The Glen Report

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GLEN RESEARCH

VOLUME 19

NUMBER 1

APRIL 2007

INSIDE

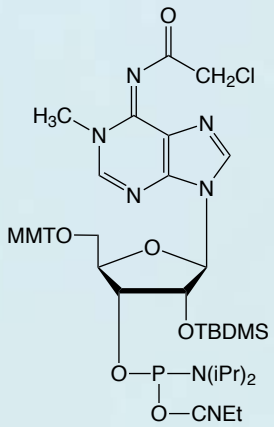
MODIFIED RNA PHOSPHORAMIDITES USEFUL IN siRNA RESEARCH AND BIOLOGICALLY SIGNIFICANT 1-METHYL-ADENOSINE

In all 20 years of Glen Research's existence, we have heard prediction after prediction that RNA synthesis was about to enjoy explosive growth. Although activity certainly increased slightly over the years thanks to studies on RNA structure and function relative to RNA viral activity, t-RNA and ribozymes (among others) growth could never have been defined as explosive. Finally, these predictions seem to be coming to fruition thanks to the advent of siRNA. Currently, researchers seem to be happy with the ready availability of unmodified siRNA but we predict that interest will continue to rise in the ability to use base, sugar and phosphate modifications to optimize siRNA activity. In this short article, we will focus on base modifications that are potentially useful in siRNA research. In the following article on Page 3, we will highlight a modified RNA base with significant biological interest and potential: 1-Methyl-Adenosine (m^1A), the phosphoramidite of which is shown in Figure 1 on this page.

A quick glance through our catalog or a browse through our web site quickly reveals a significant selection of base modified and minor RNA monomers available from stock. Here is the current list:

- Pyrrolo-Cytidine (10-3017)
- Inosine (10-3040)
- 5-Methyl-Uridine (ribo version of Thymidine) (10-3050)
- 4-Thio-Uridine (10-3052)
- 5-Methyl-Cytidine (10-3064)
- 2-Aminopurine Ribonucleoside (10-3070)
- 6-Thio-Guanosine (10-3072)
- 2,6-Diaminopurine Ribonucleoside (2-Amino-Adenosine) (10-3084)
- 5-Bromo-Uridine (10-3090)
- 5-Iodo-Uridine (10-3091)
- Amino-Modifier C6-U (10-3039)
- rSpacer (10-3914)

FIGURE 1: **1-ME-A-CE PHOSPHORAMIDITE**



We are happy to add to this list of RNA phosphoramidites the following naturally-occurring or modified bases:

- 7-Deaza-Adenosine (10-3001)
- 8-Aza-7-deaza-Adenosine (10-3083)
- Zebularine (10-3011)
- Pyridin-2-one Ribonucleoside (10-3012)
- Nebularine (Purine Ribonucleoside) (10-3041)
- PseudoUridine (10-3055)

The structures of these RNA phosphoramidites are shown in Figure 2, Page 2 and the following is a very brief introduction to each of these monomers.

Adenosine Analogues

7-Deaza-Adenosine is lacking nitrogen at the 7-position, which is replaced by carbon. The N7 position in adenosine takes part in non-Watson and Crick hydrogen bonding, which may be relevant to RNA folding and subsequent activity. This Adenosine analogue is also known as Tubercidin. 8-Aza-7-deaza-Adenosine is an isomer of Adenosine with

(Continued on Page 2)

(Continued from Front Page)

virtually identical electron density. Again, the N7 nitrogen is not available for hydrogen bonding. Nebularine or Purine Nucleoside can be viewed as an Adenosine derivative that is lacking the exocyclic amino group. This molecule allows researchers to determine the relevance of the exocyclic amine of Adenosine to RNA structure and function. If functionality is lost after substitution of Nebularine for Adenosine, then hydrogen bonding involving the exocyclic amine can be inferred, while retention of functionality infers the opposite.

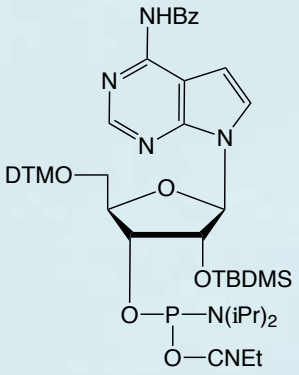
Cytidine Analogues

Ribozyme activity is substantially affected by the substitution of modified pyrimidine bases. Zebularine (pyrimidin-2-one ribonucleoside) may be regarded as a Cytidine derivative lacking the exocyclic amino group. Zebularine and Pyridin-2-one Ribonucleoside, the 3-deaza analogue of Zebularine, are prime candidates for use in evaluating ribozyme activity and function. It should be noted that Zebularine is mildly fluorescent, absorbing at 298nm and emitting at 367nm.

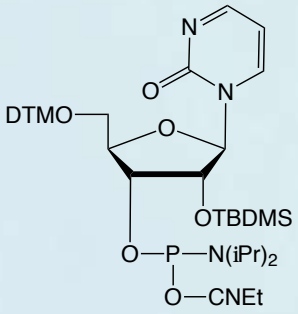
PseudoUridine

PseudoUridine is one of the most common modified nucleosides found in RNA. The availability of a phosphoramidite will allow detailed research into the effects of this modified base on RNA structure and activity.

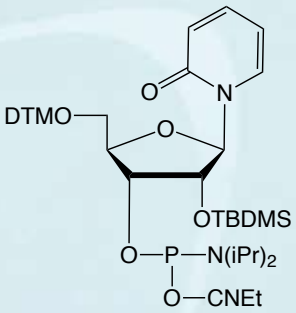
FIGURE 2: STRUCTURES OF NEW MODIFIED RNA PHOSPHORAMIDITES



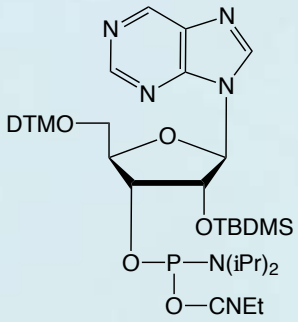
7-Deaza-Adenosine (10-3001)



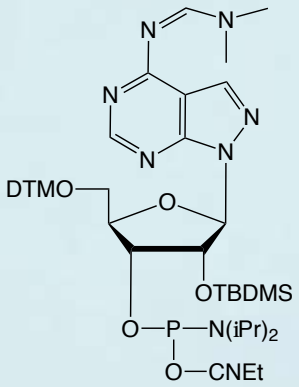
Zebularine (10-3011)



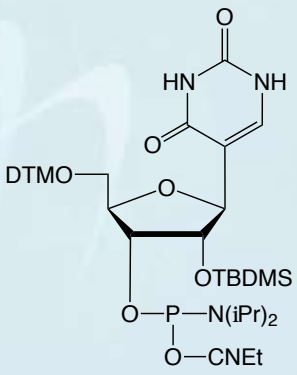
Pyridin-2-one Ribonucleoside (10-3012)



Nebularine (Purine Ribonucleoside) (10-3041)



8-Aza-7-deaza-Adenosine (10-3083)



PseudoUridine (10-3055)

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
7-Deaza-A-CE Phosphoramidite	10-3001-95	50 µmole	225.00
	10-3001-90	100 µmole	450.00
	10-3001-02	0.25g	1100.00
Zebularine-CE Phosphoramidite	10-3011-95	50 µmole	125.00
	10-3011-90	100 µmole	250.00
	10-3011-02	0.25g	650.00
Pyridin-2-one-CE Phosphoramidite	10-3012-95	50 µmole	210.00
	10-3012-90	100 µmole	420.00
	10-3012-02	0.25g	1200.00
Nebularine-CE Phosphoramidite (Purine Ribonucleoside)	10-3041-95	50 µmole	225.00
	10-3041-90	100 µmole	450.00
	10-3041-02	0.25g	1250.00
PseudoUridine-CE Phosphoramidite	10-3055-95	50 µmole	175.00
	10-3055-90	100 µmole	350.00
	10-3055-02	0.25g	995.00
8-Aza-7-deaza-A-CE Phosphoramidite	10-3083-95	50 µmole	300.00
	10-3083-90	100 µmole	600.00
	10-3083-02	0.25g	1500.00

1-METHYL-ADENINE IN NUCLEIC ACIDS

Natural nucleic acids, in particular RNA, contain a vast quantity of modified nucleosides[1] that are responsible for a large variety of chemical and potential biological functions. Some of these modifications are biologically essential, while some others are detrimental and mainly considered as lesions. In this article, we present new additions to our product portfolio and describe how similar base modifications may have completely different consequences when in RNA or DNA.

1-Methyladenosine

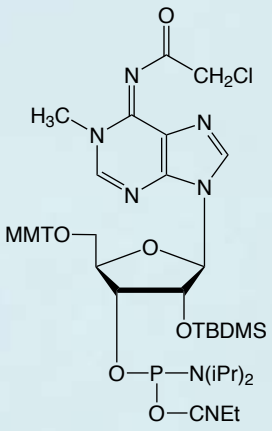
Even though nucleic acid modifications have been linked to control of gene expression at both the levels of transcription and translation through folding and alternate foldings,[2,3] much has still to be done to understand the contribution of modified nucleosides to the functional chemistry, structure and biological activity of RNA. Consequently, Glen Research continues to evaluate the potential of novel nucleoside phosphoramidites to help this research to progress. We are particularly happy to introduce here 1-Methyladenosine (m^1A) as a phosphoramidite for RNA modification.[4]

Methylation of adenosine at position 1 produces a drastic functional change in the nucleobase. 1-Methyladenosine (pK_a 8.25) is a much stronger base than adenosine (pK_a 3.5). N-1 methylation excludes participation of the adenine base in canonical Watson–Crick base pairing and provides a positive charge to the nucleobase. This modification also alters the hydrophobicity of the base, the stacking properties, the ordering of water molecules and the chelation properties. The base may become involved in non-canonical hydrogen bonding, in electrostatic interactions and, in general, it may contribute to the conformational dynamics of the tRNA.

