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INSIDE

ZIP NUCLEIC ACIDS (ZNA®) ARE POWERFUL CATIONIC OLIGONUCLEOTIDES FOR MOLECULAR BIOLOGY, DIAGNOSTIC AND THERAPEUTIC APPLICATIONS

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INTRODUCTION TO ZIP NUCLEIC ACIDS (ZNA®)

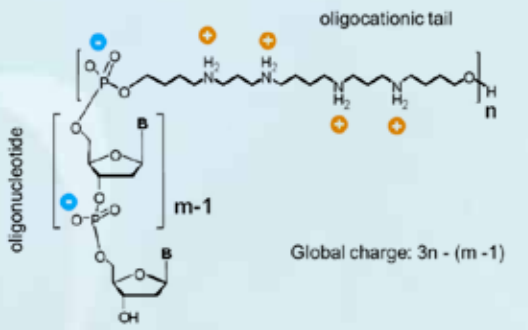
ZNA® are a novel class of modified oligonucleotides enhancing hybridization properties of nucleic acids. ZNA are oligonucleotides conjugated to cationic units. Their global charge can be modulated by the number of cationic spermine moieties grafted on each oligonucleotide. ZNA are produced using standard automated nucleic acid synthesis based on phosphoramidite chemistry. A spermine derivative (*i.e.*, spermine phosphoramidite synthon) was specifically developed as an N-protected trityl (DMT) spermine phosphoramidite that can be introduced during the oligonucleotide synthesis.[1] This spermine addition chemistry is robust and versatile.[2] In addition, coupling yields for such addition are high and comparable to the coupling yields obtained with the standard protected nucleobases, as described in a previous Glen Report.[3]

With such versatile ZNA chemistry, ZNA technology is applicable to any application involving oligonucleotides. However, the global charge of ZNA defines *per se* the fields of applications.

When negatively charged, ZNA conserve their ability to mediate specific hybridization with complementary strands and they are more suitable for PCR applications, amplification techniques, diagnostic applications or purification technologies.[4,5]

On the contrary, positively charged ZNA are more likely to mediate first a non-specific binding/interaction with polyanions through electrostatic interactions before their possible specific hybridization to a target sequence through base pairing. ZNA molecules bearing excess cationic charges were shown to have the property to stick to anionic cell surfaces and to trigger endocytosis, leading to cell transfection without the need of a transfection reagent (or a cargo).[6] As a proof-of-principle, cationic ZNA-based siRNAs were shown to perform effective and specific gene silencing in animals cells without the need of a transfection reagent to mediate the cell uptake.[7]

OLIGONUCLEOTIDE OLIGOSPERMINE CONJUGATE



INCREASE IN Tm/DUPLEX STABILITY

Hybridization studies demonstrated that spermine-mediated attraction is the main driving force for the initiation of nucleic acid binding. Then, the standard base pairing, according to Watson-Crick rules, between the target strand sequence and the complementary ZNA leads to a duplex formation. As a consequence of the cationic contribution, the thermal stability of the formed duplex is increased. The stepwise spermine conjugation raises the Tm in a smooth and linear way while preserving mismatch discrimination. According to the initial study from

(Continued on Page 2)

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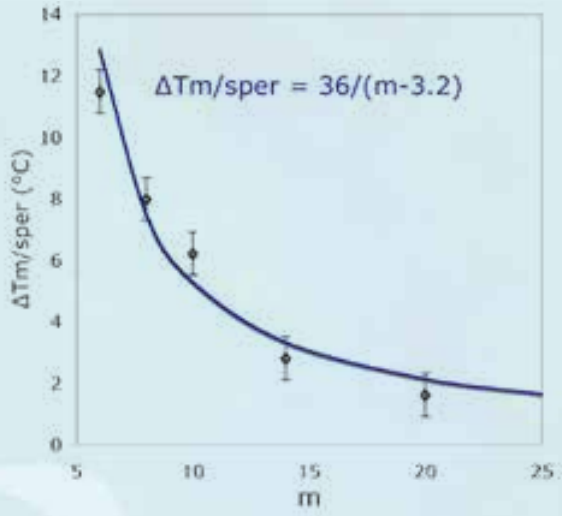
Noir, *et al.*,[8] the melting temperature (Tm) increase of ZNA brought about by spermine conjugation only depends on the length of the oligonucleotide and on the number of spermines grafted on it, providing a way to predict and finely tune the Tm of any given sequence. In this study, the impact of the number of grafted spermines per oligonucleotide on the melting temperature was determined, with 6-, 8-, 10-, 14-, or 20-mer oligonucleotides bound to complementary sequences within 45-59-mer oligonucleotides (see Figure 1).

In this initial study, the melting temperatures were measured in physiological ionic strength (150 mM sodium chloride) and at pH 7.4. Additional measurements were made in order to study the impact of the ΔTm increase per spermine in more standard PCR conditions using a PCR-like buffer (Tris-HCl 10 mM pH 8.5, KCl 50 mM, $MgCl_2$ 3 mM) and using a complementary target containing 55 bases. Three groups of sequences with various GC contents (low, medium, and high) and standard lengths of primers and probes (22 to 16 mers) were synthesized (see Table 1). For each group, the length of sequences was gradually decreased, while maintaining a similar GC content for each group and the equal stability of both 3′ and 5′ sides. Each sequence was modified with an increasing number of spermines. The maximum number of grafted spermines was determined taking into consideration the solubility of the molecule (*i.e.*, with a negative global charge). Finally, the melting temperature

FIGURE 1: **ΔTm INCREASE PER CONJUGATED SPERMINE**

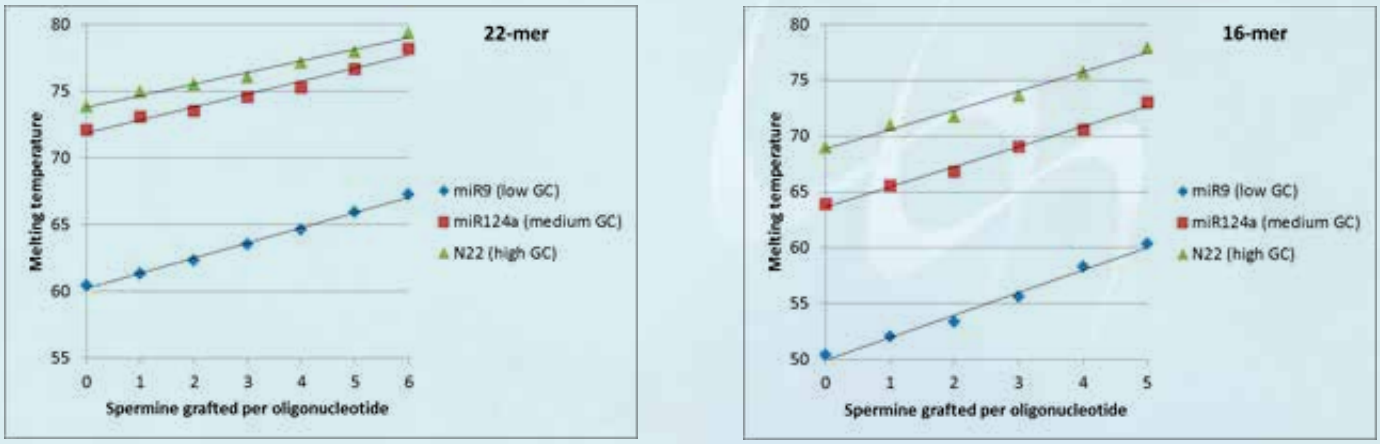


The ΔTm increase per conjugated spermine depends on the length (m) of the oligonucleotide.

