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INSIDE

SPERMINE PHOSPHORAMIDITE: A POTENT MODIFICATION WITH MANY APPLICATIONS

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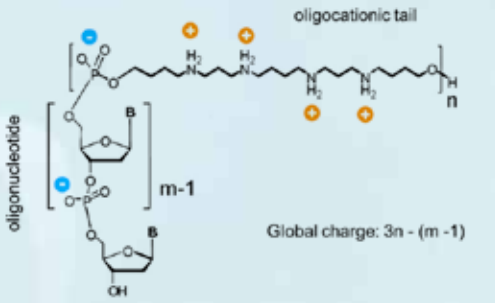
INTRODUCTION TO ZNA® OLIGOS

Synthetically derived oligonucleotides have found many uses in molecular biology, diagnostic and therapeutic applications. At the heart of the majority of these applications is the canonical Watson-Crick base-pairing of nucleobases, whereby complementary strands form duplexes with each other. However, the strength of such interactions is tempered by the mutual electrostatic repulsion of the negatively charged phosphate backbones running through each strand. This polyanionic nature of oligonucleotides also hinders their ability to enter cells and access therapeutically interesting targets that are in the cell cytosol or nucleus.

Chemists have long sought to address the issue of electrostatic repulsion to improve both the binding of an oligonucleotide to a target sequence and its cell delivery. Several approaches aimed at reducing the anionic charge can be found in the literature ranging from the replacement of the phosphate backbone with one that is amide based, such as in Peptide Nucleic Acids (PNA),[1] or the introduction of ammonium or guanidinium residues throughout the strand,[2,3] to the conjugation of polycations that preserve the hybridization specificity and nucleic acid-processing enzyme activity of the parent oligonucleotide.[4] In the case of the latter, a convenient way of introducing a polycation polymer is by the use of the spermine phosphoramidite (Figure 1, Page 2), which can be coupled in an iterative manner similar to standard phosphoramidites.[5,6] The resulting oligonucleotide-oligospermine conjugates (illustrated above and in Figure 2, Page 2) are known as "Zip Nucleic Acids" or "ZNA®",[7] a term that reflects

OLIGONUCLEOTIDE OLIGOSPERMINE CONJUGATE



the presumed mode of action of the conjugates that are believed to use the oligospermine to seek out and move along (scan) oligonucleotide strands until the probe complementary sequence is located. The oligospermine then performs the function of stabilizing the formed duplex by reducing electrostatic repulsion, thereby leading to significantly increased binding affinities. Despite this increase, ZNA® oligonucleotides still retain the ability to discriminate mismatches, doing so to the same extent as unmodified DNA.[8] Moreover, the oligospermine can be located at the 3' or 5' terminus depending on the application, producing the same beneficial effects to the binding affinity.

APPLICATIONS OF ZNA® OLIGOS

Since their inception, an increasing number of applications are being found for ZNA® that take advantage of their unique properties. For example, their use as primers for PCR and reverse transcription has been examined.[9] ZNA® oligos were found to drive PCR reactions very efficiently at higher annealing temperatures, substantially lower primer, substrate and magnesium concentrations, and faster cycling timeframes compared to DNA and LNA primer controls. The latter feature is a result of the "Zip" character of ZNA®, which also results

(Continued on Page 2)

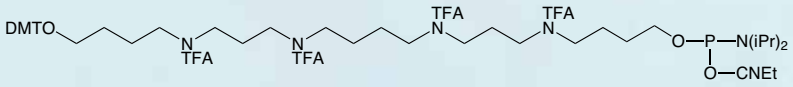
(Continued from the Front Page)

in higher probe sensitivity presumably by increasing the local concentration of the probe in solution. The primer T_m could be easily and predictably tuned simply by the addition of spermine units. Such properties make ZNA® ideal for multiplex PCR and PCR of AT-rich regions that are typically problematic due to the low T_m of the corresponding duplexes. Figure 3 shows the comparative PCR performance of ZNA® and DNA primers on an AT-rich sequence.

ZNA® oligos also have notable advantages when used as primers for reverse transcription (RT).[9] In this respect, it was shown that ZNA® primers improve the yield of RNA to cDNA conversion under standard conditions that led to earlier detection in RT-qPCR quantifications compared to DNA. As a result, this leads to more accurate quantification of low-abundant transcripts. Moreover, the primers in such applications were found to be more robust to variations in buffer environment compared to unmodified DNA primers.

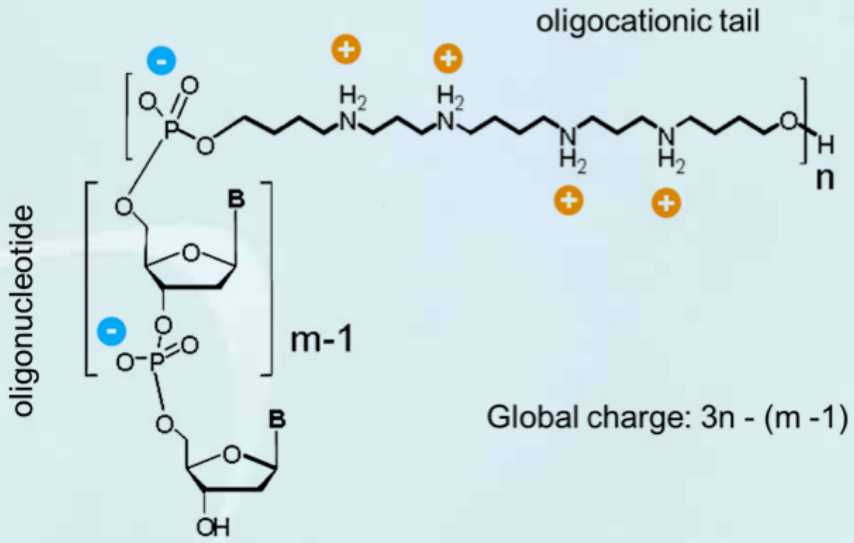
The properties of ZNA® molecules make them ideal for use as dual-labelled hydrolysis probes for qPCR. 3'-Attached oligospermine-oligonucleotide conjugates can be made shorter than their non-modified counterparts leading to improved single-nucleotide polymorphism discrimination (Figure 4, Page 3).[8] In addition, longer ZNA® of a length similar to that used for non-modified probes, exhibited reduced background fluorescence through increased quenching, thus generating improved signal-to-noise ratios. Such a finding opens the way for ZNA® to be used as non-hydrolysis probes. Due to the predictable increases in T_m afforded to the molecules by the attached spermine units, it is easier to design ZNA® probes compared to alternative oligonucleotide modifications such as minor groove binders (MGB) or LNA probes. Moreover, it was found that ZNA® tolerated a wider range of conditions than their DNA and LNA counterparts. In a recent report by Trevisan and coworkers,[10] ZNA® probes were successfully used for the *in-situ* detection of microRNAs in plants. The probes were shown to be highly sensitive and selective when compared to LNA probes and indicate a potential for exploitation of ZNA® for high-throughput miRNA profiling applications.