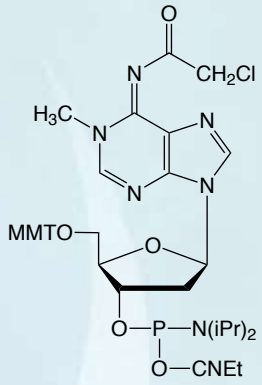
1-Methyladenosine (m^1A) is obtained in nature by post-transcriptional methylation of adenosine by methyl-1-adenosine transferase[5] and it has a special role in t-RNA folding.[6,7] Approximately 25% of all tRNAs have m^1A at position 58 in the T loop, while m^1A also often occurs at position 14 in the D loop.[8]

Reverse transcription is a central step in HIV-1 replication that represents a typical case of interplay between viral and cellular factors. HIV-1 diverts a cellular tRNA, tRNA*Lys*[3], to prime reverse transcription.

FIGURE 1: **STRUCTURES OF 1-METHYL ADENOSINE PHOSPHORAMIDITES**



1-methyladenosine (1)



1-methyl-2'-deoxyadenosine (2)

The post-transcriptional modifications of tRNALys[3] are crucial for completion of reverse transcription. In all HIV strains, methylation of A58 is required to allow productive strand transfer during (+) strand DNA synthesis.[9]

The function of modified nucleosides is usually studied by comparison of the properties of modified tRNAs (purified from wild-type organisms) with that of unmodified tRNAs (obtained by in vitro transcription) and of partially modified tRNAs (from modification mutants).

Because m^1A is a basic nucleoside, which can easily undergo a Dimroth rearrangement to yield N6-methyladenosine, the design of a system for its incorporation into synthetic oligonucleotides is not easy. Here we are happy to propose a protocol first described by Sergey Mikhailov, Piet Herdewijn and coworkers[4] from the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow (Russia) and the Laboratory of Medicinal Chemistry, Rega Institute, Katholieke Universiteit Leuven (Belgium). These groups described the synthesis of the phosphoramidite of protected 1-methyladenosine (1) and its successful incorporation into RNA. The availability of this m^1A phosphoramidite will now enable researchers to synthesize substrate or prototype more easily.

This new RNA minor bases is a 2'-TBDMS protected phosphoramidite. Synthesis tests have been performed on ABI 39x synthesizers and using either standard (N-Bz-A/N-iBu-G/N-Ac-C) or the mild deprotection phosphoramidites (N-Pac-A/N-iPr-Pac-G). We tested several coupling times and several activators and found that 0.25M 5-Benzylthio-1H-tetrazole in acetonitrile gave the best coupling efficiency. With this activator we obtained 96% coupling with a coupling time of 15 minutes, while we barely obtained over 90% coupling after 15 minutes with 1H-tetrazole.

Deprotection of the bases is then carried out in 2M methanolic ammonia at room temperature for 17 - 48 hours depending on the base composition of the oligo. Mikhailov and co-authors[4] found that 60 hours at RT is sufficient to cleave the N2-isobutyryl protecting group of the guanine base. However, we prefer the UltraMild approach in order to avoid any degradation of the fragile m^1A.[10] Also, we would recommend the use of the UltraMild Cap A reagent containing phenoxyacetic anhydride (Pac_2O). This modification removes the possibility of exchange of the iPr-Pac protecting group on the dG with acetate from the regular acetic anhydride capping mix. After deprotection, the solution was decanted from the support, the methanolic ammonia was evaporated, and the residue was treated with 1.5 mL of a 1M TBAF solution for 18 hours at room temperature.

1-Methyl-2'-deoxyadenosine

As described above, alkylation may be desirable or may be a problem. Alkylating agents are abundant in the environment and are also generated inside cells. Such agents introduce various and numerous lesions in DNA and some are even carcinogenic in mammals. To counteract the effects of these lesions, most organisms express several mechanisms for repairing alkylation

damage in DNA.

In brief, cellular polynucleotides are alkylated by endogenous components, such as *S*-adenosylmethionine, or after reacting with two general classes of environmental and laboratory chemicals. SN1 agents include alkylnitrosourea (e.g., MNU, ENU) and *N*-alkyl-*N*-nitro-*N*-nitrosoguanidine (e.g., MNNG) that react with the N7 position of guanine, N3 of adenine, O6 of guanine, O2 or O4 of pyrimidines, and the non-phosphodiester oxygen atoms of the phosphate backbone. In contrast, SN2 agents such as methyl methanesulfonate and dimethyl sulfate react primarily with the N1 position of adenine (1-Methyl-2'-deoxyadenosine) and N3 of cytosine.[11]

To overcome the mutagenic and toxic effects of these modifications, cells produce a variety of DNA repair enzymes. For example, E.coli *ogt* and *ada* genes encode O6-methylguanine methyltransferases, while *ada* also encodes O4-methylthymine methyltransferase and methylphosphotriester methyltransferase activities, and *alkB* encodes an oxidative demethylase that directly reverses 1-alkyladenine and 3-alkylcytosine lesions in DNA or RNA.[12,13]

The availability of m^1A 2'-deoxynucleoside (m^1dA) phosphoramidite (2) will now enable researchers to synthesize substrate or prototype more easily.

The m^1dA phosphoramidite coupled very well (>99%) at all coupling times tested with 0.45M 1H-Tetrazole in acetonitrile as activator, so a standard coupling method can be used for this amidite. We conducted a series of deprotection experiments on the deoxynucleoside to confirm the literature results and to see if we could use an alternate deprotection reagent. Since this m^1dA is sensitive to rearrangement to m^6dA when using standard NH_4OH deprotection methods, we recommend the use of UltraMild phosphoramidites for the synthesis of oligonucleotides containing this sensitive modified base. We have found that deprotection of oligos containing m^1dA using 2M anhydrous ammonia in methanol gave good results and a 24h deprotection time seems to be optimal. Again, the use of the UltraMild Cap A containing phenoxyacetic anhydride (Pac_2O) is also to be recommended.

Other DNA Repair Substrates

This addition to our catalogue is just another example of our commitment to provide the researcher interested in DNA damage and repair with the largest range of products in this field. We would like to review these products as follows.

As mentioned above, DNA exposed to reagents like nitrosourea or dimethylsulfate result in different sorts of alkylation lesions. Most of these lesions modify the base pairing properties of the nucleobase (e.g., DNA polymerases readily insert T opposite O6-methyl-dG). We offer amidites that enable the incorporation of O6-methylguanine, N-6 methyladenine and O4-methylthymine.

The 8-oxo purine monomers allow investigation of the structure and activity of oligonucleotides containing an 8-oxo mutation, which is formed naturally when DNA is subjected to oxidative conditions or ionizing radiation.

5,6-Dihydro pyrimidines are naturally occurring compounds that are structural components of alanine transfer RNA. Dihydrouracil and the hydroxy pyrimidines are major base damage products formed by exposure of DNA to ionizing radiation.

8-Amino-G is formed along with 8-oxo-G as the major mutagenic lesions formed in DNA damage caused by 2-nitropropane. 2-Nitropropane is an industrial solvent and a component of paints, dyes and varnishes, and is also present in cigarette smoke.

Thymine glycol (5,6-dihydroxy-5,6-dihydrothymine) is formed when thymine is subjected to oxidative stress, including ionizing radiation. Oxidation of the 5,6 double bond of Thymidine generates two chiral centers at C5 and C6. The cis-5R,6S form is generated as the predominant product along with the other diastereomer, the cis-5S,6R form. The presence of thymidine glycol in DNA has significant biological consequences and many organisms possess specific repair enzymes for the excision of this lesion.

2-Aminoimidazolone (Iz) and its hydrolysis product imidazolone (Z) are major oxidation products of G. Access to these two potential lesions is not possible during oligonucleotide synthesis because they are so base-labile. A suitable precursor, 8-methoxy-dG (8-OMe-dG), to dIz has now been described. The conversion of 8-OMe-dG to dIz takes place by irradiation of the oligonucleotide (1 mM) in 50 mM sodium cacodylate buffer, pH 7, in the presence of riboflavin (50 µM) for 2 minutes on a transilluminator (366 nm), under aerobic conditions at 4°C. Interestingly for a photochemical reaction, the conversion is virtually quantitative.

Hydrolysis of nucleoside residues in DNA occurs naturally to generate abasic sites. Most commonly, dA sites are hydrolyzed causing depurination and leading to abasic residues. A new chemical method allows the generation of abasic sites in double and single stranded oligonucleotides using very mild specific conditions and with very low probability of side reactions. The abasic phosphoramidite allows oligonucleotide synthesis under standard conditions. Following standard deprotection, the silyl protecting groups of the residue are removed with aqueous acid. (This can be done in conjunction with trityl removal in the last step of a DMT-on purification.) The diol so formed is then treated with aqueous sodium periodate to form an aldehyde plus formaldehyde. The aldehyde then immediately cyclizes to its preferred structure, the abasic cyclic sugar (dR). dSpacer has also been used successfully as a mimic of the highly base-labile abasic site.

Finally, one of the major sources of DNA damage in all organisms is the UV component of sunlight. The predominant reaction induced by UV light on DNA is dimerization of adjacent pyrimidine bases leading to cyclobutane dimers (CBDs). The dimers

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
1-Me-A-CE Phosphoramidite	10-3501-95	50 µmole	190.00
	10-3501-90	100 µmole	380.00
	10-3501-02	0.25g	975.00
1-Me-dA-CE Phosphoramidite	10-1501-95	50 µmole	125.00
	10-1501-90	100 µmole	250.00
	10-1501-02	0.25g	750.00

formed in the most significant quantity are the cis-syn cyclobutane dimer of two thymine bases. Although formed routinely, these dimer products are efficiently excised and repaired enzymatically (nucleotide excision repair) or the dimerization is reversed by photolase enzymes. These lesions have been connected to the formation of squamous cell carcinomas. In addition, humans who lack ability to repair CBD lesions with high efficiency may be genetically predisposed to Xeroderma Pigmentosa (XP), a disease characterized by extreme sensitivity to sunlight and high frequency of skin cancer. Polymerases encountering unrepaired CBD lesions are quite error-prone, leading to incorrect base insertions and subsequent mutations.

References:

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TECHNICAL BRIEF - PRECAUTIONS DURING PACKAGING OF PHOSPHORAMIDITES AND TRANSFER INTO ALTERNATIVE VIALS

As all of our customers are no doubt aware, phosphoramidites are moisture-sensitive. Indeed, by far the most common cause of poor performance of a phosphoramidite is the presence of moisture.

