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DCI - A LOGICAL ALTERNATIVE ACTIVATOR

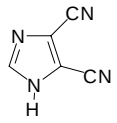
Introduction

The main reason for the success of phosphoramidite chemistry is the fact that the phosphoramidite monomer, while it is stable in acetonitrile for weeks, is readily activated to form an intermediate which reacts rapidly and efficiently with the 5'-hydroxyl group of the extending oligonucleotide. The efficiency of this coupling step during oligonucleotide synthesis correlates directly with the final yield of product. A great deal of research effort has been focused on developing activators which will increase the rate and efficiency of the coupling reaction.

The most commonly used activator, both historically and presently, is 1H-tetrazole (1). The proposed mechanism of activation with tetrazole is shown in Scheme 1.[1,2] It is interesting that tetrazole acts as a weak acid to protonate the phosphoramidite while also being the nucleophile which displaces the diisopropylamino group. The protonation is fast and reversible, while the nucleophilic displacement with tetrazole, also reversible, is the rate-determining step. The tetrazolide, so formed, is the reactive intermediate which allows rapid and efficient coupling with the 5'-hydroxyl group.

More acidic tetrazole derivatives would be expected to speed up phosphoramidite activation by faster protonation. And so, it transpires that 5-(4-nitrophenyl)-1H-tetrazole (2)[3], 5-methylthio-1H-tetrazole (3), and 5-ethylthio-1H-tetrazole (4), among others, have found favor as "turbo" activators. Ethylthiotetrazole is especially popular[4,5] for use in RNA synthesis, where the coupling step is made more sluggish by the presence of the bulky silyl protecting group on the adjacent 2'-position.

A major drawback to the acidity of tetrazole and the quest for more acid versions has been revealed in a study on larger scale synthesis.[6] This study revealed that tetrazole is sufficiently acidic to deprotect to a small extent the trityl group in the monomer solution, leading to a small amount of dimer formation. Coupling of the dimer phosphoramidite leads to the presence of longer oligos in the crude product mixture.



DCI - 4,5-Dicyanoimidazole

DCI

The synthesis group at NeXstar Technology Products, working on the preparation of highly modified RNA derivatives, found that the use of tetrazole as activator led to very low yields of full-length products. Addition of 0.1M methylimidazole (NMI) to the activator solution as a buffer to tetrazole's acidity led to much higher yield of full-length product.[7] This result indicates that tetrazole is, in fact, slightly too acidic and led to a search for an alternative activator. As can be seen from the mechanism of activation in Scheme 1, the rate determining step is the nucleophilic displacement of the diisopropylamino group from the protonated

(Continued on Page 2)

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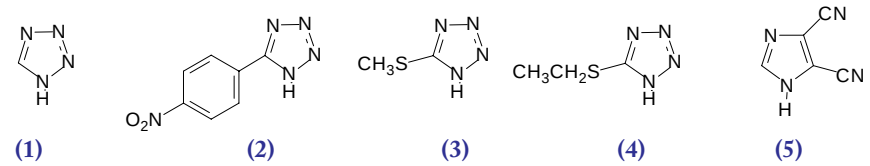
intermediate. This led to the search for a less acidic but more nucleophilic activator and the discovery of 4,5-dicyanoimidazole (DCI) (5) as the first true alternative activator to tetrazole and its derivatives. DCI offers the following advantages over tetrazole:

- DCI is less acidic with a pKa of 5.2, compared with a pKa of 4.8 for tetrazole (which is the same as acetic acid).
- DCI is more soluble than tetrazole allowing a 1.1M solution in acetonitrile, while tetrazole is saturated at 0.5M in acetonitrile.
- DCI is more nucleophilic than tetrazole and doubles the coupling rate relative to tetrazole.

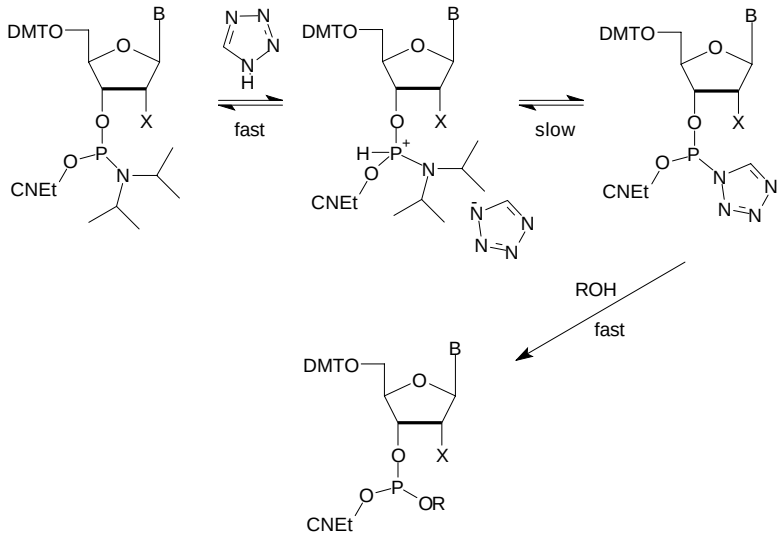
The biggest difference between DCI and tetrazole manifests itself at larger scales with a low monomer excess. For example, a 34mer oligoribonucleotide, including 2'-fluoropyrimidine residues, was prepared on a 1 mmole scale with 2 equivalents of monomer using 0.45M tetrazole, 0.45M tetrazole + 0.1M NMI, or 1M DCI as activator. No full length product was detected with tetrazole activation, while a low yield (13%) of product was observed with the activator containing NMI. With DCI the full-length product was observed in 54% yield. This is a remarkable demonstration of the increased effectiveness of DCI.

