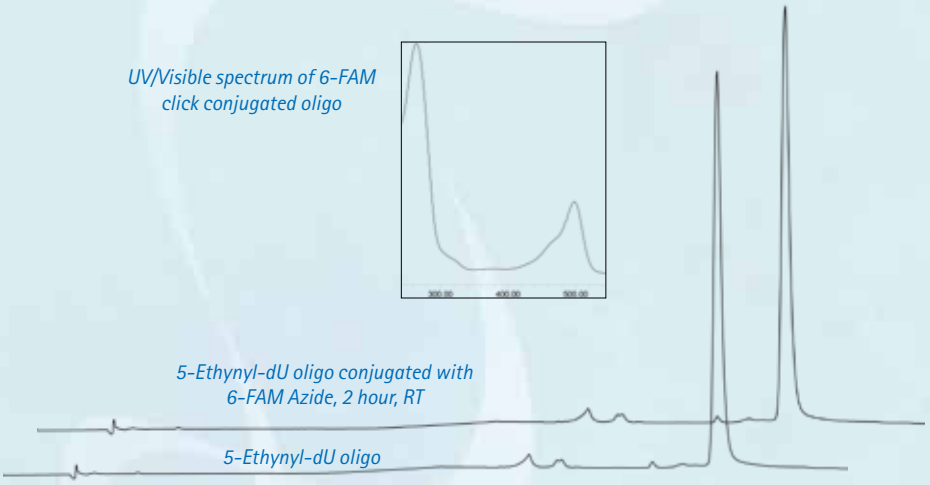


FIGURE 5: **RP HPLC ANALYSIS - CONJUGATION OF 5-ETHYNYL-dU OLIGO WITH 6-FAM AZIDE**



DBCO COPPER-FREE CLICK CHEMISTRY

1. TEMPO-TEG - 5'-DBCO labelled oligo
2. Dissolve 100nmole of DBCO labelled oligo in PBS, pH 7.4.
3. Add 4µL of 100mM TEMPO-TEG Azide in DMSO.
4. React for 1 hour at room temperature.
5. Add 60µL of 3M Sodium Acetate and mix well.
6. Add 1mL cold ethanol and mix well.
7. Chill for 30 minutes at (-20°C to 70°C).
8. Centrifuge for 10 minutes at 13,500 rpm.
9. Decant, wash twice with cold ethanol and dry.

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3. C.H. Wunderlich, et al., *ACS Chem Biol*, 2013, In Press.

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
TEMPO Azide	50-2007-92	25 µmole	115.00
	50-2007-90	100 µmole	350.00
TEMPO-TEG Azide	50-2008-92	25 µmole	135.00
	50-2008-90	100 µmole	400.00

NEW PRODUCT - 5-FORMYL-DC III AND THE SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING ALL

First came the genetic code mediated by the four regular DNA bases. But beyond genetics lies the recently explored space of epigenetics. Our little corner of this space is dedicated to oligonucleotide synthesis and, for us, epigenetics predominantly means methylated cytosine variants and their incorporation into oligonucleotides. Figure 1 shows the structures of dC and its epigenetically significant variants. The C5 methylated version of dC, 5-Me-dC, is oxidized stepwise to 5-hydroxymethyl-dC (5-hm-dC), 5-formyl-dC (5-f-dC) and 5-carboxy-dC (5-ca-dC) (Figure 1).

In 2009, Glen Research introduced the 5-hm-dC phosphoramidite (1) and that product has proved to be very useful even though it requires very forcing conditions (ammonium hydroxide at 75 °C for 17 hours) for complete deprotection. In 2011, we published an article by Markus Mueller and Thomas Carell of the Ludwig-Maximilians University in Munich, which detailed the processes involved in cytosine methylation and demethylation. At that time, we introduced a second monomer for hm-dC (2), and monomers for f-dC (3) and ca-dC (4). The structures of these monomers are shown in Figure 2. Using these monomers, researchers were able to prepare oligonucleotides containing one or several of these individual bases but could they produce an oligo containing all of these bases? Unfortunately, the answer was – no – but why?

First, ca-dC (4) is not compatible with deprotection using ammonium hydroxide or AMA which would lead to a mixture of the desired carboxylic acid and incorrect amides. So, an oligo containing all four of these methylated analogues has to be deprotected using sodium hydroxide in aqueous methanol to be compatible with ca-dC. Fortunately, hm-dC II (2) is also compatible with sodium hydroxide deprotection, as indeed is f-dC (3).

However, the structure of the f-dC monomer (3) requires that the fully deprotected oligo be treated with 50mM aqueous sodium periodate to generate the formyl group (Figure 3). This treatment has been reported to be incompatible with hm-dC (1).[1]

Clearly a new f-dC structure is required. The previous effort to achieve this goal was to prepare the 5-formyl-dC monomer with no protection on the formyl group. Although this product almost solved the problem, the reactivity of the unprotected formyl group precluded making the more complex oligonucleotides that researchers needed.

After preparing and testing a variety of candidates, the Carell group was able to design a monomer for f-dC which seems to meet all of the requirements to prepare an oligo containing all of the methylated variants.[1] The structure of the f-dC III monomer (5) is shown in Figure 2.