A critical component of Glen Research's quality management system (QMS) is the standard operating procedure (SOP) covering packaging of amidites into vials ready to be attached to synthesizers. When a new batch of a phosphoramidite is produced and approved by QC, the most urgent task is to package it efficiently. The only way to preserve its integrity and maintain a high coupling efficiency even after months of storage, is to package this bulk into end-user vials within a few days. At this point, we use historical sales data along with information on pending orders to determine which pack sizes and vial types to elect to weigh. In particular for our more exotic products, we dispense the majority into standard slider (ABI type) vials. These vials are shipped with a clean, dry empty vial/bottle corresponding to the synthesizer type.

We are, of course, able to do special packaging if it is in phase with our production and QC schedules.

It is important to remember that some phosphoramidites are white or colored powders after purification and isolation while other products do not crystallize and stay as viscous oils. These oil-products have specific features and risks. Among them are the Spacers, Chemical Phosphorylation Reagents and the vast majority of non-nucleosidic modifiers.

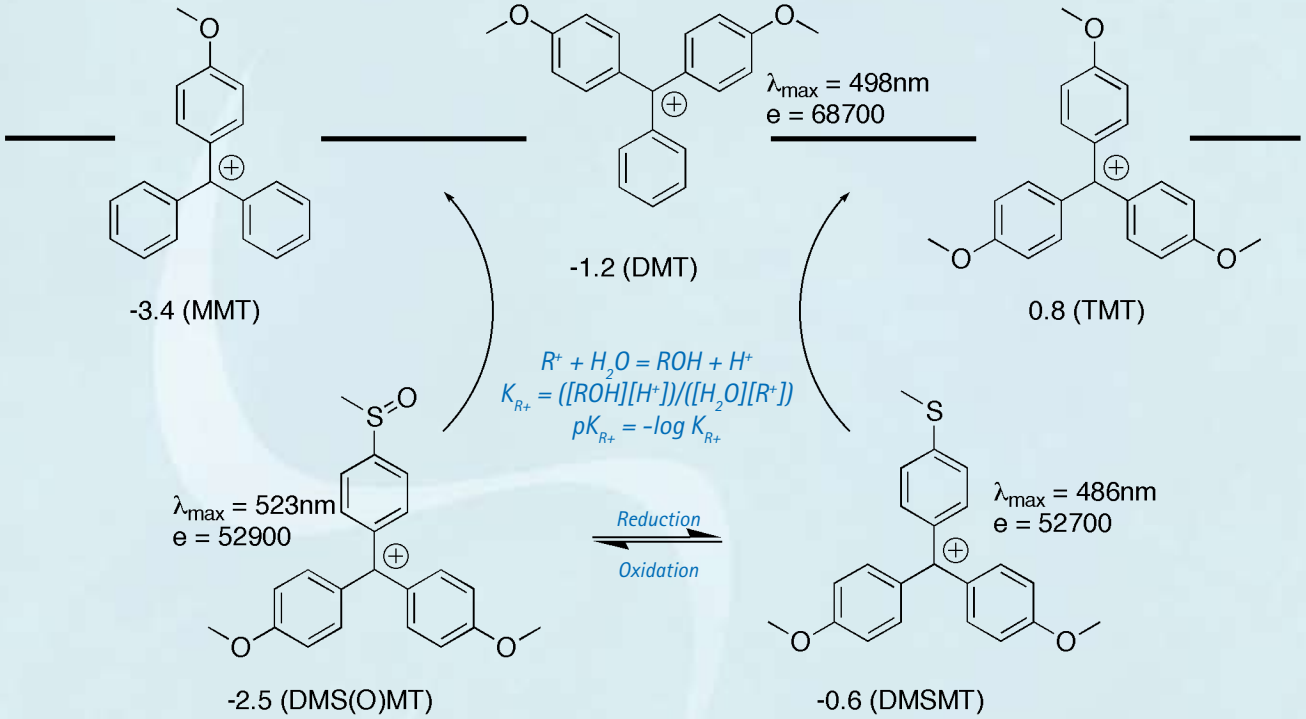
Due to their nature, these oil-products form a thin film once transferred and dried in end-user vials and are impossible to transfer, without first dissolving them again. Also, oils are just as hygroscopic as powders. If we were to redissolve these oils for transfer to user-specified vials, we could no longer guarantee that the product would accurately conform to the data on the Certificate of Analysis. Consequently, we have never transferred such products and they are always shipped together with an appropriate empty bottle, corresponding to the customer's synthesizer type.

Also it is important to remember that, sometimes, oils are difficult to dissolve. And again, they can pick up moisture as fast as powders. So we recommend doing any synthesis with these products using fresh diluent (anhydrous acetonitrile, less than 25 ppm of water). Using a fresh disposable (or dried glass) syringe, draw the acetonitrile through the septum of the diluent bottle and inject this acetonitrile into the "oil-amidite" vial through the septum, to avoid moisture exposure. Then swirl occasionally over several minutes until the oil is completely dissolved and forms a homogenous solution, which occurs when no wavy lines of concentration gradient are observed. Some of the more viscous oils may require between 5 and 10 minutes. Use care to maintain anhydrous conditions. Then transfer the solution to the alternate vial type (when necessary), again using a syringe, and make sure the final pre-dried recipient vial (from Glen Research or pre-dried by the user) is opened at the very last minute.

For use of an expensive amidite on an Expedite synthesizer, you may prefer to use a vial with an internal conical shape that makes small sample handling easy and convenient and allows maximum delivery of your amidite solutions. V-vials from Wheaton or Reacti-vials from Pierce are available in 2, 3 or 5 mL size and with the 20-400 neck that is directly compatible with the Expedite amidite port.

Finally a description of our products (powder or oil) is mentioned on the Certificate of Analysis that we provide with each product. Specific information about dissolution and special instructions are written on this Certificate, when applicable. So please do not forget to check this important information before using the product.

FIGURE 1: STABILITY OF TRITYL CATIONS (pK_{R+}) DISCUSSED IN THIS ARTICLE



1. AMINO-MODIFICATION: MATURITY AT LAST?

It is well over 20 years since the first amino-modifier prototypes were described on a modified base[1] and at the 5′ terminus[2]. However, even after all these years, we have to acknowledge that there is still no ideal amino-modifier currently available commercially. Glen Research is now pleased to offer customers a mature version of the amino-modifier C6 phosphoramidite.

1.1. Aminolink and Microarrays/DNA chips

Amino-modified oligos are popularly used for microarray manufacturing since DNA chip technology has become such an indispensable tool for life sciences.[3,4] Fabrication is based on *in situ* synthesis on silicon chips or the more accessible approach of post-synthetic immobilization of oligos onto activated surfaces, predominantly glass slides. Companies and oligo synthesis houses use high-throughput processing to prepare libraries of tens of thousands of amino-modified oligos which are then spotted on surfaces to yield microarrays. The spotting process uses either piezo-based dispensers or the more popular pin-based spotters. Procedures have been optimized mainly in terms of immobilization chemistries, with the most popular methods relying on the reaction of amino-modified oligos with surfaces derivatized with moieties such as isothiocyanate or epoxy[5] groups. The quality of the product as well as the yield of these amino-modified oligos is vitally important since re-synthesis on this scale is time-consuming and expensive. Clearly, for this kind of high-volume throughput, purification of all these oligos using conventional methods, like HPLC or PAGE, is out of the question - although high-throughput reverse phase (RP) purification in cartridges and plates has proved to be useful.

1.2. Cartridge Purification

The most popular amino-modifier used today contains an amine protected by a trifluoroacetyl (TFA) group, e.g., Amino-Modifier C6-TFA (10-1916). This modifier has the advantages of being inexpensive and reliable. However, it has flaws in that it does not allow the use of trityl-on purification techniques or even an estimate of the coupling yield by the trityl cation measurement. Moreover, since the removal of a TFA protecting group happens simultaneously with the deprotection of an oligo, side reactions such as Michael addition of acrylonitrile (formed from elimination of cyanoethyl protecting groups) take place, reducing the yield of the aminated product (http://www.glenresearch.com//ProductFiles/Technical/TB_avoidaminealkylation.html).

A reagent more suitable for preparation of thousands of amino-modified oligos contains an amine protected with a trityl group, e.g., Amino-Modifier C6 (10-1906). Triphenylmethyl groups (trityls) are a popular family of protecting groups, used in oligonucleotide chemistry for hydroxyl (DMT) and amino (MMT) protection, and removable by mild acidic treatment. Conveniently, trityl cations have large extinction coefficients allowing stepwise coupling yields to be measured easily. Alternatively, due to the hydrophobicity of trityls, separation of the full-length product, still bearing the tritylprotecting group, from capped failure sequences can be carried out, with subsequent acidic removal of the DMT or MMT protection as the final step.

Manufacturing large numbers of oligonucleotides requires cheap and fast purification techniques, ruling out HPLC and PAGE as too expensive and time-consuming. The popular RP cartridge purification method (e.g., PolyPak) does not allow detritylation of the monomethoxytrityl (MMT) group from protected amino-modified oligonucleotides in high yield on the cartridge. The reason for this situation is that acidic cleavage of the N-trityl bond is an equilibrium reaction. On

an RP cartridge, where the trityl cation is not physically separated from the amine, the process results in substantial (up to 50%) reattachment of the MMT-group back onto the amine. Subsequent elution of the amino-modified oligonucleotide results in a product that is up to 50% inactive. Interestingly, in addition to this reattachment problem, MMT-amino-modified oligos are quite unstable when stored in aqueous ammonia, gradually losing the MMT group over time.

Recently, we have seen some interest in the dimethoxytrityl equivalent to the amino-modifier C6, presumably in an attempt to overcome some of the problems associated with the MMT version. In our hands, this phosphoramidite is less stable than its MMT cousin and we have concerns about long-term storage. Although the product couples perfectly well in synthesis, there was substantial loss of DMT from the amine during deprotection EVEN at room temperature. Our view is that the DMT-amino-modifier C6 is too labile for routine use but it could prove to be useful in situations requiring very mild removal of the amine protecting group.