TABLE 1: **SEQUENCES USED IN THE Tm STUDY.**

Name	Sequence	GC content	Tm
Anti-miR9-22	TCATACAGCTAGATAACCAAAG	36.4%	59.5°C
Anti-miR9-20	TCATACAGCTAGATAACCAA	35%	57.8°C
Anti-miR9-18	TCATACAGCTAGATAACC	38.9%	54.6°C
Anti-miR9-16	TCATACAGCTAGATAA	31.3%	49.2°C
Anti-mir124a-22	TGGCATTCACCGCGTGCCTTAA	54%	70.7°C
Anti-mir124a-20	GGCATTCACCGCGTGCCTTA	60%	69.2°C
Anti-mir124a-18	CATTCACCGCGTGCCTTA	55.6%	64.5°C
Anti-mir124a-16	ATTCACCGCGTGCCTT	56.3%	64°C
N-22	ACCTGGCTCCCTCGCCTGTCAC	68.2%	73.1°C
N-20	ACCTGGCTCCCTCGCCTGTC	70%	71.7°C
N-18	ACCTGGCTCCCTCGCCTG	72.2%	70.1°C
N-16	ACCTGGCTCCCTCGCC	75%	68.1°C

FIGURE 2: **Tm INCREASE PER CONJUGATED SPERMINE.**



The Tm increase per conjugated spermine measured in PCR buffer conditions depends on the length (m) of the oligonucleotide, 22-mer (Left) and 16-mer (Right), and on the GC content.

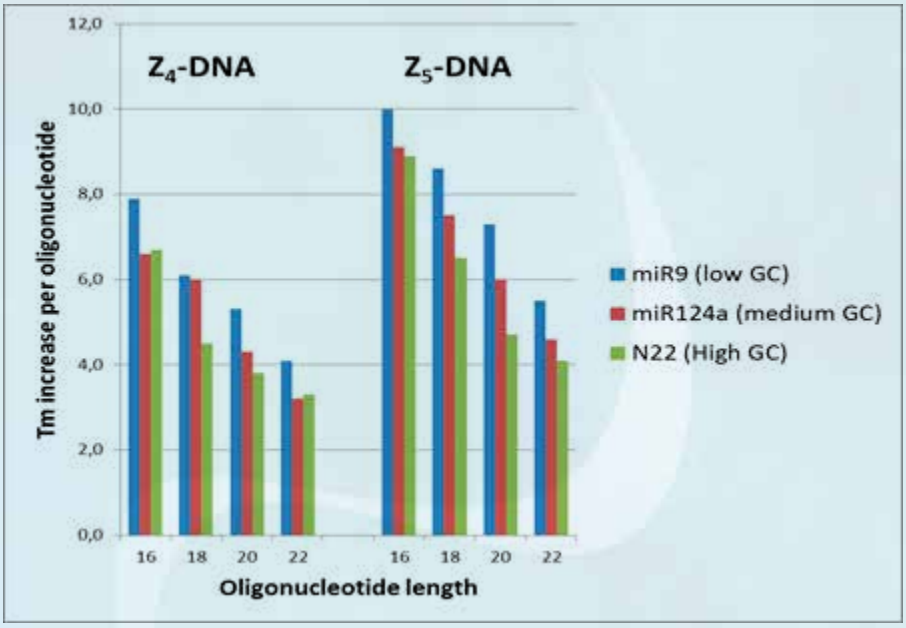
was measured.

Tm measurements in PCR buffer conditions indicate that the Tm increase is linear and is a function of the number of spermines grafted to the oligonucleotide, as shown in Figure 2, which presents the results obtained with 16- and 22-mers. The linear increase per conjugated spermine was also confirmed for the 18- and 20-mers. This study also demonstrates that the Tm increase induced by the spermine grafting is lower in GC-rich oligonucleotides. It also provides additional explanation further to prior studies[4] about why, when ZNA are used as primers, the maximal improvement effect on the amplification is obtained with AT-rich sequences.

Many recent reports have described the use of ZNA as potent primers and probes for PCR applications, which are mostly based on Z4- and Z5-conjugated oligonucleotides with lengths of 16 to 22 nucleotides. Figure 3 is a summary of the ΔTm increase per oligonucleotide in these selected classes of ZNA and shows that the Tm remains predictable, approximately following the previous published rules[8] even in PCR buffer conditions. The Tm increase per oligonucleotide remains a function of the number of spermines grafted onto the oligonucleotide, is dependent on the oligonucleotide length, and is slightly dependent on the GC content. However, the Tm impact per spermine can be affected by the pH which depends on the buffer used and on the salt content having a shielding effect on the cationic charge. Since the composition of PCR Master Mixes is rarely provided, it is therefore recommended to find the optimal conditions and design for the ZNA. The Mg^{2+} concentration is critical for ZNA primers' activity (ideally 1.5 mM). However, the addition of EDTA can improve the ZNA primers' performances by depleting the excess of free Mg^{2+} in some PCR Master Mixes.[4]

Recent publications highlight the high sensitivity of ZNA when used as probes such as dual-labelled hydrolysis probes for microRNAs,[9] polymorphism/mutation[10] or detection of rare disease-associated transcripts[11] by qPCR. A representative probe structure consists of 5'-FAM, 18/24-mer probe length, 3'-Z4/5, and 3'-BHQ-1.

FIGURE 3: **SUMMARY OF THE ΔTm INCREASE EXPECTED.**



Summary of the ΔTm increase expected for Z4- and Z5-DNA related to the oligonucleotide length and GC content.

The versatile chemistry of ZNA offers various possibilities of primer and probe chemical structures, such as dual-labelled probes. The intrinsic properties of ZNA drive their potency in PCR applications. Their properties lead to an increased affinity with nucleic acids due to low electrostatic repulsion, an increased hybridization and stability with the target sequence (Tm increase), a preserved capacity of single base discrimination (target specificity), and a preserved activity as substrates of polymerases.

SELF-DELIVERING CATIONIC OLIGONUCLEOTIDES

Oligonucleotides cannot enter living cells by themselves due to their negative charge. To date, oligonucleotides are formulated into particles using cationic polymers, such as Polyplus-transfection reagent jetPEI® or lipid compounds to improve their delivery, or when conjugated to cationic peptide moieties such as cell penetrating peptides. The ZNA chemistry was used to produce cationic antisense[12] or siRNA[7] conjugates via automated solid phase synthesis. These positively charged oligonucleotides are defined as molecular conjugates whose size is close to that of oligonucleotides - a few nanometers. Such cationic oligonucleotides are able to bind the cell membrane, inducing an endocytosis and transport in the cytoplasm.[6] The study of the influence of the global charge of the conjugate (spermine/phosphate ratio) showed carrier-free intracellular delivery for oligonucleotide conjugates having a net positive charge (see Figure 4, Page 4). In addition, when compared to cationic lipid mediated delivery, a diffuse cytoplasmic pattern of cationic oligonucleotides indicates an efficient access to the cytoplasm as molecules and not as aggregates. This observation suggests an endosome escape of cationic oligonucleotides based on the proton sponge mechanism associated with the individual amine pK's of the oligospermine moieties.

Activity results have also confirmed the requirement for an overall positive charge of the conjugated siRNA (Z25 to Z35 conjugates) in many cellular models and for many targets.[7] A specific gene silencing was obtained at sub-micromolar concentration (25 to 100 nM) in the presence of serum and the RNAi mechanism was confirmed by a 5'-RACE assay as well as the use of control siRNAs.

