The Glen Report

22825 DAVIS DRIVE • STERLING, VIRGINIA • 20164

GLEN RESEARCH

VOLUME 23

NUMBER 2

DECEMBER 2011

INSIDE

GLEN RESEARCH EPIGENETIC BASES REPORT

M. Mueller and T. Carell
Center for Integrated Protein Science (CiPSM)
Department of Chemistry
Ludwig-Maximilians-University Butenandtstr. 5–13
81377 Munich, Germany

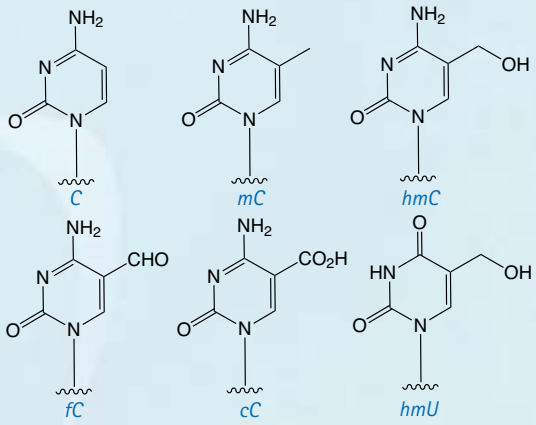
Heritable control of gene expression is the field of epigenetic research. DNA and histone modifications can switch a gene from its 'on' to the 'off' state and *vice versa*. Until 2009, the knowledge about DNA modifications was limited to the introduction of a methyl group at C5 of the cytosine base, which converts 2'-deoxyCytidine (dC) into 5-methyl-2'-deoxyCytidine (mdC). (See Figure 1 for structures of the modified pyrimidine bases discussed in this article.) This methylation occurs predominantly in CpG islands (areas with high occurrence of the CG motif) of promoters. mdC is introduced by specialized DNA methyltransferase (DNMT) enzymes.

In 2009, two reports[1,2] described the discovery of 5-hydroxymethyl-2'-deoxyCytidine (hmdC), a novel dC modification in Purkinje neurons and embryonic stem cells. Later, a third report found this modification to be strongly enriched in brain tissues associated with higher cognitive functions.[3] This new dC modification is generated by the action of α-ketoglutarate dependent TET enzymes (ten eleven translocation), which oxidizes mdC to hmdC, as shown in Figure 2. This finding stimulated discussion about active demethylation pathways that could occur, e.g., *via* base excision repair (BER), with the help of specialized DNA glycosylases. Alternatively, one could envision a process in which the hydroxymethyl group of hmdC is further oxidized to a formyl or carboxyl functionality followed by elimination of either formic acid or carbon dioxide[4,5] (Figure 2).

A number of recent publications provides data that support both pathways. It was discovered that hmdC could be deaminated by activation-induced deaminase (AID) enzymes to provide 5-hydroxymethyl-2'-deoxyUridine (hmdU). This compound was shown to be excised by the SMUG-1 DNA glycosylase[6] (Figure 2). After initial failure to detect any further oxidized hmdC derivatives in somatic tissues[4], newly developed mass spectrometric technologies, in combination with the available reference compounds, finally enabled researchers to gather strong support for the putative oxidative demethylation pathway. These methods and standards enabled the discovery of 5-formyl-2'-deoxyCytidine (fdC) in differentiating embryonic stem cells.[7] Recently, a similar technology also led to the discovery of 5-carboxyl-2'-deoxyCytidine (cdC)[8,9], but the amount of fdC and cdC measured differs largely in all three reports.

Research is currently ongoing to unravel the true levels and fate of these further oxidized dC bases in somatic tissues and in different stem cells. Even along the oxidative pathway, base excision processes have been proposed to play a major role with two reports showing that thymidine DNA glycosylase (TDG) accepts both fdC and

FIGURE 1: CYTOSINE ANALOGUES



(Continued on Page 2)

(Continued from Front Page)

cdC as substrates.[8,10] A possible oxidative demethylation pathway would clearly rely on the existence of a dedicated decarboxylase that is able to convert cdC back into dC. Such an intriguing decarboxylation would enable nature to remove the 5-methyl group in mdC without introducing DNA strand-breaks that accompany any BER based base removal (Figure 2).

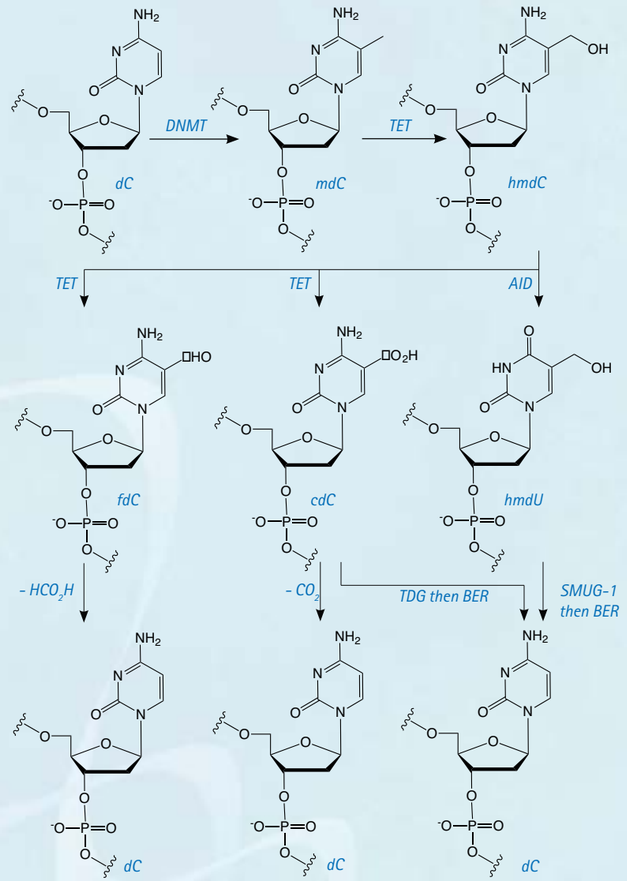
Glen Research has supported this research since its inception by providing the building blocks for the synthesis of oligonucleotides containing all the new dC derivatives - hmdC, fdC and cdC. The first generation hmdC-phosphoramidite, (1) in Figure 3, was fairly very well accepted but required harsh deprotection conditions. Therefore, a new building block developed by Carell and co-workers[11] is now being offered, (2) in Figure 3. This new monomer can be deprotected using potassium carbonate in methanol, making this second generation phosphoramidite compatible with standard phosphoramidite chemistry using UltraMild conditions. Oligonucleotides using standard base protection and this second generation hmC monomer can also be deprotected using sodium hydroxide in aqueous methanol.