FIGURE 1: STRUCTURE OF SPERMINE PHOSPHORAMIDITE



(1) Spermine Phosphoramidite

FIGURE 2: OLIGONUCLEOTIDE OLIGOSPERMINE CONJUGATE



Depiction of ZNA® molecule whereby the oligospermine tail is attached to the 5' of a DNA oligomer. The net charge is determined by the formula 3n-(m-1), where m is the oligonucleotide length and n the number of spermine units.

FIGURE 3: COMPARATIVE PCR PERFORMANCE OF ZNA® AND DNA PRIMERS ON AN AT-RICH SEQUENCE

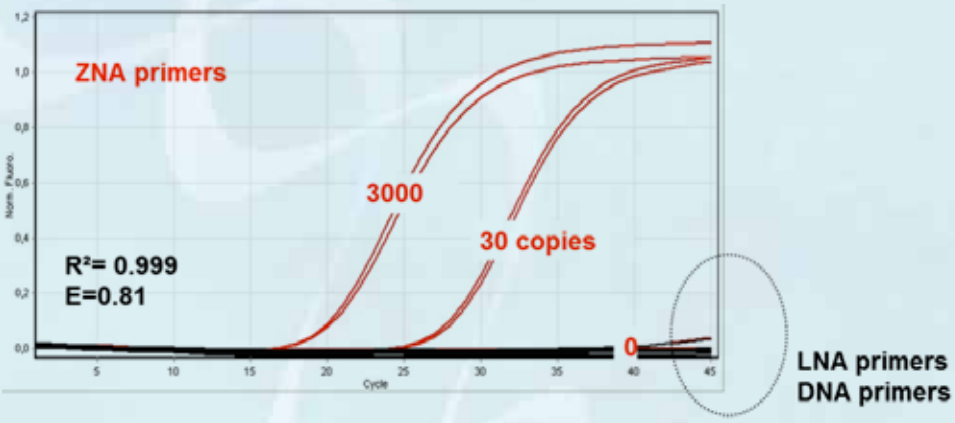


Figure 3: ZNA® primers (Red) used for AT-rich regions in comparison to the corresponding LNA and DNA primers (Black) at 100nM primer concentration and varying copies of initial template following a standard 2 step protocol (95°C, 10s followed by 60°C, 30s).

ZNA® OLIGOS AS ANTISENSE AND ANTIGENE AGENTS

As the efficiency of cell-uptake of oligonucleotides is improved by their conjugation to cationically charged moieties,[11] it follows that polyspermine modification also renders ZNA® more amenable to cell delivery. This point has been exploited, for example, by the group of David Corey who assessed the use of ZNA® as antisense and antigene agents.[12]

In-vitro assays revealed that oligospermine modification of control DNA and LNA/DNA mixmers had a significant beneficial impact on antisense activity when delivered with cationic lipid. For instance, a previously nonfunctional DNA molecule attained antisense activity through the addition of 6 spermine units. More interestingly, free delivery (without cationic lipid mediated transfection) yielded IC_{50} values in the mid-nanomolar range (Table 1).

A similar outcome was seen with ZNA® oligos used for the antigene application. Oligospermine-ribonucleotide conjugates have also been reported as a way to transport siRNA into cells. Nothisen and coworkers[13] synthesized several 5'-oligospermine-sense strand conjugates and determined that an siRNA net cationic charge was necessary to effect efficient cell uptake and gene silencing in the submicromolar range. These encouraging results were also accompanied by an indirect confirmation of the siRNA mechanism, namely that switching the oligospermine to the 5'- of the antisense strand resulted in loss of activity.

ZNA®IN OLIGO SYNTHESIS

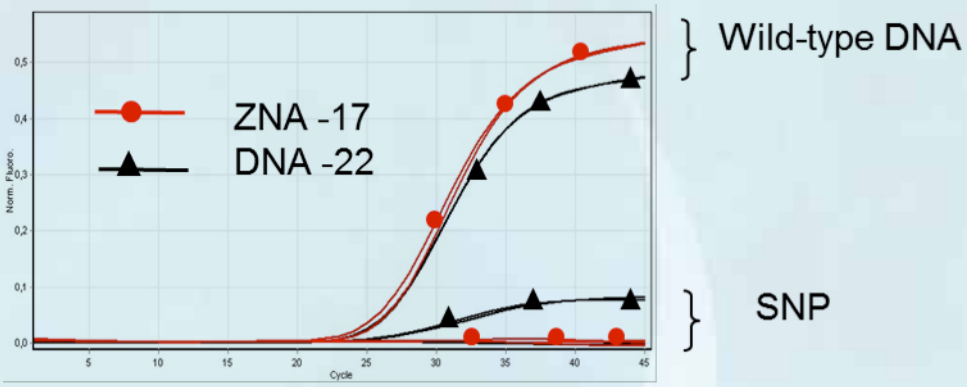
Due to their versatility, compatibility with a variety of other modifications and the ability to fine tune their properties, there is a growing interest in ZNA®. Consequently, Glen Research has now added the spermine phosphoramidite to their catalog.

The spermine amidite can be efficiently incorporated using a 3 minute coupling time, and can be deprotected with standard deprotection conditions in ammonium hydroxide, however some loss of the spermine modification has been observed with extended heating. To avoid acrylonitrile addition to the spermine units, it is necessary to first treat the CPG with 10% DEA in acetonitrile prior to cleavage from the support. Five minutes at room temperature was found to be sufficient time.

Due to the modified properties of the ZNA®molecules, standard reversed-phase purification conditions, i.e., 0.1 M TEAA/ acetonitrile, tend to produce broad, smeared peaks. However, it is possible to purify the crude oligos using Glen-Pak™ cartridges (http://www.glenresearch.com/Technical/GlenPak_UserGuide.pdf) as well as RP-HPLC using a Waters X-Bridge column in combination with a high pH buffer system (5 mM TEA in 25 mM TEAA/acetonitrile). High pH is also necessary to purify ZNA® with anion exchange.[5,6] In this regard, addition of 10% acetonitrile and warming the column to 35°C can both have beneficial effects on resolution.

With an increase in spermine content, the solubility of ZNA® oligonucleotides may be noticeably less than unmodified DNA or RNA counterparts. This is typically observed when re-dissolving dried-down purified ZNA® in water. In this case, dropwise addition of 50 mM ammonium hydroxide brings ZNA® molecules into solution. Alternatively, dissolving ZNA® oligos in concentrated phosphate buffered saline (2.5x PBS, pH 7.4) has also been found to resolve solubility issues.[12]

FIGURE 4: IMPROVED SINGLE-NUCLEOTIDE POLYMORPHISM (SNP) DISCRIMINATION WITH SHORT ZNA® PROBES



A 17mer DNA conjugated with 4 spermines (Red) shows better mismatch discrimination compared to an unmodified DNA 22mer (Black).