1.3. The New Amino-Modifier Phosphoramidite

One way around these problems is to use a modified trityl with controlled pK_{R+}, where pK_{R+} is defined by the following formulae:

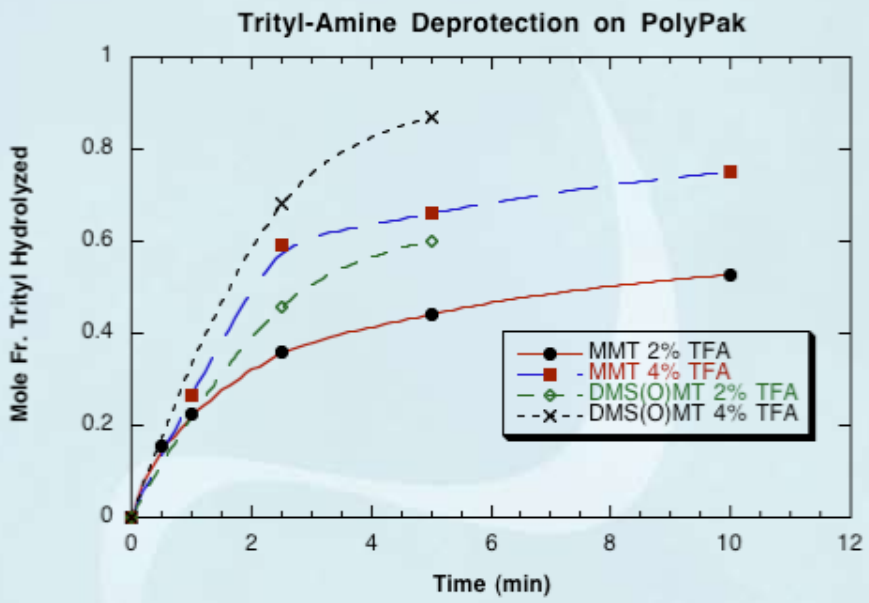
$R^+ + H_2O = ROH + H^+$

$K_{R+} = ([ROH][H^+])/([H_2O][R^+])$

$pK_{R+} = -\log K_{R+}$

4,4'-Dimethoxy-4''-thiomethoxytrityl (DMS(O)MT; sulfoxy-form) cation is more stabilized than MMT^+, and so the DMS(O)MT-protected amino group is easier to deprotect compared to the MMT-protected one, as shown in Figure 1 on Page 6. The sulfoxy derivative survives conditions of oligonucleotide synthesis and can either be cleaved with standard deblock solution, or left intact for an HPLC purification. At the same time, the DMS(O)MT group is fully compatible with cartridge purification. When detritylation on a cartridge is carried out, the $DMS(O)MT^+$, which is more stable than MMT^+, does not reattach itself to an amine. The new aminolink phosphoramidite reagent, 5'-DMS(O)MT-Amino-Modifier C6, utilizing this new trityl based protecting group is shown in Figure 5 (1) on Page 8. The reagent is stable in solution in acetonitrile

FIGURE 2: **RATE OF HYDROLYSIS OF MMT AND DS(O)MT WITH 2% AND 4% AQUEOUS TFA**



Protocol:

1. *0.2-0.25 µmole 5'-amino-modifier C6-T6 oligo in NH4OH/H2O (1:3) loaded on PolyPak.*
2. *Failures eluted with 10% Acetonitrile/0.1 M TEAA followed by H2O wash to remove buffer.*
3. *Oligo detritylated by flowing TFA solution through PolyPak for specified time.*
4. *Acid neutralized by flowing 0.1 M TEAA solution through PolyPak.*
5. *Trityl-ON and trityl-OFF oligo eluted with 50% Acetonitrile/0.1 M TEAA.*
6. *Trityl-ON/Trityl-OFF ratio determined by RP HPLC analysis of eluted oligo.*

FIGURE 3: **RP HPLC OF DMS(O)MT-ON AND POLY-PAK PURIFIED AMINO-MODIFIED OLIGO**

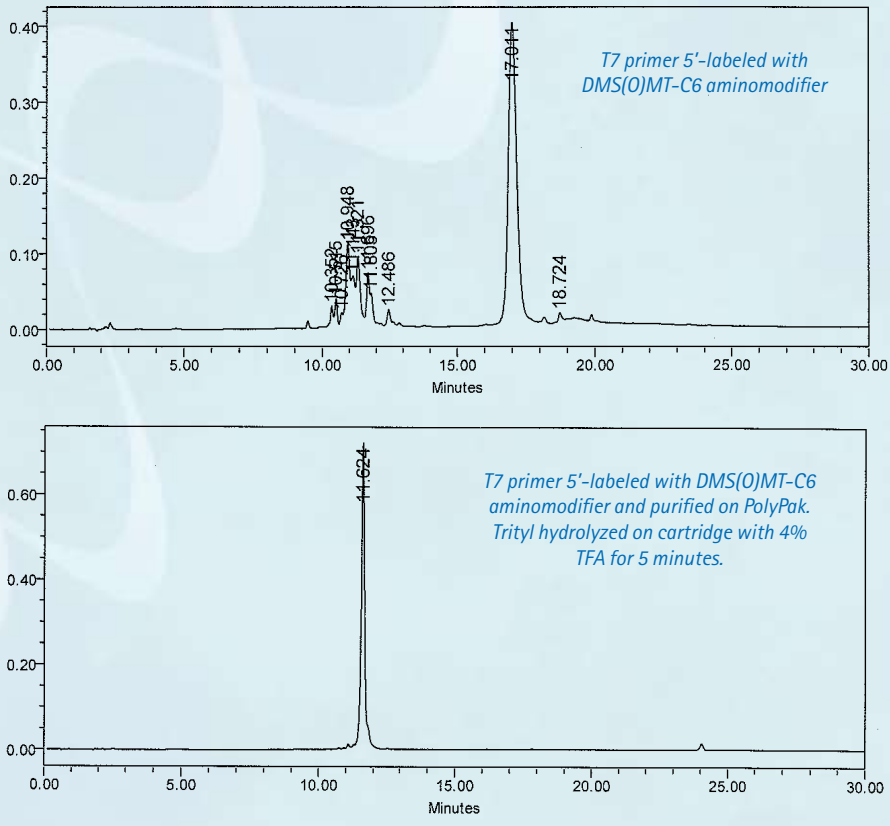
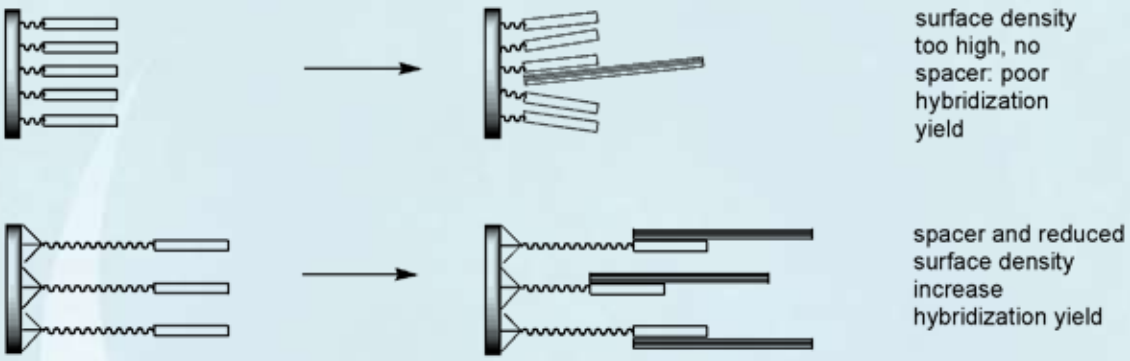


FIGURE 4: IMPROVING SPACIAL DISTRIBUTION INCREASES HYBRIDIZATION EFFICIENCY AND SIGNAL DETECTION



at room temperature for at least two weeks. UV quantification for release of the new protecting group is possible. Extinction coefficients (L/(mol x cm), shown in Figure 1 on Page 6, were measured in 2% TFA/DCM. In PolyPak detritylation experiments followed by HPLC measurements, the new reagent gave more than 20% improvement in deprotection yields compared to an MMT-protected amino group labeled oligo (4% TFA, 5 min exposure time). A DMS(O)MT-protected amino-modified oligonucleotide was stored in NH_4OH/H_2O (1:3) at room temperature for 6 days and exhibited no loss of trityl.

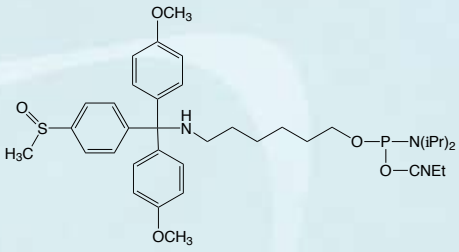
Figure 2 on Page 7 illustrates the rate of deprotection of DMS(O)MT-protected amino-modified oligonucleotides on PolyPak cartridges using 2% and 4% aqueous trifluoroacetic acid (TFA). It was found that complete deprotection of DMS(O)MT is achieved after 5 minutes. Under the same conditions, MMT was less than 50% deprotected.

The efficiency of the process is further illustrated in Figure 3 on Page 7. In this experiment, the T7 primer was 5'-labeled with DMS(O)MT-C6 aminomodifier and purified on a PolyPak cartridge. The DS(O)MT trityl was hydrolyzed on the cartridge with 4% TFA for 5 minutes. The upper chromatogram shows the RP HPLC of the DMS(O)MT-on primer and the lower shows the purified amino-modified primer.

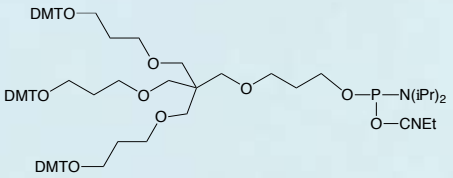
2. TREBLERS: INTERFACING OLIGO SYNTHESIS AND NANOTECHNOLOGY

Several new applications for dendrimers in the oligonucleotide field have emerged since Glen Research commercialized branching reagents in 1999.[6] Examples include multiple fluorescent labeling[7], controlled delivery of antisense oligos[8], more efficient conjugation of oligos with nano-gold[9], and more efficient quenching for molecular beacon applications[10]. The new trebler described below, now available from Glen Research, possesses features that will assist in some of these applications and thus further expand the array of Glen Research's building blocks.

FIGURE 5: PHOSPHORAMIDITE STRUCTURES



DMS(O)MT-Amino-Modifier C6 (1)



Long Trebler (2)

2.1. 'Long' Trebler Phosphoramidite

The **Trebler Phosphoramidite** currently available from Glen Research contains a phosphoramidite 'arm' that is somewhat crowded by the three adjacent DMT-bearing branches. This leads to increased coupling times (recommended coupling time: 10-15 min) and lower coupling efficiency.