Our studies with DCI show that 0.25M is the optimal concentration for routine small-scale synthesis (< 15 µmole), using normal synthesis cycles. We are therefore providing solutions at that concentration but we will also offer the raw material so that researchers can prepare more or less concentrated solutions should they desire. Distribution of DCI by Glen Research is done in collaboration with NeXstar Technology Products.

FIGURE 1: ACTIVATOR STRUCTURES



SCHEME 1: MECHANISM OF PHOSPHORAMIDITE ACTIVATION



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
DCI, crystalline	30-3050-07	0.7g	25.00
	30-3050-15	1.5g	50.00
	30-3050-25	25g	500.00
Applied Biosystems Instruments			
0.25M DCI in Acetonitrile	30-3150-45	45mL	50.00
	30-3150-52	200mL	120.00
	30-3150-57	450mL	250.00
	30-3150-62	2L	950.00
Expedite Instruments			
0.25M DCI in Acetonitrile	30-3152-66	60mL	60.00
	30-3152-52	200mL	120.00
Beckman Instruments			
0.25M DCI in Acetonitrile	30-3150-52	200mL	120.00

References:

(1) O. Dahl, J. Nielsen, and B.H. Dahl, *Nucleic Acids Res.*, 1987, **15**, 1729-1742.
(2) S. Berner, K. Muhlegger, and H. Seliger, *Nucleic Acid Res.*, 1989, **17**, 853-864.
(3) B.S. Sproat, A.I. Lamond, B. Beijer, P. Neuner, and U. Ryder, *Nucleic Acids Res.*, 1989, **17**, 3373.
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(6) A.H. Krotz, P.G. Klopchin, K.L. Walker, G.S. Srivatsa, D.L. Cole, and V.T. Ravikumar, *Tetrahedron Lett.*, 1997, **38**, 3875-3878.
(7) C. Vargeese, J. Carter, J. Yegge, S. Krivjansky, A. Settle, E. Kropp, K. Peterson, and W. Pieken, *Nucleic Acids Res.*, In Press.

PREPARING OLIGONUCLEOTIDES FOR ANTISENSE EXPERIMENTS

The last 20 years have brought an amazing advance in the use of oligonucleotides in molecular biology. With the development of phosphoramidite chemistry and the application of solid phase synthesis techniques in the early 80s, oligonucleotide synthesis has grown from a technique restricted to a few specialized labs to one available to almost any researcher. As a result, oligonucleotides have found increasing use in research, diagnostic and therapeutic applications. One area of particular interest has been that of antisense research. Antisense oligonucleotides are designed to be complementary to critical regions on mRNA of a targeted gene. They act by binding to mRNA and blocking the translation of sequence information into protein synthesis. This is accomplished either directly through translation arrest, or indirectly through the activation of RNase H, an enzyme which degrades RNA in RNA/DNA duplexes.

Since they are used either *in vitro* or *in vivo* , oligonucleotides for antisense experiments present a number of unique challenges in their design, synthesis and purification. Changes in the design of oligonucleotides for antisense experiments include backbone modification to block degradation by nucleases and base or sugar modification to increase hybridization Tm and specificity. Examples are phosphorothioate backbone modification to increase nuclease resistance and use of propynyl pyrimidines and 2'-OMe or 2'-fluoro sugars to increase hybridization Tm. Most oligonucleotides used in antisense experiments have phosphorothioate backbones.

An often overlooked area is that of preparing oligonucleotides for antisense experiments. Some of the common reagents used in synthesis and purification can be quite toxic to cells and must be removed prior to use either in tissue culture or in animal studies. Crude oligonucleotides can be contaminated with residual synthesis solvents as well as the deprotection by-products of the base and phosphate protecting groups. Purified oligos will exist as the salt of the buffer used for their purification. The most common technique used for the purification of phosphorothioate oligos is reverse phase chromatography of the trityl-on oligonucleotide using either a reverse phase cartridge or HPLC column. Oligonucleotides purified by reverse phase chromatography typically use triethylammonium acetate (TEAA) buffers and are isolated as the triethylammonium salt. While the presence of triethylamine in oligonucleotides used in enzyme systems is generally not deleterious to their activity, it can be quite toxic to cells grown in tissue culture.

There are several methods for preparing oligos for antisense experiments that eliminate the above effects.

Ethanol Precipitation:

Crude oligos are best prepared by two EtOH precipitations from sodium acetate. This will remove organic contaminates as well as yield the oligo as the sodium salt. Following EtOH precipitation the oligo can be dissolved in buffer and filtered through a 0.22 micron sterile filter before use.

1) Dissolve the crude oligo in 0.3 M sodium acetate-100 A_{260}units/mL, 1 mL for 1µmole or 0.4 mL for 0.2µmole syntheses.
2) Add 3 times the volume of 95% EtOH, vortex and store at -20 °C for at least 30 minutes. Centrifuge at high speed for 10 minutes.
3) Carefully remove supernate with pipet being careful not to disturb the pellet.
4) Resuspend the oligo in an original volume of 0.3 M sodium acetate, and repeat EtOH precipitation.
5) After removing supernate carefully rinse pellet with 95% EtOH. Centrifuge at high speed for 10 minutes.
6) Pipet off the supernate and dry the pellet in a Speed-vac.
7) Dissolve the oligo in H_2O or buffer of choice, filter through a sterile filter and quantify by absorbance at 260 nm.

Poly Pak Purification:

Antisense oligos can easily be purified on Poly Pak cartridges using a slightly modified procedure to convert to the sodium salt.

1) Process oligos as normal through the TFA detritylation step.
2) Rinse the cartridge with 3 mL 10 mM NaOH containing 0.2 M NaCl. (This will convert the phosphorothioate backbone from the acid form to the sodium salt.)
3) Wash the cartridge again with 2 mL H_2O and elute the oligo with 1 mL of 20 % acetonitrile in H_2O. (For purification of 1 µmole syntheses using Poly Pak II cartridges, double the volumes of all solutions.)