TABLE 1: ANTISENSE ACTIVITY OF ZNA®OLIGONUCLEOTIDES

Oligonucleotide	IC50 (nM) with cationic lipid	IC50 (nM) without cationic lipid
DNA	>100	>400
Z3-DNA	>100	>400
Z6-DNA	30.8	194
Z9-DNA	81.9	119
LNA(T)	30.5	>400
Z3-LNA(T)	16.4	>400
Z6-LNA(T)	30.2	>400
Z9-LNA(T)	19.1	160

Antisense Activity of ZNA®oligonucleotides in the presence and absence of cationic lipid.[12] *Spermine (Z) additions to DNA (gctgctgctgctgctgctg) directed towards the CAG repeat of mutant huntingtin (HTT) mRNA triggered antisense activity with and without cationic lipid mediated transfection. The same sequence with LNA substitutions for Thymidine, LNA(T), also showed significant improvement in assisted and free delivery.*

INTELLECTUAL PROPERTY

"Spermine phosphoramidite" synthon is the subject matter of U.S. Patent Application No. 12/086.599, European Patent Application No. EP20060847298 and foreign equivalents for which Polyplus-transfection is the co-owner. Product is sold for research purposes only. Product shall not be used to manufacture oligospermine-oligonucleotide conjugates for use in diagnostics, clinical or commercial applications including use in humans. There is no implied license to manufacture oligospermine-oligonucleotide conjugates for diagnostic, clinical or commercial applications, including but not limited to contract research.

Please contact Polyplus-transfection at licensing@polyplus-transfection.com to obtain a license for such use.

REFERENCES:

1. Nielsen, P.E. et al. *Science* **1991**, *254 (5037)*, p.1497–1500.
2. Michel, T. et al. *Nucleic Acids Res.* **2003**, *31*, p. 5282-5290.
3. Barawkar, D.A.; Bruice, T.C. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, p.11047-11052.
4. Fraley, A.W. et al. *J. Am. Chem. Soc.* **2006**, *128*, p.10763-10771.
5. Pons, B. et al. *ChemBioChem* **2006**, *7*, p1173-1176.
6. Voirin, E. et al. *Nature Protocols* **2007**, *2*, p.1360-1367.
7. Noir, R. et al *J. Am. Chem. Soc.* **2008**, *130*, p.13500-13505.
8. Paris, C. et al. *Nucleic Acids Res.* **2010**, *38*, e95.
9. Moreau, V. et al. *Nucleic Acids Res.* **2009**, *37*, e130.
10. Trevisan, S. et al. *Plant, Cell & Environment* **2012**, *35*, p. 1137-1155.
11. Juliano, R. et al *Nucleic Acids Res.* **2008**, *36*, p.4158–4171.
12. Gagnon, K.T. et al. *J. Am. Chem. Soc.* **2011**, *133*, p.8404-8407.
13. Nothisen, M. et al. *J. Am. Chem. Soc.* **2009**, *131*, p.17730-17731.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Spermine Phosphoramidite	10-1939-95	50 µmole	145.00
	10-1939-90	100 µmole	270.00
	10-1939-02	0.25g	525.00

ZNA® is a registered trademark of Polyplus-transfection SA.

NEW PRODUCT - S-Bz-THIOL-MODIFIER C6-DT

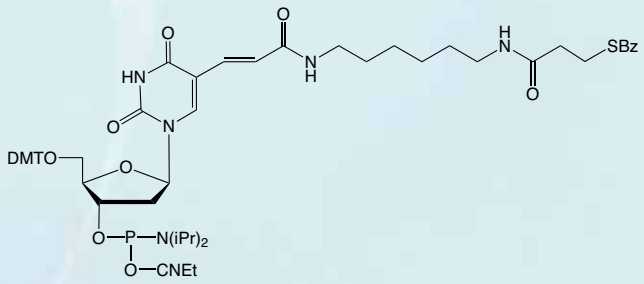
Glen Research has a variety of options available for thiol-modification of the 3' or 5' terminus of oligonucleotides. However, the options available for thiol-modification within the sequence are much more limited. We do offer a complete range of amino-modified nucleoside phosphoramidites and oligonucleotides containing these can be modified using a hetero bifunctional crosslinker to convert the amino groups to thiols. The original crosslinker used for this purpose was Traut's Reagent which simply converts an amino linkage to a thiol linkage. However, post-synthesis conjugation reactions are tricky to carry out and are sometimes not very efficient.

A better solution to this problem would be to modify an amino nucleoside, such as Amino-Modifier C6-dT, to a protected thiol prior to phosphoramidite production. Hence, we are pleased to introduce S-Bz-Thiol-Modifier C6-dT (1) to join the ranks of thiol-modifiers for oligonucleotide synthesis.

Thiol-Modifier C6-dT can be added as usual at the desired locations within a sequence. A coupling time of 3 minutes is recommended. No other changes from normal synthesis protocols are needed.

Prior to cleavage from the synthesis column, the support must be treated with 10% diethylamine (DEA) in acetonitrile to eliminate the cyanoethyl protecting groups from the phosphate backbone and to remove the acrylonitrile formed. This has to be done before cleavage and deprotection since the deprotected thiol would be very susceptible to alkylation by the acrylonitrile.

FIGURE 1: **STRUCTURE OF S-Bz-THIOL-MODIFIER-DT**



(1) S-Bz-Thiol-Modifier C6-dT

Cleavage and deprotection are then carried out using AMA for 2 hours at room temperature. Deprotection with ammonium hydroxide is not recommended for oligonucleotides containing Thiol-Modifier C6-dT since significant degradation is observed.

If oligonucleotides containing Thiol-Modifier C6-dT are to be used immediately for conjugation, 100mM tris(2-carboxyethyl) phosphine (TCEP) should be added to the AMA solution. Dithiothreitol (DTT) can also be used to keep the thiol in its reduced form but conjugation efficiencies are slightly higher (2-3%) if TCEP is used.

Oligonucleotides containing Thiol-Modifier C6-dT can be purified using RP cartridges, such as Glen-Pak™ cartridges, but the DMT group should not be removed on the cartridge with 2% TFA. Rather, the oligo should be eluted DMT-ON and the DMT group removed with aqueous acetic acid.

If oligonucleotides containing Thiol-Modifier C6-dT need to be stored prior to use, they should be reduced with 100mM TCEP in water to reverse any disulfide formation and then desalted into the conjugation buffer. The conjugation with an appropriate maleimide or iodoacetamide can then be carried out as normal.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
S-Bz-Thiol-Modifier C6-dT	10-1538-95	50 µmole	195.00
	10-1538-90	100 µmole	375.00
	10-1538-02	0.25g	845.00

Glen-Pak™ is a trademark of Glen Research Corporation.

NEW PRODUCTS: AB 3900 COLUMNS CONTAINING 1000Å CPG

The AB 3900 synthesizer platform is designed to run using columns containing polystyrene (PS) supports. At the time the synthesizer was launched, only the four standard bases were available as PS filled columns, thus creating an immediate demand for PS modifiers. We have supplied a number of modified supports on PS for this very reason. While these are popular with many of our customers, there has been ongoing demand for high quality, CPG filled columns for use on this instrument platform.

Until now, using CPG supports on the AB 3900 required a number of changes to both the instrument settings and synthesis software. In order to achieve proper reagent flow and contact times, the purge and chamber pressure settings needed to be altered. Software settings also had to be changed to give shorter push and wait times, purge increment/vial parameters, and REACT modes. All of this, as well as a significant amount of development work, was required when using CPG to obtain synthesis quality similar to that of PS.