In addition, a second generation fdC-phosphoramidite, (4) in Figure 3, developed by Carell and co-workers is also being introduced.[12] This second generation fdC monomer is fully compatible with standard phosphoramidite chemistry. Synthesis of fdC-containing oligonucleotides with this new building block is now possible without an additional oxidative post synthesis deprotection step (Figure 4) that was required for the earlier version, (3) in Figure 3.

Both building blocks were recently used to create hmdC and fdC containing oligonucleotides for detailed mutagenicity studies. In contrast to previous results, which indicated a mutagenic potential of fdC[13], it has now been shown[12] that hmdC, fdC and cdC are not mutagenic, which turns these bases into perfect epigenetic coding units.

Deprotection conditions for all the phosphoramidite monomers included in this report are included in Table 1.

FIGURE 2: CYTOSINE METHYLATION AND DEMETHYLATION PATHWAYS



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
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
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 II-CE Phosphoramidite	10-1512-95	50 µmole	495.00
	10-1512-90	100 µmole	975.00
	10-1512-02	0.25g	2800.00

References:

1. S. Kriaucionis, and N. Heintz, *Science*, 2009, **324**, 929-30.
2. M. Tahiliani, et al., *Science*, 2009, **324**, 930-935.
3. M. Münzel, et al., *Angewandte Chemie-International Edition*, 2010, **49**, 5375-5377.
4. D. Globisch, et al., *PLoS One*, 2010, **5**, e15367.
5. S.C. Wu, and Y. Zhang, *Nat Rev Mol Cell Biol*, 2010, **11**, 607-20.
6. J.U. Guo, Y.J. Su, C. Zhong, G.L. Ming, and H.J. Song, *Cell*, 2011, **145**, 423-434.
7. T. Pfaffeneder, et al., *Angewandte Chemie-International Edition*, 2011, **50**, 7008-7012.
8. Y.F. He, et al., *Science*, 2011, **333**, 1303-1307.
9. S. Ito, et al., *Science*, 2011, **333**, 1300-1303.
10. A. Maiti, and A.C. Drohat, *J Biol Chem*, 2011, **286**, 35334-8.
11. M. Münzel, D. Globisch, C. Trindler, and T. Carell, *Org Lett*, 2010, **12**, 5671-3.
12. M. Münzel, et al., Improved Synthesis and Evaluation of Oligonucleotides Containing 5-Hydroxymethylcytosine, 5-Formylcytosine and 5-Carboxylcytosine. In *Chemistry - A European Journal*, 2011, in press.
13. N. Karino, Y. Ueno, and A. Matsuda, *Nucleic Acids Res.*, 2001, **29**, 2456-63.

FIGURE 4: FORMYL-dC FORMATION

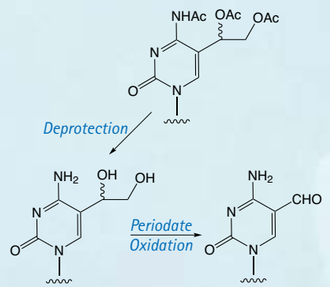


FIGURE 3: PHOSPHORAMIDITE MONOMERS

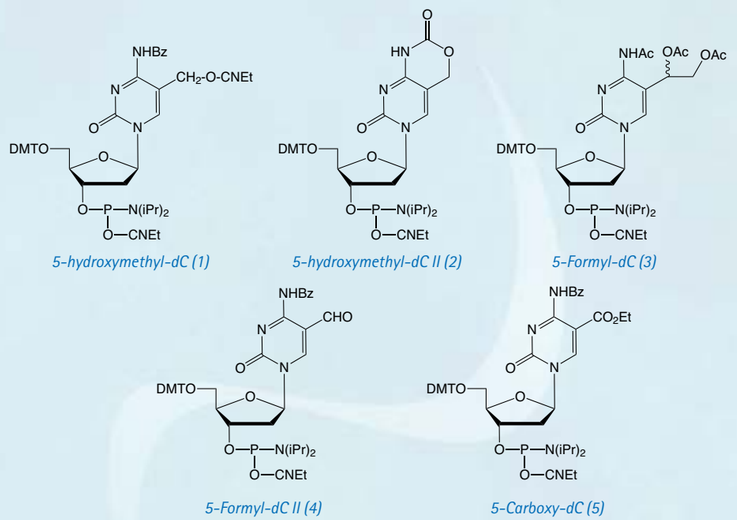


TABLE 1: DEPROTECTION CONDITIONS FOR dC ANALOGUES

Building Block*/ Deprotection Conditions	NH_4OH or AMA	NaOH	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 (NaOH deprotection is not compatible with DMF protecting groups)	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 4	Standard deprotection followed by periodate oxidation, see Figure 4	Standard deprotection followed by periodate oxidation, see Figure 4
5-Formyl-dC II (4)	Conc. NH_4OH, 25°C, 17 h Initially yields imine, which is hydrolysed by water	0.4 M NaOH in methanol/water 4:1, 25°C, 17 h (NaOH deprotection is not compatible with DMF protecting groups)	Benzoyl protecting group is not compatible with UltraMild deprotection conditions
5-Carboxy-dC (5) *See Figure 3 for structures	Yields amides as well as desired carboxylic acid	0.4 M NaOH in methanol/water 4:1, 25°C, 17 h (NaOH deprotection is not compatible with DMF protecting groups)	Not compatible

NEW PRODUCT – 5′-AMINOOXY-MODIFIER 11

The most common conjugation reaction type by far is the reaction between an amino-modified oligonucleotide and an activated carboxylic acid to form a stable amide linkage. This relatively fast and efficient conjugation reaction is carried out at around pH9 where the exocyclic amines of the bases are inactive. However, the reaction between an amine and an aldehyde or ketone is much less useful since the imine that is formed is not stable under acidic or basic conditions and has to be reduced to a secondary amine using a borohydride reagent, such as sodium cyanoborohydride. Aminooxy modifiers may offer an alternative conjugation reaction with aldehydes and ketones since they form stable oximes.

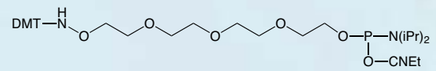
The use of nucleophilic aminooxy modifiers for DNA conjugations was first described in the mid 1990's.[1,2] A number of improvements in oxime conjugation chemistry has led to a more reliable and direct approach for the use of aminooxy modifiers in conjugations with DNA.[3,4] The oxime formed from the reaction of alkyloxyamines with aldehydes creates a stable covalent bond. In comparison, the imine formed by the conjugation of primary amines with aldehydes is not stable to acidic or basic conditions and requires subsequent reduction with borohydrides to form stable amine conjugates, as shown in Scheme 1.

Glen Research is pleased to offer 5′-AminoOxy-Modifier 11 (1), based on a tetraethylene glycol linkage for improved solubility and for reducing the potential negative impact on hybridization of the oligo. For synthesis, we recommend using 1H-Tetrazole with a 3 minute coupling time.