HPLC Purification:

Phosphorothioate oligos can be purified by reverse phase or anion exchange chromatography. Reverse phase purification of trityl-on oligos is routinely done on C-18 silica or polymer columns, followed by detritylation of the pooled fractions with acetic acid and EtOH precipitation to remove DMT alcohol, acetic acid and excess salts.

TEAA buffer with an acetonitrile gradient can be used for occasional purifications provided that triethylamine is removed. This can be accomplished by multiple EtOH precipitations following detritylation of the pooled trityl-on fractions. Three EtOH precipitations will remove TEA to ppm concentrations when analyzed by ion chromatography. For labs purifying either a large number of phosphorothioate oligos or large scale syntheses, RP chromatography can be done using sodium acetate[1] or ammonium acetate[2] buffers.

For oligos that have a high dG content or have internal complementary structure, denaturing chromatography on polymer based columns using 10 mM NaOH acetonitrile gradients has been used effectively.

Anion exchange chromatography has also been used to purify phosphorothioate oligos. Due to the increased hydrophobic nature of phosphorothioates, polymer-based strong anion exchangers work best. Chromatography is usually done using denaturing buffers such as 20 mM NaOH with a gradient to 1.5 to 2.0 M NaCl.

Ion exchange chromatography has also been used to separate fully thioated from partially thioated oligos and to quantify the degree of thioation.[3]

References:

(1) V.T. Ravikumar, M. Andrade, T. Wyrzykiewicz, A. Scozzari, and D.L. Cole, *Nucleosides & Nucleotides*, 1995, **14**, 1219-1226.
(2) A.A. Padmapriya, J. Tang, and S. Agrawal, *Antisense Res. Dev.*, 1994, **4**, 185-199.
(3) B.J. Bergot and W. Egan, *Journal of Chromatography*, 1992, **599**, 35-42.

CYTOFECTIN GSV TRANSFECTION PROTOCOL

Introduction

Introducing DNA into cells with cationic lipids can be a powerful tool for examining the roles of genes in biological systems. For example, antisense oligonucleotides, delivered into cells with cationic lipids, have become standard tools for the elucidation of gene function.[1,2] Until now, the problems associated with lipid-mediated delivery have been poor transfection efficiency and/or high levels of cytotoxicity. Cytofectin GSV alleviates both of these problems, delivering DNA efficiently to a broad spectrum of cell lines in the presence of serum containing growth media. Cytofectin GSV is a formulation of two moles of a cationic lipid, cytofectin GS, with one mole of the zwitterion L-α dioleoyl phosphatidyl-ethanolamine (DOPE*). Cytofectin GSV delivers DNA with or without serum. (Cytofectin GS corresponds to Gilead Sciences' GS 3815 which is a more stable formulation of the cytofectin GS 2888 (Figure 1) reported by Lewis, *et al.*[3]) Like GS 2888 vesicles, Cytofectin GSV can deliver plasmids as well as oligonucleotides. It should be noted that Cytofectin GSV works best with adherent cells and performs less well with cells grown in suspension culture.

The following is a protocol for transfection of antisense phospho-rothioate oligodeoxynucleotides using Cytofectin GSV into adherent cells grown on 100 mm plates (Figure 2). It was provided by Gilead Sciences where this system has been used for gene inhibition with C-5 propyne antisense oligonucleotides in low nanomolar concentrations.[3-7] Gilead's contribution to this project is gratefully acknowledged.

Stability and Storage Conditions

1. Store Cytofectin GSV at 4°C; mix well before use. It is stable for at least three months. For a longer term, store at -70°C. Cytofectin GSV is a liquid formulation of 2 moles of the cationic lipid, Cytofectin GS, and one mole of DOPE and shipped as a 2 mg/mL lipid in sterile water. The formula weight of Cytofectin GS is 782 and of DOPE is 744.

2. Store oligos at -20°C. If the oligo will be used frequently, store it at 4°C.

Determination of Appropriate Oligo and Lipid Concentrations

Other transfection reagents only work in a narrow range based on the final charge ratio of the lipid/DNA complex. Cytofectin GSV, however, works across a much broader range of charges, eliminating the need for numerous calculations. We suggest beginning with 2.5 µg/mL of lipid for transfecting oligos and 10 µg/mL for plasmid transfections, and titrating the oligo within the ranges below.

1. Oligo concentration range:
   500 nM-1 nM depending on the potency of the oligo used.
   Phosphorothioate oligos:
   500 nM-100 nM
   Propyne modified oligos:
   30 nM-1 nM

2. Plasmid concentration range:
   1.0 - 2 µg/mL.

3. Lipid concentration range:
   1.0 - 10 µg/mL.

Plating cells

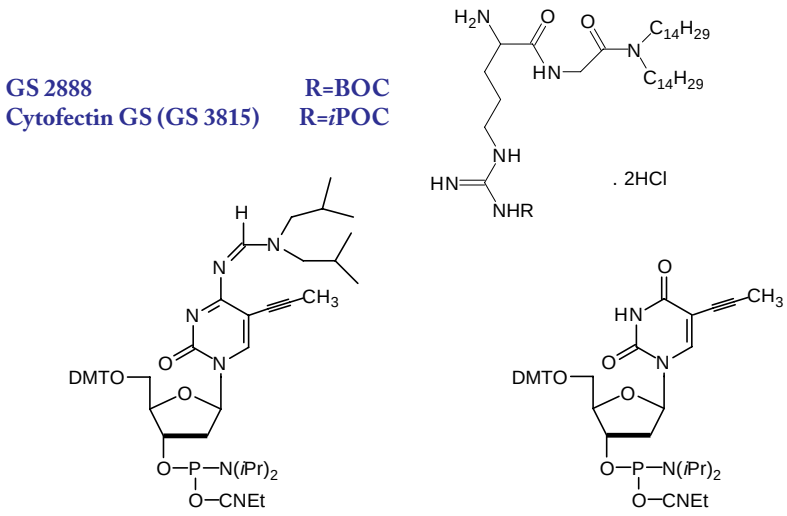
In 100 mm plates, plate cells so they achieve 60-80% confluence on the day of transfection.