Glen Research's AB 3900 CPG columns are fully compatible with the AB 3900 family of synthesizers and require **no** alterations to existing preset programs or instrument settings. The column assemblies have been adjusted structurally to allow for proper reagent hold up and contact times. They also contain high quality 1000Å CPG supports for optimal synthesis results.

Glen Research's AB 3900 columns bring the lower cost of CPG to this platform while maintaining the high synthesis efficiency of 1000Å CPG. Our columns offer the following key attributes:

- No need to change instrument settings
- No need to change software parameters
- Easier handling post -synthesis compared to PS
- High quality 1000Å CPG for optimal synthesis results

Ordering information for our CPG columns for use on the AB 3900 synthesizer is shown.

AB 3900 1000Å CPG COLUMNS

MODIFIED CPG SUPPORTS

In addition, our entire catalog of modified supports will now be available in this column format. However, only 200 nmole columns will be available initially in this format since the typical loading of our 1000Å supports is too low to allow 1 µmole columns to be prepared in this column. Simply find the modified CPG needed from our catalog and add an "A" to the standard catalog number to order for the AB 3900 instrument.

KEY POINTS

- *Modified CPG columns only available in 200 nmole size.*
- *Add an "A" to the standard catalog number to order.*

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
dA-CPG 1000	20-2101-65	200X40nmole	600.00
AB 3900 columns	20-2101-62	200X200nmole	650.00
	20-2101-61	200X1µmole	875.00
Ac-dC-CPG 1000	20-2115-65	200X40nmole	600.00
AB 3900 columns	20-2115-62	200X200nmole	650.00
	20-2115-61	200X1µmole	875.00
dmf-dG-CPG 1000	20-2129-65	200X40nmole	600.00
AB 3900 columns	20-2129-62	200X200nmole	650.00
	20-2129-61	200X1µmole	875.00
dT-CPG 1000	20-2131-65	200X40nmole	600.00
AB 3900 columns	20-2131-62	200X200nmole	650.00
	20-2131-61	200X1µmole	875.00

NEW PRODUCT - DIBENZOCYCLOOCTYL (DBCO) COPPER-FREE CLICK CHEMISTRY

INTRODUCTION

The copper(I) catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) is the most prominent example of a group of reactions known as click reactions, as shown in Figure 1. This cycloaddition reaction is characterized by its high efficency, mild reaction conditions, and by its tolerance of the broad range of functional groups found in DNA.[1] The most important characteristic of the CuAAC reaction is its unique bioorthogonality, as neither azide nor terminal alkyne functional groups are generally present in natural systems. Despite the attractive features of CuAAC-based click chemistry, the presence of copper may be a hindrance to bioorthogonal conjugation in living cells or whole organisms.

Recently, developers of bioorthogonal techniques have begun to consider the use of highly strained alkynes to obviate the need for copper catalysis in click conjugation reactions. Cyclooctyne is the smallest cyclic octyne that can be isolated. Because of the severe deformation of the alkyne from its desired linear geometry, cyclooctynes are highly reactive towards azides without the need for copper catalysis.

FIGURE 1: CuAAC REACTION

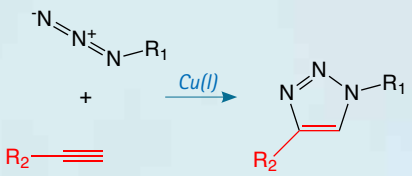
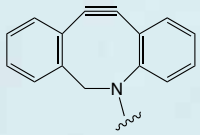
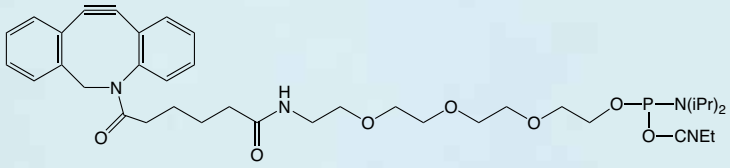


FIGURE 2: DIBENZOCYCLOOCTYL (DBCO)

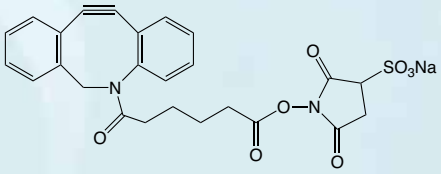


DBCO

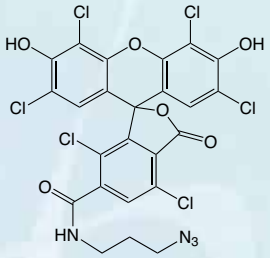
FIGURE 3: PRODUCT STRUCTURES



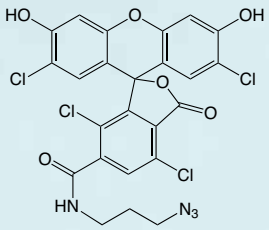
(1) 5'-DBCO-TEG Phosphoramidite



(2) DBCO-sulfo-NHS Ester



(3) 6-HEX Azide



(4) 6-TET Azide

COPPER-FREE CLICK

At Glen Research, our goal was to offer a copper-free click phosphoramidite reagent with the following properties:

- Simple to use
- Stable in solution on the synthesizer
- Stable to ammonium hydroxide and AMA
- Excellent click performance in 17 hours or less at room temperature

From the variety of cyclooctyne-based copper-free click reagents so far described, we have chosen to offer compounds based on a dibenzo-cyclooctyne (DBCO) structure, shown in Figure 2.[2-4] Further, we chose to separate this very hydrophobic moiety from the phosphoramidite and subsequent oligo with a triethyleneglycol (TEG) spacer. The structure of the 5'-DBCO-TEG Phosphoramidite (1) is shown in Figure 3. In addition, we have chosen to offer a soluble DBCO-sulfo-NHS ester sodium salt (2) for post-synthesis conjugation reactions with amino-modified oligonucleotides and proteins.

SYNTHESIS AND DEPROTECTION

A coupling time of 10 minutes was found to be optimal for the phosphoramidite (1). It was found that DBCO-modified oligos were stable to deprotection with ammonium hydroxide for 2 hours at 65°C or overnight at room temperature, which would allow the use of regular phosphoramidites, including dmf-dG but not ibu-dG. Deprotection with AMA for 2 hours at room temperature showed only slight degradation of the cyclooctyne, making the modification compatible with ibu-dG if Ac-dC is used. DBCO-modified oligos are also compatible with UltraMild deprotection conditions.

PURIFICATION

5'-DBCO-modified oligos are sufficiently hydrophobic that they may be purified using Glen-Pak™ cartridges and omitting the TFA step normally used to remove the DMT group.

CLICK REACTION

5'-DBCO-modified oligos may be conjugated with azides in organic solvents, such as DMSO, or aqeous buffers. Depending on the azide used, the reaction will go to completion in 4-17 hours at room temperature. Simple desalting on a Glen Gel-Pak™ leads to a product with virtually quantitative conjugation efficiency.

Procedure:

1. Dissolve 1mg Azide per 150 µL of DMSO.
2. Add azide solution to 10 OD of 5'-DBCO oligo in 100 µL of Water.
3. Incubate reaction at room temperature overnight.
4. Desalt conjugated oligo on a Glen Gel-Pak™ to remove organics.