NUCLEASE STABILITY

More importantly, the oligospermine tail protects the cationic oligonucleotide from degradation. This was observed with conjugates containing a low number of spermines (Z5-siRNA) where a limited degradation was shown even after 24 h incubation in serum, contrasting with unmodified siRNAs which were significantly degraded (Figure 5). Self-delivering and biologically active phosphodiester-based siRNAs containing 30 spermine moieties were also shown to be highly stable in presence of serum.[7] The spermine tail effect against the siRNA degradation was also confirmed in an RNase A assay (Figure 5). In these examples of siRNA conjugates, the cationic tail was grafted on the 5'-end of sense strands but it is expected that a spermine tail at either end of the oligonucleotide will improve the nuclease stability.

CONCLUSION

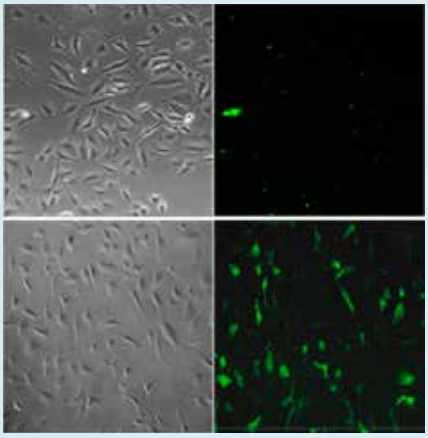
By selecting the number of cationic units, the global charge of the ZNA molecules can be modulated which defines their field of applications. When negatively charged, ZNA are potent tools for molecular biology and diagnostic applications. Their design is essentially based on the expected and predictable Tm of the oligonucleotide which depends on the number of conjugated cationic units. When positively charged, the cationic conjugates become self-delivering oligonucleotides into cells and resistant to nucleases which make them very attractive molecules for antisense or RNA interference applications.

Intellectual Property

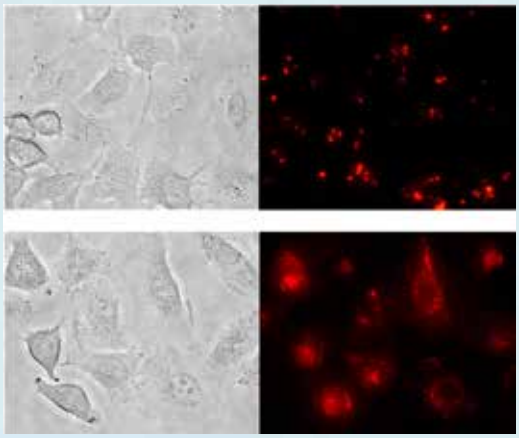
"Spermine phosphoramidite" synthon is the subject matter of U.S. Divisional Patent Application No. 14/745,871, European Patent No. 1 973 927 and foreign equivalents for which Polyplus-transfection is the co-owner. Product is sold for research purposes only.

Product shall not be used to manufacture oligonucleotide-oligospermine conjugates for use in diagnostics, clinical

FIGURE 4: **SELF-DELIVERING CATIONIC OLIGONUCLETIDES, ANTISENSE (LEFT) AND siRNA (RIGHT).**

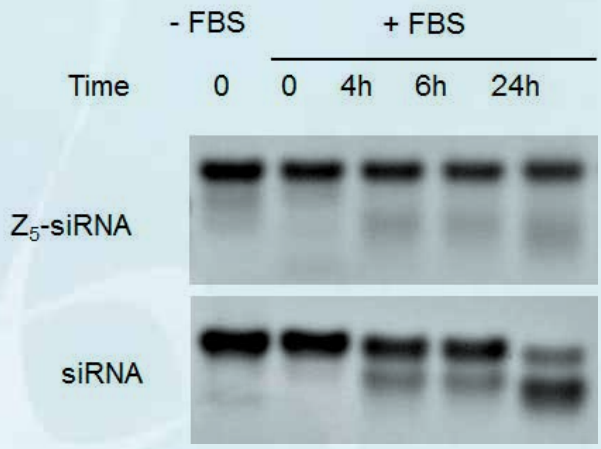


Intracellular delivery of 5'-Fluo-N_{19} (upper) and 5'-Fluo-Z_{18}-N_{19} (lower) in HeLa after 2 h incubation at 2 µM and 500 nM, respectively.

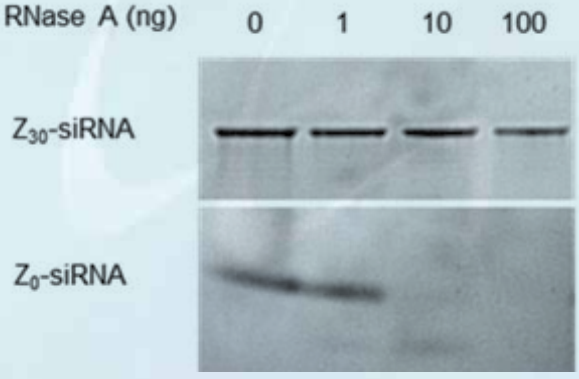


Intracellular delivery of 5'-Rho-siRNA with cationic lipid INTERFERin (upper) and 5'-Rho-Z_{25}-siRNA in A549 cells after 24 h incubation at 10 nM and 100 nM, respectively.

FIGURE 5: **THE OLIGOSPERMINE TAIL PROTECTS THE siRNA CONJUGATES FROM DEGRADATION**



Analysis on Tris-Tricine polyacrylamide 10–20% gel of siRNA or Z_5-siRNA (50 pmol) incubated in the presence of 10% serum at 37°C.



Analysis on SDS–Tris-Tricine polyacrylamide 10–20% gel of siRNA or Z30-siRNA (50 pmol) incubated in the presence of RNase A for 2 h at 37°C.

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. For a commercial license to Polyplus-transfection Intellectual Property, please visit the Polyplus-transfection webpage dedicated to Licensing Opportunities.

GLEN RESEARCH NOTE

We would like to take this opportunity to thank our colleagues at Polyplus for preparing this article and are delighted to share it with our readers. This report updates the research described in a previous Glen Report.[3]

Glen Research has been able to offer Spermine Phosphoramidite (1) to our customer base wordwide, under licence from Polyplus, since 2012. Over the intervening period, we have been able to help our research customers to simply and reliably prepare oligospermine 5'-conjugates of the structure shown in Figure 7 and also equivalent structures at the 3'-terminus. We certainly expect that publication of this update in this Glen Report will demonstrate the utility of spermine technology in an even broader array of research endeavors.