Oligo/Lipid Complex-4 mL Final Volume

1. Vortex oligos well and dispense appropriate amount into Eppendorf tubes. (The oligos do not need to be kept sterile.)

2. Transfer the tubes to a tissue culture hood, and add Opti-MEM media (Life Technologies Inc.) to each tube to a final volume of 200 µL (*i.e.*, to 4

FIGURE 1: STRUCTURES OF CYTOFECTIN GS and C-5 PROPYNYL PYRIMIDINES



pdC-CE Phosphoramidite pdU-CE Phosphoramidite

FIGURE 2: DETERMINATION OF TRANSFECTION VOLUME

Plate size	Transfection Volume	Volume of 2 mg/mL Lipid
6-well plate	1 mL	1.25 µL
60 mm plate	2 mL	2.5 µL
100 mm plate	4 mL	5µL

µL of a 10 µM oligo stock, add 196 µL Opti-MEM). Opti-MEM can be replaced by non-serum containing minimum essential medium (MEM).

3. Aliquot the lipid (in a tissue culture hood) into a 12-well ***polystyrene*** tissue culture plate.

Note: Use of polystyrene plates is essential as the oligo/lipid complex binds to polypropylene.

4. Add Opti-MEM to each well to a final volume of 200 µL (i.e., to 10 µL of a 1 mg/mL lipid stock add 190 µL Opti-MEM).

5. Add 200 µL of oligo/Opti-MEM mixture in the Eppendorf tube from Step 2 to the corresponding well in the 12-well polystyrene tissue culture plate containing the lipid/Opti-MEM mixture.

200 µL oligo/Opti-MEM
+ 200 µL lipid/Opti-MEM
= 400 µL

FIGURE 3: PHOTOMICROGRAPH OF CELL TRANSFECTED WITH PLASMID

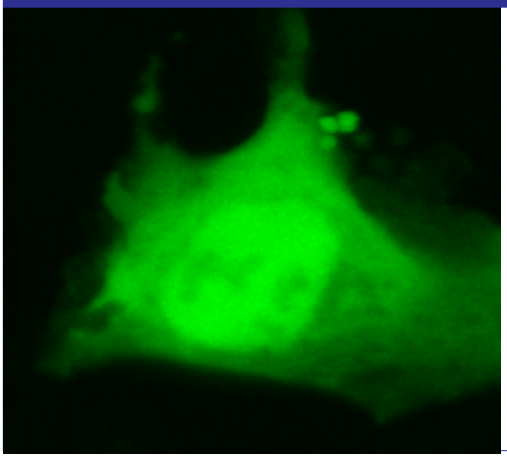


Image from confocal microscopy of a cell transfected with a plasmid using Cytofectin GS and the procedures described here. We thank Tom Keller, Oregon Health Sciences University for providing us with this and other similar images.

6. Incubate 10 to 15 minutes at RT.

7. Add 3.6 mL complete growth media (10% FBS and antibiotics do not affect transfection efficiency) and mix well for a total transfection volume of 4 mL.

Transfection

1. Remove media from cells and replace with the 4 mL of the oligo/lipid transfection mixture. Do not wash cells at any time with PBS or Tris buffered saline before or after the addition of the transfection mix as this may affect transfection efficiency.

2. Incubate transfection mixture with cells for 4-6 hours, then add a further 4 mL of complete media. Do not remove the lipid/oligo transfection mixture after 4-6 hours unless intolerable toxicity is observed.

3. Dose for 24-48 hours before

PREPARATION OF CYTOFECTIN GS VESICLES[1]

Materials

a. Cytofectin GS: (Glen 70-3815-70)

b. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in CH_3Cl (10 mg/ml): Avanti Polar Lipids, Inc., Alabaster, Alabama, # 850725* or equivalent.

Method

1. To one tube containing 2 mg of cytofectin GS 3815 lipid add 0.1 ml of a 10 mg/ml solution of DOPE in chloroform. Vortex to dissolve Cytofectin GS. This will yield a final molar ratio of Cytofectin GS to DOPE of 2:1.

2. Evaporate the chloroform in a speed-vac or under an argon stream to form a thin lipid film in the tube.

3. Add 1.5 ml sterile water to the film to form a lipid suspension. Vortex the suspension for 5 min. This yields a final lipid concentration of 2 mg/ml.

4. Freeze-thaw the tubes 6 times (dry ice/37 °C bath).

5. Store lipid preparation at 4 °C. Material retains activity for ≥3 months. Also material can be stored at -70 °C and will retain activity for ≥1 year.

6. The liposomes can be used directly or sized by filtration through a polycarbonate membrane (50-200 nm pore sizes; Liposofast; Avestin, Ottowa, Canada). 100 nm vesicles were found to be optimal in studies by Gilead Sciences.

Uptake analysis of fluorescent oligonucleotides in African green monkey kidney cells (CV1), at Gilead Sciences, showed that an unsized formulation, prepared by the freeze-thaw method, of 2:1 (mole:mole) ratio of Cytofectin GS to DOPE was optimal for delivery. This formulation is referred to as Cytofectin GSV (Glen 70-3815-79).

