Glen Research

REPORT

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Antisense Oligonucleotides

Recently, we attended a meeting in Rockville, MD cosponsored by the National Cancer Institute and the National Institute for Allergy and Infectious Diseases. Entitled, Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression: Therapeutic Implications, the conference provided chemists and biologists from academia, research institutes and industry with a forum to discuss research into the synthesis and biological activity of antisense oligonucleotides.

Frequent examples of the use of oligonucleotides to inhibit viral replication and translation of mRNA have been described in the literature. One of the main problems in this approach is that oligodeoxynucleotides are hydrolyzed by nuclease enzymes when they enter infected cells. A variety of modified oligonucleotides with nuclease resistant linkages and with enhanced antiviral activity were discussed. These modified oligonucleotides included phosphorothioates, phosphorodithioates, phosphotriesters, methyl phosphonates, and oligo-[α]-deoxynucleotides. Although the variety of linkages is wide, the majority of biological data presented used phosphorothioates.

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Probes and Affinity Supports

In previous issues of the *Glen Research Report*, we have focused on reagents and techniques used to functionalize synthetic oligonucleotides at the 5'- and/or 3'-terminus. These techniques have been successfully applied to the synthesis of oligonucleotides labelled with biotin, fluorescent tags, or enzymes, and to the preparation of affinity supports. However, we could offer no method for attachment of labels at specific points along a defined sequence or for the direct synthesis of affinity supports. Both of these situations had already been addressed by Molecular Biosystems, Inc. (MBI) of San Diego and elegant solutions to both had been developed. We are now happy to make available two products designed to address these tricky situations. Amino-Modifier-dT (1) can be added in place of a Thymidine residue during oligonucleotide synthesis. After deprotection, the primary amine which is separated from the oligonucleotide by a 10 atom spacer arm, can be labelled or attached to an enzyme. Oligo-Affinity Support (OAS) (2) allows the synthesis of an oligonucleotide which can be deprotected while remaining attached to the support. The synthetic oligonucleotide support can then be used directly for the purification of DNA binding proteins.

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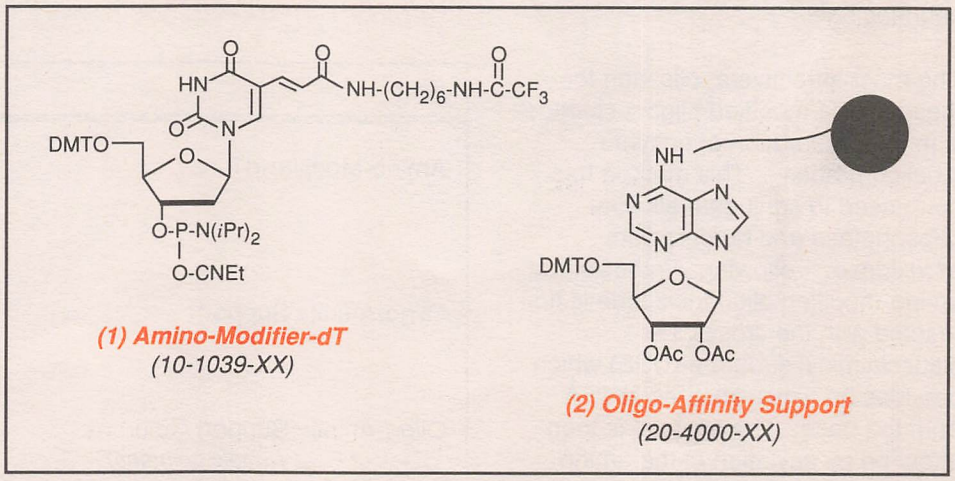

(1) Amino-Modifier-dT
(10-1039-XX)

(2) Oligo-Affinity Support
(20-4000-XX)

Figure 1: *Structure and Catalog Numbers of New Products*

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Amino-Modifier-dT

While a variety of products for introducing primary amines at the 5'- and/or 3'-terminus of synthetic oligonucleotides are commercially available, there is currently a scarcity of products for introducing primary amines at specific points along the sequence. Such products must be designed to be compatible with the techniques of automated synthesis and must not affect normal hybridization. Amino-Modifier-dT has been established to meet all the required criteria. The modified deoxynucleoside is produced from 2'-deoxyUridine via organometallic intermediates[1]. The compound allows the incorporation of a primary amine with a 10 atom spacer at any T site within an oligonucleotide. One to all of the T sites can be modified in this way without significantly affecting hybridization.