The next generation Trebler (**Long Trebler Phosphoramidite**) contains an extended phosphoramidite arm, thus markedly reducing the problem of steric hindrance. The new reagent gives higher coupling yields and requires shorter coupling times. Large pore size CPG supports (1000Å and 2000Å) should be selected when using this phosphoramidite. The new trebler phosphoramidite, Long Trebler Phosphoramidite (2), is shown in Figure 5.

3. SOME SUGGESTED APPLICATIONS FOR MICROARRAYS

3.1. Improved Surface and Spatial Density

Spatial factors greatly affect the quality of DNA chips. When the probes are too far apart, there would not be enough fluorescence signal to be detected. With probes positioned too close to one another on a surface, there would not be enough room for the target DNA to squeeze through and hybridize - again compromising the quality of the detection. Reducing the surface loading chemically improves the hybridization yield and signal quality, as does linking the oligos to a microarray

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-DMS(O)MT-Amino-Modifier C6	10-1907-90	100 µmole	60.00
	10-1907-02	0.25g	200.00
Long Trebler Phosphoramidite	10-1925-90	100 µmole	200.00
	10-1925-02	0.25g	300.00

surface through long spacers. Up to 150 times improvement in hybridization yield compared to oligos directly attached to a surface at high density can be achieved by optimizing spacing and surface density.[11]

A recently published approach, illustrated in Figure 4, involves a multistep procedure whereby an aminated surface is treated with two-sided bulky structures.[12-14] One side of such a structure non-covalently sticks to several reactive sites on the surface, whereas the opposite part of the structure has just one reactive group of its own, thus effectively reducing the surface loading. The method relies on non-covalent binding and requires commercially available glass slides to be additionally modified.

The same result can be achieved in a simpler and more controllable way by employing phosphoramidites available from Glen Research. Upon completion of an oligo synthesis on a large pore CPG (1000Å or 2000Å), the 5' end can be derivatized with spacer modifiers 9 or 18, followed by a trebler amidite and an amino-modifier amidite. Following standard deprotection, the resulting trebler amino-modified probe will be spotted on to an activated glass surface. The probe will not only immobilize faster courtesy of multiple amino groups, but will also take up more surface area to generate increased spacing, thereby improving the yield in hybridization experiments.

For further technical data on these products please contact: John Thornback at john.thornback@btinternet.com

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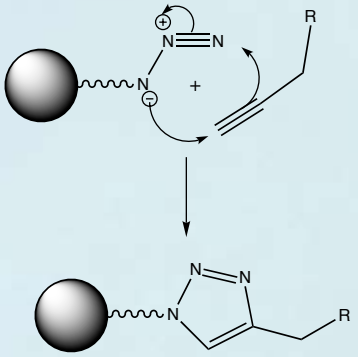
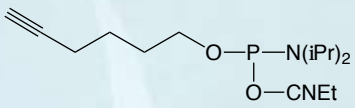
14. S.J. Oh, et al., *Nucleic Acids Res*, 2005, **33**, e90.

FIGURE 1: **CLICK CHEMISTRY STRUCTURES**



[3+2] Cycloaddition or 'Click'



5'-Hexynyl Phosphoramidite (1)

The ability to perform a specific conjugation in biological samples is a daunting task. The presence of the complex variety of functional groups leads to almost unavoidable cross-reactivity and non-specific labeling. However, one reaction which shows remarkable selectivity is the [3+2] cycloaddition between azides and alkynes, as shown in Figure 1.

Huisgen originally described the reaction of azides and alkynes to form 1,2,3-triazoles,[1] but the incredible utility of the reaction was not borne out until Sharpless reported[2] that the addition of copper (I) iodide (CuI) led to a million-fold increase in the rate of the cycloaddition. The reaction was found to be so exquisitely regioselective and efficient at even the most mild conditions that Sharpless coined the term 'Click Chemistry' to describe it. This chemistry has been used to label specific proteins from cell lysates with essentially no nonspecific labeling[3] and can even be coaxed to occur at specific locations in a microarray with 10 micron resolution by applying a potential of -300 mV.[4]

Given the potential of this chemistry, we are happy to introduce the 5'-Hexynyl Phosphoramidite (1). Oligonucleotides containing the 5'-Hexynyl moiety are stable to standard deprotection conditions and exhibit a slightly increased retention time on RP HPLC. A number of azide labels are commercially available for click conjugation to a 5'-hexynyl-modified oligonucleotide.

Azide-modified labels and tags are commercially available, but how easy is it to access azido-labeled oligonucleotides? Azides are not compatible with oligonucleotide synthesis using phosphoramidites so a post-synthesis displacement reaction is likely to be required. We found we were able to quantitatively convert a 5'-iodo-modified oligonucleotide (prepared using 5'-Iodo-dT) to the corresponding 5'-azide. Specifically, after synthesis of the oligonucleotide, the CPG was transferred to a vial and heated at 55 °C for 1 hour in a solution of NaN_3 in DMSO (~5 mg/mL). After rinsing the CPG with DMSO and ACN, the oligo was deprotected in AMA for 15 minutes at 65 °C to afford the 5'-azido-modified oligonucleotide. When this oligo was reacted in the presence of 0.6 mM CuI in DMSO/water with the hexynyl-labeled oligo, IEX HPLC confirmed the conjugation of the two oligonucleotides.

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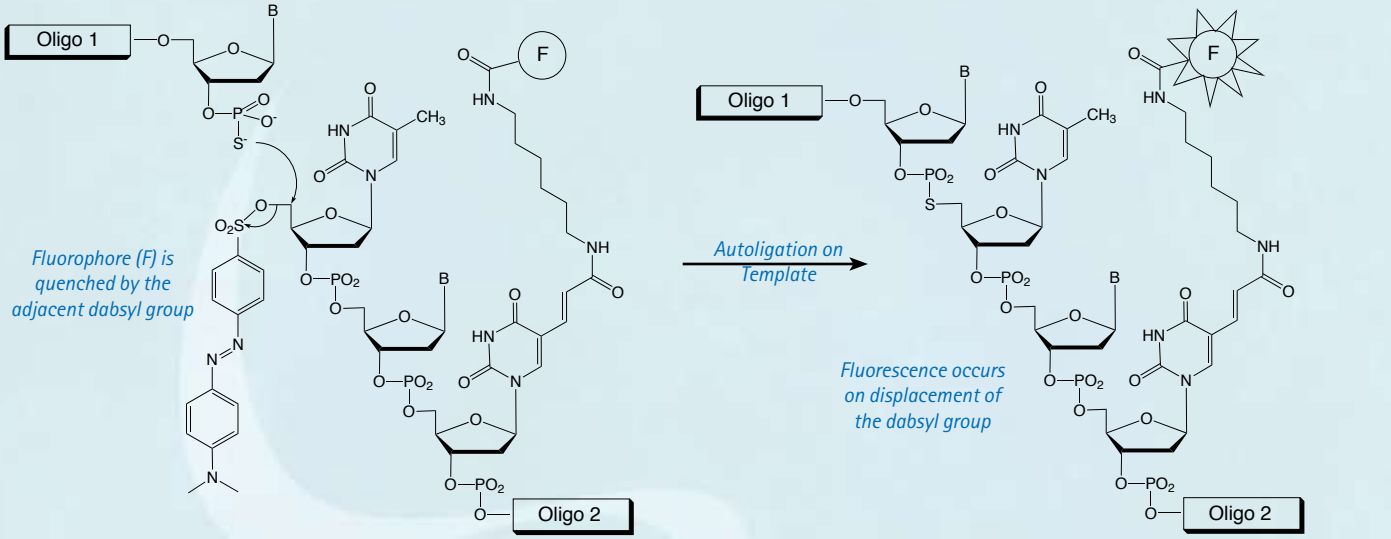
ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-Hexynyl Phosphoramidite	10-1908-90	100 µmole	60.00
	10-1908-02	0.25g	200.00

QUENCHED AUTOLIGATION (QUAL) PROBES

FIGURE 1: **AUTOLIGATION PROCESS OCCURS ON A COMPLEMENTARY TEMPLATE TO GENERATE THE FLUORESCENT SPECIES**

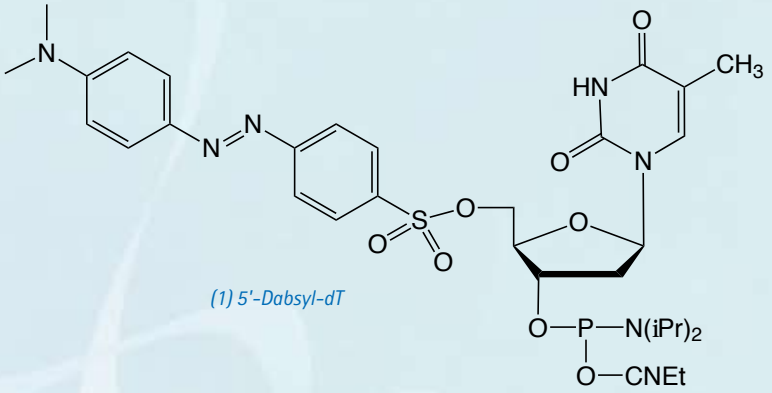


The search for improvements in oligonucleotide probe-based assays has continued over the last few years.[1,2] One diagnostic probe strategy that has caught our attention is the one developed by the Kool lab at Stanford University, which they have named quenched autoligation (QUAL) probes.[3] QUAL probes consist of two oligonucleotides, the first containing a nucleophilic group at the 3'-terminus, while the second has an electrophilic group at the 5'-terminus. When the probe pair finds the target, the oligos line up with the 3'-terminus of the first directly adjacent to the 5'-terminus of the second. A Kool autoligation reaction then takes place to combine the two oligos into a single probe. As usual, the 3' nucleophilic group is the 3-thiophosphate easily prepared using 3'-phosphate CPG with a sulfurizing step in the first cycle. In this case, the electrophilic group is a 5'-dabsyl group, which is an excellent leaving group as well as a fine quencher of fluorescence. The second oligo, therefore, contains a fluorophore which is quenched by the dabsyl group. A popular choice for fluorophore is fluorescein-dT but it is easy to imagine that a variety of fluorophores could be attached to any of the commercially available amino-modified nucleoside phosphoramidites. The process is illustrated in Figure 1.