The cleanest approach we have found for solution phase conjugation is to synthesize the oligo DMT-ON and complete the deprotection with ammonium hydroxide or AMA. After drying, the conjugation can be completed in 80% acetic acid to simultaneously remove the DMT group and catalyze the oxime formation. Subsequent gel-filtration using a Glen Gel-Pak removes the acetic acid and unreacted aldehydes. This progression of oxime formation with cinnamaldehyde is illustrated in Figure 2.

The aminooxy conjugation can also be performed on the column after removal of the 5′-DMT group, provided the label is stable to the subsequent deprotection conditions.[4]

FIGURE 1: **STRUCTURE OF 5′-AMINOOXY-MODIFIER**



(1) 5′-AminoOxy-Modifier 11

SCHEME 1: **COMPARISON OF THE REACTION OF AMINOOXY AND AMINO GROUPS WITH AN ALDEHYDE**

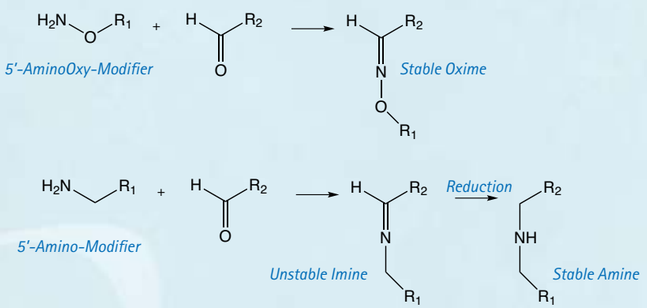
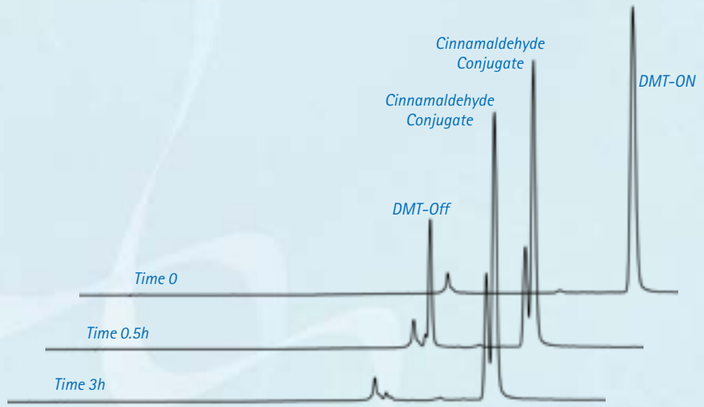


FIGURE 2: **RP HPLC ANALYSIS OF REACTION OF 5′-AMINOOXY-MODIFIED WITH CINNAMALDEHYDE**



Protocol:

1. Synthesize the 5′-AminoOxy modifier, DMT-ON.
2. Cleave and deprotect with AMA for 10 minutes at 65°C.
3. Dry oligo.
4. Dissolve oligo in 0.2mL water.
5. Add 10 equivalents of aldehyde in a suitable solvent, 100µL.
6. Add 0.8mL acetic acid and mix well.
7. React for 60 minutes at room temperature.
8. Quench with 0.1M TEAA, 1mL.
9. Purify on Glen Gel-Pak or equivalent.

References:

1. B. Cebon, et al., *Australian Journal of Chemistry*, 2000, **53**, 333-339.
2. E. Trevisiol, A. Renard, E. Defrancq, and J. Lhomme, *Tetrahedron Lett*, 1997, **38**, 8687-8690.
3. O.P. Edupuganti, E. Defrancq, and P. Dumy, *J Org Chem*, 2003, **68**, 8708-8710.
4. T.S. Zatsepin, D.A. Stetsenko, M.J. Gait, and T.S. Oretskaya, *Tetrahedron Lett*, 2005, **46**, 3191-3195.

ORDERING INFORMATION

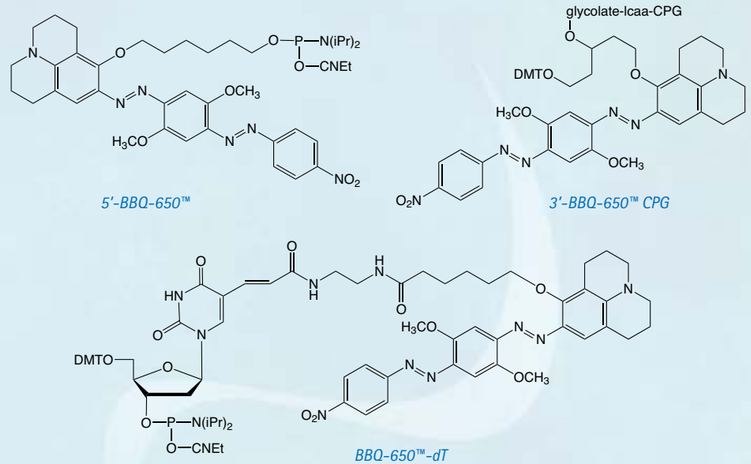
Item	*Catalog No.*	*Pack*	*Price($)*
5′-AminoOxy-Modifier 11	10-1919-95	50 µmole	140.00
	10-1919-90	100 µmole	265.00
	10-1919-02	0.25g	895.00

NEW PRODUCTS - BLACKBERRY® QUENCHER (BBQ-650™)

A dark quencher is a non-fluorescent chromophore that absorbs fluorescence energy from a neighboring fluorophore, thereby preventing emission of fluorescent light. In Fluorescence Resonance Energy Transfer (FRET), a dark quencher then relaxes to its ground state by emitting energy as heat. In static quenching, e.g., in molecular beacons, the dark quencher forms a ground state complex with the fluorophore, preventing emission of fluorescence until hybridization disrupts the complex. Glen Research already offers a variety of quenchers for a wide range of applications. However, quenching at longer wavelengths has always been problematical since molecules absorbing at >600nm tend to have extended π systems that make them very susceptible to degradation by the conditions of oligonucleotide synthesis and deprotection.

We are now happy to begin offering several products containing the BlackBerry® Quencher (BBQ-650™). Based on an 8-alkoxyjulolidine structure that is a potent π electron donor, BBQ-650 exhibits a broad absorption profile from 550nm to 750nm, centered at 650nm. This range offers more effective quenching of some of our popular long wavelength dyes like TAMRA, Redmond Red, Cy dyes and DyLight dyes. Moreover, BBQ-650 is stable to regular deprotection, but requires mild deprotection with AMA at room temperature. We offer BBQ-650 products for the 3′ and 5′ termini, as well as BBQ-650-dT for inclusion within the oligonucleotide sequence.