References:

1. B. Pons, M. Kotera, G. Zuber, and J.P. Behr, *Chembiochem*, 2006, **7**, 1173-6.
2. E. Voirin, J.-P. Behr, and M. Kotera, *Nat. Protocols*, 2007, **2**, 1360-1367.
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6. M. Nothisen, M. Kotera, E. Voirin, J.S. Remy, and J.P. Behr, *J Amer Chem Soc*, 2009, **131**, 17730-1.
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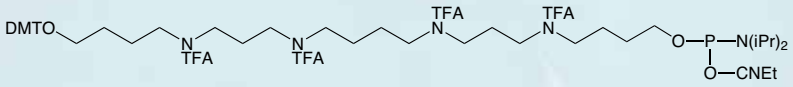
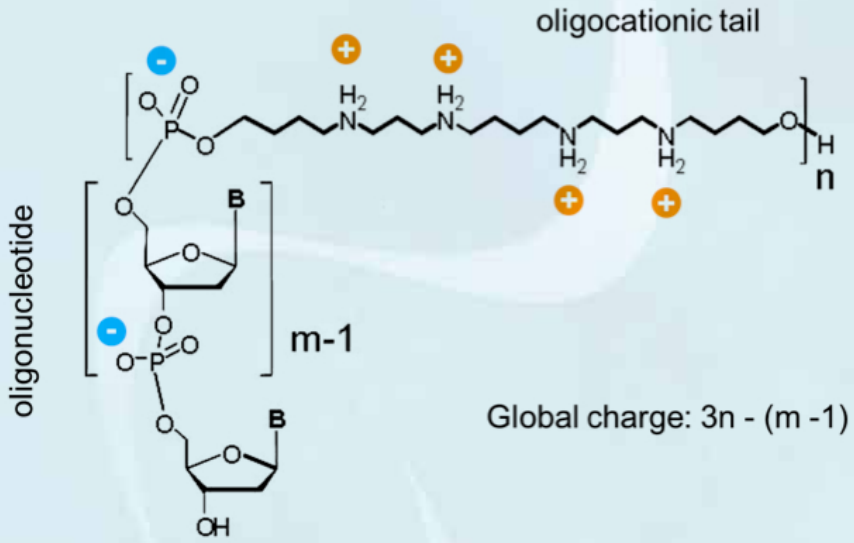
9. H. Kandemir, et al., *Neurosci Lett*, 2014, **580**, 158-62.
10. Z. Iranmanesh, et al., *Asian Pac J Cancer Prev*, 2015, **16**, 1919-24.
11. B. Alaiyan, et al., *BMC Cancer*, 2013, **13**, 196.
12. K.T. Gagnon, et al., *J Amer Chem Soc*, 2011, **133**, 8404-7.

***FIGURE 6:* STRUCTURE OF SPERMINE PHOSPHORAMIDITE**



(1) Spermine Phosphoramidite

***FIGURE 7:* OLIGONUCLEOTIDE OLIGOSPERMINE CONJUGATE**



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

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.

TECHNICAL BRIEF –1-ETHYNYL-dSPACER IN THE STUDY OF INTER-STRAND CROSSLINKS

INTRODUCTION

Simple and efficient labelling of oligonucleotides with a variety of labels is now routine through Click Chemistry.[1] Earlier Glen Reports have highlighted several approaches for efficient labelling of oligonucleotides with Click Chemistry.[2-6] All of our alkyne modifiers have proved to be excellent substrates for Click Chemistry in the presence of the water-soluble Click ligand, THPTA.

1-Ethynyl-dSpacer CE Phosphoramidite (1) is a new alkyne modifier that has the potential for producing and studying site-specific DNA inter-strand crosslinks. This modifier is stable, possesses high coupling efficiency, retains the standard phosphodiester backbone, is stable to standard deprotection techniques, and is an excellent substrate for Click Chemistry.

In the previous Glen Report,[4] we described the use of 1-Ethynyl-dSpacer. We reported on the Click reaction of this modifier with a variety of azides to form a selection of 1,2,3-triazole pseudo-nucleoside analogues of general structure as shown in Figure 1. There are undoubtedly many potential uses for these labelled pseudo-nucleosides but, in the present Technical Brief, we highlight our experience with oligonucleotides modified with psoralen and coumarin and their potential for studying inter-strand crosslinks (ICL).

INTER-STRAND CROSSLINKS (ICL)

Naturally occurring ICL originate from a variety of exogenous and endogenous sources that include alkylating agents, ionizing radiation, metabolism and environmental exposure.[7] Cellular repair of ICL has been linked to an assortment of DNA repair enzymes. Genomic defects associated with ICL are implicated in cancer and confer hypersensitivity to cross-linking agents. A cost effective path for generating ICL is essential for studying these repair mechanisms. There are many approaches for generating ICL including non-specific agents as well as site-specific approaches.[8-11]

In this Brief, we describe the use of 1-Ethynyl-dSpacer for the site-specific incorporation of 4'-azidomethyl-4,5,8-

FIGURE 1: 1-ETHYNYL-dSPACER AND ITS CLICK REACTION WITH PSORALEN AND COUMARIN AZIDES

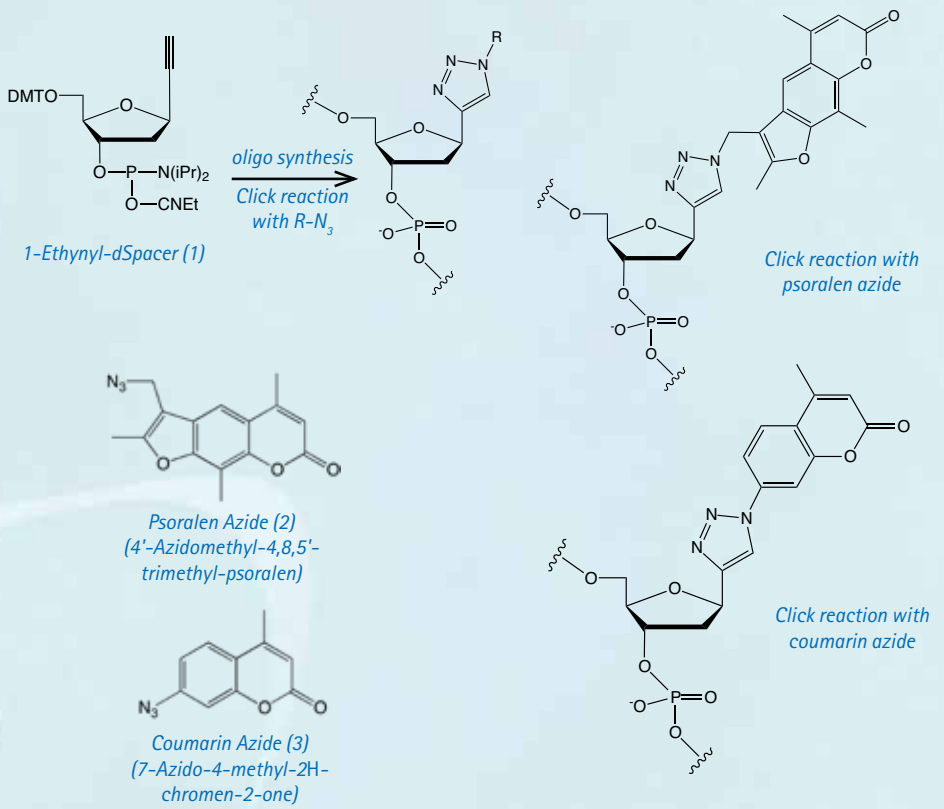


TABLE 1: DUPLEXES USED IN THIS STUDY

Duplex 1, X=Psoralen

```
        3'-G CTG AXT GAT AT-5'
5'-TAA TAC GAC TCA CTA TAG GG 3'
```

Duplex 2, X=Psoralen

```
        3'-G CTG XGT GAT AT-5'
5'-TAA TAC GAC TCA CTA TAG GG 3'
```

Duplex 3, X=Psoralen

```
        3'-G CTG XGT GAT AT-5'
5'-TAA TAC GAC TAA CTA TAG GG 3'
```

Duplex 4, X=Psoralen

```
        3'-G CTG AXT GAT AT-5'
5'-TAA TAC GAC TAA CTA TAG GG 3'
```

Duplex 5, X=Psoralen

```
        3'-G CTG AGT GAT AX-5'
5'-TAA TAC GAC TCA CTA TAG GG 3'
```

Duplex 6, X=Coumarin

```
        3'-G CTG XGT GAT AT-5'
5'-TAA TAC GAC TCA CTA TAG GG 3'
```

Duplex 7, X=Coumarin

```
        3'-G CTG AXT GAT AT-5'
5'-TAA TAC GAC TCA CTA TAG GG 3'
```

Duplex 8, X=Coumarin

```
        3'-G CTG AGT GAT AX-5'
5'-TAA TAC GAC TCA CTA TAG GG 3'
```

trimethylpsoralen (2) or 7-azido-4-methyl coumarin (3) as possible approaches for generating photoinducible internal and terminal ICL. Psoralen and coumarin undergo photo-induced cycloaddition with pyrimidine nucleosides on exposure to long wavelength UV light (300-350nm).[12,13] In some cases, these ICL are reversible with short wavelength UV light (254nm).

