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INSIDE

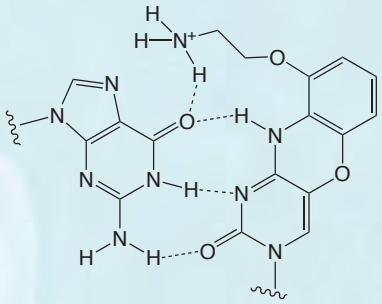
AP-DC - A CYTOSINE ANALOGUE CAPABLE OF CLAMP-LIKE BINDING TO GUANINE - G-CLAMP

The ability to modify but, more specifically, to enhance binding between nucleotide bases has always been of critical importance in many avenues of DNA and RNA research. The cytosine – guanine (C-G) base pair with 3 hydrogen bonds is already much stronger than the adenine – thymine (A-T) base pair, which has 2 hydrogen bonds. In this article, we focus on the C-G base pair and a remarkable cytosine analogue, which has been popularly named "G-clamp".

G-clamp[1] is a tricyclic Aminoethyl-Phenoxazine 2'- deoxyCytidine analogue (AP-dC). The nucleoside structure and its relationship to dC are shown in Figure 1, Page 2. The heterocyclic phenoxazine structure provides a stable basis for the protonated amine of an aminoethyl chain to interact with the O6 position of a complementary dG molecule, as shown in Figure 2, Page 2. In principle, therefore, G-clamp should stabilize a duplex due to its ability to interact with both the Watson-Crick and Hoogsteen faces of the target G. In practice, G-clamp did indeed have a very dramatic effect on duplex stabilization. A single G-clamp incorporation in a poly-pyrimidine decamer resulted in a spectacular 18 °C enhancement of the melting temperature of the duplex relative to a control containing 5-Me-dC at the same point.[1]

Since the G-clamp relies on the hydrogen bonding of its protonated amine to the O6 position of the G target, specificity should, in theory, be enhanced, and indeed it is. The sequence containing a single G-clamp residue exhibited better discrimination between the perfect match with G in comparison to the mismatches with A, C and T.[1] In further experiments, it was shown[1] that the enhanced binding of G-clamp was not affected by the nature of the bases flanking the target G. It was also shown that the interaction was with G rather than the phosphate backbone when it was determined that the ionic strength of the buffer had little effect on the binding and when uncharged linkages flanked the enhanced base pair.

G...AP-C BASE PAIR



G ---------- AP-C

MELTING EXPERIMENTS WITH 19-MER OLIGOS

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5'-CCT-ATA-GTX-AGT-CGT-ATT-A-3'
3'-GGA-TAT-CAY-TCA-GCA-TAA-T-5'
```

X	Y	Tm(°C)	ΔTm(°C)
dC	dG	57.5	
AP-dC	dG	65.0	+ 7.5
AP-dC	dA	50.0	-15.0
AP-dC	dC	48.0	-17.0
AP-dC	dT	49.5	-15.5

The specific interaction between G-clamp and the guanine residue should also be unaffected by the nature of the duplex. The melting temperature of a B-form DNA-DNA duplex containing G-clamp was enhanced to about the same extent as an A-form DNA-RNA hybrid.[1]

Oligonucleotides containing G-clamp have been evaluated[2] for sequence-context dependence, activity mismatch, sensitivity, RNAse-H cleavage, and hybridization kinetics in antisense experiments. In previously optimized systems, oligophosphorothioates (S-ON) containing a single G-clamp residue increased the potency of the antisense oligonucleotide even in comparison to the most potent C5-propynyl-modified S-ON previously tested by the researchers.[2] Indeed, they conclude that the G-clamp modification is a highly potent, mismatch-sensitive cytosine analog that will find

(Continued on Page 2)

(Continued from Front Page)

applications in elucidating gene function, in validating gene targets, and in developing more potent antisense oligonucleotides.

Furthermore, a single incorporation of AP-dC at the 3' terminus was shown[3] to protect phosphodiester oligonucleotides from 3'-exonuclease digestion.

Our own experiments confirm that AP-dC not only increases hybridization efficiency but, at the same time, the increase is additive over more than one addition of AP-dC. We have also confirmed that AP-dC increases mismatch descrimination. These results are listed in Table 1 and Table 2.

These findings clearly support the conclusion that AP-dC (G-clamp) is a very important addition to the modified nucleosides that enhance hybridization. The product is clearly remarkable for being so stable to conditions of oligonucleotide synthesis and deprotection. We especially envisage its use in the development of short, easily prepared oligonucleotides for use in highly specific single nucleotide polymorphism (SNP) and other *in vitro* diagnostic assays.

We are indebted to Isis Pharmaceuticals for including AP-dC-CE Phosphoramidite, (1) in Figure 1, to our License Agreement.

References:

1. K.Y. Lin and M.D. Matteucci, *J Am Chem Soc*, 1998, **120**, 8531-8532.
2. W.M. Flanagan, et al., *Proc Nat Acad Sci USA*, 1999, **96**, 3513-3518.
3. M.A. Maier, et al., ***Biochemistry***, 2002, **41**, 1323-1327.

Intellectual Property

FIGURE 1: DC, AP-DC AND AP-DC-CE PHOSPHORAMIDITE

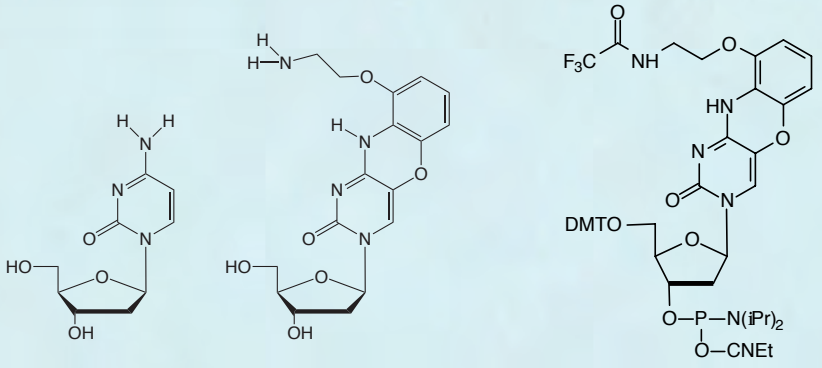


FIGURE 2: G - C AND G - AP-C BASE PAIRING

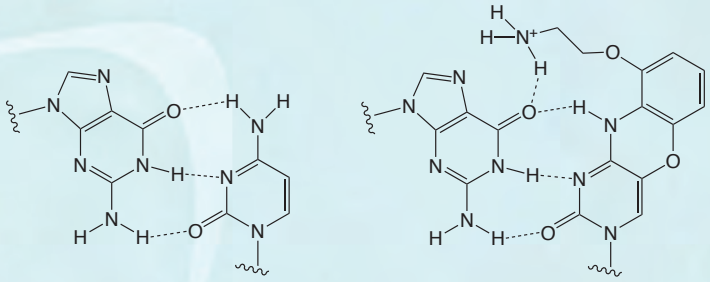


TABLE 1: MELTING EXPERIMENTS WITH SHORT POLY-PYRIMIDINE OLIGOS

5'-T**Z**T-C**X**C-TCT-C-3'
3'-AGA-G**Y**G-AGA-GAG-AAA-AA-5'

Z	X	Y	Tm(°C)	ΔTm(°C)
dC	dC	dG	39.0	
dC	5-MedC	dG	41.0	+ 2.0
dC	AP-dC	dG	60.0	+21.0
AP-dC	dC	dG	54.0	+15.0
AP-dC	AP-dC	dG	74.0	+35.0

X	Y	Tm(°C)	ΔTm(°C)
AP-dC	dG	60.0	
AP-dC	dA	32.5	-27.5
AP-dC	dC	30.5	-29.5
AP-dC	dT	29.5	-30.5

Tm measurements were carried out as described in Reference 1.