* Avanti Polar Lipids, Inc.
700 Industrial Park Drive
Alabaster, AL 35007
Phone: 1-800-227-0651 or 205-663-2494
Fax: 1-800-229-1004 or 205-663-0756
Email: avanti@quicklink.net

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analyzing cells or preparing protein extracts. Exact timing of the experiment will depend on the $t_{1/2}$ of the protein of interest. The $t_{1/2}$ of phosphorothioate oligos is ~35 hours; phosphodiester oligos have a $t_{1/2}$ of 20 minutes.

In Table 1, we have listed cell lines which have been successfully transfected with Cytofectin GSV.

References:

(1) Wagner, R. W. Gene inhibition using antisense oligodeoxynucleotides. *Nature*, 1994, **372**, 333-5.

(2) Wagner, R. W., Matteucci, M. D., Lewis, J. G., Gutierrez, A. J., Moulds, C. and Froehler, B. C. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science*, 1993, **260**, 1510-3.

(3) Lewis, J. G., Lin, K.-Y., Kothavale, A., Flanagan, W. M., Matteucci, M. D., DePrince, R. B., Mook, J. R. A., Hendren, R. W. and Wagner, R. W. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl. Acad. U.S.A.*, 1996, **93**, 3176-3181.

(4) Flanagan, W. M., Su, L. and Wagner, R. W. Elucidation of cell cycle gene function using C-5 propyne antisense oligonucleotides. *Nature Biotechnology*, 1996, **14**, 1141-1145.

(5) Flanagan, W. M., Kothavale A. and Wagner, R. W. Effects of oligonucleotide length, mismatches and mRNA levels on C-5 propyne-modified antisense potency. *Nucleic Acids Research*, 1996, **24**, 2936-2941.

(6) Coats, S., Flanagan, W. M., Nourse, J. and Roberts, J. M. Requirement of P27kip1 for restriction point control of the fibroblast cell cycle. *Science*, 1996, **272**, 877-880.

(7) St. Croix, B., Florenes, V. A., Rak, J. W., Flanagan, M., Bhattacharya, N., Slingerland, J. M. and Kerbel, R. S. Impact of the cyclin dependent kinase inhibitor P27kip1 on adhesion-dependent resistance of tumor cells to anticancer agents. *Nature Medicine*, 1996, **2**, 1204-1210.

Note:

Cytofectin GS and C-5 propynyl pyrimidine nucleoside phosphoramidites are made available by Glen Research under exclusive license from Gilead Sciences.

TABLE 1: CELL LINES TRANSFECTED WITH CYTOFECTIN GS

Cell Line	Species	Type
Transfected Successfully		
Cos-7	African Green Monkey	Kidney
CV-1	African Green Monkey	Kidney
BalbC-3T3	Mouse	Embryo fibroblast
Rat-2	Rat	Embryo
HeLa	Human	Cervical carcinoma
NHDF	Human	Dermal fibroblast
MCF-7	Human	Breast carcinoma
A549	Human	Lung carcinoma
T24	Human	Bladder carcinoma
HCT116	Human	Colon carcinoma
H460	Human	Lung carcinoma
WiDR	Human	Colon carcinoma
HT-29	Human	Colon carcinoma
CasKi	Human	Cervical carcinoma
SiHa	Human	Cervical carcinoma
Transfected with Limited Success		
MDA-MB	Human	Breast
MES	Human	Ovarian carcinoma

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
pdC-CE Phosphoramidite	10-1014-90	100 µmole	85.00
	10-1014-02	0.25g	245.00
	10-1014-05	0.5g	490.00
pdC-lcaa-CPG 500	20-2014-01	0.1g	75.00
1 µmole columns	20-2114-41	Pack of 4	250.00
0.2 µmole columns	20-2114-42	Pack of 4	150.00
pdU-CE Phosphoramidite	10-1054-90	100 µmole	65.00
	10-1054-02	0.25g	195.00
	10-1054-05	0.5g	390.00
pdU-lcaa-CPG 500	20-2054-01	0.1g	75.00
1 µmole columns	20-2154-41	Pack of 4	250.00
0.2 µmole columns	20-2154-42	Pack of 4	150.00
Cytofectin GS *(Raw Material, Requires DOPE for Formulation)*	70-3815-70	2 mg	95.00
	70-3815-71	10X2 mg	900.00
Cytofectin GSV *(Vesicles Formulated with DOPE to 2 mg/mL lipid)*	70-3815-79	1 mL	105.00
	70-3815-78	10X1 mL	1000.00

NEW FLUORESCENT REAGENTS - TAMRA CPG, FLUORESCEIN-dT

The interest in and uses for fluorescently-labelled oligonucleotides continues to increase by leaps and bounds. Glen Research is happy to have participated in this growth by being among the first companies to make available reagents for modifying oligos for subsequent labelling with fluorescent dyes. More recently, we have been in the forefront of supplying fluorescent dyes attached to supports for 3'-labelling, as well as to phosphoramidites for sequence and 5'-labelling. We are now happy to introduce two new fluorescent products: 3'-TAMRA CPG (1) and fluorescein-dT (2) (Figure 1).

Glen Research is dedicated to bringing the newest techniques and products to the research market. Many of our new products are covered by patent, or groups have shared intellectual property with us. In this *Glen Report* alone, six new products are offered under license for which a license fee has been paid and royalties will accrue. We fully support the role of patents in our field. In the area of fluorescent labelling, many applications of the products are covered by patent. We would request that our customers make themselves aware of any patents covering the use and processing of labelled oligonucleotides and take appropriate action with the patent holders.