The efficacy of this procedure has recently been illustrated in intact cells. Using fluorescence microscopy or flow cytometry, QUAL probes were able to distinguish single nucleotide differences in the 16S rRNA sequences of three closely related bacteria. Even a single mismatch was revealed by a significant decrease in fluorescence signal.[4] By combining QUAL with FRET, Abe and Kool were able to substantially reduce the background signal arising from the release of the dabsyl group. In this way, they were able to detect and quantify three different messenger RNAs, as well as 28S ribosomal RNA, by flow cytometry.[5]

Glen Research is happy to offer 5'-Dabsyl-dT CE Phosphoramidite (1) to allow the simple synthesis of 5'-O-dabsyl QUAL probes.

FIGURE 2: **STRUCTURE OF 5'-DABSYL-DT**



ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-Dabsyl-dT	10-1532-90	100 µmole	250.00
	10-1532-02	0.25g	775.00
2'-Se-Me-U-CE Phosphoramidite	10-3730-95	50 µmole	175.00
	10-3730-90	100 µmole	350.00
	10-3730-02	0.25g	975.00

References:

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SELENIUM DERIVATIZATION OF NUCLEIC ACIDS FOR X-RAY CRYSTAL STRUCTURE DETERMINATION

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Determination of the three-dimensional structures of RNA molecules, RNA-protein and DNA-protein complexes with high resolution is invaluable for gaining understanding of biological systems at the molecular level. X-ray crystallography is the most direct and powerful tool for structure determination of these macromolecules. However, besides the difficulties related to crystallization, heavy atom derivatization for phase determination has been a long-standing problem in nucleic acid X-ray crystallography that has largely slowed down structural determination of new structures and folds. The conventional approaches for DNA and RNA derivatization, such as heavy-atom soaking and co-crystallization, have proved to be much more difficult for nucleic acids than for proteins, probably because nucleic acids often lack specific binding sites for metal ions. In addition, the radiation stability and structure perturbation have reduced the usefulness of the halogen derivatization (such as Br and I).

Recently, we have successfully demonstrated a novel derivatization strategy via selenium replacement of oxygen in nucleic acids.[1-8] Unlike conventional halogen derivatization (Br or I), where halogens are primarily placed on the 5-position of deoxyuridine (a mimic of thymidine), selenium can be selectively introduced to a variety of positions via oxygen replacement (e.g., 2'-, 3'-, 5'-ribose oxygen, furan ring oxygen, non-bridging phosphate oxygen, or oxygen on nucleobases).[1-8] Choice of positioning can avoid disruption of structure and function caused by modification. We have found that the 2'-Se-derivatized DNA structure (Fig. 1) reveals that the 2'-Se-furanose displays the 3'-endo sugar pucker, which is consistent with the sugar pucker of A-form DNAs and RNAs, and the 2'-methylseleno group is placed in the minor groove of the duplex.

In addition, we found that the local backbone torsion angles and solvent hydration patterns were altered in the DNA structure with the Br derivatization.[1] Furthermore, while the native and Br-derivatized DNAs needed over several weeks to form reasonable-size crystals, we observed

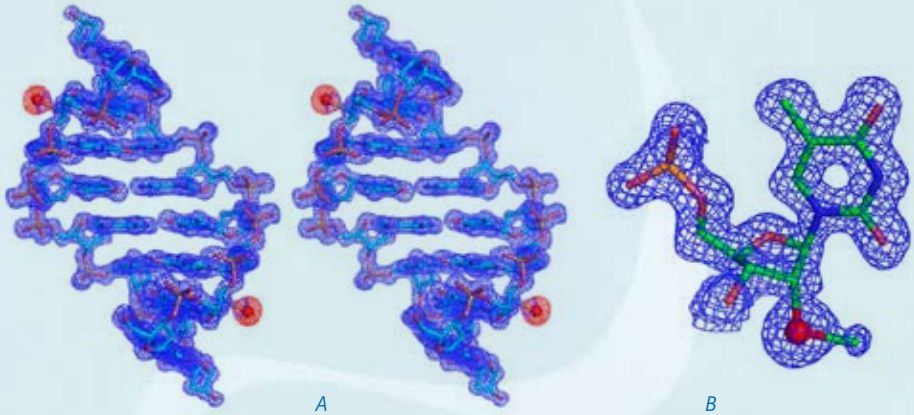

Figure 1: Electron density maps and structures of the Se-derivatized DNA duplexes. Red balls represent Se. (A). The Se-DNA structure [(GdUSeGTACAC)2, Stereo view] at 1.28 Å resolution (1Z7I).[1] Contour is at the 1.2σ level. (B). The structure and the electron density map of 2'-Se-thymidine in the Se-derivatized DNA [(GTSeGTACAC)2, 1.40 Å resolution, 2HC7].[2]

that the Se-derivatized DNAs grew crystals overnight with high diffraction quality,[1,2] suggesting that the Se-derivatization facilitated crystal formation. In addition, the Se-derivatized DNA sequences crystallized under a broader range of buffer conditions, and generally had a faster crystal growth rate. We have observed Se-DNA crystal formation in a few days or even overnight, while the crystals of the corresponding native DNAs need two to three months to grow. Our experimental results indicate that the selenium derivatization of DNAs may largely facilitate the determination of nucleic acid X-ray crystal structures in phasing and high-quality crystal growth. Moreover, our results suggest that the Se derivatization can be a better alternative to the conventional Br derivatization. This Se derivatization strategy via the atom-specific substitution will significantly facilitate X-ray crystal structure studies of nucleic acids and their protein complexes.

See Ordering Information on Page 10.

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LARGE SCALE SYNTHESIS UPDATE

USE OF UNIVERSAL SUPPORTS TO PREVENT SOME MAJOR IMPURITIES IN OLIGO SYNTHESIS

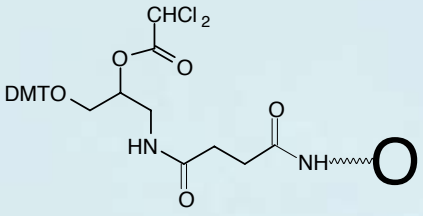
With the development of applications requiring large quantities of oligonucleotides, overall yield and synthesis optimization have been investigated in detail. Major impurities have been tracked, analyzed and characterized.

One of these typical impurities can be seen when polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis is used for analyzing oligos. For example, when looking at a PAGE gel using the UV-shadowing technique,[1,2] the band corresponding to the full-length oligonucleotide appears as a large, intensely dark band migrating at the expected position according to its length (n). Bands of various intensities migrating faster than the intended oligonucleotide usually accompany this major band. These bands correspond to failure sequences (n-1, n-2, etc). However, bands migrating much slower than the desired oligonucleotide may also be observed.[3] More surprisingly, the somewhat common impurity band has a mobility corresponding to the approximate size expected from a dimer (2n) of the intended oligonucleotide. This impurity is also observed on HPLC, eluting after the expected peak. These impurity products, also known as high molecular weight impurities, have recently been studied and characterized by two groups: one at ISIS[4], and one at the University of Bordeaux (France)[5].

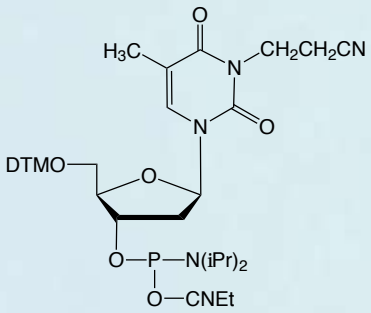
Both groups made similar discoveries: the bands migrating like dimers of the expected oligonucleotide are in fact branched impurities consisting of two oligonucleotide chains, one linked through the exocyclic amino group of the 3'-terminal nucleoside and the other through the 3'-terminal hydroxyl group via a phosphodiester or a phosphorothioate linkage, depending on the oxidizing process used.

The data presented in these reports indicate that phosphorothioate and phosphodiester oligonucleotides synthesized using A, C, or 5-Me-C derivatized solid supports can contain high molecular weight impurities of the type described. The problem appears more pronounced with 5-Me-2'-OMe-C supports. It is certainly possible to remove these impurities by purification. Or, as pointed out by Cazenave,[6] a post-synthetic treatment with neat triethylamine trihydrofluoride would selectively and efficiently cleave any phosphoramidate linkage and convert any N-branched oligonucleotide back to the desired compound.

FIGURE 1: STRUCTURES OF UNIVERSAL SUPPORT II AND N3-CYANOETHYL-DT



(1) Universal Support II



(2) N3-Cyanoethyl-dT

This treatment would also improve the recovery of oligonucleotides obtained from the N-unprotected phosphoramidite method[6,7,8] that despite considerable progress in the discovery of O-selective activators, still produces detectable amounts of N-branched oligonucleotides. However, such an approach definitely reduces the overall synthesis yield. In other words, for a given amount of purified products, one will have to use more phosphoramidite and other reagents. Consequently, the overall cost per purified unit will be higher.

A more efficient approach is to develop chemistries that avoid impurity formation. As presented in the Kurata paper,[4] the use of a UNIVERSAL SUPPORT is an excellent approach to circumvent occurrence of these 2n-1 and related impurities.

Glen Research offers several possible solutions and we believe that our Universal Support II (US II) is today the only truly universal support.[9] As described in a paper we published in 2005,[10] US II performed the best of the group of commercially available universal supports, generating the highest yields of oligonucleotides under the mildest conditions. Only US II is appropriate for the production of DNA oligos, long and short, as well as those requiring mild deprotection. It is also compatible with the synthesis of RNA and siRNA.

Another important advantage of a universal support is that it reduces the need to keep inventory of many pre-loaded supports bearing unnatural nucleosides or

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
Polystyrene Support			
Universal Support II PS2	27-5010-01	0.1g	15.00
	27-5010-02	0.25g	35.00
	27-5010-10	1.0g	95.00
96 Well Format			
0.2 µmole columns	27-5110-92	Pack of 96	250.00
40 nmole columns	27-5110-95	Pack of 96	250.00
ÄKTA oligopilot			
0.2 µmole columns	27-5110-P2	Pack of 10	100.00
1.3 µmole columns	27-5110-P1	Pack of 10	150.00
N3-Cyanoethyl-dT	10-1531-90	100 µmole	200.00
	10-1531-02	0.25g	600.00
ÄKTA oligopilot Deblocking Mix 3% DCA/toluene	40-4240-71	1L	145.00

labels (LNA, RNA, modified nucleosides). For example, the use of USII polystyrene columns is the best way to synthesize RNA or LNA molecules on an AB 3900 synthesizer. This approach is also useable for synthesis in both orientations, 3'->5' as well as 5'->3'.