Amino-Modifier-dT reacts in a manner identical to normal monomers and the trifluoroacetyl protecting group on the primary amine is removed during the standard ammonium hydroxide deprotection step. Purification of the modified synthetic oligonucleotide is accomplished using standard procedures: purification cartridge, HPLC or gel electrophoresis.

The resulting amino-oligonucleotides have been labelled with a selection of reporter molecules, including biotin and fluorescent labels, for use as probes, as PCR primers, or in DNA sequencing[2].

The most attractive application for these amino-modified oligonucleotides is in the preparation of enzyme-labelled probes[3]. This method has been used to conjugate alkaline phosphatase and horseradish peroxidase. Following synthesis, the amino-modified oligonucleotide is first reacted with the crosslinker disuccinimidyl subarate (DSS) which provides an active NHS ester 25Å from the base. The enzyme is then attached by acylation of the amino groups with the NHS ester. This procedure results in a product composed of one oligonucleotide per enzyme. The activity of the enzyme portion of the conjugate is equivalent to that of the starting enzyme.

A further application of these modified oligonucleotides has been to use the amino groups to attach them to solid supports, allowing sandwich hybridizations and affinity separations[4].

Oligo-Affinity Support

Researchers are able to choose from a wide selection of techniques for attaching oligonucleotides to solid supports. However, these techniques usually require synthesis and purification of an amino-modified oligonucleotide, followed by its covalent linkage to an appropriately activated solid support. While these techniques are relatively straightforward, there still exists a need to prepare affinity supports simply and directly.

The Oligo-Affinity Support (OAS)[5] consists of fibers with an inner core of Teflon®, covalently coated with an organic layer of functionalized copolymers. A 25-carbon spacer arm terminating in adenosine with a DMT group at the 5'-position is attached to the support. Since the linkage to adenosine is stable to acid and base, the oligonucleotide formed by the standard synthesis protocol remains attached to the support after deprotection.

For use as an affinity support for the purification of DNA binding proteins[6], one oligonucleotide was synthesized and deprotected using ammonium hydroxide on the support. The complementary strand was then bound through normal hybridization. The affinity support was used to purify a sequence specific DNA binding protein 100 fold to near homogeneity. The Teflon support showed very low levels of non-specific binding of proteins. It also exhibited the further advantage of being composed of non-friable flexible fibers which do not shrink or swell. The matrix proved to be stable and reusable at least ten times.

Although the most likely use for the OAS is in the preparation of affinity supports, ligations and kinase reactions can also be carried out.

Teflon is a registered trademark of E.I. Dupont de Nemours & Company (Inc.).

References:

1. J.L. Ruth, C. Morgan, and A. Pasko, *DNA*, 1985, **4**, 93.
2. J.A. Brumbaugh, L.R. Middendorf, D.L. Grone, and J.L. Ruth, *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 5610.
3. E. Jablonski, E.W. Moomaw, R.H. Tullis, and J.L. Ruth, *Nucl. Acids Res.*, 1986, **14**, 6115.
4. J.L. Ruth, R.D. Smith, and R. Lohrmann, *Fed. Proc.*, 1985, **44**, 1622.
5. R. Lohrmann, L. Arnold, J.L. Ruth, *DNA*, 1984, **3**, 122.
6. C.H. Duncan and S.L. Cavalier, *Anal. Biochem.*, 1988, **169**, 104.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price ($)
Amino-Modifier-dT	10-1039-90	100µM	240.00
	10-1039-02	0.25g	480.00
	10-1039-05	0.5g	960.00
	10-1039-10	1.0g	1920.00
Oligo-Affinity Support	20-4000-01	0.1g	120.00
	20-4000-02	0.25g	285.00
	20-4000-10	1.0g	1060.00
Oligo-Affinity Support Columns *(Pack of Four 1µmole columns)*	20-4100-41	Pk/4	200.00