TABLE 2: MELTING EXPERIMENTS WITH 19-MER OLIGOS

5'-CCT-ATA-GT**X**-AGT-CGT-ATT-A-3'
3'-GGA-TAT-CA**Y**-TCA-GCA-TAA-T-5'

X	Y	Tm(°C)	ΔTm(°C)
dC	dG	57.5	
AP-dC	dG	65.0	+ 7.5
AP-dC	dA	50.0	-15.0
AP-dC	dC	48.0	-17.0
AP-dC	dT	49.5	-15.5

Tm measurements determined by thermal denaturation in a Jasco V530 spectrophotometer. Each oligo was at a concentration of 1 µM in 5mM Tris, 0.1 M NaCl pH 7.4

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
AP-dC-CE Phosphoramidite	10-1097-95	50 µmole	230.00
(G-Clamp)	10-1097-90	100 µmole	460.00
	10-1097-02	0.25g	1175.00

TRIMER PHOSPHORAMIDITES – TOOLS FOR FINE-TUNING PROTEIN FUNCTION

Introducing mutations in existing proteins can be used to fine-tune almost any desired property, such as improved stability to high temperatures, denaturants, or non-aqueous solvents; higher affinity binding to a target molecule; increased rates of enzymatic reactions; or changes of specificities. However, generating and finding these improved proteins can be a difficult task. Now for the first time, new tools are available to radically improve the efficiency of this process – Trimer Phosphoramidites.[1-4]

Covering all 20 amino acids, Trimer Phosphoramidites (Figure 1) allow mutation of a gene at the codon level rather than at individual bases. Therefore, unlike other methods of mutagenesis, Trimer Phosphoramidites lead to no codon bias, no frame-shift mutations, and no production of stop codons, making them highly efficient tools for the exploration of sequence space in protein regions that are important for function.

Trimer phosphoramidites can be added during synthesis using standard DNA synthesis chemistry. A Reaction Factor (RF) has been determined for each Trimer to compensate for differences in their relative rate of reaction during coupling. It is therefore possible to introduce an equimolar mix of all 20 amino acid codons, or subsets thereof, at any location within the sequence.

Degenerate Oligonucleotides

Of course, there are cheaper alternatives for introducing mutations, but they are far less effective and may cause considerably more expense in downstream screening for desired mutations. One of the most popular choices is to make pools of degenerate oligonucleotides, which can be incorporated into the genes as cassettes or by PCR by using the degenerate oligo as a primer.[5] Degenerate oligonucleotides are synthesized as a mixture of A/C/G/T phosphoramidites (N) at the site of the codons to be mutated. Problems arise, though, from using an equimolar solution of each base. First there is a coding bias. Out of the 64 possible codon combinations of A, C, G and T, 18 code for leucine, arginine or serine, but only 2 for tryptophan or methionine. As a result, only 3% of the mutagenic oligonucleotides will contain methionine or tryptophan, and over 28% will contain either leucine, arginine or serine. In addition, the three nonsense codons will lead to chain termination in 4.7% of the sequences. There are ways to help this situation. For instance, using two degenerate mixes of bases, N and G/C, on the DNA synthesizer to insert NNG/C into the sequence will halve the number of the most degenerate codons, but still code for all 20 amino acids. However, still 59% of the clones will code for just eight amino acids and 3% will have a stop codon inserted. The generation of redundant sequences and stop codons makes searching a clonal library inefficient.

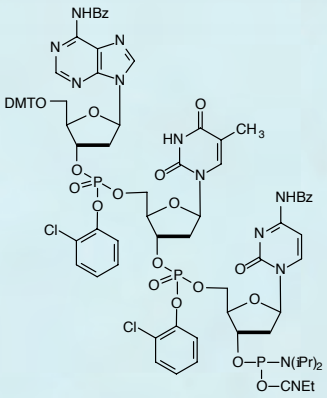
Trimer phosphoramidites offer an elegant solution[6] that circumvents these problems of codon bias, frame-shift mutations and stop-codon production – even in nonsaturating conditions.[7]

Mutagenesis Strategy

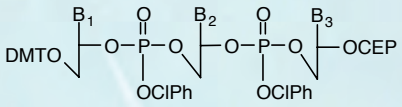
Once a mutagenic oligonucleotide has been synthesized using Trimer Phosphoramidites, there are a variety of means of introducing it into a plasmid for construction of a library. A very efficient means of mutagenesis has been reported[8] as shown in Figure 2. A synthetic oligonucleotide (solid line) is annealed to a circularized dU-containing ssDNA template (dashed line) that was obtained from *dut⁻/ung⁻ E. coli* strain that lacks Uracil DNA glycosylase. The mismatched variable region is flanked by perfectly complementary sequences (A). Covalently closed circular DNA (CCC-ds DNA) is obtained by the action of the T7 polymerase and T4 ligase (B). When the CCC-ds-DNA is introduced into *ung⁺ E. coli*, the template DNA, containing dU, is preferentially destroyed, leading to the enrichment of the mutant plasmid (C).

We thank Sachdev Sidhu, Ron Godiska and Paul Gaytan for reviewing this document and for their many helpful suggestions.

FIGURE 1: STRUCTURE OF TRIMER PHOSPHORAMIDITES

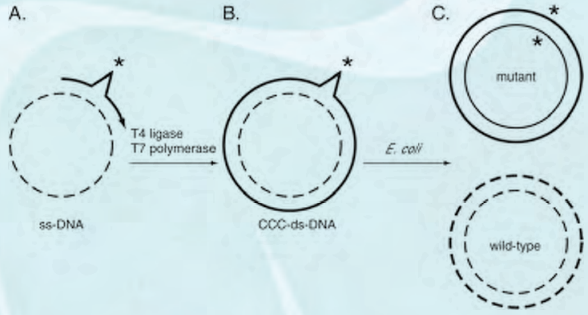


Detailed Structure of ATC



General Structure of Trimer Phosphoramidites, where B=A^{bz}, C^{bz}, G^{ibu}, T

FIGURE 2: INTRODUCTION OF MUTAGENIC OLIGONUCLEOTIDE INTO A PLASMID



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1. A. Kayushin, M. Korosteleva, and A. Miroshnikov, *Nucleos Nucleot Nucleic Acids*, 2000, **19**, 1967-1976.
2. A. Kayushin, et al., *Nucleos Nucleot*, 1999, **18**, 1531-1533.
3. A.L. Kayushin, et al., *Nucleic Acids Res*, 1996, **24**, 3748-3755.
4. T. Mauriala, et al., *J Pharm Biomed Anal*, 2004, **34**, 199-206.
5. G. Zon, et al., *Nucleic Acids Res*, 1985, **13**, 8181-96.
6. C. Neylon, *Nucleic Acids Res*, 2004, **32**, 1448-59.
7. J. Sondek and D. Shortle, *Proc Natl Acad Sci U S A*, 1992, **89**, 3581-5.
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SULFURIZING REAGENT II - STABLE IN SOLUTION AND OPTIMIZED FOR RNA SULFURIZATION

The use of a sulfurizing reagent during the regular synthesis cycle using phosphoramidite chemistry has revolutionized the production of phosphorothioate oligonucleotide analogues. Undoubtedly, this ease of preparation of phosphorothioates has made this oligonucleotide modification by far the most common in research. Glen Research was one of the first sources of the sulfurizing reagent, 3H-1,2-benzodithiol-3-one 1,1-dioxide, popularly known as Beaucage Reagent (1).[1] This sulfurizing reagent has found common use in the face of a plethora of rival reagents over the years because of its high efficiency, fast reaction time, and widespread availability.