The aldehyde is protected as an acetal group and the exocyclic amino group is protected with the 4-methoxy-benzoyl group. The choice of the acetal protecting group is critical since the group must be easily removed while surviving the conditions of oligonucleotide synthesis. It was found that the acetal formed from propane-1,3-diol offered the optimal characteristics. It was also found that the regular N4-benzoyl protecting group was too labile in f-dC and its partial loss during oligonucleotide synthesis

FIGURE 1: dC AND VARIANTS

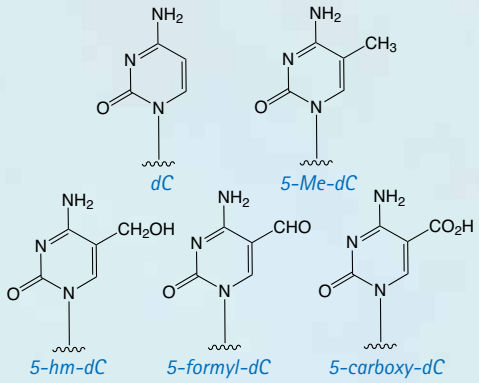


FIGURE 2: PHOSPHORAMIDITE MONOMERS

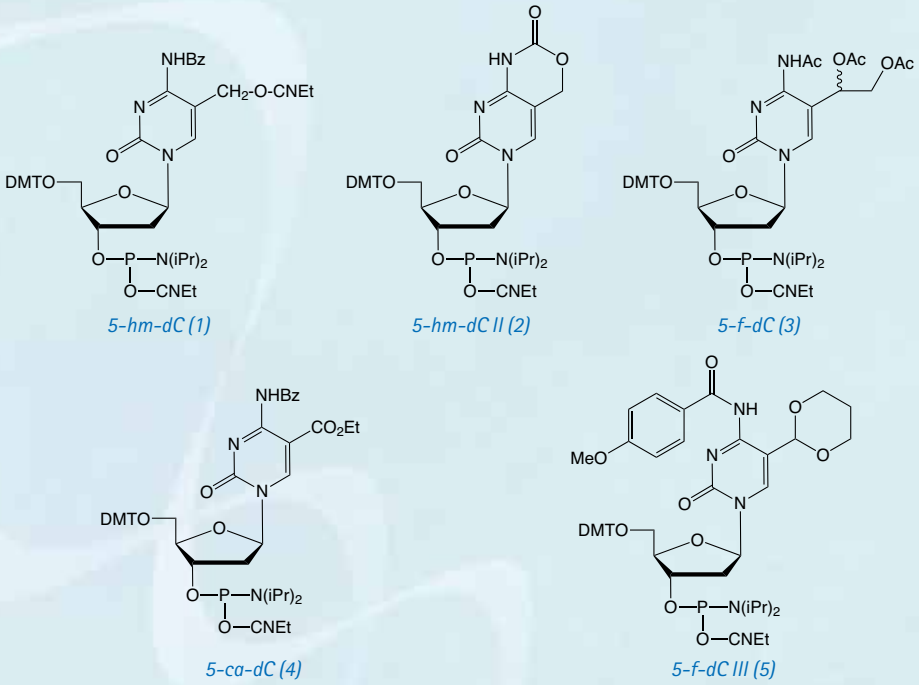
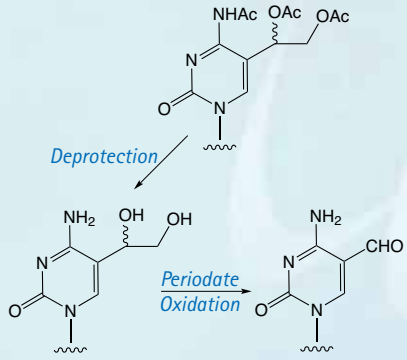


FIGURE 3: FORMYL-dC FORMATION

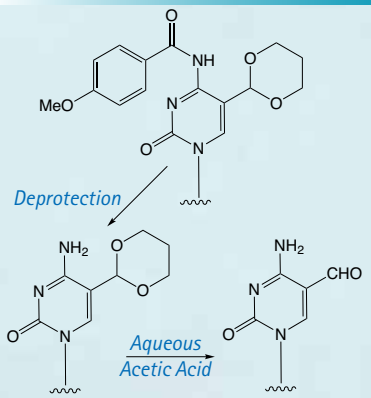


led to some chain branching. So, the more resistant 4-methoxy-benzoyl group was chosen in its place.

Using the f-dC III monomer, an oligo containing all four of these methylated bases can be simply prepared. The synthesis conditions are normal and the deprotection is carried out using 0.4M sodium hydroxide in methanol/water 4:1 (v/v) for 17 hours at room temperature. *(Please note that this deprotection scheme is not compatible with the use of dmf-protected dG in the oligo. However, iBu-dG is fully deprotected under these conditions.)* After deprotection with sodium hydroxide, the oligo must not be isolated by simple evaporation. Instead, the sodium hydroxide can be neutralized using a suitable buffer or the oligo can be ethanol precipitated.