NEW PRODUCTS - 6-HEX AND 6-TET AZIDES

We illustrate the efficiency of the copper-free click reaction using 6-HEX Azide, one of two additions to our selection of azides for click chemistry, in Figure 5.

USE OF DBCO-SULFO-NHS ESTER

Dissolve DBCO-sulfo-NHS Ester at a concentration of 5.2mg per 60 µL (~0.17M solution) in water or anhydrous DMSO. Use this stock solution to conjugate with amino-modified oligos in sodium carbonate/ bicarbonate conjugation buffer (pH=9).

Labelling Procedure

For a 0.2 µmole synthesis of an amino-modified oligo:

1. Dissolve oligo in 500 µL of conjugation buffer.
2. Add 6 µL of DBCO-sulfo-NHS Ester solution (~ 6 fold excess).
3. Vortex mixture and incubate at room temperature for 2-4 hours or overnight.
4. Desalt conjugated oligo on a Glen Gel-Pak™ to remove salts and organics.

References:

1. V.V. Rostovtsev, Green, L.G., Fokin, V.V. and Sharpless, K.B., *Angew Chem Int Ed,* 2002, **41**, 2596-2599.
2. P. van Delft, *et al., Org Lett,* 2010, **12**, 5486-9.
3. P. van Delft, *et al., Synthesis-Stuttgart,* 2011, 2724-2732.
4. I.S. Marks, *et al., Bioconjugate Chemistry,* 2011, **22**, 1259-1263.

6-HEX AND 6-TET AZIDES

Glen Research has committed to adding to our product line azide analogues of our most popular tags, in addition to interesting reagents which are not compatible with routine oligonucleotide synthesis. HEX and TET are two of our most popular fluorescein-based dyes for labelling oligonucleotides. We are happy to offer 6-HEX (3) and 6-TET (4) Azides for use in click conjugations.

In Figure 5, the high conjugation efficiency of 6-HEX Azide is illustrated. Virtually 100% efficiency was achieved in a copper-free click with an oligo modified at the 5' terminus using 5'-DBCO-TEG Phosphoramidite.

Glen-Pak™ and Glen Gel-Pak™ are trademarks of Glen Research Corporation.

***FIGURE 4:* COPPER-FREE CONJUGATION OF DBCO-OLIGO WITH HEX AZIDE**

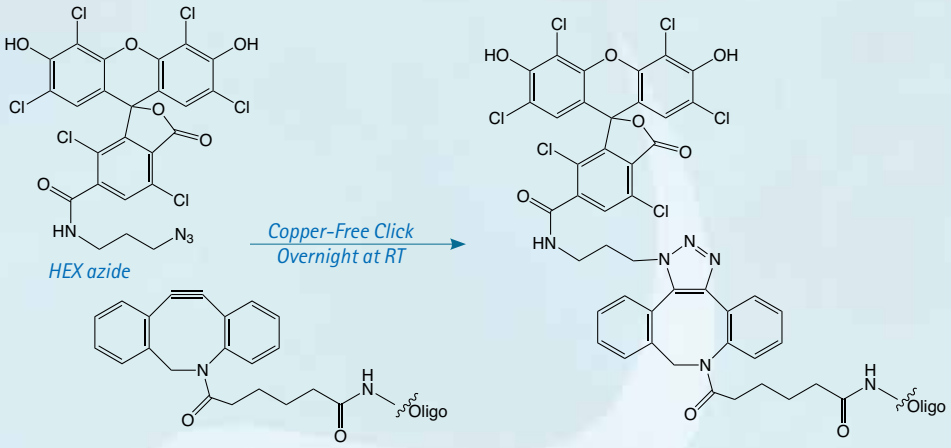
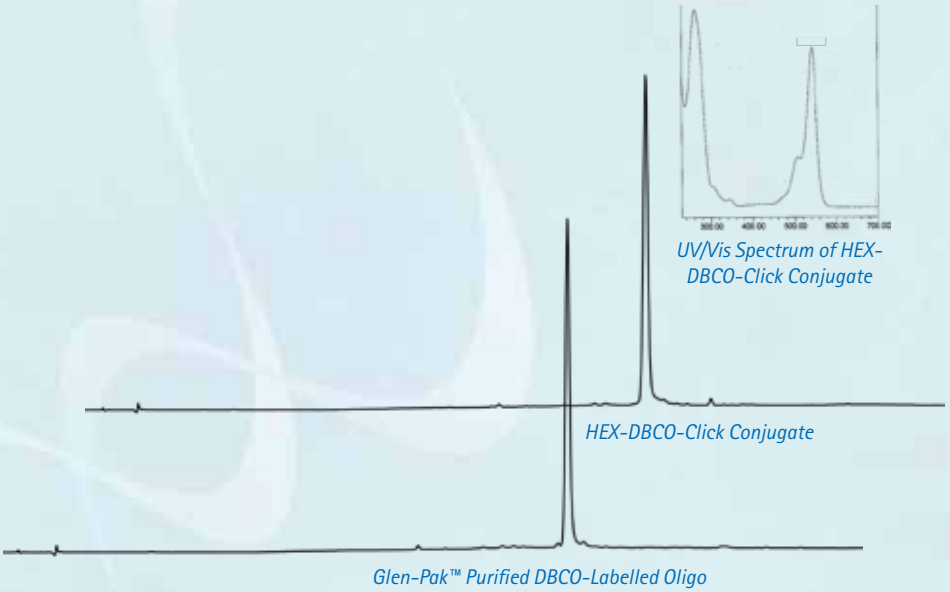


***FIGURE 5: RP HPLC OF COPPER-FREE CONJUGATION OF* DBCO-OLIGO WITH HEX AZIDE**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-DBCO-TEG Phosphoramidite	10-1941-95	50 µmole	125.00
	10-1941-90	100 µmole	230.00
	10-1941-02	0.25g	775.00
DBCO-sulfo-NHS Ester	50-1941-23	5.2mg	60.00
(Dissolve 5.2mg in 60µL water or DMSO)	50-1941-24	52mg	300.00
6-HEX Azide	50-2005-92	25 µmole	150.00
	50-2005-90	100 µmole	450.00
6-TET Azide	50-2006-92	25 µmole	150.00
	50-2006-90	100 µmole	450.00

Labelling or immobilization of oligonucleotides by conjugation reactions requires the presence of functional groups with unique reactivity towards suitably activated dyes, reporter groups or surfaces. Most commonly, amino-modified oligonucleotides are reacted with active esters of various compounds, thereby forming stable amide conjugates. For the introduction of such amino groups at the 5' terminus of oligonucleotides, 5' amino-modifiers are employed. They consist of a phosphoramidite moiety and a suitably protected amino group connected by linkers of various length and structure. Depending on the planned purification, amino linkers with acid or base labile amine protecting groups (for DMT-ON or DMT-Off applications, respectively) can be used. The most popular among the base labile protecting groups is the trifluoroacetyl (TFA) group, which is cleaved by ammonia or methylamine.