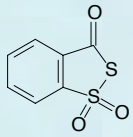
The one mild flaw we have found with Beaucage Reagent is that, although it is quite stable in acetonitrile solution in a silanized amber bottle, it is has relatively poor stability in solution once installed on the DNA synthesizer. Consequently, we have not been able to supply a sulfurizing solution, preferring to supply the powdered reagent along with an appropriate silanized bottle. The customer then weighs an appropriate amount of reagent into the silanized bottle and adds acetonitrile at a concentration of 1g/100mL. Over the years, we have considered other sulfurizing reagents but we were not able to find another reagent that exhibits the same fast sulfurization kinetics along with improved stability on the synthesizer.

RNA Sulfurization

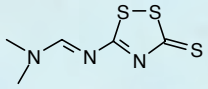
The most common usage for oligonucleotide phosphorothioates has been in the production of antisense oligodeoxynucleotides destined for use in identifying or modifying gene expression. Now, phosphorothioate linkages are popping up in the RNA world and sulfurizing RNA linkages with reagents like Beaucage Reagent has proved to be much more difficult than DNA linkages.

The phosphorothioate (PS) linkage is a not-so-expensive way of increasing the stability of nucleic acids and increasing nuclease resistance of RNA. Now, it has been shown[2] that fully PS oligos can promote the delivery of siRNA in cell culture. This siRNA uptake is sequence-independent and the length seems to vary between 30 and 70 nucleotides depending on the cell line. Even though this method is not yet as efficient as the cationic lipids, it opens the way to possible new methods. Reasons that may explain this are not understood at this time.

FIGURE 1: STRUCTURES OF SULFURIZING REAGENTS



Sulfurizing Reagent (Beaucage) (1)



Sulfurizing Reagent II (DDTT) (2)

Another paper[3] describes a method for the inactivation of micro RNA (miRNA) that may help to elucidate their functions. It uses 2'-OMe-RNA oligonucleotides (23-mers, complementary to a target miRNA) with a cholesteryl group at the 3´terminus and phosphorothioates at positions 1 and 2 at the 5´end and at the last four positions at the 3´end. These oligos are called antagomirs. These molecules promote the cleavage of complementary miRNAs and thus should allow analysis of their function. The role of the PS linkages presumably is the stabilization against degradation in the mouse experiments as it is standard in the antisense field in such in vivo situations.

And finally, a recent paper[4] shows that PS does not systematically abolish siRNA activity, opening the way for some potentially less expensive stabilization of such molecules. Incorporation of 2'-OMe (in the sense strand) in combination with PS linkages should confer to siRNA increased resistance to degradation by nucleases, as well as prolonged serum retention. And it is also possible that such easy modification of siRNA may increase the specificity by eliminating sense strand recruitment in the RISC complex and thus reducing a source of off-target effect.

Sulfurizing Reagent II

A new sulfurizing reagent must, therefore, exhibit all the good properties of Beaucage Reagent while adding good stability in solution on the synthesizer AND offering strong ability to sulfurize RNA linkages. We are happy to offer Sulfurizing Reagent II, 3-((Dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-3-thione, DDTT (2).

Use of Sulfurizing Reagent II in RNA Synthesis

Our experiments demonstrate that a 0.05 M solution of Sulfurizing Reagent II is recommended for the synthesis of RNA phosphorothioates. A sulfurizing time of 2-4 minutes generated oligophosphorothioates of high quality. This was true for both TOM-RNA and TBDMS-RNA monomers. As shown in Figure 2, Beaucage Reagent was significantly more sluggish than Sulfurizing Reagent II. Representative HPLC analyses[5]

FIGURE 2: CYCLE EFFICIENCY USING SULFURIZING REAGENTS AND U-TOM-RNA MONOMER

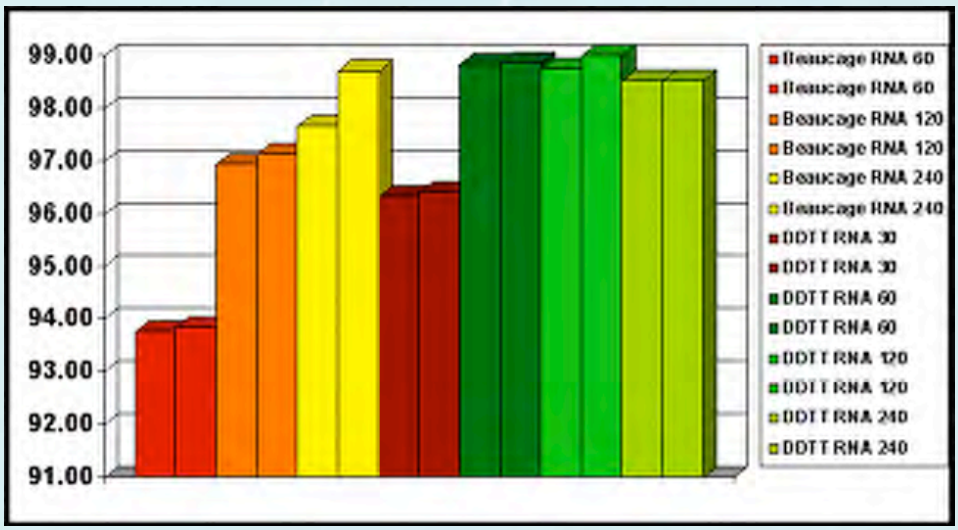
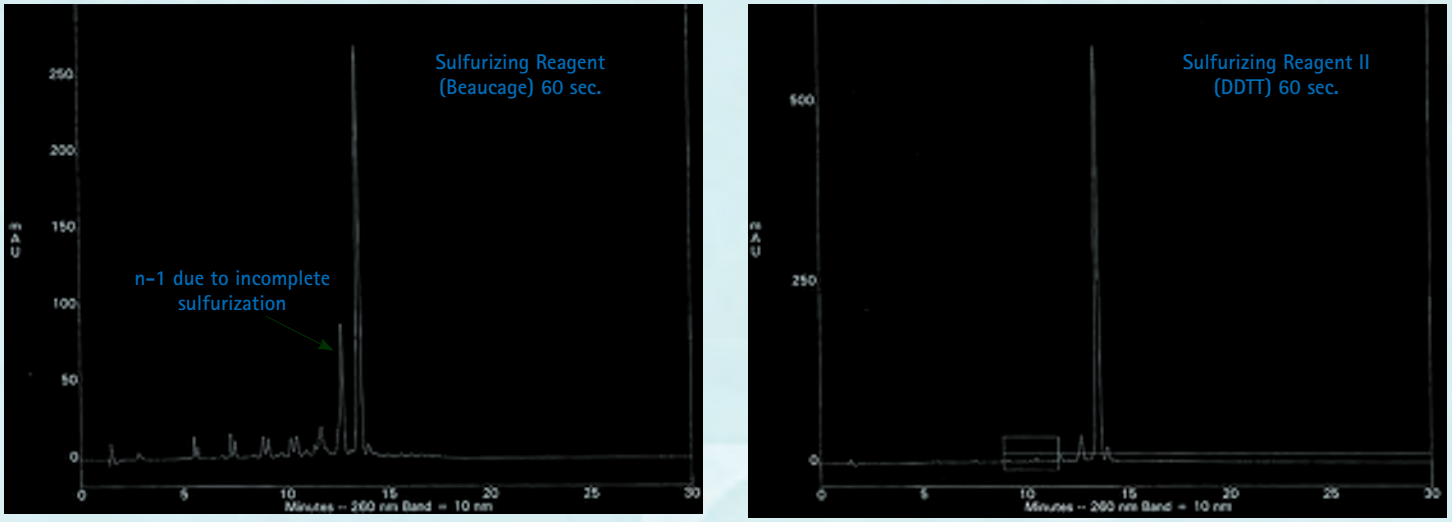


FIGURE 3: ION EXCHANGE HPLC ANALYSIS OF RNA OLIGOS SULFURIZED WITH SULFURIZING REAGENTS



Column: Dionex B100, 4.6mm x 250mm
Buffers: A- 10mM $NaClO_4$, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4 ; B- 600mM $NaClO_4$, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4
Flow rate: 1mL/Min

of RNA oligos are shown in Figure 3. The chromatogram on the left was obtained from sulfurizing U-TOM-RNA linkages for 60 seconds with Beaucage Reagent. The large n-1 peak is due to incomplete stepwise sulfurization and accumulation of deletions. The chromatogram on the right was an identical synthesis except using Sulfurizing Reagent II. Individual RNA sequences, especially those containing stretches of purine nucleoside residues are more difficult to sulfurize irrespective of the reagent used. To obtain a high degree of sulfurization with those oligonucleotides, a 0.1 M solution of Sulfurizing Reagent II and/or extended contact time may be required.