For synthesizing in 96-well (or more) plates, the use of a universal support eliminates the tedious and risk-prone delivery of support loaded with the appropriate base to each well. Plates loaded with universal support can be prepared in advance.

The reagent used for the cleavage/dephosphorylation step of Universal Support II (anhydrous ammonia in methanol) is commercially available and the procedures described with the product are fully compatible with high throughput synthesis.

This product is available on CPG (bulk, columns for 96 well plates, Expedite or ABI 39x format), on polystyrene (columns for AB 3900 synthesizer) and a new higher functionality polystyrene (US II PS2) (bulk, columns for 96 well plates and ÄKTA oligopilot).

More information on cleavage and deprotection using this support is available on our web site http://www.glenres.com/ProductFiles/20-5010.html

Note: This product is covered by US Patent No.: 6,770,754 and European Patent No.: 1404695.

References:

1. S.M. Hassur and H.W. Whitlock, *Anal Biochem*, 1974, **59**, 162-164.
2. P. Hendry and G. Hannan, *Biotechniques*, 1996, **20**, 258-264.
3. A. Andrus, *Gel-capillary electrophoresis analysis of oligonucleotides. In Protocols for Oligonucleotide Conjugates*, S. Agrawal, Ed. Humana Press: Totowa, NJ, 1993; pp 277-300.
4. C. Kurata, et al., *Bioorganic & Medicinal Chemistry Letters*, 2006, **16**, 607.
5. C. Cazenave, K. Bathany, and B. Rayner, *Oligonucleotides*, 2006, **16**, 181.
6. S.M. Gryaznov and R.L. Letsinger, *Tetrahedron Letters*, 1992, **33**, 4127-4128.
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9. A.V. Azhayev and M.L. Antopolsky, *Tetrahedron*, 2001, **57**, 4977-4986.
10. A. Azhayev, et al., *GEN 2005*, **25** (5). http://www.glenres.com/ExtraPages/25GEN5BPAdvance.pdf

N3-CYANOETHYL-DT

Synthesis of oligonucleotides on a large scale has become increasingly important in a variety of fields, including therapeutics and diagnostics. 2-Cyanoethyl (CE) phosphoramidites are produced on vast scales, are consequently inexpensive, and are by far the reagents of choice for oligonucleotide synthesis. The CE protecting groups are removed from the phosphate backbone by ß-elimination under basic conditions, generating acrylonitrile which is an alkylating agent and known carcinogen. A common side reaction is the alkylation of dT residues by acrylonitrile to form N3-cyanoethyl-dT.[1] A recent publication describes[2] the use of nitromethane as a scavenger to inhibit the alkylation of dT during deprotection. It is possible to ß-eliminate the CE protecting groups using, for example, diethylamine in acetonitrile while the oligonucleotide is otherwise protected on the solid support. Another option is to deprotect with methylamine which can also act as a scavenger of acrylonitrile but which requires the use of acetyl-protected dC in the oligonucleotide.

Glen Research is pleased to offer N3-cyanoethyl-dT-CE phosphoramidite for use as a standard and to aid in the detection of small quantities of this side reaction in synthetic oligonucleotides that have been produced on a large scale.

References:

1. D.C. Capaldi, et al., *Org. Process Res. Dev.*, 2003, **7**, 832-838.
2. T. Umemoto and T. Wada, *Tetrahedron Lett*, 2005, **46**, 4251-4253.

REAGENTS FOR AKTA OLIGOPILOT SYNTHESIZER - UPDATE

With the development of applications requiring larger quantities of oligonucleotides, the ÄKTA oligopilot™ (GE Healthcare Life Sciences) has become increasingly popular with our customer base. Also the ÄKTA oligopilot can be used for synthesis under cGMP guidelines, so it is used in many diagnostic and pharmaceutical companies as well as contract manufacturing organizations.

According to GE Healthcare, the batch operation and documentation of this synthesizer are in accordance with the requirements of Good Manufacturing Practice (GMP), Good Laboratory Practice (GLP) and in full compliance with 21 CFR part 11. For additional information on the operating features of the ÄKTA™ oligopilot, please contact GE Healthcare. The high quality of Glen Research products and our production, which is performed under a strict Quality Management System, including full traceability, fits perfectly with this system. Consequently, we have extended our range of products to offer reagents for the ÄKTA oligopilot.

One key feature of the ÄKTA oligopilot is its use of THF-free solutions. In addition, many ÄKTA users prefer the 3% DCA in Toluene deblocking mix since it eliminates the disposal issue of dichloromethane. We have added this deblock solution to our catalog (see Page 12). In addition, we can supply 1.3 µmol cassettes that will fit on the ÄKTA-10 for smaller scale synthesis, which is helpful for early development synthesis. We can fill these cassettes with all of our available polystyrene supports for 3'-modification, including:

3'-PT-Amino-Modifier C6 PS
3'-TAMRA-PS
3'-Dabcyl-PS
Universal Support II PS

All of our phosphoramidites in vials designed for ABI 39x/3900 synthesizers will also fit on the slider connection of the ÄKTA oligopilot. And if necessary, we can provide special pricing for packing multi-gram quantities of some products in GL45 bottles, upon request. In this case, we suggest that interested customers contact info@glenres.com in order to evaluate the feasibility and the possible timeline as this is coordinated with our production schedule.

NHS-CARBOXY-DT – EXPANDING THE NHS ESTER PHOSPHORAMIDITE REPERTOIRE

Introduction

When Glen Research was founded in 1987, the oligonucleotide market was very different. In these days, researchers were just delighted to have ready access to unmodified DNA oligos at a reasonable price but, in our view, the biggest change has been the adoption of strategies that use modified oligonucleotides and the ready availability of these oligos. Modification with amines and thiols at the 3' and 5' termini, as well as within the sequence, have supported explosive growth in fields such as labeling and array construction.

The most common strategy is for oligonucleotides to be modified with a nucleophile – e.g., amine or thiol – and then reacted with the corresponding N-hydroxy-succinimide (NHS) ester or maleimide of the desired label. However, the opposite approach can also be taken in which the oligonucleotide is modified with an electrophilic NHS ester and is conjugated while still on the support with a nucleophile – such as an amino-, hydrazide-, or hydroxylamine-modified label (On-Column labeling). Afterward, the labeled oligonucleotide is cleaved from the support and deprotected. In 2002, we introduced the Carboxy-Modifier C10 (1) which is the phosphoramidite derivative of an N-hydroxysuccinimidyl (NHS) ester of a decanoic acid. This has proven to be a popular item for the greater flexibility it gives the researcher.

On-Column Labeling

There are a number of advantages to On-Column labeling of oligonucleotides. Foremost is probably the much-enhanced speed and convenience of the conjugation which is amenable to high-throughput production of labeled probes. For example, as a model system, we used Dansyl Cadaverine in DMSO containing 0.5% diisopropylethylamine to label oligonucleotides quantitatively in less than 2.5 minutes at room temperature. The unreacted Dansyl Cadaverine was conveniently rinsed from the column – a great improvement over time-consuming and labor-intensive desalting of an oligo over a NAP or Sephadex column.

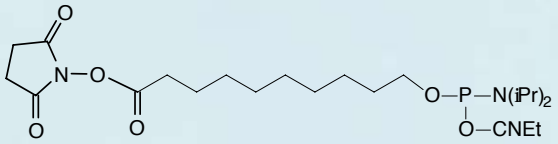
A further advantage of On-Column labeling is the minimal amount of label that is required to conjugate an NHS-derivatized oligonucleotide. This is especially useful when conjugating with an expensive molecule or antigen. With as little as 1.3 equivalents, we have observed quantitative labeling in less than 2 hours at room temperature. The reason is straightforward – in standard solution phase NHS ester chemistry, the reaction of the NHS ester with the primary amine is competing against the hydrolysis of the NHS ester since the reaction is typically performed in aqueous buffer at pH 9. As a result, On-Column labeling uses roughly 20-25% of the amount of label required for solution-phase conjugation reactions. Furthermore, with On-Column labeling, the unreacted amino-modified label can be collected and reused for further conjugations.

The only caveat for On-Column labeling is that the conjugated label HAS to be able to withstand the deprotection and DNA synthesis chemistries without degradation or branching off the label.

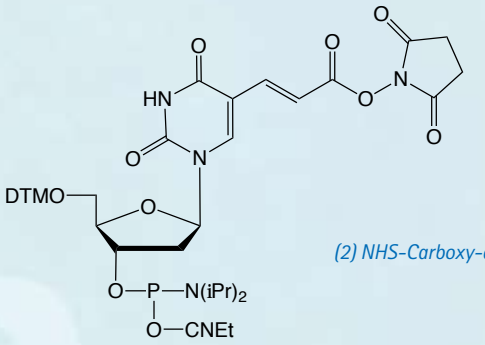
Given these useful qualities, we have decided to expand our repertoire of NHS ester derivatives to include the NHS-Carboxy-dT Phosphoramidite (2). By making a dT analog of the Carboxy-Modifier C10, it is possible to label one or multiple sites within an oligonucleotide. This opens up the possibility to label any number of different dyes or molecules within an oligonucleotide when the phosphoramidite is unavailable. Doing so is straightforward and may be done manually off the synthesizer or even in a fully-automated manner on the DNA synthesizer.

An obvious question is whether the synthesis of the oligo could be completed and then the NHS-Carboxy-dT residues within the sequenced labeled. The answer is yes – but the results are mixed. After the NHS-Carboxy-dT has undergone an additional 18 coupling cycles, approximately 50% of the NHS ester has been hydrolyzed. We believe that this is primarily due to hydrolysis during oxidation. For this reason, we recommend using a low-water content oxidizer during synthesis, such as 40-4230-xx or 40-4032-xx, which we have found improves the stability of the NHS ester.