TAMRA CPG

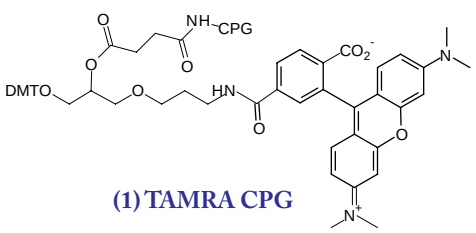
Many researchers have requested that we offer more products containing tetramethylrhodamine (TAMRA) to supplement the NHS ester we currently supply. Because TAMRA is not stable to ammonium hydroxide, the procedure to cleave and deprotect the labelled oligonucleotide must be considered as the first priority. The Fmoc protecting group on our 3'-Amino-Modifier CPGs can be removed specifically with DBU/acetonitrile or piperidine/DMF, releasing the primary amine. By labelling this amine with TAMRA-NHS ester, it is possible to generate an oligo with TAMRA at the 3'-terminus. Using the UltraMILD monomers and deprotection with potassium carbonate in methanol, TAMRA oligonucleotides can be fairly conveniently isolated. To further streamline the preparation of TAMRA oligos, we now offer the support 3'-TAMRA CPG (1).

A recent publication[1] describes an alternative TAMRA support, along with a procedure[2] to allow the use of regular monomers rather than our UltraMILD set. Cleavage and deprotection is carried out using *t*-butylamine/methanol/water (1:1:2).

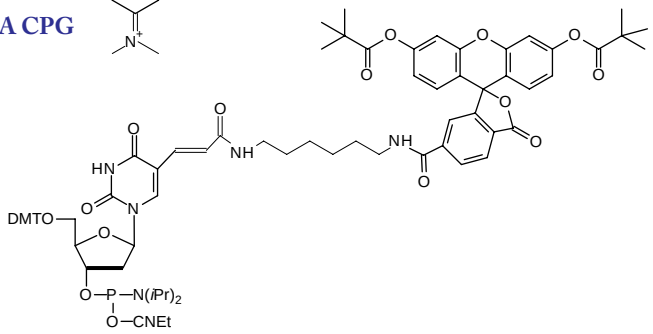
Fluorescein-dT

Amino-Modifier C6-dT has become the monomer of choice for modifying oligonucleotides within the sequence. We have been asked for some time to supply this monomer pre-labelled with fluorescent dyes. Now, we are pleased to offer fluorescein-dT (2). This monomer can be inserted into the desired sequence as a replacement for a dT residue. Normal cleavage and deprotection with ammonium hydroxide readily generates the fluorescein labelled oligo.

FIGURE 1: STRUCTURES OF NEW FLUORESCENT PRODUCTS



(1) TAMRA CPG



(2) Fluorescein dT

References:

(1) B. Mullah and A. Andrus, *Tetrahedron Lett*, 1997, **38**, 5751-5754.

(2) S.L. Woo, S.M. Menchen, and S. Fung, 1993, US Patent No. 5,231,191.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
3'-TAMRA CPG 500	20-5910-01	0.1g	120.00
	20-5910-10	1.0g	995.00
1 µmole columns	20-5910-41	Pack of 4	200.00
0.2 µmole columns	20-5910-42	Pack of 4	120.00
Fluorescein-dT	10-1056-95	50 µmole	180.00
	10-1056-90	100 µmole	325.00
	10-1056-02	0.25g	675.00

UNIVERSAL SUPPORT REPLACES INDIVIDUAL COLUMNS

Current procedures in oligonucleotide synthesis require that the solid support contains the first nucleoside which is destined to become the nucleoside at the 3'-terminus of the synthetic oligonucleotide. This situation therefore requires that an inventory of all four regular nucleoside supports must be maintained. At the same time, oligonucleotides with unusual nucleosides, available as phosphoramidites but not as supports, at the 3'-terminus can not be readily prepared. However, the most worriesome aspect of this situation is the potential for a mistake to be made in the selection of the column containing the 3'-nucleoside. This potential for error may be fairly low in regular column-type synthesizers, but it is especially significant in the new generation of parallel synthesizers where 96 or 192 wells may contain all four supports in a defined grid.

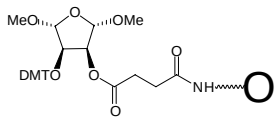
Universal supports are routinely used for preparing modified oligonucleotides with amine, thiol, or phosphate groups at the 3'-terminus. In these cases, it is standard to add the nucleoside which is destined to become the 3'-terminal nucleoside as the first monomer addition. This strategy always leads to a 3'-modifier attached with a phosphodiester group to the 3'-terminal nucleoside. A universal support for preparing regular oligonucleotides must allow the elimination, during the deprotection step, of the terminal phosphodiester linkage along with the group originally attached to the support.

A support which has the potential to be truly universal for the production of oligonucleotides containing a 3'-hydroxyl group has been described recently.[1] The structure is shown in Figure 1. The detritylation of the support is carried out under normal conditions, as is the addition of the first nucleoside monomer. The remainder of the oligonucleotide synthesis also proceeds without any changes from the regular cycles.

A proposed mechanism for the base-mediated elimination of the terminal phosphodiester group in the oligonucleotide is shown in Figure 2. This is the crucial step in this strategy. The elimination must proceed promptly under conditions comparable with routine deprotection strategies, using reagents which are also standard. Preferably the reagents should be volatile for simple evaporation (ammonium hydroxide or aqueous methylamine) or readily desalted (aqueous sodium hydroxide).

The most likely options for routine use are shown in Table 1. The conditions necessary for complete elimination of the terminal phosphodiester linkage, although aggressive, are totally compatible with the isolation techniques used commonly for oligonucleotides. However, it is fair to say that they are not compatible with base-sensitive minor nucleosides.

FIGURE 1: UNIVERSAL SUPPORT



(1)

The Universal Support (1) is sold under license from Zeneca PLC.