Use of Sulfurizing Reagent II in DNA Synthesis

Sulfurizing Reagent II was compared to Beaucage Reagent in the synthesis of DNA phosphorothioates. The quality of the products was identical with both reagents. In DNA synthesis, the cycle efficiency seemed to be optimal for Sulfurizing Reagent II when using a 60 second sulfurizing time.

Solubility and Stability of DDTT

The solubility of Sulfurizing Reagent II in mixtures of anhydrous pyridine and acetonitrile or anhydrous pyridine and THF is relatively limited and increases with the increasing concentration of pyridine. Some useful compositions are listed in Table 1. Under these conditions, Sulfurizing Reagent II forms stable solutions that do not display any loss of functional activity or precipitation of the reagent for a period of over 6 months. To prepare solutions of the desired concentration, we recommend first dissolving Sulfurizing Reagent II in the calculated amount of pyridine, which may require mild heat, followed by diluting the obtained solution with the required volume of acetonitrile or THF.

We are happy to offer Sulfurizing Reagent II as a powder and as a 0.05M solution in pyridine/acetomitrile.

References:

1. R.P. Iyer, W. Egan, J.B. Regan, and S.L. Beaucage, *J. Amer. Chem. Soc.*, 1990, **112**, 1253-1254.
2. M. Overhoff and G. Sczakiel, *EMBO Rep*, 2005, **6**, 1176-81.
3. J. Krutzfeldt, et al., *Nature*, 2005, **438**, 685-9.
4. B.A. Kraynack and B.F. Baker, *RNA*, 2006, **12**, 163-76.
5. B.J. Bergot and W. Egan, *Journal of Chromatography*, 1992, **599**, 35-42.

TABLE 1: **SOLUBILITY OF SULFURIZING REAGENT II**

Concentration of Sulfurizing Reagent II	Solvents and their ratio (v/v) Pyridine:ACN	Pyridine:THF
0.1M	100:0	40:60
0.06M	50:50	-
0.05M	40:60	20:80
0.03M	30:70	-
0.02M	20:80	0:100

Notes: Dissolve Sulfurizing Reagent II in pyridine first and then add acetonitrile (ACN) or tetrahydrofuran (THF). Molecular Weight is 205.31

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Sulfurizing Reagent II (DDTT)	40-4037-10	1.0g	50.00
	40-4037-20	2.0g	100.00
(Dissolve at a concentration of 1g/100mL to form an approximate 0.05M solution)			
Sulfurizing Solution			
0.05M Sulfurizing Reagent II in pyridine/acetonitrile	40-4137-51	100mL	100.00
	40-4137-52	200mL	200.00
	40-4137-57	450mL	450.00
3'-Cholesteryl-TEG CPG	20-2975-01	0.1g	120.00
	20-2975-10	1.0g	995.00
1 µmole columns	20-2975-41	Pack of 4	200.00
0.2 µmole columns	20-2975-42	Pack of 4	120.00

CAPS FOR INCREASED DUPLEX STABILITY AND BASE-PAIRING FIDELITY AT TERMINI

Glen Research is pleased to offer two new products for the synthesis of oligonucleotides. The new products allow for the preparation of hybridization probes with increased affinity for complementary sequences. Both are phosphoramidites that can be readily introduced via automated DNA synthesis at the end of solid phase syntheses. The caps favor the formation of stable Watson-Crick duplexes by stacking on the terminal base pair (Figure 1). Melting point increases of over 10 °C per modification can be realized for short duplexes.[1,2]

The caps fit canonical Watson-Crick base pairs and do not stack well on mismatched base pairs. This leads to increased base pairing selectivity at the terminal and the penultimate position of oligonucleotides featuring the caps. Base pairing fidelity is usually low at the termini, where fraying occurs frequently in the absence of caps. The beneficial effects of the caps are also realized when longer target strands are bound, so there is no need for blunt ends for the duplexes formed.[1,2] The caps, when attached to the terminus of an oligonucleotide, also facilitate purification as their lipophilicity leads to prolonged retention on reversed phase columns or cartridges. Finally, capping of termini may discourage the degradation of oligonucleotides by exonucleases. Figure 2 shows the structures of the phosphoramidites producing the caps.

Trimethoxystilbene

Stilbenes have been successfully employed for covalently bridging the termini of oligonucleotide hairpins.[3] The trimethoxystilbene cap that is now available is the result of a recent study that focused on stilbenes that are covalently linked to only one of the two strands forming a duplex.[1] The three methoxy substituents interact with the 2'-methylene group of the nucleoside in the target strand (Figure 3), as shown in a recent high resolution structure.[4] Together with the stacking on the terminal base pair, this leads to much-improved mismatch discrimination. The effect is observed for any of the four possible base pairs at the terminus.[1]

When employed for hybridization probes immobilized on a glass surface in the form of a DNA microarray, the trimethoxystilbene cap increases the signal for the fully complementary target strand.[1] This feature is particularly important for A/T-rich sequences that often cause false negatives. The selective stabilization of neighboring Watson-Crick base pairs helps to suppress cross hybridization that would otherwise lead to stronger false positive results.[1]

Figure 4 shows a recent example of a hybridization result involving a DNA microarray, where enhanced target signal as well as improved mismatch discrimination at the very terminus of the probe:target duplex are demonstrated.