In this set of experiments, we synthesized a group of test oligonucleotides incorporating 1-Ethynyl-dSpacer (12-mer) and a set of complementary target oligonucleotides (20-mer) (Table 1). All oligonucleotides were purified DMT-ON using standard Glen-Pak protocols. The test oligonucleotides included site-specific internal modification or a 3'-terminus modification. Each test oligonucleotide containing 1-Ethynyl-dSpacer was conjugated with high efficiency to either the psoralen azide (2) or the coumarin azide (3) via standard Click Chemistry and purified by simple ethanol precipitation. Representative chromatograms with attendant UV spectra of the products are shown in Figure 2.

The test and target oligonucleotides were hybridized to form the duplexes shown in Table 1. Each duplex was exposed to long wavelength light (365nm) from a handheld UV lamp for up to 4 hours at room temperature to generate cross-linked oligonucleotides. The formation of ICL was evaluated using a denaturing IEX HPLC method capable of resolving each oligo as well as the cross-linked duplex. Duplexes 1 and 5 were evaluated for reversal of crosslinking by exposure to short wavelength UV light.

DISCUSSION

The level and rate of crosslinking varied depending on the location of the modification (Table 2). Some crosslinking was observed in as little as 15 minutes for all duplexes although most duplexes required significantly longer exposure time for maximum yield. As modifiers for oligonucleotide synthesis, psoralen labels are typically added to the 5'-terminus with the canonical target sequence as 5'-TpA-3'.[13] Surprisingly, the best result (Duplex 1) with Psoralen was obtained with the internal sequence of 5'-CpT-3' and 1-Ethynyl-dSpacer opposite dC, resulting in 77% ICL formation in as little as 15 minutes. Overall ICL yield rose to 87% after 2 hours with a maximum of 89% after a 4-hour exposure. In comparison, when psoralen was incorporated opposite dT (Duplex 2), the initial rate was reduced and the duplex required 4 hours for a maximum yield of 81%. Despite the low initial rates when opposite dT, high final yields for both duplexes were still observed and are most likely a result of two potential nucleoside targets (dT and dC) for crosslinking with Psoralen.

After substituting dC with dA in the

FIGURE 2: **RP HPLC ANALYSIS OF 12-MER OLIGOS CLICK CONJUGATED WITH PSORALEN AND COUMARIN**

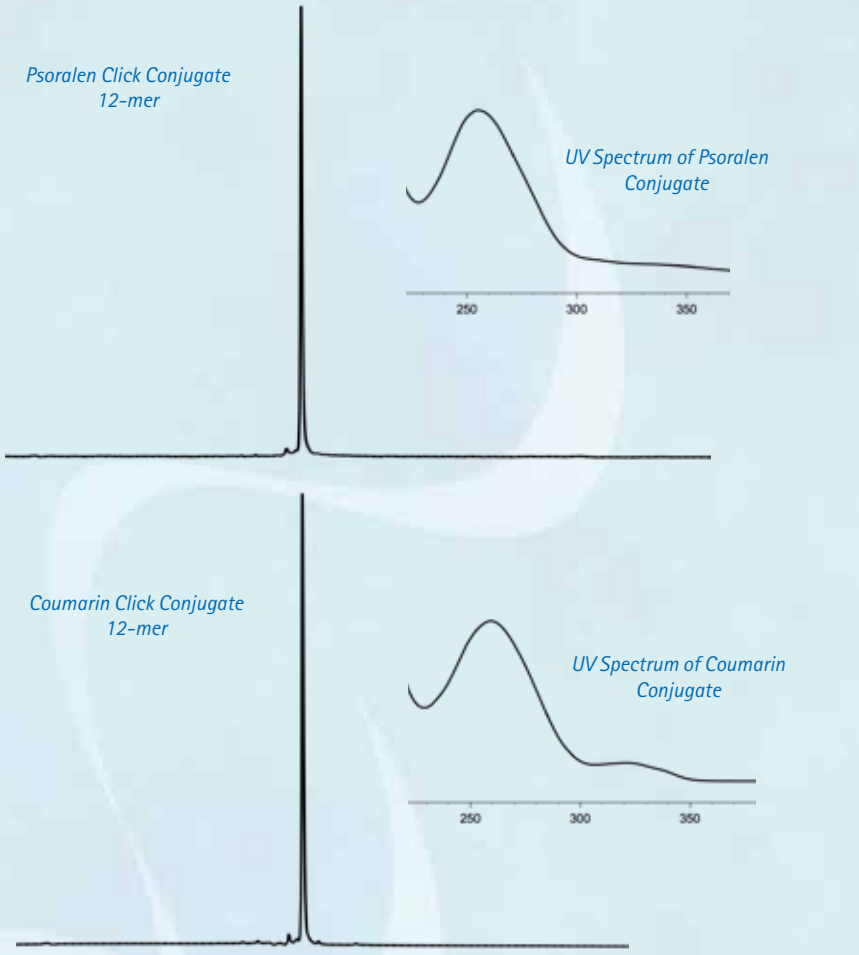


TABLE 2: **DUPLEXES USED IN THIS STUDY**

Modification	Duplex	Site	Opposite	15 min.	60 min.	120 min.	240 min.
Psoralen	Duplex 1	Internal	TC	77%	87%	87%	89%
Psoralen	Duplex 2	Internal	TC	10%	51%	68%	81%
Psoralen	Duplex 3	Internal	TA	22%	50%	62%	64%
Psoralen	Duplex 4	Internal	TA	52%	68%	72%	76%
Psoralen	Duplex 5	Terminal	TA	77%	78%	78%	74%
Coumarin	Duplex 6	Internal	TC	3%	10%	17%	26%
Coumarin	Duplex 7	Internal	TC	3%	5%	14%	25%
Coumarin	Duplex 8	Terminal	TA	8%	23%	40%	58%

target sequence (Duplexes 3, 4), the relative initial rates were reduced to 22% and 52% at 15 minutes compared to the ideal sequence 5'-TpC-3' (Duplex 1). The reduced initial yields may result from the removal of a potential crosslink with cytosine.

With a terminal 1-Ethynyl-dSpacer modifier, the psoralen was incorporated opposite dA in the terminal sequence 5'-TpA-3' (Duplex 5). A 15 minute exposure resulted in 77% ICL formation. Prolonged exposure of 4 hours, however, resulted in reduced final yields suggesting additional side reactions of psoralen. Indeed, in the absence of target, psoralen-containing oligonucleotides were rapidly degraded. Terminal modifications may provide steric freedom from unwanted side reactions. In contrast, internal incorporations may be shielded through base stacking that prevents subsequent side reactions.