FIGURE 1: **BBQ-650™ PHOSPHORAMIDITE MONOMERS AND SUPPORT**

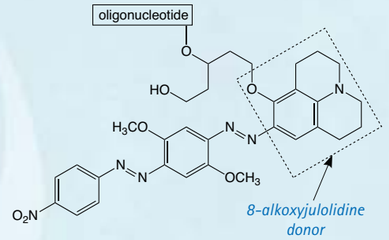


BLACKBERRY® QUENCHER HIGHLIGHTS

- Quenches the fluorescence of long wavelength dyes
- Quenches in FRET and contact mode
- Absorbance maximum at ~650nm
- Quenching range – 550-750nm
- Compatible with standard oligo synthesis chemistry
- Compatible with regular deprotection but requires mild deprotection with AMA at room temperature
- Available for 3′, 5′, and internal substitution

BlackBerry® Quencher technology: US Patent 7,879,986. The purchase of BlackBerry® Quencher reagents includes a limited license to use these reagents exclusively for research and development purposes. They may not be used for clinical or diagnostic purposes and they may not be re-sold, distributed, or re-packaged without prior agreement and consent of Berry & Associates, Inc. Subsequent sale of products that are derived from BlackBerry® Quencher reagents is permitted so long as the following written disclaimer is included in written and electronic catalogs, in commercial advertisement, and in packages with containers of such derivative products: *"BlackBerry is a trademark of Berry & Associates, Inc. Products derived from BlackBerry® Quencher reagents are sold exclusively for research and development use by the purchaser. They may not be used for clinical or diagnostic purposes without prior agreement and consent of Berry & Associates, Inc."*

FIGURE 2: **3′-BBQ-650™ OLIGO**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5′-BBQ-650™ Phosphoramidite	10-5934-95	50 µmole	160.00
	10-5934-90	100 µmole	305.00
	10-5934-02	0.25g	925.00
BBQ-650™-dT	10-5944-95	50 µmole	280.00
	10-5944-90	100 µmole	545.00
	10-5944-02	0.25g	925.00
3′-BBQ-650™ CPG	20-5934-01	0.1g	190.00
	20-5934-10	1.0g	1500.00
1 µmole columns	20-5934-41	Pack of 4	300.00
0.2 µmole columns	20-5934-42	Pack of 4	80.00
10 µmole column (ABI)	20-5934-13	Pack of 1	575.00
15 µmole column (Expedite)	20-5934-14	Pack of 1	825.00

ON-RESIN SYNTHESIS OF MALEIMIDO-OLIGONUCLEOTIDES

Albert Sánchez, Enrique Pedroso and Anna Grandas
Departament de Química Orgànica and IBUB
Facultat de Química, Universitat de Barcelona
Martí i Franquès 1-11
08028 Barcelona, Spain

INTRODUCTION

Two conjugation methodologies make use of maleimide groups, the maleimide-thiol Michael reaction and the Diels-Alder cycloaddition. Maleimides cannot be linked to the oligonucleotide while still anchored to the solid support because of their incompatibility with standard deprotection conditions, which make use of nucleophilic bases such as ammonia.

So far the most common alternative to prepare maleimido-oligonucleotides has been to react, in solution, bifunctional reagents incorporating a maleimide and a carboxyl group (or the corresponding active ester) with amino-derivatized oligonucleotides.[1] However, reaction yields are not reproducibly high.

Maleimide protection provides a simpler, straightforward solution, since it allows the maleimide building block to be introduced on-resin into the protected oligonucleotide.[2] For this purpose, phosphoramidite derivative (**1**) has been developed. This reagent incorporates a maleimide-2,5-dimethylfuran cycloadduct (*exo* isomer), which is stable to ammonium hydroxide at room temperature. This phosphoramidite (**1**) can be incorporated into DNA and RNA with both phosphate and phosphorothioate linkages.[3]

MALEIMIDO-OLIGONUCLEOTIDES

The oligonucleotide chain must be assembled using "ultra mild" phosphoramidites to avoid heating with ammonia. Then, following oligonucleotide elongation, the protected maleimide can be introduced at the 5' end using phosphoramidite (**1**) and standard phosphite triester chemistry. Treatment with concentrated aqueous ammonia at room temperature removes protecting groups on the oligonucleotide chain, affording stable [protected maleimido]-oligonucleotides that can be purified as normal.

For the preparation of maleimido-oligoribonucleotides, deprotection of 2'-hydroxyl groups is carried out by reaction with Et3N·3HF (8 h, room temperature) before removal of the maleimide protecting group.

A retro–Diels-Alder reaction deprotects the maleimide. The [protected maleimido]-oligonucleotide is first dried by coevaporation with toluene (3x). Further addition of toluene (the amount that would be required to yield a 25 µM solution) provides a suspension that is heated in a metal block for 3-4 h at 90 °C, and solvent is then removed *in vacuo*. The resulting residue is the target maleimido-oligonucleotide. Maleimide deprotection must be carried out immediately before use in conjugation reactions, because maleimido-oligonucleotides are not very stable.[2]

FIGURE 1: **INCORPORATION OF 5'-MALEIMIDE-MODIFIER INTO OLIGONUCLEOTIDES**

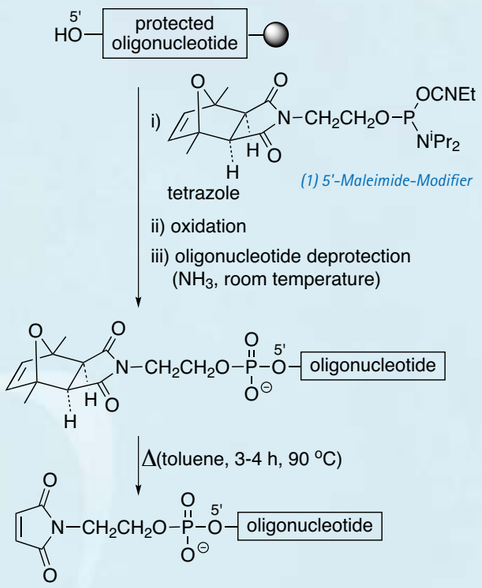
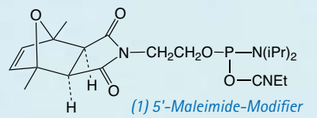


FIGURE 2: **5'-MALEIMIDE MODIFIER**



(1) 5'-Maleimide-Modifier

INTELLECTUAL PROPERTY

5'-Maleimide Modifier Phosphoramidite (1) is protected by a patent application and is offered by Glen Research under a non-exclusive license agreement from the University of Barcelona.