To remove the acetal protecting group, as shown in Figure 4, the oligo is treated with acetic acid/water 80:20 (v/v) for 6 hours at 20 °C. It should be noted that both time and temperature are critical for complete removal of the acetal protecting group with absence of side reactions.

FIGURE 4: **FORMYL-dC FORMATION**



These highly modified oligos can be simply purified by Glen-Pak purification. The normal procedure can be followed with the exception that the removal of the 5'-DMT group with 2% aqueous trifluoroacetic acid should be omitted. The DMT group is then removed during the acetic acid/water procedure described above for removal of the acetal protecting groups.

The work of the Carell group described here is a superb illustration of the use of protecting groups in oligonucleotide synthesis. We are happy to offer these products for sale and to acknowledge that bringing this set of monomers to market would not have been possible without their talent and help.

Reference:

1. Schroder, A. S.; Steinbacher, J.; Steigenberger, B.; Gnerlich, F. A.; Schiesser, S.; Pfaffeneder, T.; Carell, T., Synthesis of a DNA Promoter Segment Containing All Four Epigenetic Nucleosides: 5-Methyl-, 5-Hydroxymethyl-, 5-Formyl-, and 5-Carboxy-2'-Deoxycytidine. *Angewandte Chemie-International Edition* **2014**, 53 (1), 315-318.

TABLE 1: **DEPROTECTION CONDITIONS FOR dC ANALOGUES**

Building Block/ Deprotection Conditions*	*NH_4OH or AMA*	*NaOH (not compatible with dmf-dG)*	*K_2CO_3*
5-Hydroxymethyl-dC (1)	Ammonium hydroxide at 75° C for 17 hours.	Not compatible	Not compatible
5-Hydroxymethyl-dC II (2)	Yields urea derivatives – possibility to introduce different ureas post-synthetically by deprotection with suitable amine	0.4 M NaOH in methanol/water 4:1, 25°C, 17 h	0.05M K_2CO_3 in anhydrous methanol, 25°C, 17 h using UltraMild conditions
5-Formyl-dC (3)	Standard deprotection followed by periodate oxidation, see Figure 3	Standard deprotection followed by periodate oxidation, see Figure 3	Standard deprotection followed by periodate oxidation, see Figure 3
5-Formyl-dC III (5)	Conc. NH_4OH, 25°C, 17 h followed by acetal removal, see Figure 4	0.4 M NaOH in methanol/water 4:1, 25°C, 17 h followed by acetal removal, see Figure 4	4-Methoxy-benzoyl protecting group is not compatible with UltraMild deprotection conditions
5-Carboxy-dC (4) **See Figure 2 for structures*	Yields amides as well as desired carboxylic acid	0.4 M NaOH in methanol/water 4:1, 25°C, 17 h	Not compatible

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5-Hydroxymethyl-dC II-CE Phosphoramidite	10-1510-95	50 µmole	345.00
	10-1510-90	100 µmole	670.00
	10-1510-02	0.25g	2100.00
5-Formyl-dC III-CE Phosphoramidite	10-1564-95	50 µmole	360.00
	10-1564-90	100 µmole	700.00
	10-1564-02	0.25g	1800.00
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

CYANINE DYES - A PERSONAL PERSPECTIVE

John B. Randolph, Ph.D.
Glen Research Corporation

With the patents on cyanine dyes having come to a close, this seemed to be a good time to reminisce about the development of this unique class of fluorophores and their rise in popularity. This article is from my perspective having spent many years in the lab of Dr. Alan Waggoner who first developed and commercialized them.

The Waggoner lab was part of an NSF-funded Science and Technology Center, the Center for Light Microscope Imaging and Biotechnology. The Center, deep in the bowels of the Mellon Institute of Carnegie Mellon University, was a mixture of chemists, biologists, computer scientists, and physicists all working together with the goal of developing new technologies and methods to study cell functions and processes. It was a uniquely collaborative environment in which chemists would synthesize dyes and label biomolecules, which in turn were used by biologists to study cellular functions on fluorescence microscopes developed by physicists and automated by computer scientists to collect and store data.

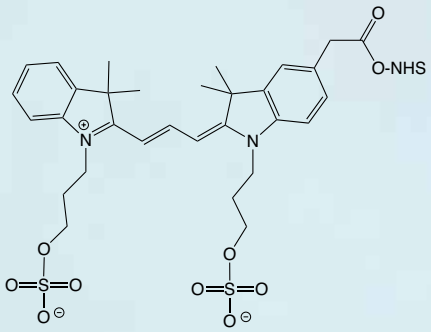
The Waggoner lab focused primarily on the development of new fluorophores and their first major success was a paper on the synthesis and properties of carboxymethylindocyanine dyes as succinimidyl esters.[1] This paper was important in a number of respects - first it demonstrated the properties of cyanine dyes quite well:

- the ability to tune their fluorescence emission by changing the number of carbons in the polymethine chain
- their relative insensitivity to solvent polarity and pH
- their high quantum yields of fluorescence and extinction coefficients.