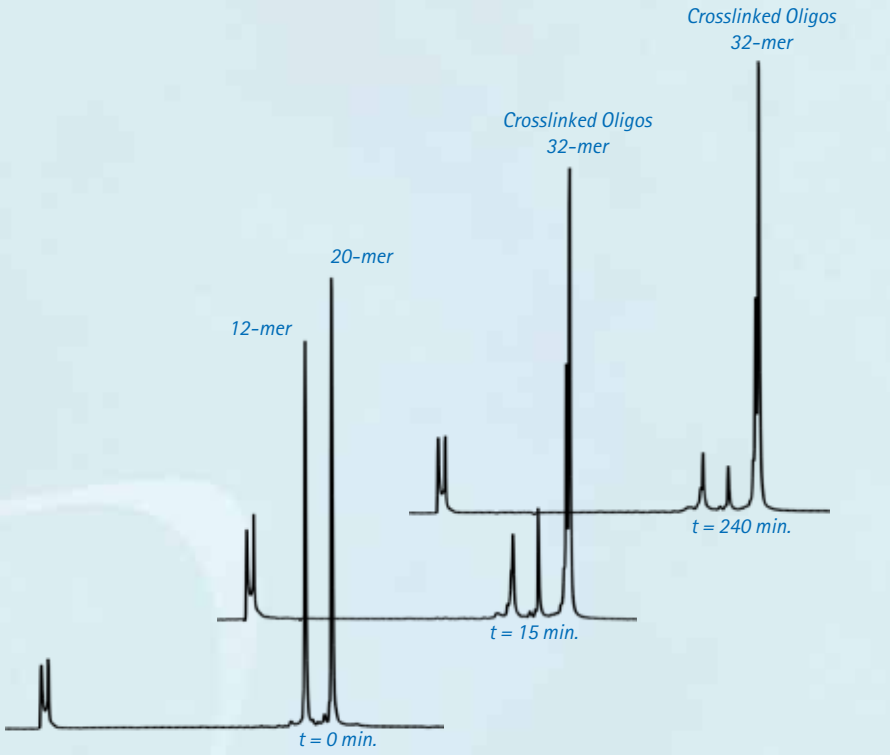
Psoralen crosslinks are often reversible after exposure to short wavelength UV light (254nm). Duplexes 1 and 5 were evaluated for potential reversal for up to 4 hours. Some reversal, 19% and 43% respectively, was observed after exposure and prolonged exposure times (>4 hours) may be required.

Coumarin conjugated oligonucleotides also formed ICL after exposure to long wavelength UV light (Duplexes 1, 2, 3). The initial and final ICL rates were significantly lower than psoralen-containing oligonucleotides. Interestingly, the coumarin-modified duplexes generated a less complex HPLC profile suggesting the formation of a single product. Extending the overall exposure time should result in a more extensive conversion.

SUMMARY

1-Ethynyl-dSpacer, in combination with psoralen and coumarin azides, has been shown to be a potential research tool for studying ICL. As a simple Click tool, 1-Ethynyl-dSpacer works exceptionally well to introduce a 1,2,3-substituted-triazole residue into an oligonucleotide as a pseudo nucleobase, while preserving the natural phosphodiester backbone. With the selection of a suitable azide such as psoralen or coumarin, the modifier can be used to incorporate photo-inducible and site-specific ICL. The versatility of 1-Ethynyl-dSpacer is highlighted by the fact that a single 1 µmole synthesis can be used with multiple azides to generate and evaluate several different structures.

***FIGURE 3: IEX HPLC ANALYSIS OF* CROSSLINKING REACTION OF DUPLEX 1 OVER TIME**



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ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
1-Ethynyl-dSpacer CE Phosphoramidite	10-1910-95	50 µmole	180.00
	10-1910-90	100 µmole	340.00
	10-1910-02	0.25g	1250.00
Psoralen Azide	50-2009-92	25 µmole	115.00
	50-2009-90	100 µmole	350.00
THPTA Ligand (Water soluble)	50-1004-92	25 µmole	50.00
	50-1004-90	100 µmole	180.00

CHOLESTERYL-TEG PHOSPHORAMIDITES AND SUPPORT NOW CERTIFIED BSE/TSE FREE

Cholesterol remains one of the most popular forms of labelling of oligonucleotides, especially those destined for use in therapeutics, including antisense and siRNA.[1,2] Most oligonucleotides are hydrophilic, so they are constantly challenged in their ability to permeate cell membranes. In order to improve cellular uptake, one strategy is to conjugate oligonucleotides with hydrophobic molecules that are non-toxic and cholesterol is one of the most popular choices.

In 1993, Glen Research introduced Cholesteryl-TEG phosphoramidite (1) and the corresponding CPG support (2). Later, in 2008, we introduced the simplified version 5'-Cholesteryl-TEG phosphoramidite (3), designed solely for 5' labelling of oligonucleotides.

In those early days, the cholesterol to make these products was sourced from sheep's wool and the products were perfectly useable for their intended research and development purposes. However, it has become clear that this cholesterol source was unacceptable in situations where product oligonucleotides were required to be certified as being free from BSE/TSE contamination. Bovine spongiform encephalopathy (BSE) is a transmissible spongiform encephalopathy (TSE) that affects cattle. There is also a spongiform encephalopathy associated with sheep, called scrapie, that has been shown to induce BSE in cattle.

We are happy to report that our raw material cholesterol is now from a plant source and our cholesterol products can now be certified as free from BSE/TSE contamination.

Glen Research also offers other hydrophobic labels, 5'-α-Tocopherol-TEG Phosphoramidite (4) and 5'-Stearyl Phosphoramidite (5) , to improve the cellular uptake of oligonucleotides.

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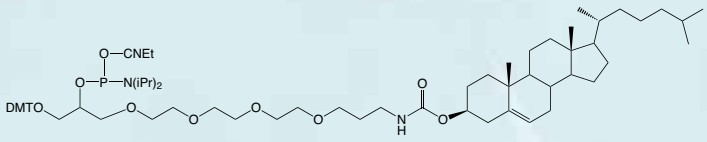
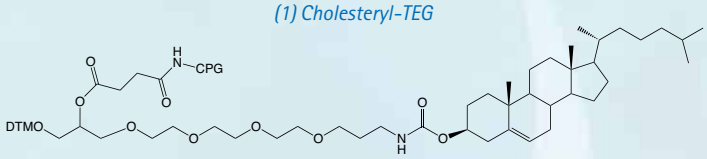
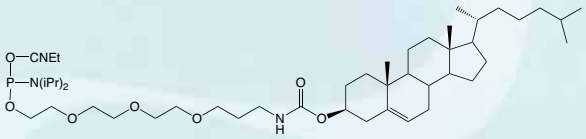
FIGURE 1: **STRUCTURES OF CHOLESTERYL AND LIPOPHILIC PHOSPHORAMIDITES AND SUPPORT**