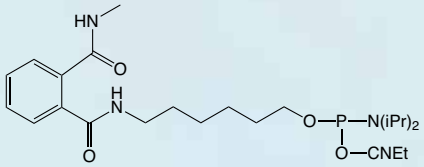
We are now introducing a new set of amino modifiers (Figure 1)[1], which are protected by a novel phthalic acid diamide (PDA) protecting group. In contrast to the TFA protected amino modifiers, which are viscous oils, the analogous PDA protected compounds are granular powders. This important property of these compounds allows straightforward handling, storage and aliquoting and leads to a significant increase in stability. For example, at room temperature and under a normal atmosphere, the solid PDA protected amino modifiers are completely stable for at least 7 days. In sharp contrast, the corresponding TFA protected compounds are almost completely degraded under these conditions.

Deprotection with methylamine in gas phase or aqueous solution or AMA leads to fast and complete removal of the PDA protecting group, as shown in Figure 2. However, ammonium hydroxide will not drive the reaction to completion and only partial deprotection occurs.

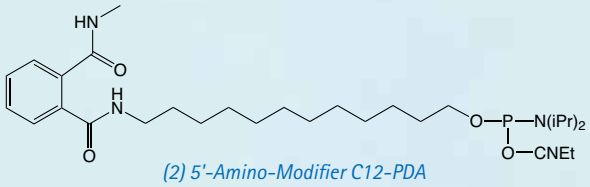
Glen Research is pleased to offer initially three PDA Amino-Modifiers. Our 5'-Amino-Modifier C6 is our most popular modifier so we offer 5'-Amino-Modifier C6-PDA (1). For longer linkers, we offer the hydrophobic 5'-Amino-Modifier C12-PDA (2) and the hydrophilic 5'-Amino-Modifier-TEG-PDA (3).

The high conjugation efficiency of amino-modified oligonucleotides prepared using PDA 5'-Amino-Modifiers is demonstrated in Figure 3. A T12 oligonucleotide was modified using 5'-Amino-Modifier C12-PDA. Following deprotection using AMA, very high coupling efficiency was observed. Further, the 5'-amino group was shown to have excellent conjugation potential by reaction with biotin NHS Ester in the normal fashion. Essentially quantitative conjugation efficiency was observed (Figure 3).

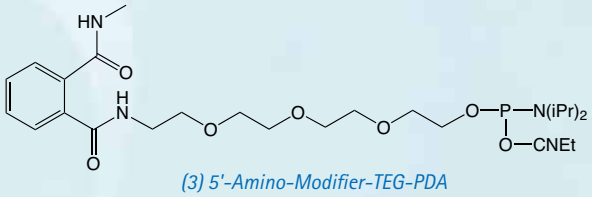
FIGURE 1: **STRUCTURES OF PDA AMINO-MODIFIER PHOSPHORAMIDITES**



(1) 5'-Amino-Modifier C6-PDA

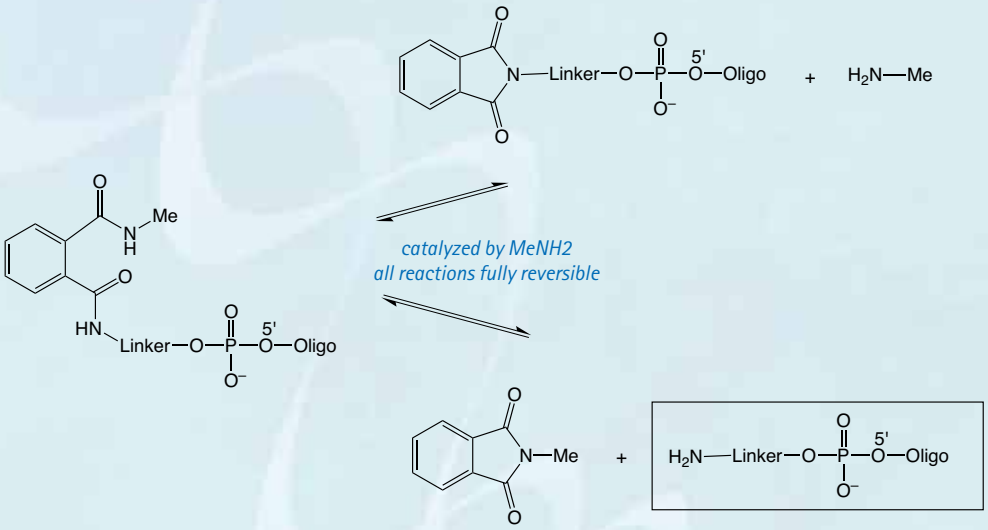


(2) 5'-Amino-Modifier C12-PDA



(3) 5'-Amino-Modifier-TEG-PDA

FIGURE 2: **DEPROTECTION OF PDA AMINO-MODIFIED OLIGONUCLEOTIDES**



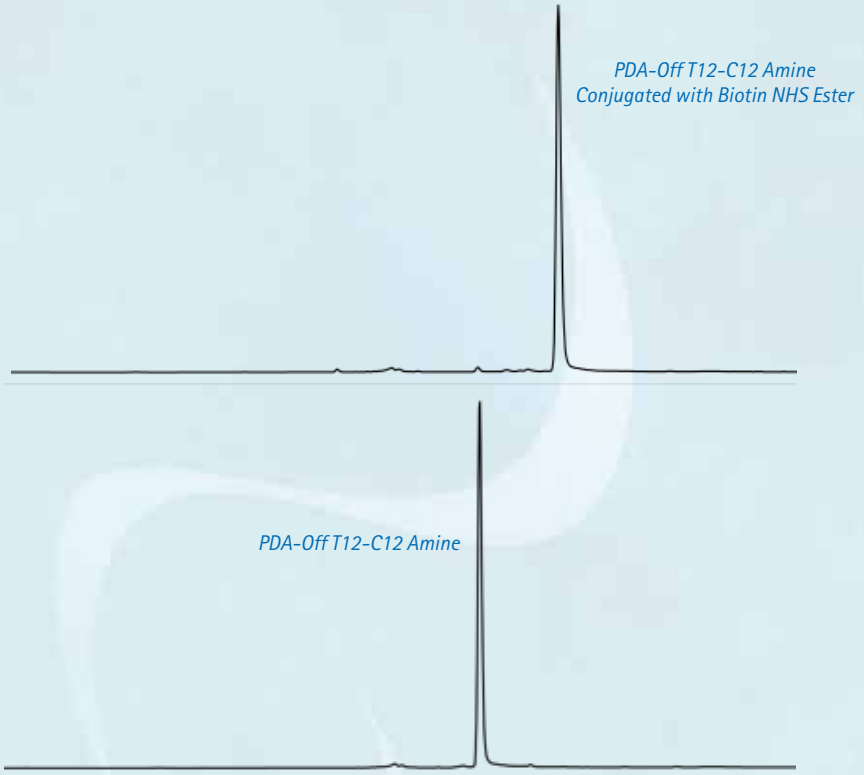
We thank Stefan Pitsch for his help in preparing this article. We also thank him for developing a product to address the problems of existing amino-modifiers by providing a new range of products with better stability and handling properties since they are granular powders rather than viscous oils.