REFERENCES

1. B. A. R. Williams, J. C. Chaput in *Current Protocols in Nucleic Acid Chemistry*, unit **4.41** (M. Egli, P. Herdewijn, A. Matsuda, Y. Sanghvi, editors), John Wiley & Sons, USA, 2010, pp. 4.41.1-4.41.20.
2. A. Sánchez, E. Pedroso, A. Grandas, *Org. Lett.* 2011, **13**, 4364-4367.
3. A. Sánchez, E. Pedroso, A. Grandas, *Bioconjugate Chem.*, in press.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-Maleimide-Modifier Phosphoramidite	10-1938-90	100 µmole	70.00
	10-1938-02	0.25g	335.00

NEW PRODUCT - NPOM-CAGED-DT

Photocaging has developed into an attractive and simple procedure for achieving exquisite control over complex biological processes through photoactivation by UV light. The term 'caging' refers to the attachment of a photolabile protecting group to a biologically active molecule at specific locations, effectively rendering the molecule inactive. After irradiation by light at the wavelength required to remove the protecting group, the biological properties of the 'uncaged' molecule are restored. Glen Research's interest lies in the caging of nucleoside phosphoramidites used to prepare caged oligonucleotides whose function is restored after uncaging by UV light at a wavelength that causes no DNA damage. In this article, we focus on the approach of Deiters and co-workers at North Carolina State University and introduce a caged monomer to allow our customers to follow their lead.

The particular form of photochemical DNA activation used by the Deiters's group has been detailed in two recent review articles.[1,2] In their approach, the nucleobase is caged with the photolabile group, 6-nitropiperonyloxymethyl (NPOM), which can be removed in a few minutes using UV light at 365nm.[3] This wavelength and the low intensity of the light used minimizes possible irradiation-induced damage to DNA, which typically occurs at wavelengths below 300nm.[4,5]

NPOM-Caged dT-CE Phosphoramidite (1) is shown in Figure 1. By attaching the NPOM group to the N3 position of thymidine, the ability of the nucleotide to undergo base-pair formation is inhibited, thus preventing regular hybridization. NPOM-caged dT is the most popular photocaged product to be introduced into DNA to date, although a caged dG monomer has also been described.[6]

Deiters and coworkers have demonstrated an astonishing array of applications of this strategy in the fields of PCR and polymerase activity[7], antisense and gene silencing[8], regulation of restriction endonuclease activity[9], and enzyme-free mutagenesis[10]. More recent publications have described activation and deactivation of DNAzyme and antisense activity controlling gene expression in mammalian cells[11], and photochemical control of DNA decoy function to regulate gene transcription[12].

Oligonucleotides containing NPOM-caged dT are easily prepared by standard oligonucleotide synthesis, with a 10 minute coupling time being optimal for NPOM-caged dT. Cleavage and deprotection of oligonucleotides containing NPOM-caged dT are also carried out under conventional conditions.

As a general design rule, oligonucleotides containing NPOM-caged dT every five or six bases do not hybridize to their complementary strand. However, fewer additions may be tolerated and efficient hybridization was still observed when only a single NPOM-caged dT residue was included.[7] Photo-uncaging of an oligonucleotide is easily carried out with UV light at 365 nm for seconds to minutes and this can be readily achieved with a UV transilluminator, a hand-held UV light, or a fluorescence microscope to uncage the oligo in a specific location within the cell.

We thank Professor Deiters for his conversations regarding the potential of caged monomers, for his encouragement to make NPOM-caged dT available to our customers, and for taking the time to review this article.

FIGURE 1: **SYNTHESIS AND DEPROTECTION USING NPOM-CAGED-dT**

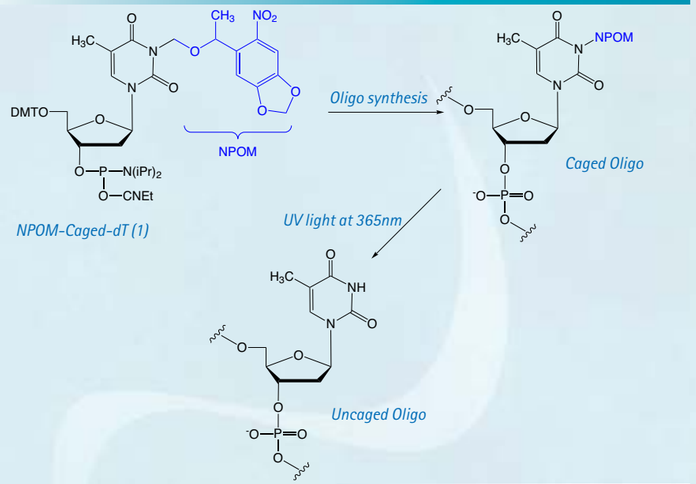
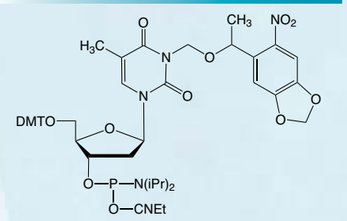


FIGURE 2: **STRUCTURE OF NPOM-CAGED-dT**



NPOM-Caged-dT

References:

1. D.D. Young, and A. Deiters, *Org Biomol Chem*, 2007, **5**, 999-1005.
2. A. Deiters, *Curr Opin Chem Biol*, 2009, **13**, 678-86.
3. H. Lusic, D.D. Young, M.O. Lively, and A. Deiters, *Org Lett*, 2007, **9**, 1903-6.
4. J. Olejnik, E. Krzymanska-Olejnik, and K.J. Rothschild, *Nucleic Acids Res*, 1996, **24**, 361-6.
5. J. Cadet, and P. Vigny, The photochemistry of nucleic acids. In *Bioorganic Photochemistry*, H. Morrison, Ed. John Wiley & Sons: New York, NY, 1990; Vol. 1, pp 179-184.
6. H. Lusic, M.O. Lively, and A. Deiters, *Mol Biosyst*, 2008, **4**, 508-11.
7. D.D. Young, W.F. Edwards, H. Lusic, M.O. Lively, and A. Deiters, *Chem Commun*, 2008, 462-4.
8. D.D. Young, H. Lusic, M.O. Lively, J.A. Yoder, and A. Deiters, *ChemBioChem*, 2008, **9**, 2937-40.
9. D.D. Young, J.M. Govan, M.O. Lively, and A. Deiters, *ChemBioChem*, 2009, **10**, 1612-6.
10. D.D. Young, H. Lusic, M.O. Lively, and A. Deiters, *Nucleic Acids Res*, 2009, **37**, e58.
11. D.D. Young, M.O. Lively, and A. Deiters, *J Am Chem Soc*, 2010, **132**, 6183-93.
12. J.M. Govan, M.O. Lively, and A. Deiters, *J Am Chem Soc*, 2011, **133**, 13176-82.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
NPOM-Caged-dT-CE Phosphoramidite	10-1534-95	50 µmole	185.00
	10-1534-90	100 µmole	355.00
	10-1534-02	0.25g	895.00

GLEN-PAK™ PURIFICATION - THEN AND NOW

Since their introduction in December of 2007, the standard Glen-Pak™ DNA and RNA cartridges have become the preferred method of DMT-ON oligonucleotide purification for many of our customers. In that same time period, the range of our Glen-Pak product line has expanded to meet the demand for multiple processing formats as well as varying applications. This article is a review of the many different Glen-Pak options now available for purification of DNA and RNA, ranging from our smallest scale 30mg, 96 well plate to our mid-scale Glen-Pak DNA Cartridge 3G.