In addition, this paper served to popularize the now ubiquitous "NHS ester" for labelling amines on proteins, oligonucleotides and antibodies. The commonly used reactive moieties at the time - sulfonyl chlorides and isothiocyanates - were often non-specific when labelling and prone to hydrolysis during storage.

However, this early incarnation of what would become Cy3 and Cy5 had issues. To increase the water solubility of the dyes, the starting trimethylindolenine

FIGURE 1: N-BUTYLSULFONATED CYANINE DYE

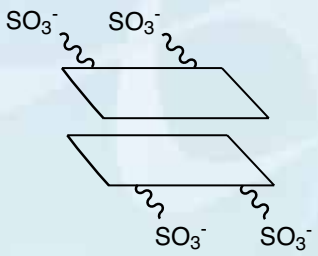


N-Butylsulfonated cyanine NHS Ester

was reacted with butane sulfone to afford an N-butylsulfonated cyanine dye upon condensation, as shown in Figure 1.

Unfortunately, despite their very high water solubility, the butylsulfonated cyanine dyes tended to form dye dimers. Dye dimers (and higher-order H-aggregates) form due to van der Waal and London forces - the result of the hydrophobic and polarizable nature of the dyes. The formation of dye dimers can readily be observed by a simple UV/Vis absorbance spectrum as the appearance of an absorption band that is blue-shifted compared to the monomeric dye - as well as

FIGURE 2: DYE DIMER

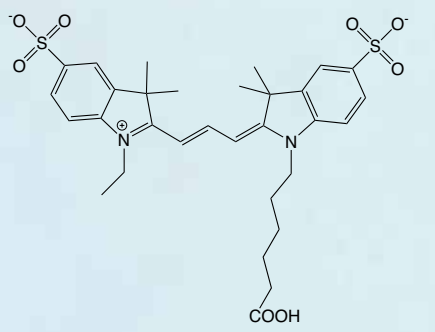


Stacking of dye dimer

a precipitous drop in fluorescence since the dye dimer is non-fluorescent. In retrospect, the reason is quite obvious - the central aromatic planar ring structures could stack with the charged sulfonates hardly interacting, as shown in Figure 2.

Recognizing this, a new cyanine dye was designed with sulfonates coupled directly to the indolenine rings that would prevent dye-dye interactions by both electrostatic and steric repulsions, as shown in Figure 3. Dr. Ratan Mujumdar succeeded in synthesizing this new set of sulfoindocyanine dyes in what would become one of the most highly cited papers to come out of the Waggoner

FIGURE 3: SULFOINDOCYANINE DYE



Sulfonates on the indolenine rings prevent dye dimer formation

lab with 689 citations at present.[2] The resulting sulfoindocyanine dyes allowed high labelling densities on antibodies and proteins without fluorescence quenching, making them some of the brightest dyes known.

Understanding the potential of these dyes, Alan Waggoner and Lansing Taylor founded Biological Detection Systems (BDS) to commercialize the dyes. Not long afterward, Amersham Biosciences acquired BDS to obtain the rights to the Cyanine dye portfolio of fluorophores. Amersham was subsequently purchased by GE - folding it into their Medical Systems Division that would become GE Healthcare BioSciences.

The new sulfoindocyanine dyes proved extremely useful in a variety of applications due to their high fluorescence brightness and their tendency to show very limited non-specific binding. This allowed the detection and quantification of RNA in single cells by flow cytometry using Fluorescent In Situ Hybridization (FISH)[3]; the determination of cytoplasm viscosity by ratiometric fluorescence emission of Cy3/Cy5,[4] and my own work on the stability, specificity and fluorescence brightness of multiply-labelled fluorescent DNA probes.[5]

In more recent work, the sulfonated Cy3 and Cy5 dyes were used in the development of STORM - Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy - which harnesses the 'blinking' quality of the cyanine dyes to reconstruct an image down to an amazing 20 nm resolution - far below the diffraction limit.[6]

While the sulfoindocyanine dyes have wonderful properties, they do have a shortcoming - they cannot be made into stable phosphoramidites which would allow oligonucleotides to be labelled on the DNA

synthesizer. As a result, they require a post-synthetic labelling of an amino-modified oligo, subsequent desalting, and typically RP HPLC or PAGE purification.

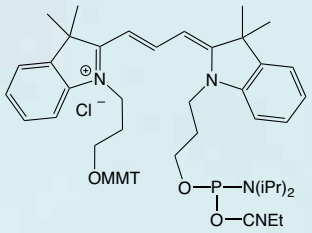
With the rapid rise of TaqMan PCR analysis and the burgeoning demand for cyanine labelled probes, non-sulfonated versions of the cyanine dyes were introduced by Pharmacia and supplied over the last decade by Amersham, then GE Healthcare BioSciences and, of course, Glen Research under license. These cyanine dyes were compatible with phosphoramidite chemistry which allowed the production of PCR probes directly on the DNA synthesizer, making automation a possibility. As a consequence, TaqMan and FRET probes became more accessible.

Glen Research continues to provide a full portfolio of cyanine dyes with only a change in description. We now describe the dyes as Cyanine 3, Cyanine 5, Cyanine 3.5 and Cyanine 5.5. In addition, we are happy to introduce new supports allowing the facile production of 3' cyanine dye-labelled oligonucleotides.