Rapid Trityl Colour Analysis Using a Fibre-Optic Probe

Richard T. Pon, Ph.D.
Director, Regional DNA Synthesis Laboratory
University of Calgary, Calgary, Alberta, Canada, T2N 4N1

The orange trityl cation released during the acidic deprotection step of oligonucleotide synthesis provides a rapid and quantitative signal of the efficiency of a solid-phase synthesis. Visual inspection of the colour intensity can readily detect gross failures (coupling <70%) but evaluation of more subtle changes requires an analytical approach. In our laboratory we have developed a quick yet quantitative trityl colour assay using a Brinkmann PC 800 colorimeter[1]. This digital colorimeter uses a flexible fibre-optic dipping probe which eliminates the need for sample transfer into cuvettes. This probe, along with a filter which measures absorbance off the peak maximum permits measurements to be made in the original collection tubes without the need for serial dilution. The PC-800 is much less expensive than a spectrophotometer and much faster to use.

Trityl colours are collected in polypropylene tubes (12mL) and diluted with 5% dichloroacetic acid/1,2- dichloroethane solution[2] (5mL), dispensed from a bottle-top dispenser. The exact volume is not critical as long as each tube is diluted identically. After mixing , the colours are measured using the standard 2cm path-length stainless steel probe supplied with the colorimeter. Measurement with either 470nm or 545nm filters for 0.2 or 1 micromole scale syntheses, respectively, will give absorbance readings within the desired range. Each sample can be measured in only a few seconds and quantitation of every coupling step in the synthesis can be quickly accomplished. Stepwise coupling yields can then be easily calculated by comparing successive values. Alternatively, molar quantities can be estimated using respective extinction coefficients of 1.3 and 7.9 mL micromole^{-1} cm^{-1}, for the absorbances at 545 and 470nm.

Although measuring the absorbance off the peak maximum of 505nm is convenient, the absorbance versus concentration relationship is not linear (see Figure). For precise work a calibration curve can be constructed, however for most applications the nonlinearity can be neglected as long as measurements are made in the most linear part of the curve (< 0.8 absorbance units).

In our laboratory, this assay is routinely used on a daily basis to monitor the sequences produced. A simple computer program converts the absorbance data into step yields and also lists the average yields for each base. This regular monitoring allows us to quickly detect small decreases in coupling yields which may be indicative of pending instrument or reagent failure. This type of assay is also extremely useful for evaluating new procedures or synthesis programs because it allows much easier quantitation of every coupling step than analysis with a conventional spectrophotometer.

Notes:

1. The PC 800 Probe Colorimeter and accessories are available from Brinkmann Instruments, Inc., Cantiague Road, Westbury, NY 11590; (516) 334-7500.

2. In our laboratory, 1,2-dichloroethane is preferred over dichloromethane for the detritylating reagent because it is less volatile and less toxic. However, the above procedure will also work with solutions of di- or trichloroacetic acid in dichloromethane.