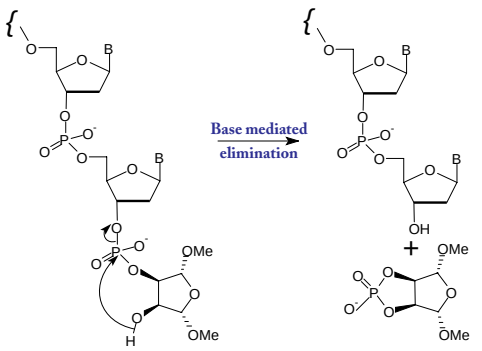
Reference:

(1) S. Scott, P. Hardy, R.C. Sheppard, and M.J. McLean, *Innovations and Perspectives in Solid Phase Synthesis, 3rd International Symposium,* **1994**, Ed. Roger Epton, Mayflower Worldwide, 115-124.

TABLE 1: ELIMINATION CONDITIONS

Reagent	Conditions
Ammonium hydroxide	80°C/17h
Ammonium hydroxide/ 40% Methylamine (AMA)	55°C/17h
40% Methylamine	55°C/5h
0.5M NaOH in methanol/water (1:1)	RT/1h

FIGURE 2: MECHANISM OF ELIMINATION



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
500Å Bulk Support			
Universal Support 500	20-5000-01	0.1g	11.00
	20-5000-02	0.25g	25.00
	20-5000-10	1.0g	95.00
ABI Format (not LV)			
1 µmole columns	20-5100-41	Pack of 4	60.00
0.2 µmole columns	20-5100-42	Pack of 4	40.00
40 nmole columns	20-5100-45	Pack of 4	40.00
10 µmole column (TWIST Format)	20-5100-13	Pack of 1	150.00
Biosearch Format			
1 µmole columns	20-5200-41	Pack of 4	60.00
0.2 µmole columns	20-5200-42	Pack of 4	40.00
40 nmole columns	20-5200-45	Pack of 4	40.00
15 µmole column (TWIST Format)	20-5200-14	Pack of 1	150.00

Q-SUPPORTS REDUCE CLEAVAGE TIME TO 2 MINUTES

Oligonucleotides are routinely prepared on supports to which the first nucleoside is attached via a succinate linkage. Over the years, the succinate linkage has demonstrated stability during the synthesis process but has sufficient lability to be cleaved quickly in the deprotection step. However, if the cleavage step is carried out with ammonium hydroxide manually or on the synthesizer, it consumes one hour of precious time while releasing only about 80% of the oligonucleotide. This step is, therefore, a bottleneck in the productivity of many synthesis groups.

Is it possible to find a replacement to the succinate group which offers good stability to the synthesis reagents while offering a much faster cleavage step? The oxalate group has been shown to be very labile during cleavage but its stability to the normal synthesis reagents is not good, requiring changes for successful use. In a practical but elegant study[1] of various bifunctional carboxylic acids, Richard Pon's group identified hydroquinone-O,O'-diacetic acid as the most satisfactory alternative to the succinate group. Nucleosides with this linker arm (Q-linker) are attached to supports with the same ease as the succinyl linker arm.

The cleavage time in ammonium hydroxide at room temperature was found to be a mere 2 minutes, but what about the stability during synthesis? How significant was premature cleavage of oligonucleotide on the synthesizer because of the basic reagents in the capping mixes and oxidizer? Pon showed that the Q-linker is stable to the capping reagents but very slightly labile to the oxidizer (8% cleavage in overnight exposure which would correspond to about 2,000 normal synthesis cycles).

Ever the skeptics, we set out to test the significance of premature cleavage by preparing sixteen 20mer oligonucleotides on a 0.2 µmole scale, eight with succinate and eight with Q-linkers. The succinate supported oligos were cleaved for 1 hour at room temperature, while those on the Q-support were cleaved for 2 minutes. Both sets were then deprotected normally with ammonium hydroxide. The results are collected in Table 1. The Q-supports actually gave 5% better yields of product than the succinate supports. Oligo purities were equivalent in both sets.

The Q-linker is absolutely compatible with all hydrolytic cleavage procedures, but especially mild procedures like potassium carbonate in methanol. Pon also showed that it is preferable for RNA supports, improving the cleavage time for 2'-silyl protected nucleoside supports from 2 hours (60-65% cleavage) to 5 minutes (95% cleavage). The Q-linker is perfectly compatible with polystyrene supports.

We are happy to offer Q-supports under license from the University of Calgary. Initially, we are offering Q-linkers on 500Å CPG in 0.2 and 1 µmole scales. The four most popular nucleosides are offered, as well as the Universal Support.

TABLE 1: Q/SUCCINATE COMPARISON

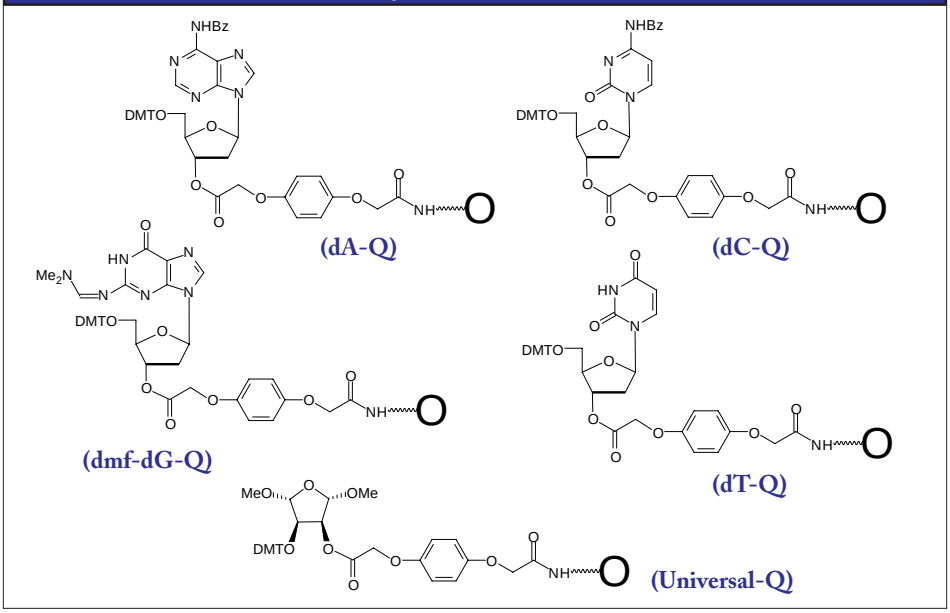
Q-Support (2 minutes cleavage)	Succinate (60 minutes cleavage)
132 ODU*	125 ODU*