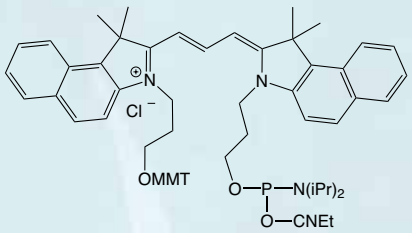
References:

1. Southwick, P. L.; Ernst, L. A.; Tauriello, E. W.; Parker, S. R.; Mujumdar, R. B.; Mujumdar, S. R.; Clever, H. A.; Waggoner, A. S., Cyanine dye labelling reagents–carboxymethylindocyanine succinimidyl esters. *Cytometry* 1990, **11** (3), 418-30.
2. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S., Cyanine dye labelling reagents: sulfoindocyanine succinimidyl esters. *Bioconjug Chem* 1993, **4** (2), 105-11.
3. Yu, H.; Ernst, L.; Wagner, M.; Waggoner, A., Sensitive detection of RNAs in single cells by flow cytometry. *Nucleic Acids Res* 1992, **20** (1), 83-8.
4. Luby-Phelps, K.; Mujumdar, S.; Mujumdar, R. B.; Ernst, L. A.; Galbraith, W.; Waggoner, A. S., A novel fluorescence ratiometric method confirms the low solvent viscosity of the cytoplasm. *Biophys J* 1993, **65** (1), 236-42.
5. Randolph, J. B.; Waggoner, A. S., Stability, specificity and fluorescence brightness of multiply-labelled fluorescent DNA probes. *Nucleic Acids Res* 1997, **25** (14), 2923-2929.
6. Rust, M. J.; Bates, M.; Zhuang, X. W., Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nature Methods* 2006, **3** (10), 793-795.

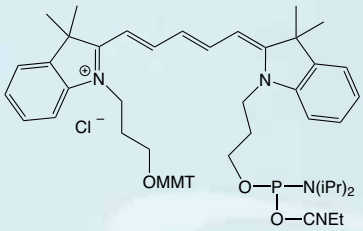
***FIGURE 4:* CYANINE DYE STRUCTURES**



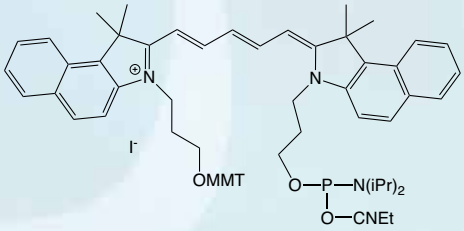
Cyanine 3



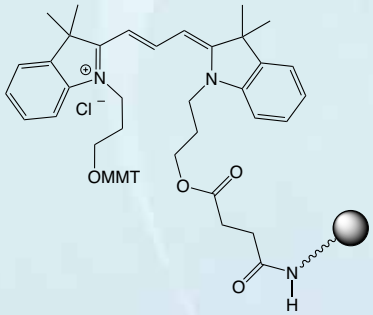
Cyanine 3.5



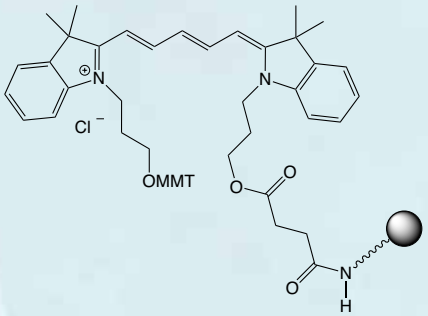
Cyanine 5



Cyanine 5.5



Cyanine 3 CPG



Cyanine 5 CPG

ORDERING INFORMATION

Item	*Cat. No.*	*Pack*	*Price ($)*
Cyanine 3 Phosphoramidite	10-5913-95	50 µmole	205.00
	10-5913-90	100 µmole	375.00
	10-5913-02	0.25g	925.00
Cyanine 3.5 Phosphoramidite	10-5914-95	50 µmole	220.00
	10-5914-90	100 µmole	400.00
	10-5914-02	0.25g	925.00
Cyanine 5 Phosphoramidite	10-5915-95	50 µmole	205.00
	10-5915-90	100 µmole	375.00
	10-5915-02	0.25g	925.00
Cyanine 5.5 Phosphoramidite	10-5916-95	50 µmole	245.00
	10-5916-90	100 µmole	450.00
	10-5916-02	0.25g	925.00
Cyanine 3 CPG	20-5913-01	0.1g	160.00
	20-5913-10	1.0g	1250.00
1 µmole columns (TWIST format only)	20-5913-41	Pack of 4	250.00
0.2 µmole columns	20-5913-42	Pack of 4	70.00
Cyanine 5 CPG	20-5915-01	0.1g	160.00
	20-5915-10	1.0g	1250.00
1 µmole columns (TWIST format only)	20-5915-41	Pack of 4	250.00
0.2 µmole columns	20-5915-42	Pack of 4	70.00