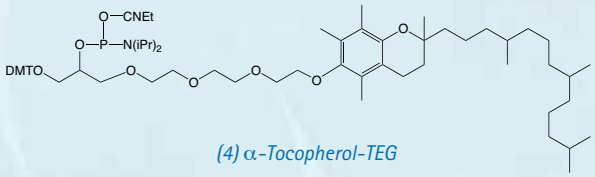
(1) Cholesteryl-TEG



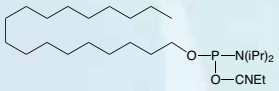
(2) 3'-Cholesteryl-TEG CPG



(3) 5'-Cholesteryl-TEG



(4) α-Tocopherol-TEG



(5) 5'- Stearyl

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Cholesteryl-TEG Phosphoramidite	10-1975-95	50 µmole	140.00
	10-1975-90	100 µmole	265.00
	10-1975-02	0.25g	545.00
5'-Cholesteryl-TEG Phosphoramidite	10-1976-95	50 µmole	95.00
	10-1976-90	100 µmole	175.00
	10-1976-02	0.25g	525.00
3'-Cholesteryl-TEG CPG	20-2975-01	0.1g	85.00
	20-2975-10	1.0g	700.00
1 µmole columns	20-2975-41	Pack of 4	140.00
0.2 µmole columns	20-2975-42	Pack of 4	84.00
10 µmole column (ABI)	20-2975-13	Pack of 1	210.00
15 µmole column (Expedite)	20-2975-14	Pack of 1	315.00
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

NEW PRODUCT - DEPURINATION RESISTANT dA-CE PHOSPHORAMIDITE

INTRODUCTION

Depurination is defined as the cleavage of the glycosidic bond attaching a purine base to the sugar moiety. Electron withdrawing acyl protecting groups like benzoyl and isobutyryl on the purine amino group(s) destabilize the glycosidic bond, whereas electron donating formamidine protecting groups stabilize the glycosidic bond. The use of formamidine protecting groups to eliminate depurination was first proposed[1,2] in the 1980s and dmf-dG is an example of this strategy in routine use today.

The consequence of depurination during oligonucleotide synthesis is the loss of the purine base to form an internucleotide linkage containing the abasic sugar at that position. This site is stable during further synthesis cycles but, upon deprotection with basic reagents, the oligonucleotide is cleaved at that position leading to two shorter fragments. The fragment towards the 5' terminus still contains the DMT group. If DMT-ON purification is being used, the depurinated fragments are co-purified along with the full length product as truncated oligonucleotides.

dA is actually more susceptible to depurination than dG and the dA monomer most commonly used is protected with the destabilizing benzoyl group (1). So why has this not been a factor in routine oligonucleotide synthesis? Undoubtedly, depurination does occur in regular oligonucleotide synthesis but at an extremely low level due to the exquisite balancing of the steps in the synthesis cycle. Nevertheless, our data show that if a $dA_{20}dT$ oligo is tortured by changing the acid deprotection step to 15 minutes per cycle, then the oligo is almost completely degraded and only a small amount of full length product is observed.

Depurination may not be very significant in routine synthesis but it is still a problem in certain other circumstances, as noted below:

Synthesis of long oligos[3]

In this scenario, the exposure of protected dA sites to acid during the deprotection step, although brief each cycle, accumulates as the synthesis proceeds. Trichloroacetic acid (TCA), which is the standard acid used in routine small scale oligo synthesis, is quite strong, with a pKa of approximately 0.7. For the synthesis of long oligos, an alternative is to use a deblocking agent with a higher pKa. The most common choice is dichloroacetic acid (DCA), which has a pKa of 1.5. However, the risk of depurination using TCA must be balanced with the risk of incomplete deprotection using DCA. Depurination may lead to shorter fragments truncated at the 3' terminus but incomplete deprotection would lead to deletion mutations in a family of n-1, n-2, etc., oligos and this may be an even worse scenario for such applications as gene construction. This situation would benefit from a depurination resistant dA monomer.

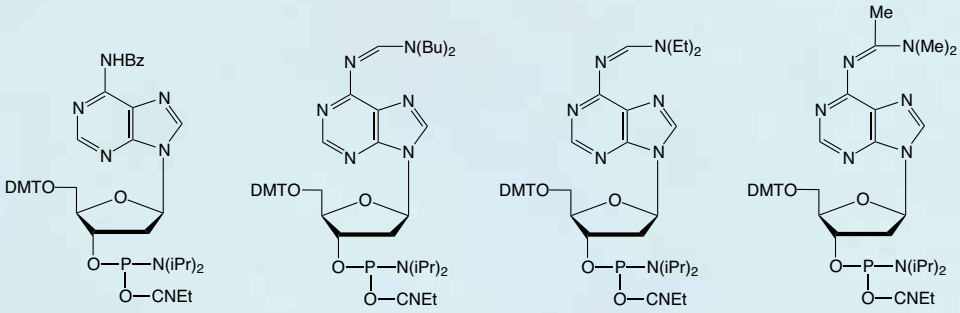
Chip-Based Synthesis

The synthesis of microarrays on silicon wafers requires the synthesis methodology that works so well on the 3-dimensional porous surface of CPG to be adapted to a 2-dimensional non-porous surface. If the synthesis strategy requires acid to be used for detritylation, there is the potential for surface effects to cause increased concentration of the acid, potentially leading to increased levels of depurination. Indeed, this very situation on Agilent's DNA microarray synthesis platform led to the conclusion that depurination is the limiting factor in the ability to synthesize long oligos (up to 150mers) – even using DCA as the detritylating reagent.[4] In this case, depurination had to be controlled by adjusting the fluidics of the flow cell and by quenching the detritylation solution with oxidizer solution. A depurination resistant dA monomer may also significantly improve this situation.

Large-Scale Synthesis

The extended reaction times of large-scale synthesis require careful consideration of depurination potential. On the other hand, cycle optimization has been so successful and costs are so tightly controlled that a more expensive depurination resistant dA monomer may well have been rejected previously.

FIGURE 1: **STRUCTURES OF PROTECTED dA CE PHOSPHORAMIDITES**



Bz-dA (10-1000) (1) *dbf-dA (10-1500) (2)* *def-dA (10-1504) (3)* *dma-dA (10-1505) (4)*

TABLE 1: **TIME REQUIRED FOR COMPLETE DEPROTECTION**

Protecting group on dA	30% Ammonium Hydroxide at 65°C	Ammonium Hydroxide/40% Methylamine (AMA) at 65 °C
Benzoyl	<2 hours	<10 minutes
dibutylformamidine	2 hours	20 minutes
diethylformamidine	2 hours	20 minutes
dimethylacetamidine	4 hours	80 minutes

DEPURINATION RESISTANT dA

We have discussed this topic in the past while introducing the dbf-dA monomer (2). This dibutylformamidine protected dA seemed a likely candidate for the synthesis of long oligos but it did not perform as well as we expected and was discontinued. We surmise that the problem occurred during deprotection when the dibutylformamidine group may have been initially hydrolyzed to form dibutylamine, which is both involatile and capable of forming a salt with the phosphate backbone.

In response, we have gone back to basics and prepared the diethylformamidine (def) (3) and the dimethylacetamidine (dma) (4) protected dA monomers. In the former case, diethylamine would be formed on deprotection and this is highly volatile. In the latter case, dimethylacetamide is fairly involatile but would be unlikely to form a salt with the phosphate backbone.

Rate of deprotection

In the first instance, we looked at the relative rates of deprotection and they are collected in Table 1. As expected, def-dA was rapidly deprotected in ammonium hydroxide and AMA. Surprisingly, we found that dma-dA was deprotected quickly in ammonium hydroxide but it took 80 minutes at 65° in AMA for complete deprotection. The time course of this deprotection is shown in Figure 2.

Depurination Resistance

As a further experiment, we carried out a torture synthesis of $dA_{10}dT$ using 3% TCA/DCM and with detritylation steps increased to 15 minutes to simulate the synthesis of an oligo approximately 15 times longer. The resulting oligonucleotides were purified DMT-ON with GlenPak cartridges to remove any oligonucleotides that did not contain 5'-DMT species. The resulting oligonucleotides were then analyzed by ion exchange HPLC, as shown in Figure 3.