FIGURE 1: STACKING OF CAP ON TERMINAL BASE PAIR

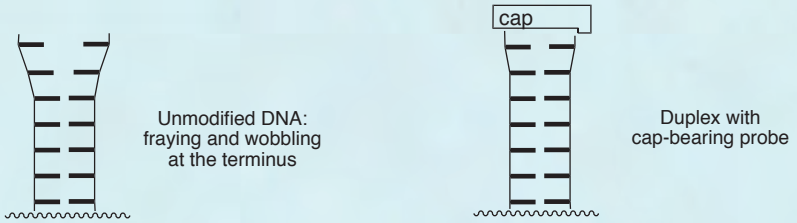
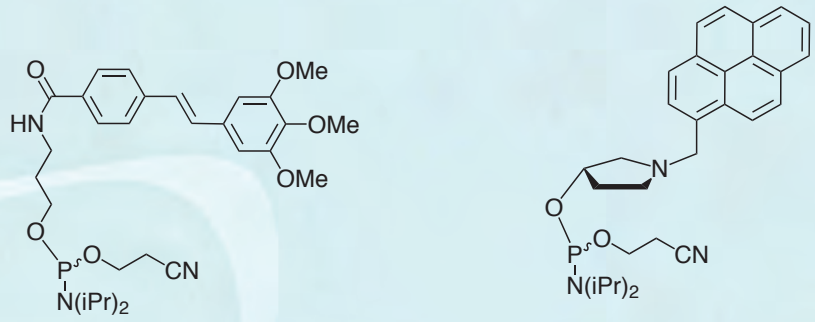


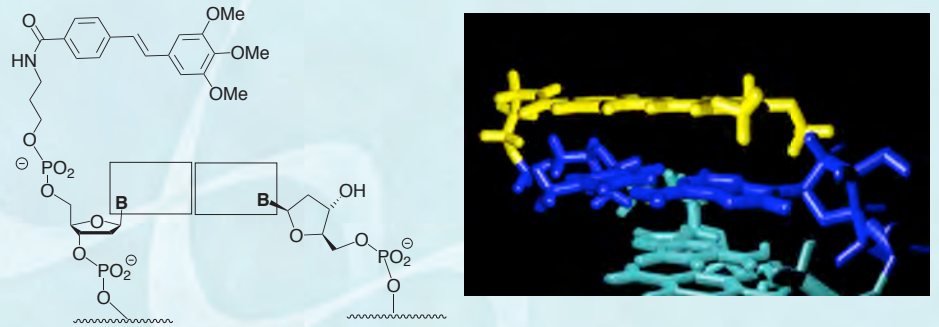
FIGURE 2: STRUCTURES OF CAP PHOSPHORAMIDITES



Trimethoxystilbene (1)

Pyrenylmethylpyrrolindol (2)

FIGURE 3: STRUCTURE OF DNA DUPLEX WITH TRIMETHOXYSTILBENE CAPS



(a) Two-dimensional structure of duplex with trimethoxystilbene cap. (b) Three-dimensional structure of DNA duplex with trimethoxystilbene caps, as determined by NMR and restrained molecular dynamics.

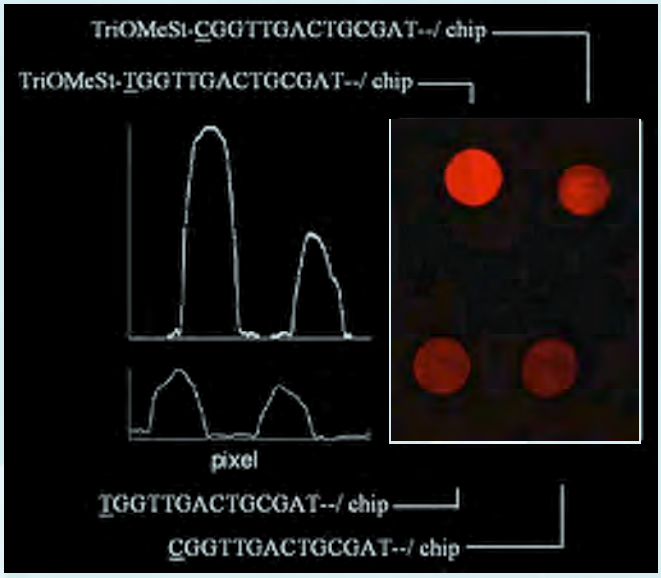
Pyrenylmethylpyrrolindol

This phosphoramidite (2), when employed in the last step of an oligonucleotide synthesis, will produce a cap that is more lipophilic than the trimethoxystilbene. The aromatic stacking moiety is linked to the terminus of the DNA through a more rigid, cyclic linker than in the case of (1). This feature may prove advantageous for researchers interested in exploiting the special photophysical properties of the pyrenyl substituent. The pyrrolindol linker is stereoregular, leading to a single product that can be readily purified by HPLC. The pyrenyl

cap is the lead compound discovered in a recent combinatorial study that evaluated over 40 different caps.[2] The cap proved particularly successful for hybridization probes with a 5′-terminal deoxyadenosine residue.[2] Again, its duplex-stabilizing effect does not require blunt ends. The tertiary amino group can be expected to be protonated at physiological pH, producing a cationic functionality that may help to attract target strands electrostatically. The five membered ring presenting the pyrenyl stacking unit mimics the deoxyribose of natural nucleosides, making duplexes terminating in this cap more similar in shape to unmodified DNA than those capped with the trimethoxystilbene.

We are indebted to Professor Clemens Richert, Institute of Organic Chemistry, University of Karlsruhe, for sharing with us the information included in this article.

FIGURE 4: HYBRIDIZATION WITH AND WITHOUT TRIMETHOXYSTILBENE CAPS



Results of hybridization experiment with 14mer DNA probes immobilized[5] on a DNA microarray and target strand 5′-Cy3-ATCGCAGTCAACCA-3′ (incubation at 70 °C for 45min, SSC buffer, pH 7.0; fluorescence readout). The fluorescence scan is shown on the right and the integration on the left. The top row features probes with 5′-attached trimethoxystilbene caps, the bottom row the same probes lacking the caps. Spots in one row differ by the 5′-terminal nucleobase. Capture efficiency and mismatch discrimination are significantly improved in the presence of the cap.

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ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5′-Trimethoxystilbene Cap Phosphoramidite	10-1986-90	100 µmole	195.00
	10-1986-02	0.25g	495.00
5′-Pyrene Cap Phosphoramidite	10-1987-90	100 µmole	195.00
	10-1987-02	0.25g	495.00

SYNTHESIS OF BRANCHED DNA WITH A COMB STRUCTURE

Introduction

By far the most common approach to DNA diagnostics is amplification of the target sequence to produce enough copies for a signal to be observed using conventional detection systems. However, an alternative approach does exist - direct analysis of the target DNA by signal amplification. This latter technique requires that the synthetic oligonucleotide should contain the primary sequence attached to many identical copies of the secondary sequence. It is the detection of the many copies of the secondary sequence that serves to amplify the signal. The resulting branched oligonucleotide has been aptly described as comb-like, with the primary sequence in the handle and the secondary sequences being the teeth of the comb.[1] Using this strategy, as few as 50 molecules of the HIV-1 genome have been quantified in human serum.[2]

A branching monomer is clearly required to construct comb-like oligonucleotides. For a monomer to generate a branch point in an oligonucleotide sequence, it requires an intermediate with 3 hydroxyl groups. The first hydroxyl is used to generate the phosphoramidite that is necessary for coupling. The second is protected with a DMT group for normal deblocking with acid prior to chain elongation. The third should be protected with a group that is stable during the synthesis of the primary sequence, but is easily removed after completion of the primary sequence, allowing the secondary sequences to be prepared by further synthesis cycles on the original synthesis column. The developers of the comb system from Chiron Corporation evaluated[3] several protecting groups for the third hydroxyl and chose levulinyl (Lev), which is specifically removed using a reagent containing hydrazine hydrate*, acetic acid and pyridine. Although non-nucleosidic structures were evaluated, the authors[3] chose to use the dC derivative, (1) in Figure 1, as the branching monomer.

Synthesis of Simple Branch Structures

This product is very straightforward to use in the synthesis of branched oligos with only a few branch points, as shown in Table 1. If a non-branching control is desired, simply deprotect in ammonium hydroxide as required by the protecting groups on the nucleobases.

Synthesis of Complex Comb Structures

The synthesis of complex structures is much more involved but the authors[4] offer some suggestions to streamline the process, as shown in Table 2. Using this general procedure, the authors were able to prepare comb oligonucleotides containing as many as 50 branch sites.