1. Developed by Stefan Pitsch and ReseaChem GmbH (S. Berger), Patent pending.

KEY POINTS

- *Oligonucleotides containing PDA-amino-modified oligonucleotides must be treated with aqueous methylamine or AMA for complete deprotection.*
- *If ammonium hydroxide must be used, reaction at 55°C will yield around 80% of the deprotected amino group, even with extended deprotection times.*

FIGURE 3: PDA-OFF AMINO-MODIFIED OLIGONUCLEOTIDE AND ITS BIOTIN CONJUGATE



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-Amino-Modifier C6-PDA	10-1947-90	100 µmole	30.00
	10-1947-02	0.25g	100.00
5'-Amino-Modifier C12-PDA	10-1948-90	100 µmole	65.00
	10-1948-02	0.25g	240.00
5'-Amino-Modifier-TEG-PDA	10-1949-90	100 µmole	105.00
	10-1949-02	0.25g	420.00

NEW PRODUCT - 5'-STEARYL PHOSPHORAMIDITE - AN ALTERNATIVE LIPOPHILIC CARRIER

Synthetic oligonucleotides that have been modified chemically to achieve certain biological characteristics have found uses in therapeutic development. For example, antisense oligonucleotides, siRNA, aptamers and miRNA have all been found to have qualities that justify pharmaceutical development. As always, the challenge of such development has been to deliver the oligos effectively to the desired cellular targets. A variety of delivery strategies is currently in use but a favorite has been simple modification of the oligonucleotide at the 5' terminus with a lipophilic and non-toxic carrier.

For example, cholesterol-conjugated siRNAs have been shown to silence gene expression *in vivo*. In a review article,[1] the authors described the preparation and use of siRNA modified with a variety of lipophilic carriers. They demonstrated that bile acids and long-chain fatty acid conjugates, in addition to cholesterol, help with siRNA delivery into cells with resulting silencing of gene expression. The authors note that efficient and selective uptake of these siRNA conjugates depends on interactions with lipoprotein particles, lipoprotein receptors and transmembrane proteins.[1]

Glen Research has supported these activities for several years with cholesterol and α-tocopherol carriers (1-3). We now offer a simple C18 lipid as an economical and effective carrier molecule.[2] We envisage that 5'-Stearyl Phosphoramidite (4) will become a favored lipophilic carrier for experimentation with synthetic oligonucleotides.

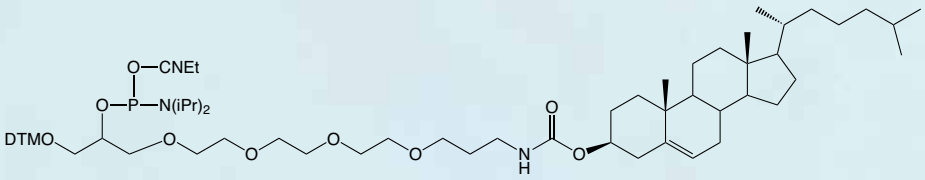
5'-Stearyl Phosphoramidite is not fully soluble in acetonitrile and must be dissolved in a mixture of acetonitrile/dichloromethane (1:3). Otherwise the coupling reaction at the 5' terminus is standard with a 3 minute coupling time recommended.

5'-Stearyl oligonucleotides can be purified using standard procedures on RP cartridges, such as Glen-Pak™ cartridges, as well as standard chromatographic techniques.

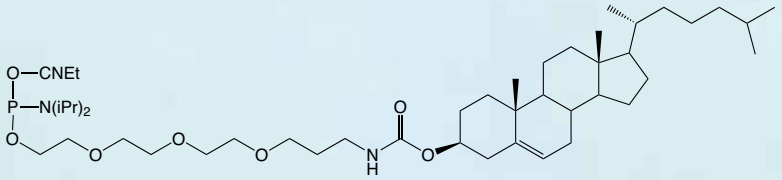
References:

1. C. Wolfrum, et al., *Nat Biotechnol*, 2007, **25**, 1149-57.
2. H. Liu, B. Kwong, and D.J. Irvine, *Angew Chem Int Ed Engl*, 2011, **50**, 7052-5.

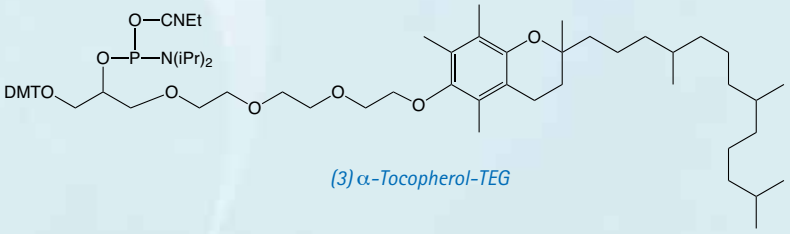
FIGURE 1: **STRUCTURES OF LIPID PHOSPHORAMIDITES**



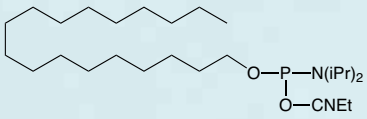
(1) Cholesteryl-TEG



(2) 5'-Cholesteryl-TEG



(3) α-Tocopherol-TEG



(4) 5'- Stearyl

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Cholesteryl-TEG Phosphoramidite	10-1975-95	50 µmole	165.00
	10-1975-90	100 µmole	295.00
	10-1975-02	0.25g	675.00
5'-Cholesteryl-TEG Phosphoramidite	10-1976-95	50 µmole	150.00
	10-1976-90	100 µmole	260.00
	10-1976-02	0.25g	675.00
α-Tocopherol-TEG Phosphoramidite	10-1977-95	50 µmole	160.00
	10-1977-90	100 µmole	300.00
	10-1977-02	0.25g	575.00
5'- Stearyl Phosphoramidite	10-1979-90	100 µmole	45.00
	10-1979-02	0.25g	180.00

UltraMild Phosphoramidites (Pac-dA, iPr-Pac-dG, Ac-dC) are versatile phosphoramidites commonly used for the incorporation of modifications that are sensitive to standard deprotection conditions. These phosphoramidites are compatible with a range of deprotection conditions. For UltraMild deprotection we recommend room temperature deprotection with ammonium hydroxide or potassium carbonate in methanol.

Acetyl-dC is also a popular monomer in its own right since it is compatible with UltraMild deprotection, as well as regular deprotection using ammonium hydroxide, and is necessary for AMA deprotection.

See our overview of deprotection schemes for further information: http://www.glenresearch.com/Technical/Deprotection.pdf

Glen Research offers one of the largest selections of phosphoramidites for DNA and RNA synthesis. A popular product is our 5-Me-dC Brancher Phosphoramidite (10-1018; (1)), which is used for synthesizing branched DNA or comb-like DNA structures.[1,2] This sequence modifier incorporates a levulinyl-protected alcohol that can serve as a branch point for a second sequence. The process for generating these structures starts by synthesizing the first sequence that incorporates the dC-brancher. After the full-length oligo is synthesized, the DMT is removed and the 5'-hydroxyl is capped with standard Cap A/B reagents. The levulinyl protecting group is then selectively removed with hydrazine hydrate in pyridine/acetic acid solution. The second sequence is then synthesized from the dC brancher.