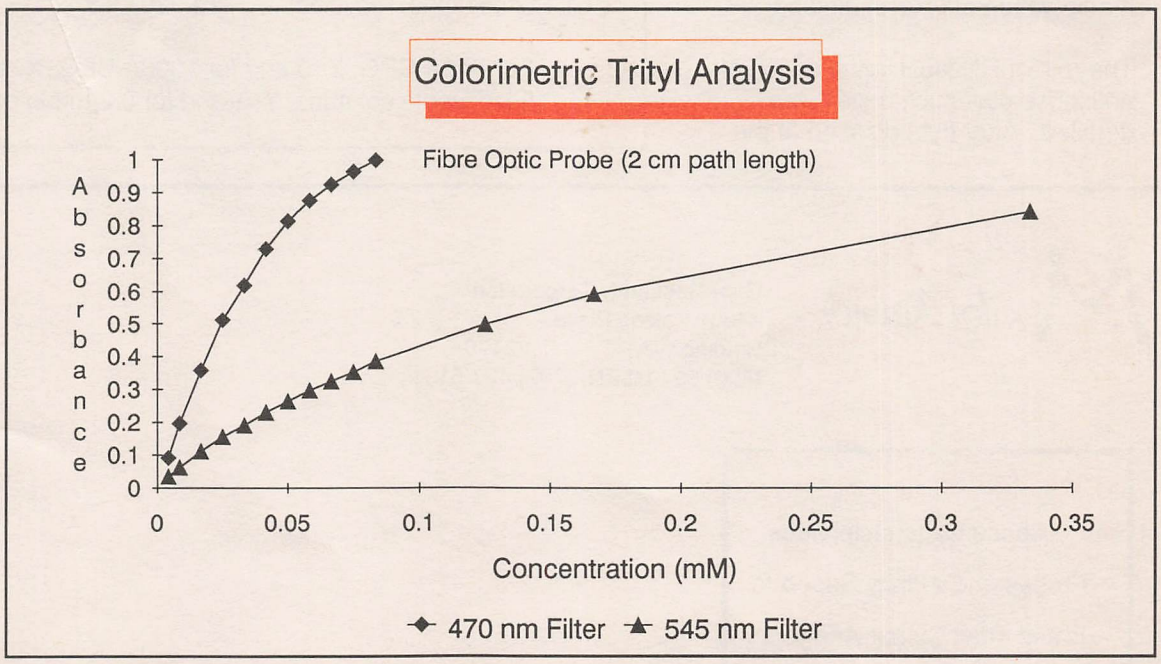

Figure: *Calibration Curve of Absorbance versus Trityl Concentration*

Antisense Oligonucleotides *(Continued from Front Page)*

Modification of oligonucleotides at the 5'- and/or 3'-terminus allows the attachment of molecules which may act as intercalating agents or as agents to cleave the complementary strand. Examples were presented demonstrating enhanced antiviral activity of acridine and anthroquinone conjugates. Similarly, intercalating agents which can be used to induce photocrosslinking, e.g., psoralen and proflavine, were also examined. Other intercalating agents, e.g., copper-phenanthroline, can be added to oligonucleotides to induce cleavage of the complementary strand. The ability to modify phosphate linkages to induce nuclease resistance and increase cell permeability, along with techniques to stabilize, crosslink or cleave duplexes, clearly opens the way for the development of highly specific therapeutic agents.

The intricacies of triple helix formation, including a third strand binding code, were reviewed by several groups. The formation of triplex DNA has been shown to inhibit several biological processes including replication and transcription. Such sequence specific intervention offers the potential for the manipulation of protein binding.

The role of RNaseH in the action of antisense oligonucleotides was detailed. After hybridization of the antisense oligonucleotide to mRNA, the RNA strand of the duplex is cleaved by RNaseH. The oligonucleotide is then recycled for further mRNA cleavage.

The state of the art of RNA synthesis, along with the potential of modified oligoribonucleotides as therapeutic agents was detailed.

Because of our belief that therapeutic agents will eventually be developed from these lines of research, we will continue to support the role of H-phosphonate chemistry in the synthesis of modified oligonucleotides. To help promote this methodology, we have reduced our H-phosphonate monomer prices as detailed below.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
dA-H-Phosphonate	10-1200-05	0.5g	60.00
dC-H-Phosphonate	10-1210-05	0.5g	60.00
dG-H-Phosphonate	10-1220-05	0.5g	60.00
dT-H-Phosphonate	10-1230-05	0.5g	60.00
dA-CPG columns, recycled	20-220X-4Y	Pk/4	36.00
dC-CPG columns, recycled	20-220X-4Y	Pk/4	36.00
dG-CPG columns, recycled	20-220X-4Y	Pk/4	36.00
dT-CPG columns, recycled	20-220X-4Y	Pk/4	36.00

Note: For 500Å CPG, X=0 and for 1000Å CPG, X=1.
For 1μmole columns, Y=1 and for 0.2μmole columns, Y=2.

Column Recycling Service

Since we are unable to supply unused columns for Milligen/Biosearch DNA synthesizers, we have recently begun to recycle columns at the request of end-users. Here's how it works. You return your used (disposable!) columns to us. We will disassemble and clean them, replace the support with fresh CPG, replace the filters if necessary, and reseal the columns. For this service, we offer a 40% discount from our normal column prices, with a final price shown below. Of course, we guarantee that the columns will not leak and that they will perform to our usual high standards or we will replace them. Sometimes recycling makes sense. If you think that this is one of these occasions, why not give it a try?

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Glen Research Corporation
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Sterling, VA 22170
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