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1. T. Horn and M.S. Urdea, *Nucleic Acids Res*, 1989, **17**, 6959-67.
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TABLE 1: SYNTHESIS OF SIMPLE BRANCHED OLIGONUCLEOTIDES

- Carry out the synthesis of the primary sequence with no changes from the regular synthesis cycle.
- Remove the final DMT group, and terminate by capping for 30 minutes.
- Remove the synthesis column from the synthesizer.
- Remove the levulinyl protecting group (Lev) selectively without cleavage of the oligonucleotide from the CPG by treatment with 0.5 M hydrazine hydrate* in 1:1 pyridine/acetic acid.
- Fit the column with syringes and push the solution back and forth across the column.
- Leave for 15 minutes at room temperature.
- Rinse the column with 1.5 mL of 1:1 pyridine/acetic acid (3x) and then 1.5 mL of ACN (3x).
- Dry the CPG support with an argon stream and proceed with the synthesis of the branching sequence.

TABLE 2: SYNTHESIS OF COMPLEX COMB STRUCTURES

Primary Sequence

- Synthesize the primary target detecting sequence on 2000 Å CPG using the standard synthesis cycle.
- Add an additional T20 to the 5' of the target sequence to distance the comb structure from the CPG surface.
- Add dC Brancher Phosphoramidite (B) using the general formula (BTT)n.
- If desired, remove the final DMT group, and terminate by capping for 30 minutes.
- Remove the synthesis column from the synthesizer and using a pair of disposable syringes, remove the Lev protection using 10 mL of 0.5 M hydrazine hydrate* in pyridine: acetic acid (1:1). Pass the solution back and forth through the column for a minimum of 15 minutes. Sequences with many branches may require a longer treatment of 90 minutes.
- Rinse the column with 1.5 mL pyridine:acetic acid (1:1) (3X) and then with 1.5 mL acetonitrile (3X).
- Dry the CPG support with an argon stream and proceed with the synthesis of the secondary "comb" sequence.

Comb Sequence

- Change the synthesis cycle to increase the amidite delivery to correspond to the increase in synthesis scale, (e.g., a 1-µmole synthesis with a 15 branch comb now becomes a 15-µmole synthesis). This can be achieved by increasing the number and time of amidite:activator deliveries.
- Increase the coupling wait time to 3 minutes to maximize coupling efficiency.

***NOTE: HYDRAZINE HYDRATE IS A VIOLENT POISON THAT IS BOTH VOLATILE AND READILY ABSORBED THROUGH SKIN. USE APPROPRIATE SAFETY PRECAUTIONS!**

Potent Inhibition of Cytosine-5-methyltransferases

The methylation of cytosine in the CpG motif of DNA almost invariably leads to a reduction in gene expression and is one of the most prevalent methods for the epigenetic regulation of genes. Cytosine-5-methyltransferases are found in everything from archaebacteria to mammals and when the regulation of cytosine-5-methyltransferases goes awry, cancer can result. DNA methyltransferases were found to be essential for cancer cell survival[1] and to work in concert with p53[2]. The mechanism of action for this family of enzymes involves attack of a cysteine thiol group on the C6 position of cytosine, leading to a transient dihydrocytosine intermediate, which then facilitates the nucleophilic attack by C5 on the activated methyl group of the S-adenosyl-L-methionine cofactor.

As with many enzymes, the intermediate can be trapped using a suicide substrate and 5-fluoro-cytosine[3] has been used extensively in this role. An alternate strategy is to use a transition-state mimic that binds to the active site with high affinity. An excellent candidate was found in 5-aza-5,6-dihydrocytosine. Despite not being covalently bound to the enzyme, it was found[4,5] to be a more potent inhibitor of cytosine-5-methyltransferases than 5-fluoro-cytosine. Fortunately, 5-aza-5,6-dihydro-dC is amenable to conversion to the phosphoramidite[6] and is compatible with standard oligonucleotide synthesis and deprotection conditions. We enthusiastically offer this monomer, (2) in Figure 1, as a new tool for use in methyltransferase research. We are pleased to highlight the work done on the chemistry and biology of 5-azacytosines and their interaction with cytosine-5-methyltransferase by Victor Marquez and co-workers.

References:

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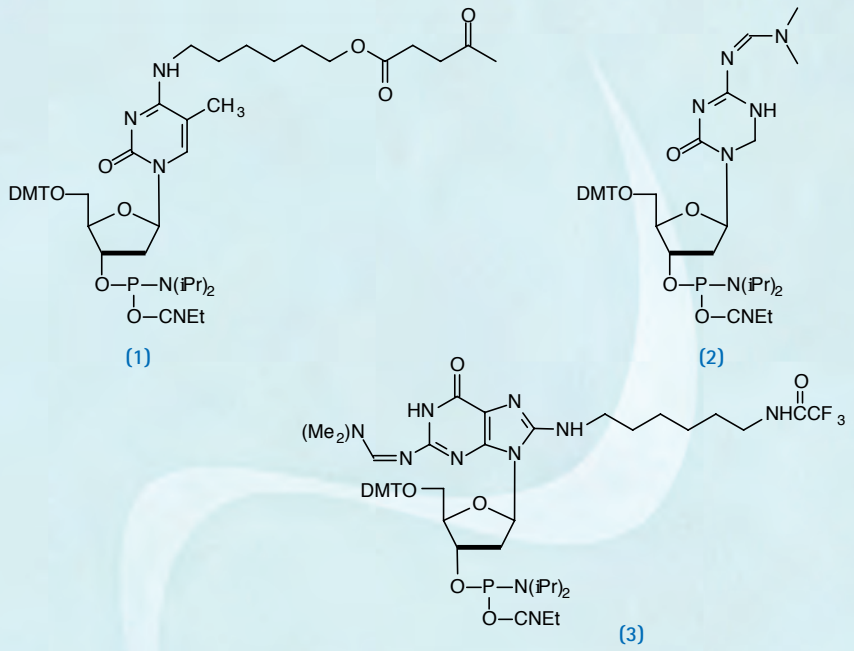
FIGURE 1: MONOMER STRUCTURES



Amino-Modifier C6 dG

When we introduced Amino-Modifier C6 dA in 2003, we were aware that the structure was not optimal and that there would be some destabilization of the base pair with dT. Indeed, the base pair was destabilized by 2°C per insertion but binding was still found to be specific for dA. We noted at the time that the optimal attachment point in purines was probably the 7-position of the 7-deaza purine and preferably using the 7-deaza-8-aza analogue. However, these analogues are very difficult to make and are consequently very expensive. In the case of Amino-Modifier C6 dA, we have been encouraged by its popularity despite these shortcomings. So we felt it would be reasonable to offer the dG analogue, Amino-Modifier C6 dG, (3) in Figure 1. This dG analogue was found to maintain specificity for dC in oligos but the melting temperature was reduced by 3°C per insertion relative to the regular dG-dC base pair. (Melting temperatures for oligos containing a mismatch with dA, dG or T opposite dG were reduced by an additional 6-7°C.) Again, these structural characteristics may not be the ideal but Amino-Modifier C6 dG maintains a fair balance of performance and price.

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
5-Aza-5,6-dihydro-dC-CE Phosphoramidite	10-1511-95	50 µmole	180.00
	10-1511-90	100 µmole	360.00
	10-1511-02	0.25g	1120.00
Amino-Modifier C6 dG	10-1099-95	50 µmole	240.00
	10-1099-90	100 µmole	480.00
	10-1099-02	0.25g	1100.00

FLUOROUS AFFINITY PURIFICATION OF OLIGONUCLEOTIDES

- A higher affinity alternative to RP cartridge purification.
- One-pass loading without ammonia removal.