FIGURE 1: **NHS-CARBOXY MODIFIER CE PHOSPHORAMIDITES**



(1) Carboxy-Modifier C10



(2) NHS-Carboxy-dT

Manual Labeling

For a manual coupling of the NHS-carboxy-dT off the synthesizer, the synthesis is paused after the addition of the NHS-carboxy-dT. The column is removed and fitted with two syringes, one containing the amino-modified label dissolved in an aprotic solvent such as ACN, DMF or DMSO containing 0.5 – 1.0% diisopropylethylamine. The solution is pushed back and forth and then allowed to sit for typically three to fifteen minutes, depending upon the label and its concentration. After the conjugation is complete, the column is rinsed with fresh solvent and given a final rinse with anhydrous ACN. The column is then placed back on the synthesizer and the synthesis resumed.

Automated Labeling

For the automated conjugation of the NHS-Carboxy-dT, it is necessary to edit the synthesis cycle such that the NHS-Carboxy-dT is labeled just after its incorporation into the oligonucleotide. The

most straightforward way to do this is to put the amino-modified label in a reserve port that is only activated during the coupling of the NHS-dT within the sequence.

Automated Protocol

For an ABI 394, for example, the NHS-Carboxy-dT phosphoramidite can be placed on port 6 and the amino-modified label in port 7. In the modified cycle, Function 31 '7 to column', can be inserted and selected to be active only when the NHS-carboxy-dT on port 6 is being coupled. Similarly, on an Expedite 8909, the NHS-Carboxy-dT phosphoramidite can be placed on ports 5-8 and the label on the auxiliary port. Cycle 5-8 (wherever the NHS-Carboxy-dT is placed) can be modified so that the Function 17 'Aux to column', can be inserted after the standard oxidation. After an appropriate waitstep of approximately 2.5-15 minutes, the unreacted label is rinsed from the column and the synthesis proceeds to the next base.

A copy of the modified ABI 394 cycle used can be found at http://www.glenres.com/NHS-labeling-cycle.pdf

On-Column Dansyl Labeling

To confirm this On-Synthesizer labeling technique, the following oligonucleotide was synthesized on an ABI 394.

5'-6TT T6T TT6 TT-3'

where 6 = NHS-Carboxy-dT. After each incorporation of the NHS-Carboxy-dT, Dansyl Cadaverine was pulled from port 7 and allowed to couple for 5 minutes. After the synthesis was completed, the oligo was deprotected in 30% NH_4OH and analyzed by RP HPLC, as shown in Figure 1. Analysis by Electrospray MS confirmed quantitative labeling by the Dansyl Cadaverine.

On-Column Cystamine Labeling

The NHS-Carboxy-dT phosphoramidite also allows for other interesting and useful modifications for which there are no base analogs available. For instance, labeling with Cystamine hydrochloride can yield an internal thiol modifier to be used for cross-linking. Shown in Figure 2 is an example of a manual conjugation of Cystamine with an end-labeled NHS-dT. Upon reduction with DTT, a single peak was observed, indicating quantitative labeling.

SCHEME 1: ON-COLUMN REACTIONS OF NHS-CARBOXY-DT

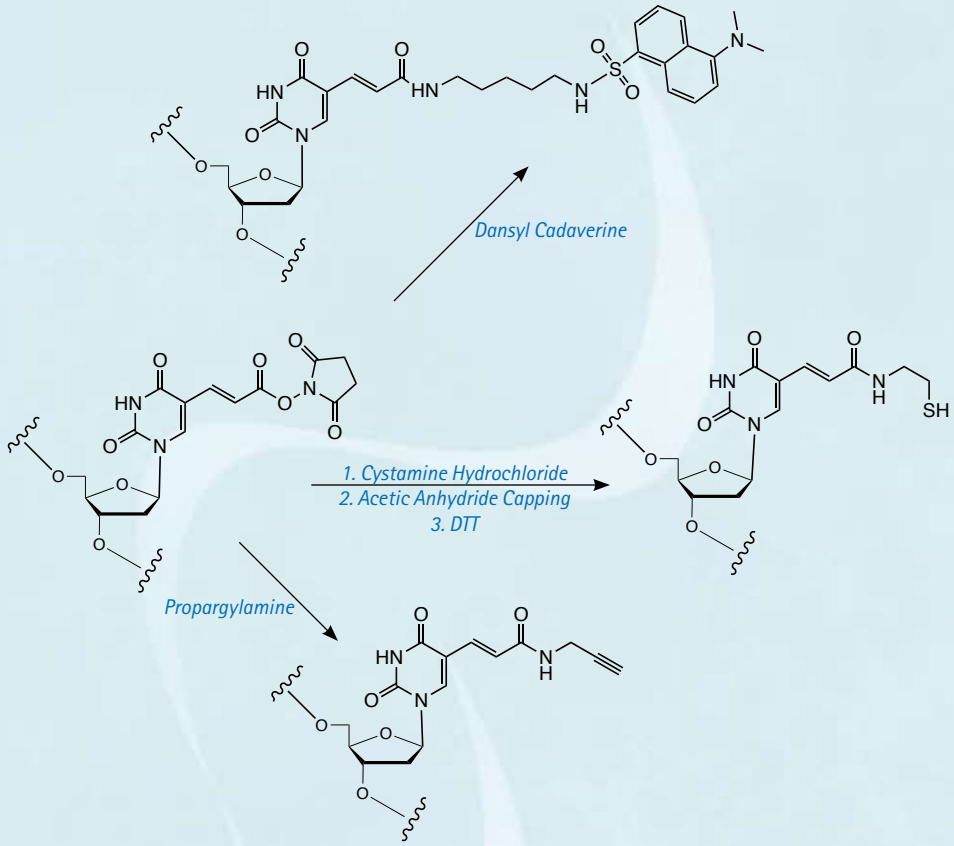


FIGURE 1: RP HPLC ANALYSIS OF DANSYL-LABELED OLIGO

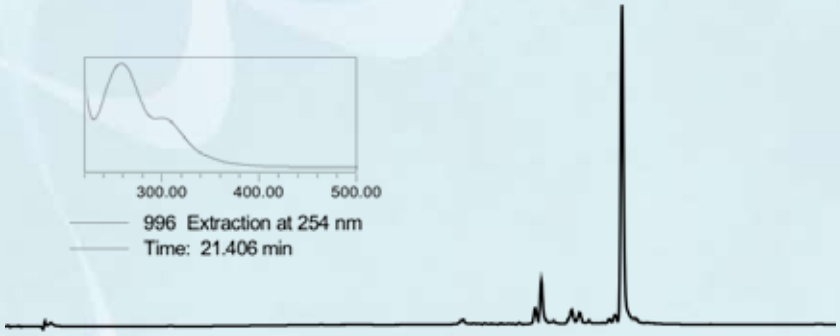


Figure 1: The result of the On-Synthesizer labeling of the oligo 5'-6TT T6T TT6 TT-3' where 6 = NHS-Carboxy-dT. Dansyl Cadaverine (4 mg/mL in 9:1 ACN/DMSO with 1% diisopropylethylamine) was pulled from port 6 and coupled for 5 minutes. The detailed procedure is described in the text.

ORDERING INFORMATION

Item	*Catalog No.*	*Pack*	*Price($)*
5'-Carboxy-Modifier C10	10-1935-90	100 µmole	50.00
	10-1935-02	0.25g	200.00
NHS-Carboxy-dT	10-1535-90	100 µmole	210.00
	10-1535-02	0.25g	550.00

(Continued from Previous Page)

These reaction schemes are detailed in Scheme 1 on Page 15. In addition, the reaction with propargylamine is shown to demonstrate the potential application to 'click' chemistry, as detailed on Page 9.

Conclusion

This technique opens up the possibility of automating the synthesis of DNA probes labeled even with dyes that usually require post-synthesis labeling. A good example would be the popular Alexa™ dyes which are not compatible with regular oligonucleotide synthesis. However, these dyes are all available as either the cadaverine or hydrazine derivative (http://probes.invitrogen.com/handbook/tables/0728.html) and one could easily envisage automated high-throughput production of probes based on these dyes. In another example, labeling with propargylamine would offer an internal alkyne for Cu(I)-catalyzed 'click' chemistry conjugation with an azide. The possibility even exists to develop procedures for conjugating oligos with peptides and PNA derivatives.

FIGURE 2: RP HPLC OF CYSTAMINE-LABELED OLIGO

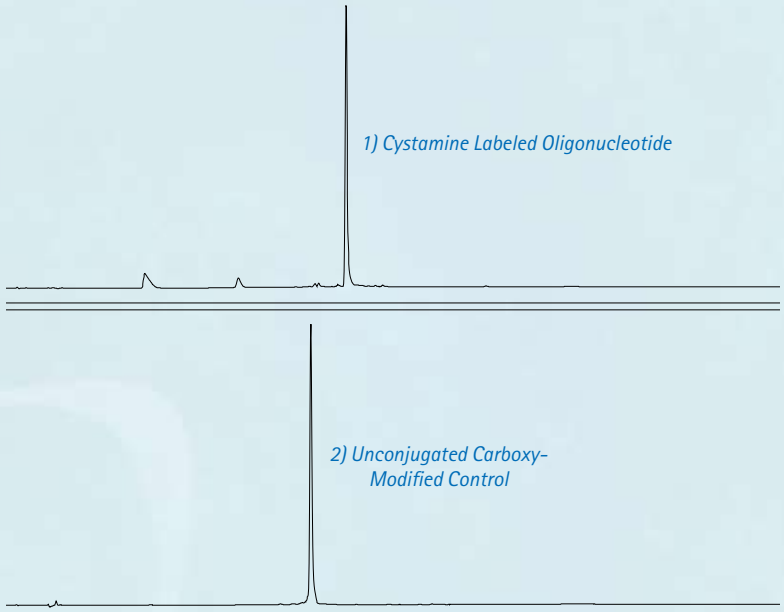


Figure 2: 1) The chromatogram of NHS-Carboxy-dT-T5 labeled with Cystamine-2HCl after reduction with DTT. Cystamine-2HCl (15 mg) was dissolved in 1 mL DMSO with 35 µL DIEA added and allowed to react for 15 minutes at room temperature. After cleavage and deprotection in ammonium hydroxide, the disulfide was reduced with DTT and the oligo analyzed by RP HPLC. The earlier eluting peaks are DTT and oxidized DTT respectively. 2) The control oligo with the NHS-Carboxy-dT hydrolyzed with 1 M NaOH 15 minutes at 65 C. Note, when labeling an oligo internally with cystamine, the oligo must be capped with acetic anhydride/N-methylimidazole after the cystamine conjugation to prevent branching off the second amine.

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