BENEFITS

Glen-Pak cartridges are highly versatile due to their ability to be used directly with common base deprotection solutions such as Ammonium Hydroxide, AMA, Tert-Butylamine/Water, and Potassium Carbonate in Methanol.

They impart a larger capacity per gram of bed volume than most standard reverse phase cartridge systems due to the highly specific capture of DMT-ON products and simultaneous exclusion of DMT-Off failure sequences during the loading step.

The cartridges are also compatible with oligonucleotides over a very broad length range. We have successfully purified DNA sequences from 2 to 150 bases in length and RNA sequences over 60 bases long.

Finally, there are multiple scales and formats available to meet downstream processing requirements ranging from single use to multi-well automation, since RNA and DNA Glen-Paks can be used with a standard, disposable syringe in addition to 96 well and chamber/port vacuum manifolds.

TO THE LIMIT

As with any cartridge purification technique, there are some inherent limitations to keep in mind.

Not all modifiers, dyes or minor bases are stable to the reagents used in the purification process (2% TFA, for example).

Some hydrophobic dyes that aid in purification when on the 5' end of an oligo (Cy3, Cy5, Dabcyl, TAMRA) tend to challenge 5'-DMT specificity during purification when placed internally or at the 3' end. Additionally, any DMT-ON failure sequences derived from a stepwise synthesis cycle or

GLEN-PAK™ OPTIONS

instrument issues may co-purify with the full-length product.

Finally, as with any chromatographic technique, challenging sequence content of an oligonucleotide such as G-quartets or designed hairpins can dramatically impact binding of the 5'-DMT to the Glen-Pak resin. As such, the Glen-Pak performance is directly correlated to the quality of the synthesis itself as well as the direct composition of the oligonucleotide.

VERSATILITY, TIPS AND TRICKS

In response to customer requests, we have added protocols to the Glen-Pak User Guide for many different oligo types. Some tips have also emerged as helpful in the routine use of Glen-Paks, as noted below.

For example, the protocol for purification of phosphorothioates as well as mixed 2'-OMe and 2'-F-RNA oligonucleotides includes a heating step (55°C for at least 15 minutes) just prior to loading of the oligonucleotide solution onto the cartridge and greatly improves yield. Customers purifying longer oligos and those with inherent secondary structure have also used this slight change to the procedure successfully.

Another example is the use of the DNA Glen-Pak for RNA desalting as an alternative to precipitation of 2' deprotected, DMT-Off RNA oligonucleotides.

As mentioned above, purification via a 5'-DMT has inherent limitations. One way to obtain highly pure oligonucleotides is to use the Glen-Pak as a preliminary purification step by omitting the TFA DMT removal step, eluting the final product DMT-ON, followed by a much more fine tuned reverse phase HPLC purification.

Finally, it has been confirmed that with the use of TEA.3HF, DMSO and the required Quenching Solution, the Glen-Pak RNA cartridge is appropriate when conducting DMT-ON purification of DNA/RNA chimeras.

GLEN-PAK SUMMARY BY SYNTHESIS SCALE

Glen-Pak DNA 30mg, 96 Well Plate (60-5400-01) - Up to 50 nmole Capacity

Designed for those customers looking for true high throughput, small scale, DMT-ON purification of oligos via either a stand-alone manifold or with their currently installed SPE robotics-based vacuum systems. Each well in the standardized 96-well filter plate is filled with 30mg of Glen-Pak DNA purification resin, which is enough to purify up to a 50 nmole scale synthesis.

For stand-alone use, the 96-well plate can be coupled to a vacuum source, a collar-based manifold, and a waste tray for loading or rinse steps. The standard sample load and rinse volumes are 500 µL with final elution volumes of 250 µL, which are collected into a 96-well deep well plate. These volumes are only suggestions based on our experience, and could be lowered with specific methods development. The plate is also compatible with previously installed 96-well filter plate and SPE manifold systems.

The advantages of using the 96-well format include:

- *Lower cost processing (less labor and unattended operation if using a robot).*
- *Less processing time per oligo when using a whole plate.*
- *Less solvent usage for every purification step.*
- *Less final elution volume to dry in preparation for analysis or use.*

Glen-Pak DNA 50mg Purification Cartridge (60-5000-96) - Up to 200 nmole Capacity

Introduced primarily for those using automated liquid handlers. It has a capacity of up to a 200 nmole scale and the same 96 well, standard configuration as the regular sized Glen-Pak DNA cartridge. The main benefit for those using liquid handlers is the shorter bed height, which allows for lower volume, single reagent additions at most of the purification steps. This cartridge is only offered in the vacuum manifold configuration due to its intended use with higher throughput applications.

Some of the benefits of the 50mg cartridge include:

- *Lower bed volume leaves more room for reagents in the column.*
- *Lower volume additions for all but the DMT removal step.*
- *Lower final elution volume of 0.5mL, which reduces sample drying time.*
- *More amenable to robotic applications due to single reagent addition capability.*

Glen-Pak DNA Purification Cartridge (60-5100-xx, 60-5200-xx) - Up to 1 µmole Capacity

Ideal for DMT-ON based purification of oligonucleotides up to a 1.0 µmole scale and comes in both hand held syringe and manifold compatible configurations. The vacuum style cartridges utilize a standardized adapter rack to allow use of 96 well manifolds and due to their luer tip, are also compatible with multi port chamber style manifolds. The syringe style gives a single use or lower throughput option with no change in protocol. Methods have been developed for 2'-OMe, 2'-F, and phosphorothioate oligonucleotides as well as for simple desalting of both DNA *and* RNA. Additional methods for 5'-Thiol, 6-FAM, CyDye and phosphorylated oligos can be found in the Glen-Pak User Guide.