- High recoveries (typically 70–100%).
- High selectivity for removal of failure sequences, even with long oligonucleotides.
- Excellent for longer oligonucleotides (e.g. 50–100+ mers); recoveries nearly quantitative.
- Does not require new techniques.

The fluorous affinity purification of oligonucleotides is a quick and simple affinity-based method for the purification of oligonucleotides that relies on the strong interaction of fluorous-tagged oligonucleotides (made with fluorous-tagged phosphoramidites, Figure 1) with the fluorinated adsorbent present in Fluoro-Pak™ columns.[1] Fluorous affinity purification is operationally similar to DMT-on purification using a reverse-phase (RP) adsorbent, e.g., RP cartridge purification, except that it involves a much stronger affinity interaction and is thus able to afford higher selectivities and recoveries, even with long oligos. DMT-on RP cartridge purification is limited to relatively short oligonucleotides, typically ≤30-40-mers, since the relative hydrophobic contribution of the lipophilic DMT group diminishes as the chain length increases, resulting in lower overall yields and a diminished selectivity of the adsorbent for the desired oligonucleotide over failure sequences.

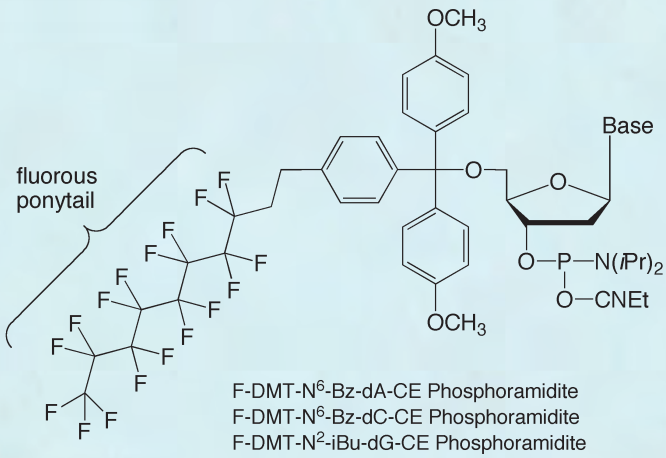
Highly fluorinated organic compounds are both hydrophobic and lipophobic, preferring instead to associate with other fluorinated substances.[2] For example, perfluorohexane is insoluble in both water and hexane. Organic molecules that have both an organic domain (e.g. an oligonucleotide) and a perfluoroalkyl domain (e.g., a linear perfluoroalkyl "ponytail") are known as fluorous molecules, and may be separated from non-fluorous molecules by interaction with fluorinated separation media such as Fluoro-Pak columns. Fluorous-fluorous interactions are strong and selective. Fluorous affinity interactions are well-documented in the chemistry literature, and are now finding application in more biological areas, e.g., proteomics[3] and now oligonucleotides.[1,4]

The fluorous affinity purification of oligonucleotides is similar to DMT-on RP cartridge purification, and we have developed protocols that are as "plug-and-play" as possible.[5] The first step is to install a single nucleotide at the 5'-terminus of the oligonucleotide using a fluorous-tagged phosphoramidite (Figure 1). The fluorous tag takes the form of a fluorous DMT group ("FDMT" group), where a fluorous ponytail is attached via an ethylene spacer to a normal DMT group. The FDMT group is designed so that it behaves just like a DMT group: the rate of detritylation is very similar to that observed for the DMT group and the absorbance maximum of FDMT cation (504 nm) is identical to DMT cation. Please note that only one coupling of a 5'-O-FDMT phosphoramidite is required; normal DMT amidites are used for the earlier steps in the synthesis. The synthesis is run in DMT-on mode, leaving the FDMT group in place. FDMT-bearing amidites are entirely soluble in acetonitrile and couple normally.

The FDMT-on oligonucleotide is cleaved from the solid support as usual and the base protecting groups are removed according to standard methods. If ammonia is used, it is not necessary to evaporate it, since Fluoro-Pak columns use a pH-stable polymeric matrix. HPLC analysis of a crude 75-mer mixture shows that the fluorous-tagged oligonucleotide is strongly retained, even on an RP-HPLC column (Figure 2).

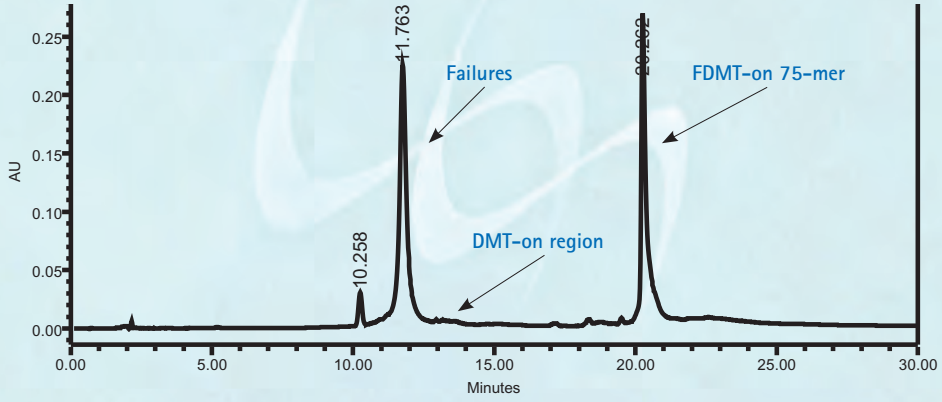
The crude deprotection solution is diluted with a salt-containing loading buffer and applied to a Fluoro-Pak column. Binding of the FDMT-tagged oligonucleotide occurs in one pass, leaving most of the failure sequences unbound. Washing with 10% acetonitrile in 0.1 M TEAA removes the rest of the failures. Additional failure washes are unnecessary, but they reveal that the fluorous-tagged oligonucleotide is still retained; no leaching from the

FIGURE 1: **FLUOROUS PHOSPHORAMIDITES FEATURING A FLUOROUS DIMETHOXYTRITYL (FDMT) GROUP**



F-DMT-N^6-Bz-dA-CE Phosphoramidite
F-DMT-N^6-Bz-dC-CE Phosphoramidite
F-DMT-N^2-iBu-dG-CE Phosphoramidite
F-DMT-dT-CE Phosphoramidite

FIGURE 2: **HPLC ANALYSIS OF A CRUDE 75-MER**



HPLC analysis of a crude 75-mer, showing that the fluorous-tagged oligonucleotide is strongly retained. Waters Spherisorb ODS-2 (5 µm, 4.6 x 150 mm, 1 mL/min), mobile phase A = 0.1 M aqueous TEAA; mobile phase B = acetonitrile.

column is observed, even with 100-mers. Such selectivity is unprecedented with DMT-on cartridge purification. On-column detritylation with TFA followed by elution of the purified oligonucleotide is then carried out. As an example, fluorous-tagged mixed-base 75-mers (200 nmol scale) were purified by fluorous affinity purification to provide 9-11 A260 units of the fully deprotected material, free from failure sequences. This represented a nearly quantitative recovery of the available FDMT-tagged oligonucleotides present in the crude ammonia deblock solutions (estimated by HPLC). Ion-exchange HPLC of the crude 75-mer with DMT removed is shown in Figure 3. The ion-exchange chromatogram of the fluorous-purified 75-mer is shown in Figure 4. The method was extended to 100-mers, which resulted in recoveries of 76-100% of the available tagged oligonucleotides, again free of failure sequences.