Recently, we received feedback from our customers that seemed to indicate an incompatibility between the UltraMild phosphoramidites or Ac-dC alone and the protocol for removing the levulinyl protecting group. We suspected the protecting groups of these monomers were prematurely removed during the hydrazine treatment of the dC Brancher, resulting in unwanted branching. The branched sequences were observed as smears on the subsequent PAGE analysis of the final products.

To investigate if the problems were caused by deprotection of the base protecting groups by the hydrazine reagent, we analyzed the stability of Ac-dC to the recommended procedure for removing the levulinyl protecting group. We duly found that the hydrazine reagent can partially deprotect the Ac-dC, leading to chain branching in subsequent coupling cycles.

We conclude that the standard phosphoramidites (Bz-dA, ibu-dG, Bz-dC, and T) should be used when making a branched oligo with our 5-Me-dC Brancher.

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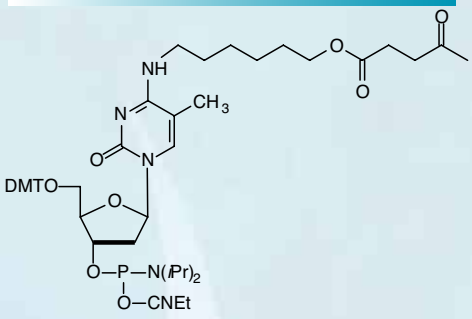
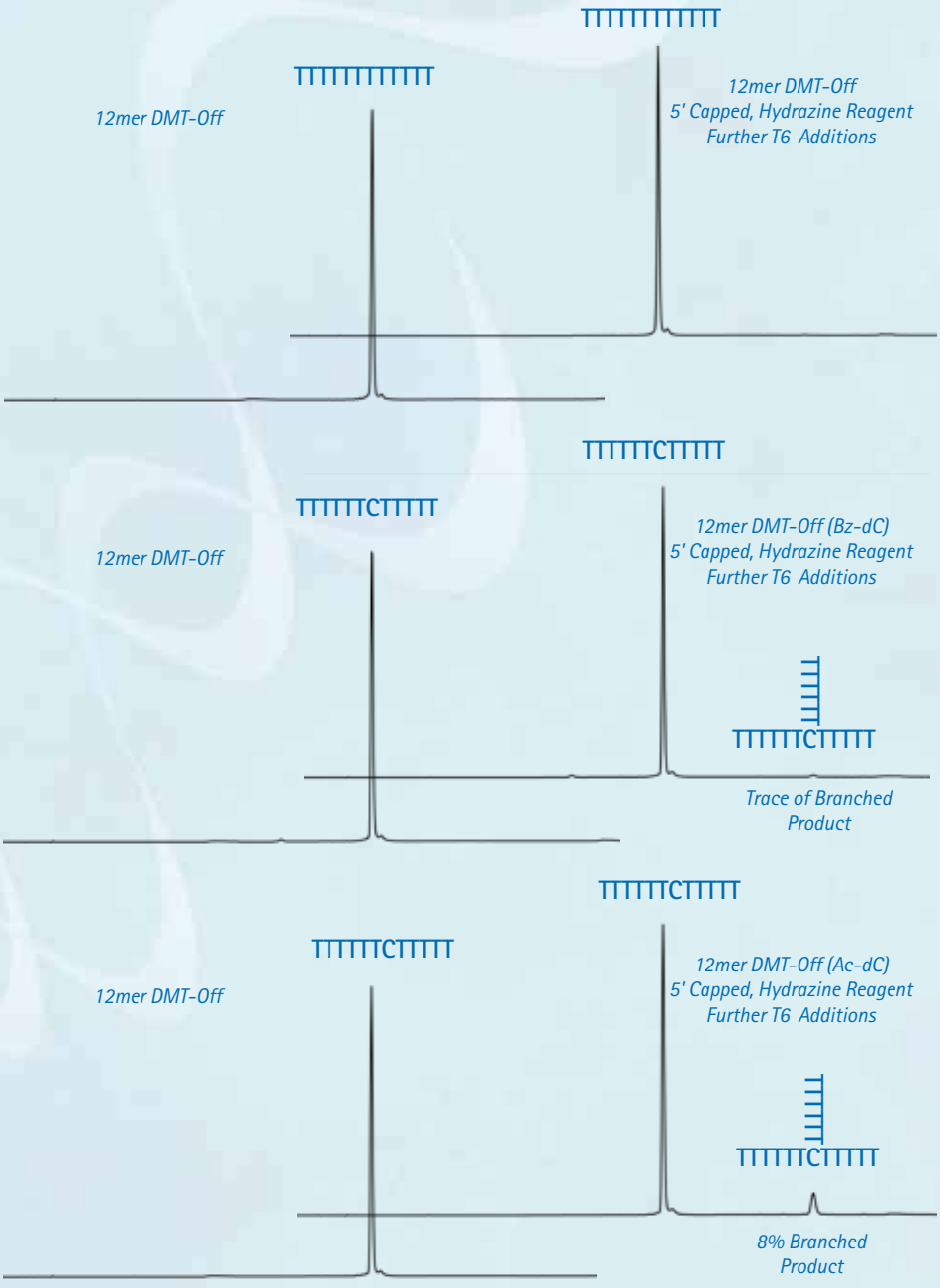
FIGURE 1: 5-Me-dC BRANCHER



(1) 5-Me-dC Brancher

FIGURE 2: RP HPLC OF SIMPLE OLIGOS TREATED WITH HYDRAZINE REAGENT



GLEN RESEARCH

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20164

(Continued from Page 11)

EXPERIMENT

1. Synthesize a control T12 oligo DMT-Off (with no dC incorporations).
2. Synthesize two 12-mer T oligos DMT-Off, one with a single internal incorporation of Ac-dC and the other containing Bz-dC.
3. Cap the 5'-OH with standard Cap A/B mix.
4. Treat oligos with 0.5M hydrazine hydrate in pyridine/acetic acid (1:1) for 15 minutes at room temperature.
5. Rinse with pyridine/ acetic acid followed by acetonitrile.
6. Continue syntheses, adding T6, DMT-ON.
7. Deprotect oligos with ammonium hydroxide.
8. Analyze on RP-HPLC.

RESULTS

Branched oligos would contain a 5'-DMT group and have a significantly longer retention time on RP-HPLC. The control T12 did not show any signs of branching as seen by the absence of any DMT containing oligo. This also indicates that the 5'-acetyl capping group was retained during the hydrazine hydrate deprotection. The Ac-dC containing oligo did have a late eluting peak on RP-HPLC that would be consistent with branching. We calculated about 8% branching after a 15 minute treatment with hydrazine hydrate. The Bz-dC containing oligo showed only a trace amount of branched oligo. The chromatograms are shown in Figure 2, Page 11.

References:

1. T. Horn, C.A. Chang, and M.S. Urdea, *Nucleic Acids Res,* 1997, **25**, 4842-4849.
2. T. Horn, C.A. Chang, and M.S. Urdea, *Nucleic Acids Res,* 1997, **25**, 4835-4841.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5-Me-dC Brancher Phosphoramidite	10-1018-90	100 µmole	205.00
	10-1018-02	0.25g	505.00