TABLE: GLEN-PAK™ SUMMARY

Description	Catalog No.	Scale	Format	Uses
Glen-Pak DNA 30mg	60-5400-01	Up to 50 nmole	96 Well Plate	High throughput Routine DNA
Glen-Pak DNA 50mg	60-5000-xx	Up to 200 nmole	Cartridge	High throughput Routine DNA Low volumes used
Glen-Pak DNA	60-5100-xx	Up to 1 µmole	Cartridge	High throughput Routine DNA Up to 150mers
Glen-Pak DNA	60-5200-xx	Up to 1 µmole	Hand Held Cartridge	Routine DNA
Glen-Pak RNA	60-6100-xx	Up to 1 µmole	Cartridge	High throughput Routine RNA
Glen-Pak RNA	60-6200-xx	Up to 1 µmole	Hand Held Cartridge	Routine RNA
Glen-Pak DNA 3G	60-5300-01	Up to 20 µmole	Cartridge	Routine DNA

Glen-Pak RNA Purification Cartridge (60-6100-xx, 60-6200-xx) - Up to 1 µmole Capacity

As with the standard Glen-Pak DNA cartridges, these products are able to purify DMT-ON RNA and RNA/DNA chimeric oligonucleotides of scales up to 1.0 µmole. The same hand held and vacuum manifold formats are available to enable both low and high throughput options. Due to its functionalized resin, the cartridge is amenable to both long and short sequences and is compatible with RNA base deprotection of both TOM and TBDMS monomers followed by TEA.3HF based 2'-deprotection. If highly pure RNA oligos are needed, this cartridge is the perfect pre-purification step should a second PAGE or HPLC processing step be desired.

With a simple, direct load of a quenched 2'-deprotection mixture, these cartridges offer one of the fastest, most convenient DMT-ON purification methods for RNA oligonucleotides available.

Glen-Pak DNA Cartridge 3G (60-5300-01)- Up to 20 µmole Capacity

Using the same resin as in the standard DNA Glen-Pak, these cartridges can purify and desalt a mid-scale oligonucleotide synthesis of between 5 and 20 µmoles using the same protocol as the standard cartridge but with a 10-fold linear increase in volume for each step. Many of our customers use this product as a DMT-ON pre-purification cartridge, as it is a simple way to remove excess failures, protecting groups, and salts prior to proceeding with a second ion exchange or reverse phase HPLC step. As with all of the Glen-Pak cartridges, an oligonucleotide can also be processed with the DMT still intact by skipping the TFA step and eluting in the same 50% Acetonitrile solution, as described in the Glen-Pak User Guide.

Accessories

Various accessories are available to enable proper use of the Glen-Pak DNA and RNA Cartridges. Both an adapter rack (60-0010-01) and seal for unused wells (60-0020-01) are made available for use with any of the vacuum style Glen-Paks on a 96 well manifold. Less than 96 purifications per run can be done using this seal. Additionally, RNA Quenching Buffer (60-4120-xx) is required to stop the 2'-deprotection reaction of an RNA oligonucleotide and prepare it for loading onto the Glen-Pak RNA cartridge.

Please see the Glen-Pak User Guide for specific protocols: http://www.glenresearch.com/Technical/GlenPak_UserGuide.pdf

TECHNICAL BRIEF - DEPROTECTION OF HMdU

5-Hydroxymethyl-2'-deoxyUridine (hmdU) is a lesion formed from Thymidine by ionizing radiation or peroxide radicals. HmdU is also interesting because of the current work on the closely related 5-hydroxymethyl-2'-deoxyCytidine monomer (hmdC) which has implications in epigenetic research.

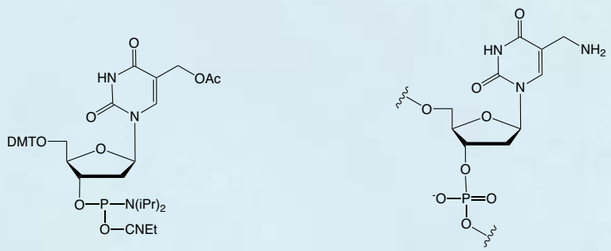
Deprotection of hmdU proceeds with hydrolysis of the acetyl protecting group from the hydroxymethyl group to generate the desired modified nucleoside. However, results generated while working on a different project led us to become concerned that the deprotection of our 5-hydroxymethyl-2'-deoxyUridine (hmdU) monomer (10-1093) (1) might not be as straightforward as we first reported. As part of our policy of continuous improvement, we took a further very close look at the deprotection procedure.

Unfortunately, our suspicions were confirmed when we found that deprotection of an oligonucleotide containing hmdU by heating with ammonium hydroxide for 2 hours at 65°C led to an impurity, as shown in Figure 2. Further analysis confirmed that this impurity was formed by the displacement of the acetate group with ammonia to form the 5-aminomethyl analogue (2). Interestingly, we found that deprotection with ammonium hydroxide at room temperature led to complete hydrolysis and none of the displacement product. The result of deprotection with ammonium hydroxide/methylamine (AMA) at 65 °C was also intriguing in that no displacement reaction was detected (Figure 3). In the case of AMA, the hydrolysis reaction is presumably so fast that the displacement has no chance to occur. Similarly, as expected, deprotection with potassium carbonate in methanol or sodium hydroxide in aqueous methanol gave a pure product identical to Figure 3.

Consequently, we have changed the instructions for the deprotection of oligos containing this modification to:

Synthesize using acetyl-protected dC (10-1015-xx) and deprotect in 30% Ammonium Hydroxide/40% Methylamine 1:1 (AMA) at 65°C for 10 minutes OR synthesize using dmf-protected dG (10-1029-xx) and deprotect in Ammonium Hydroxide for 17 hours at room temperature.

FIGURE 1: **STRUCTURE OF 5-HMdU-CE PHOSPHORAMIDITE AND 5-AMINOMETHYL-dU**



(1) 5-Hydroxymethyl-dU *(2) 5-Aminomethyl-dU Oligo*

FIGURE 2: **RP HPLC OF 5-HMdU-T6 OLIGO DEPROTECTED WITH AMMONIUM HYDROXIDE AT 55° C**

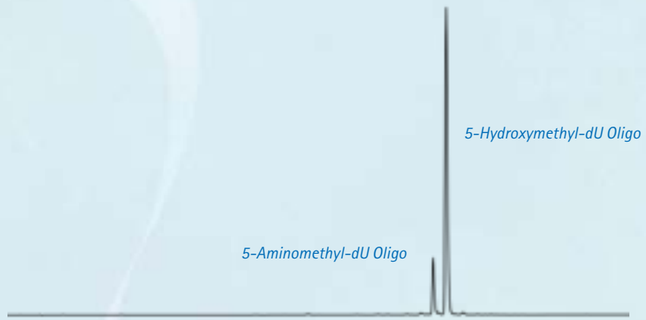


FIGURE 3: **RP HPLC OF 5-HMdU-T6 OLIGO DEPROTECTED WITH AMA AT 65° C**

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5-Hydroxymethyl-dU-CE Phosphoramidite	10-1093-90	100 µmole	225.00
	10-1093-02	0.25g	675.00