Fluoro-Pak™ and Fluoro-Pak™ II Columns

Fluoro-Pak adsorbent has fluorinated organic groups bound to a pH-stable polymeric resin and is ideal for the purification of fluorous-tagged oligonucleotides. Multiple pore and particle sizes have been evaluated in order to provide optimal performance with reasonable back-pressure. Flow may be induced by pressure or vacuum. Fluoro-Pak columns should be used with fluorous-tagged oligonucleotides; they are not designed for normal DMT-on purifications. Further, Fluoro-Pak columns were designed specifically for the fluorous purification of oligonucleotides and have not been validated for other uses, although they may provide a good alternative to traditional silica-based fluorous adsorbents. Two columns are available: Fluoro-Pak Columns, containing 75 mg of adsorbent, useful for up to 0.2 micromole purifications, and Fluoro-Pak II Columns, containing 150 mg of adsorbent, useful for 1 micromole purifications.

Additional Fluorous Monomers

In addition to the basic four fluorous deoxyribonucleoside phosphoramidites, fluorous versions of other nucleic acid synthesis reagents are in development.

A significant percentage of longer oligonucleotides are phosphorylated at the 5' terminus to allow further reactions, e.g., ligation, to take place. Fluorous Chemical Phosphorylation Reagent II (F-CPR-II), the fluorous version of CPR-II, allows the combination of fluorous affinity purification with 5'-phosphorylation.[6] In situations where all long oligos are required to be 5'-phosphates, F-CPR-II acts as the common fluorous tag for all oligos.

Detailed information on the fluorous purification method may be found in the booklet "User Guide: Fluorous Affinity Purification of Oligonucleotides", which is included with each order.[5]

We thank Dr. Will Pearson of Berry & Associates, Inc. for helpful discussions during the preparation of this article.

Intellectual Property

"Fluoro-Pak" is a trademark of Berry & Associates, Inc. Products for Fluorous Affinity Purification of Oligonucleotides: Patents applied for, Berry & Associates, Inc.

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FIGURE 3: IEX HPLC ANALYSIS OF A CRUDE 75-MER

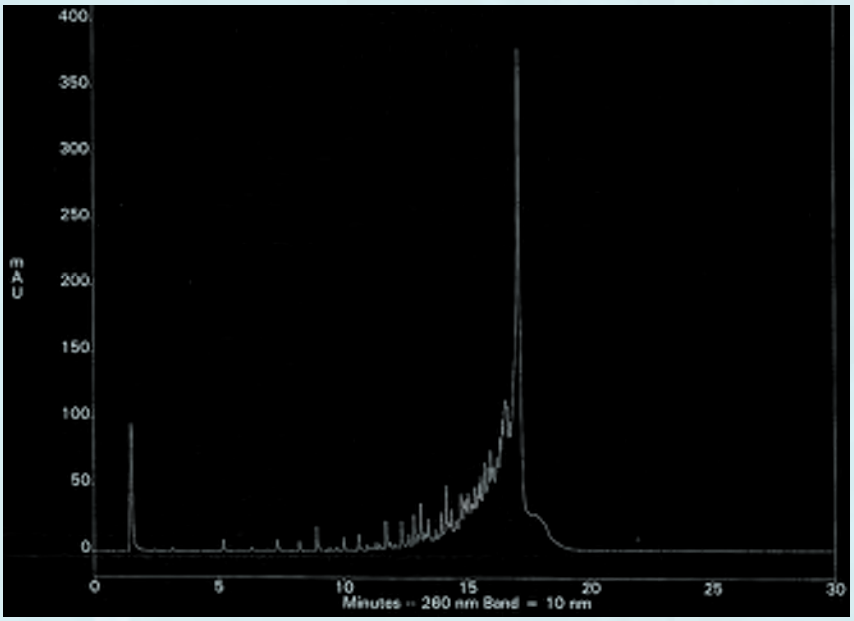
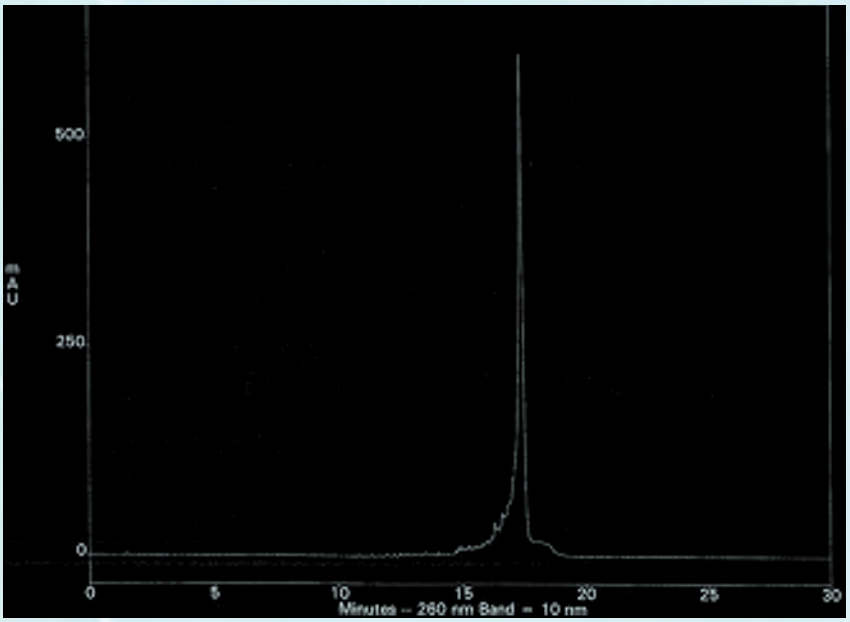


FIGURE 4: IEX HPLC ANALYSIS OF A FLUOROUS-PURIFIED 75-MER



Column: Dionex DNAPac PA200, 4 x 250mm Buffers: A- 10mM $NaClO_4$, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4 ; B- 600mM $NaClO_4$, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4 Gradient: 0-40% Buffer B at a flow rate of 1mL/min.

(Continued from Page 11)

Further, the use of these products is licensed under U.S. Patents 6,673,539, 6,156,896; 5,859,247; and 5,777,121 and one or more pending patents owned or controlled by Fluorous Technologies, Inc. CPR-II is subject to US Patent 5,959,090, assigned to Glen Research Corporation.

References:

1. Pearson, W. H.; Berry, D. A.; Stoy, P.; Jung, K.-Y.; Sercel, A. D. *J. Org. Chem.* 2005, **70**, 7114-7122.
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6. Guzaev, A.; Salo, H.; Azhayev, A.; Lönnberg, H. *Tetrahedron* 1995, **51**, 9375-9384.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Phosphoramidites			
F-DMT-dA-CE Phosphoramidite	10-1400-90	100 µmol	75.00
	10-1400-02	0.25g	140.00
F-DMT-dC-CE Phosphoramidite	10-1410-90	100 µmol	75.00
	10-1410-02	0.25g	140.00
F-DMT-dG-CE Phosphoramidite	10-1420-90	100 µmol	75.00
	10-1420-02	0.25g	140.00
F-DMT-dT-CE Phosphoramidite	10-1430-90	100 µmol	75.00
	10-1430-02	0.25g	140.00
Fluorous Chemical Phosphorylation Reagent II *(F-CPR-II)*	10-1904-90	100 µmol	95.00
	10-1904-02	0.25g	195.00
Columns			
Fluoro-Pak™columns (75 mg adsorbent for ≤200 nmol syntheses)	61-2100-10	Pack of 10	100.00
	61-2100-10 A (with Luer adaptor)	Pack of 10	100.00
Fluoro-Pak™ II columns (150 mg adsorbent, for ≤1µmol syntheses)	61-4100-10	Pack of 10	135.00
	61-4100-10 A (with Luer adaptor)	Pack of 10	135.00
Loading Buffer	61-4120-52	200 mL	35.00

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