In the case of Bz-protected dA, the oligonucleotide was substantially degraded. However, in the case of both of these new monomers, the oligonucleotide was well protected against depurination and a good yield of full length product was achieved.

FIGURE 2: **RP HPLC OF DMT-ON dT OLIGO CONTAINING 3 INSERTIONS OF DMA-dA**

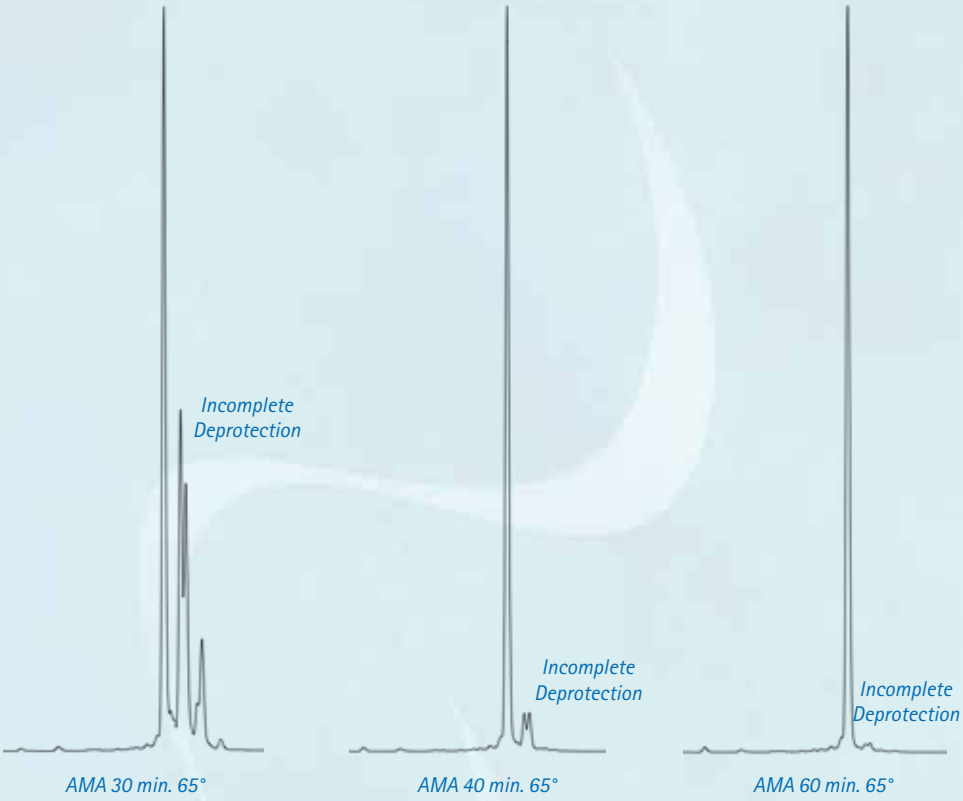
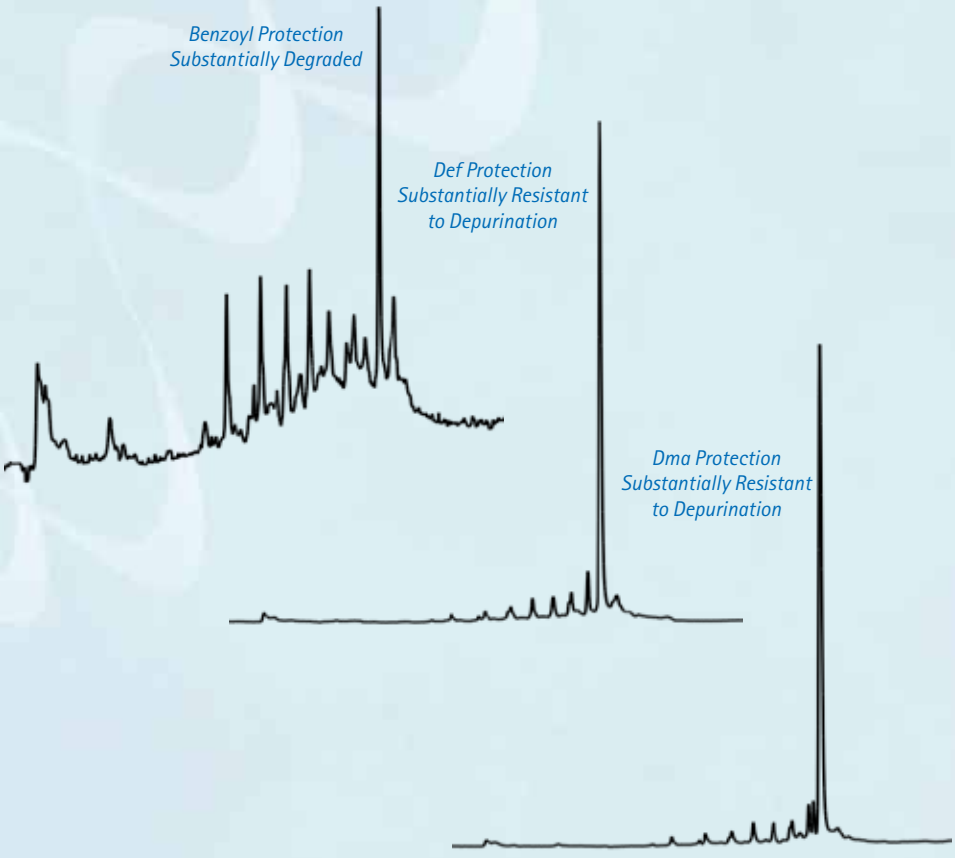


FIGURE 3: **IEX HPLC OF PROTECTED $dA_{10}dT$ TORTURED WITH TCA**



GLEN RESEARCH

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20164

CONCLUSION

In this article, we have demonstrated that the most commonly used dA-CE Phosphoramidite containing benzoyl protecting groups suffers substantial degradation by depurination after excessive exposure to TCA. However, the TCA exposure in our torture treatment of $dA_{10}dT$ (15 minutes per cycle or 150 minutes total) is equivalent to the exposure experienced by a dA residue adjacent to the 3' terminus during the synthesis of a 150mer on the LV200 cycle of an ABI synthesizer.

At the same time, we have demonstrated that two depurination resistant dA monomers, protected with diethyl-formamidine (def) and dimethylacetamidine (dma), are essentially stable to depurination during the torture testing.

Testing of the rates of deprotection of the two new monomers showed that both were rapidly deprotected in ammonium hydroxide and are fully compatible with regular deprotection strategies. However, an interesting anomaly was observed during deprotection with popular AMA at 65°. Def-protected-dA was rapidly deprotected in 20 minutes, which makes it fully compatible with regular AMA deprotection. In contrast, the dma-protected-dA was only about 50% deprotected by AMA in 20 minutes at 65°. A time course study revealed that 80 minutes were required for complete deprotection.

Due to the longer time of 80 minutes required for complete deprotection of dma-dA with AMA, we have decided to offer the def-dA for routine synthesis requiring depurination resistance. We leave open the option of supplying dma-dA for more specific applications.

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3. *The Glen Report,* 2009, **21.2**, 14-15.
4. E.M. LeProust, *et al.*, *Nucl Acid Res,* 2010, **38**, 2522-2540.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
def-dA-CE Phosphoramidite	10-1504-02	0.25g	15.00
	10-1504-05	0.5g	30.00
	10-1504-10	1.0g	60.00
dma-dA-CE Phosphoramidite Please inquire.	10-1505		