TECHNICAL BRIEF - SELECTIVE COVALENT CAPTURE OF DNA AND RNA TARGETS WITH SHIELDED COVALENT PROBES INCORPORATING A PHOTO-ACTIVATED CROSSLINKER

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Department of Bioengineering, Department of Computing and Mathematical Sciences, California Institute of Technology, Pasadena, California 91125

Nucleic acid probes are used for diverse applications *in vitro*, *in situ*, and *in vivo*. In any setting, their power is limited by imperfect selectivity (binding of undesired targets) and incomplete affinity (binding is reversible and not all desired targets are bound). These difficulties are fundamental, stemming from reliance on base pairing to provide both selectivity and affinity. Chemical modifications to base or backbone moieties within the probe can partially address these challenges, but come with their own set of drawbacks. Shielded covalent (SC) probes eliminate the longstanding tradeoff between selectivity and durable target capture, achieving selectivity via programmable base pairing and molecular conformation change, and durable target capture via activatable covalent crosslinking (Figure 1a)[1].

The SC probe concept is suitable for use with diverse crosslinker chemistries, including 3-cyanovinylcarbazole (^{CNV}K), a photoactive nucleoside analog developed by Yoshimura et al. (Figure 1b)[2, 3]. We found that the ^{CNV}K phosphoramidite coupled with high yield using the same conditions as unmodified nucleosides and is compatible with traditional ammonium hydroxide deprotection at room temperature (see Reference 1 for details). We observed crosslinking yields above 90% after UV-A activation with either high-intensity LED or low-cost fluorescent lamps. The resulting crosslinks are stable against either thermal or chemical denaturation and, if desired, can be efficiently reversed with UV-B light. The only sequence requirement for ^{CNV}K crosslinking is a single opposite-strand pyrimidine, allowing a wide choice of target sequences.

In pure and mixed samples, SC probes covalently capture complementary DNA or RNA oligonucleotide targets and reject two-nucleotide mismatched targets with near-quantitative yields at room temperature, achieving discrimination ratios of 2–3 orders of magnitude. Semi-quantitative studies with full-length mRNA targets demonstrate selective covalent capture comparable to that for RNA oligo targets. Single-nucleotide DNA or RNA mismatches, including nearly-isoenergetic RNA wobble pairs, can be efficiently rejected with discrimination ratios of 1–2 orders of magnitude. Covalent capture yields appear consistent with the thermodynamics of probe/target hybridization, facilitating rational probe design[1].

SC probes require only one modified nucleoside (the crosslinker) and can incorporate DNA, RNA or modified bases as desired. In contrast to existing probe chemistries, SC probes achieve the high sequence selectivity of a structured probe, yet durably retain their targets even under denaturing conditions. This previously incompatible combination of properties suggests diverse applications based on selective and stable binding of nucleic acid targets under conditions where base-pairing is disrupted (e.g., by stringent washes *in vitro* or *in situ*, or by enzymes *in vivo*).

Adapted with permission from Vieregg *et al.*, *J. Am. Chem. Soc.*, **135**:9691-9699, 2013.

References:

1 Vieregg, J. R., Nelson, H. M., Stoltz, B. M. & Pierce, N. A., Selective nucleic acid capture with shielded covalent probes. *J. Am. Chem. Soc.* **135**, 9691-9699, (2013).
2 Yoshimura, Y. & Fujimoto, K., Ultrafast reversible photo-cross-linking reaction: Toward in situ DNA manipulation. *Org. Lett.* **10**, 3227-3230, (2008).
3 Yoshimura, Y., Ohtake, T., Okada, H. & Fujimoto, K., A new approach for reversible RNA photocrosslinking reaction: application to sequence-specific RNA selection. *ChemBioChem* **10**, 1473-1476, (2009).

Note:

^{CNV}K is covered by US Patent 8,481,714 and other patents worldwide assigned to the Japan Advanced Institute of Science and Technology (JAIST). At the time of writing (December 2013), ^{CNV}K phosphoramidite is not available commercially.