TECHNICAL BRIEF - IMPROVED OLIGO SYNTHESIS USING 2-AMINO-DA AND CSO OXIDATION

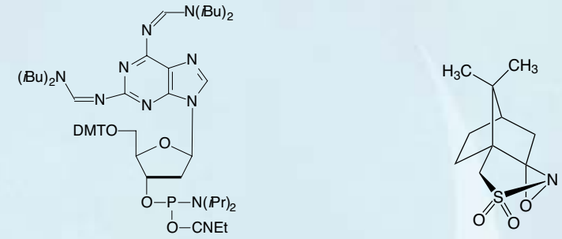
Customer feedback is one of the key resources that Glen Research uses for continuous improvement of our products. Recently, a customer informed us that he was very disappointed with the performance of our 2-Amino-dA-CE Phosphoramidite (1) when added multiple times in an oligo. When oligos were synthesized containing up to four 2-amino-dA additions, the results were just acceptable but six additions proved to have a very low yield of full length oligo. Unfortunately, we were able to confirm our customer's findings even using the optimized conditions we specify for 2-amino-dA. As shown in Figure 2, six additions of 2-Amino-dA in a mixed base 16 mer yielded a product amounting to only 13% of the oligo mixture by area of the oligo mixture when analyzed by RP HPLC. The question was then – why? - when the monomer exhibits high purity and one to three additions were achieved in high yield.

In our development work with 2-amino-dA, we investigated various activators and deblocking mixes but, at that time, we had no good alternative to iodine-containing oxidizers. Now we offer an alternative oxidizer, (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (2). 0.5M CSO in acetonitrile is a stable solution which requires a 3 minute oxidation time for regular oligonucleotide synthesis. We decided to try substituting CSO for iodine oxidation. We duly found that the synthesis of oligonucleotides with 6 additions of 2-amino-dA was enormously improved using CSO, with the percentage of full length product rising to over 60% (Figure 3).

2-Amino-dA is very susceptible to depurination if electron withdrawing protecting groups, for example acyl protection groups, are used. To stabilize the glycosidic bond, strongly electron donating formamidine protecting groups are used in this monomer. We surmise that the N2 formamidine group can activate the N3, which attacks the 3' carbon to displace the phosphonium iodide intermediate, leading to chain scission.

In situations like this where a change is required in our user instructions, we update the instructions on the analytical information and also update the Product File on our web site, in this case: http://www.glenresearch.com//ProductFiles/10-1085.html.

FIGURE 1: **STRUCTURES OF 2-AMINO-dA CE PHOSPHORAMIDITE AND CSO**



(1) 2-Amino-dA *(2) CSO*

FIGURE 2: **RP HPLC OF A MIXED BASE 16MER WITH 6 ADDITIONS OF 2-AMINO-dA AND I_2 OXIDATION**

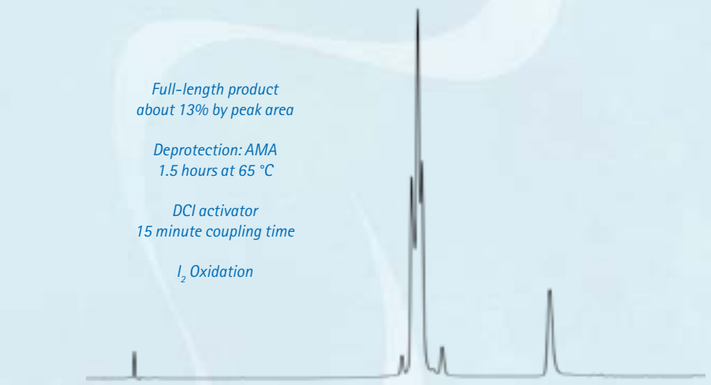
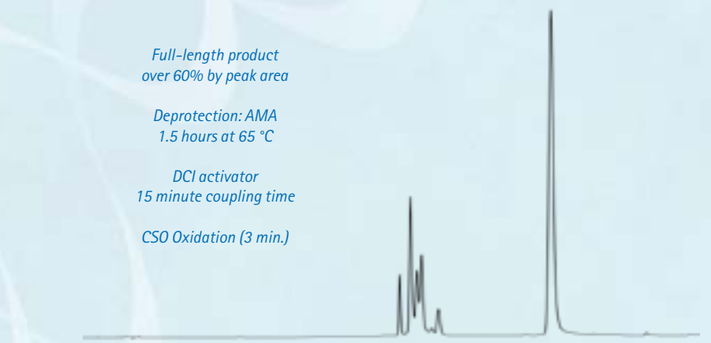


FIGURE 3: **RP HPLC OF A MIXED BASE 16MER WITH 6 ADDITIONS OF 2-AMINO-dA AND CSO OXIDATION**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
2-Amino-dA-CE Phosphoramidite (2,6-diaminopurine)	10-1085-95	50 µmole	70.00
	10-1085-90	100 µmole	125.00
	10-1085-02	0.25g	250.00
0.5M CSO in Anhydrous Acetonitrile (ABI)	40-4632-52	200mL	250.00
0.5M CSO in Anhydrous Acetonitrile (Expedite)	40-4632-52E	200mL	250.00

GLEN RESEARCH

22825 DAVIS DRIVE
STERLING, VIRGINIA
20164

NEW PRODUCTS - BULK POLYSTYRENE SUPPORTS

For some time, Glen Research has supplied our most popular modifier supports on rigid polystyrene in columns suitable for use on the AB 3900. Several of our customers have requested these supports in bulk so that they could be used in their own column or plate systems. Until now, the supplies of polystyrene have been limited but we are now delighted to offer the following polystyrene supports in bulk:

- Universal Support III PS
- Glen UnySupport PS
- 3'-Phosphate PS
- 3'-PT-Amino-Modifier C6 PS
- 3'-BiotinTEG PS
- 3'-(6-FAM) PS
- 3'-Tamra PS
- 3'-Dabcyl PS

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Universal Support III PS	26-5010-01	0.1g	16.00
	26-5010-02	0.25g	35.00
	26-5010-10	1.0g	125.00
Glen UnySupport PS	26-5040-01	0.1g	16.00
	26-5040-02	0.25g	35.00
	26-5040-10	1.0g	125.00
3'-Phosphate PS	26-2900-01	0.1g	75.00
	26-2900-10	1.0g	510.00
3'-PT-Amino-Modifier C6 PS	26-2956-01	0.1g	125.00
	26-2956-10	1.0g	1025.00
3'-BiotinTEG PS	26-2955-01	0.1g	125.00
	26-2955-10	1.0g	1025.00
3'-(6-FAM) PS	26-2961-01	0.1g	130.00
	26-2961-10	1.0g	1045.00
3'-Tamra PS	26-5910-01	0.1g	130.00
	26-5910-10	1.0g	1045.00
3'-Dabcyl PS	26-5912-01	0.1g	125.00
	26-5912-10	1.0g	1025.00