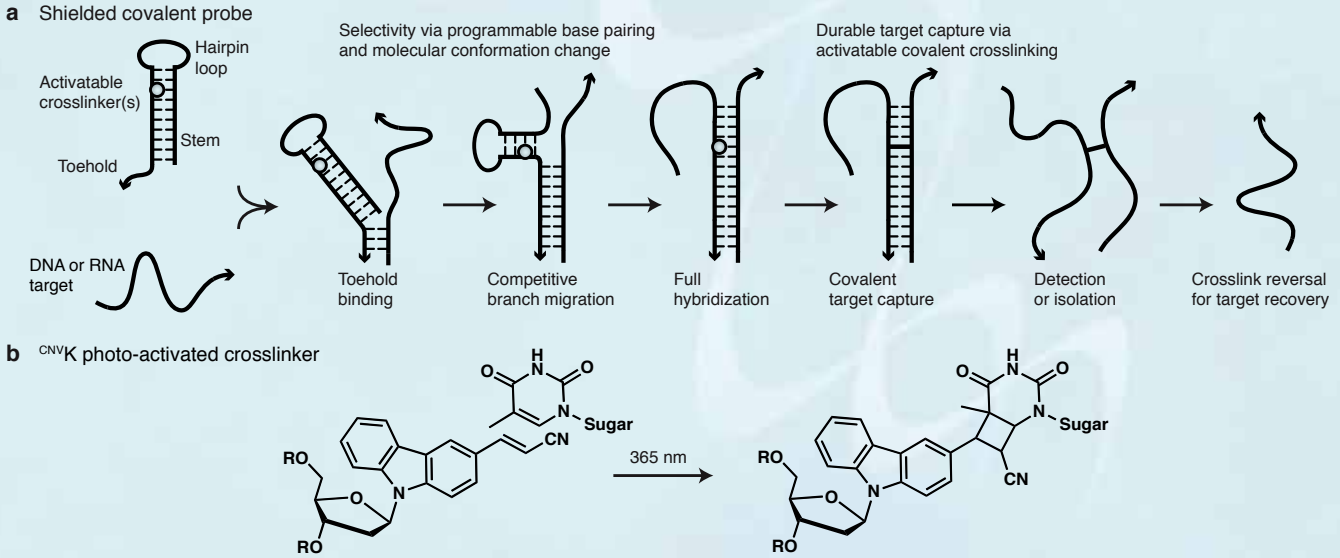


Figure 1. *Shielded covalent (SC) probes achieve high sequence selectivity and stable target capture at a temperature of choice[1]. (a) Concept. High sequence selectivity is achieved at a temperature of choice via competition between internal probe base pairs and probe/target base pairs. Durable target capture is achieved via activation of one or more covalent crosslinkers which are shielded within a duplex both before and after target hybridization, limiting side reactions. Covalent bonds are stable even when base pairing is disrupted, enabling diverse applications. (b) Photo-activated crosslinker used in the current study. The vinyl bond of the 3-cyanovinylcarbazole (^{CNV}K) nucleoside analog undergoes [2+2] cycloaddition to the double bond in an opposite-strand pyrimidine (T depicted) when exposed to 365 nm UV light, forming a stable photoadduct[2, 3]. If desired, the target can be recovered by reversing the crosslink in denaturing conditions with 311 nm UV light.*

TECHNICAL BRIEF - GLEN UNYSUPPORT NOW AVAILABLE WITH FAST CLEAVAGE

Since its introduction in 2008, Glen UnySupport™ (1) has become a very popular universal support, especially for high throughput synthesis. In this type of universal support, the 3' hydroxyl group is generated in a two step process. The first step is the relatively fast cleavage of the succinate group attaching the oligo to the support. The second step is the relatively slow dephosphorylation step to remove the 3'-phosphate linkage and generate the desired 3'-hydroxyl group. The process is illustrated in Figure 2.

We were recently asked if a faster cleaving version of Glen UnySupport could be made available since the cleavage of the succinate group in a high throughput setting sometimes necessitates the evaporation of the cleavage solution and addition of fresh Ammonium Hydroxide:MethylAmine 1:1 (AMA) or ammonium hydroxide (NH_4OH) to ensure complete deprotection and dephosphorylation of the product oligos.

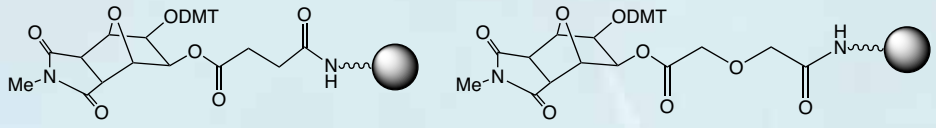
Using a diglycolate linkage in Glen UnySupport FC (2) instead of the succinate in Glen UnySupport, a significant increase in the rate of cleavage has been achieved, as demonstrated in the following experiment and illustrated in the photographs on the right.

In this experiment, two Cyanine 5-labelled T12 oligos were synthesized - one on the succinate-linked Glen UnySupport and the other on the diglycolate-linked Glen UnySupport FC. Half of the support was transferred to a second column so that the cleavage rates in ammonium hydroxide and AMA (30% ammonium hydroxide/40% methylamine 1:1 v/v) could be determined using the same, newly synthesized oligo.

1 mL syringes were filled and sealed with either a freshly-prepared solution of AMA or 30% ammonium hydroxide. The columns of oligonucleotides synthesized either on the Glen UnySupport or the Glen UnySupport FC were fitted with an empty 1 mL syringe. The filled syringe of either ammonium hydroxide or AMA was fitted to the column and the time point started, pushing the solution back and forth until the end point - 1 min, 2 min, etc. - was reached. At that time, the cleaved oligo was pushed into a collection vial and the column immediately rinsed pushing 1 mL from a second filled syringe of the cleavage solution into the collection vial. Immediately a fresh, pre-filled syringe was fitted onto the column

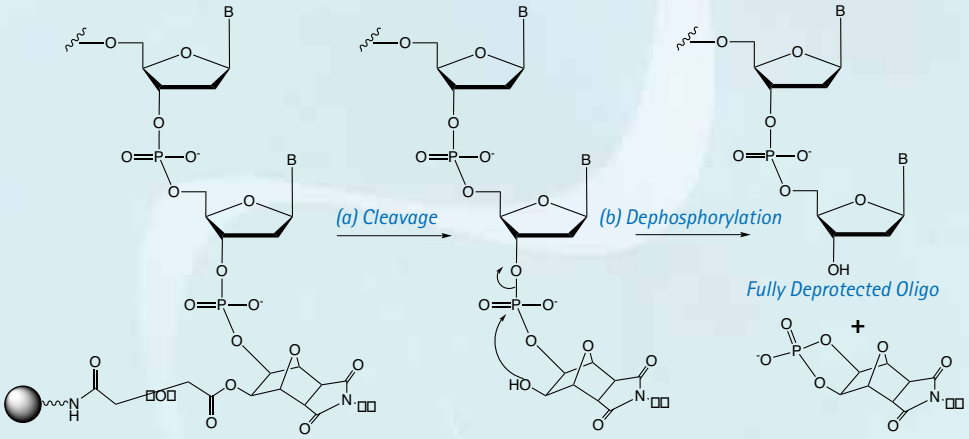
(Continued on Back Page)

FIGURE 1: GLEN UNYSUPPORT STRUCTURES



(1) Glen UnySupport *(2) Glen UnySupport FC*

FIGURE 2: CLEAVAGE AND DEPHOSPHORYLATION OF GLEN UNYSUPPORTS



Oligo Synthesized on Glen UnySupport (1) or (2)

TIME COURSE OF CLEAVAGE OF GLEN UNYSUPPORTS

Glen UnySupport

AMA

1 min 2 min 5 min 10 min

NH_4OH

10 min 20 min 30 min 40 min

Glen UnySupport FC

AMA

1 min 2 min 5 min

NH_4OH

1 min 2 min 5 min

5'-Cyanine 5 labelled T12 oligonucleotides were synthesized on Glen UnySupport and Glen UnySupport FC. Each support was deprotected with AMA or ammonium hydroxide. The supernatant was removed and transferred to a cuvette and fesh solution was added to the support. The total exposure time of each sample is shown.

FIGURE 3: DEPROTECTION AND DEPHOSPHORYLATION OF GLEN UNYSUPPORT AND GLEN UNYSUPPORT FC

Solution Phase:

Ammonium Hydroxide:MethylAmine (AMA) 1:1 for 1 hour at 65°C or Ammonium Hydroxide for 8 hours at 55°C.

For sensitive minor bases or dyes, Glen UnySupport may be eliminated with 50 mM Potassium Carbonate in Methanol in 17 hours at room temperature or with Tert-Butylamine/Water 1:3 (v/v) for 4 hours at 60°C.

Gas Phase:

Methylamine gas for 30 minutes at 65°C at 30 psi.

GLEN RESEARCH

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20164

(Continued from Page 11)

and the solution pushed back and forth until the next time point was reached. The total exposure time of each sample is shown in the illustration on Page 11.

For Glen UnySupport, the solution after 10 minutes exposure to AMA is still pale blue indicating that a minimum of 10 minutes is required for cleavage. Similarly, with ammonium hydroxide, the solution after a total of 40 minutes exposure is still pale blue and so that would be the minimum time required for cleavage.

In the same experiment with Glen UnySupport FC, the sample using AMA cleavage was pale blue after a total of only 2 minutes exposure. More surprisingly, the cleavage was essentially complete in 5 minutes using ammonium hydroxide.

It has to be stressed that these reactions are simply cleavage from the support. The oligos must still be fully deprotected, including dephosphorylation before use.

We conclude that the minimum cleavage times are as follows:

	AMA	NH_4OH
Glen UnySupport	10 min.	40 min.
Glen UnySupport FC	2 min.	5 min.

We are delighted to offer Glen UnySupport FC attached to 1000Å CPG in a variety of formats suited to high throughput synthesis, as well as in bulk for more routine use.

Intellectual Property

These products are covered by US Patent 7,202,264 owned by Isis Pharmaceuticals, Inc.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Glen UnySupport FC	22-5041-01	0.1g	11.00
(1000Å CPG)	22-5041-02	0.25g	25.00
	22-5041-10	1.0g	95.00
ABI Format (not LV)			
1 µmole columns	22-5141-41	Pack of 4	60.00
0.2 µmole columns	22-5141-42	Pack of 4	40.00
40 nmole columns	22-5141-45	Pack of 4	40.00
10 µmole column (TWIST Format)	22-5141-13	Pack of 1	100.00
AB 3900 Format			
Glen UnySupport CPG			
200 nmole columns	22-5141-52	Pack of 10	100.00
40 nmole columns	22-5141-55	Pack of 10	100.00
Expedite Format			
1 µmole columns	22-5241-41	Pack of 4	60.00
0.2 µmole columns	22-5241-42	Pack of 4	40.00
40 nmole columns	22-5241-45	Pack of 4	40.00
15 µmole column (TWIST Format)	22-5241-14	Pack of 1	150.00
96 Well Format (MerMade, etc.)			
1 µmole columns	22-5141-91	Pack of 96	375.00
200 nmole columns	22-5141-92	Pack of 96	250.00
40 nmole columns	22-5141-95	Pack of 96	250.00