*Average crude yield from eight 1µmole columns deprotected normally.

Reference:

(1) R.T. Pon and S.Y. Yu, *Tetrahedron Lett*, 1997, **38**, 3327-3330.

FIGURE 1: Q-SUPPORT STRUCTURES



ORDERING INFORMATION

Catalog No. dA	Catalog No. dC	Catalog No. dmf-dG	Catalog No. dT	Catalog No. Universal	Pack	Price($)
500Å Bulk Support						
21-2000-01	21-2010-01	21-2029-01	21-2030-01	21-5000-01	0.1g	11.00
21-2000-02	21-2010-02	21-2029-02	21-2030-02	21-5000-02	0.25g	25.00
21-2000-10	21-2010-10	21-2029-10	21-2030-10	21-5000-10	1.0g	95.00
ABI Format (not LV)						
21-2100-41	21-2110-41	21-2129-41	21-2130-41	21-5100-41	Pk/4	60.00
21-2100-42	21-2110-42	21-2129-42	21-2130-42	21-5100-42	Pk/4	40.00
Biosearch Format						
21-2200-41	21-2210-41	21-2229-41	21-2230-41	21-5200-41	Pk/4	60.00
21-2200-42	21-2210-42	21-2229-42	21-2230-42	21-5200-42	Pk/4	40.00

MORE NOVEL MONOMERS - 4-THIO-dU, 5'-AMINO-dT, 2'-F-PYRIMIDINES

4-Thio-dU

Demand for sulfur modified bases continues to expand for investigations of oligonucleotide structure, but primarily for cross-linking purposes. We are happy to broaden our line of sulfur modified nucleoside phosphoramidites with the addition of 4-thio-dU (1). We have protected this monomer as the S-cyanoethyl ether[1, 2] which is stable during synthesis and readily removed by ammonium hydroxide.

It is critical to add 50mM sodium hydrosulfide (NaSH) to the ammonium hydroxide used for deprotection. Especially if room temperature deprotection is carried out, this technique radically reduces the level of ammonolysis which would lead to undesired dC. Moreover, in critical applications, it is also desirable[3] to remove the cyanoethyl protecting group (1M DBU in acetonitrile, 3h/RT) prior to the ammonium hydroxide cleavage and deprotection.

Further reactions of oligonucleotides specifically at the sulfur residue have been described[3], allowing the incorporation of a wide variety of functional groups at these positions.

5'-Amino-dT

Applications requiring the use of peptide nucleic acids (PNA) continue to grow in popularity and the need for PNA/DNA chimeras has, consequently, become more significant. We already have offered 5'-Amino-dT where the amino group is protected as the trifluoroacetate. This was designed to be easily removed by ammonium hydroxide during the cleavage and deprotection. However, it has been brought to our attention that the free 5'-amine is capable of reacting with the thymine base during deprotection, with substantial loss of amine reactivity. We have therefore discontinued this product and now offer 5'-Amino-dT (2) protected with an MMT group. The MMT group should remain on the oligonucleotide during cleavage and deprotection steps. It should be removed in aqueous acid after removal of the ammonia. The MMT group can, of course, also be used in reverse phase purification techniques. Alternatively, the MMT group can be removed on the synthesizer to allow amine-specific reactions to be carried out in the synthesis column.

2'-F-Pyrimidines

2'-Deoxy-2'-fluoro-nucleosides adopt an RNA-type sugar conformation, presumably due to the high electronegativity of fluorine. Because of this sugar conformation, RNA duplexes (A-form) are generally more thermodynamically stable than DNA duplexes (B-form). As expected, the addition of 2'-F-RNA residues to oligodeoxynucleotides progressively increases the thermal stability of their duplexes with RNA. The stabilization is additive at approximately 2° per residue. This compares favorably with 2'-OMe-RNA at around 1.5° and RNA at 1.1° per residue. In the meantime, base pair specificity remains intact.[4]

2'-F-RNA phosphodiester linkages are not nuclease resistant, although the corresponding phosphorothioate linkages are highly resistant.

Researchers usually design antisense oligonucleotides to form duplexes with RNA which are substrates for RNase H. Uniformly modified 2'-F-RNA/RNA duplexes are not substrates for RNase H. However, it is straightforward to prepare chimeric 2'-F-RNA/DNA phosphorothioate oligonucleotides which exhibit enhanced binding to the RNA target, are substrates for RNase H, and are highly nuclease resistant.

Following many requests from our customers, we are now introducing 2'-F-C (3) and 2'-F-U (4) phosphoramidite monomers. If we are able to supply the corresponding A and G monomers at a reasonable price, we will introduce them later.

5,6-Dihydro-dU and dT

Cellular DNA is constantly being damaged by oxidation and alkylation, by free radicals, and by ultraviolet and ionizing radiation. The body has therefore evolved a number of repair enzyme systems to excise and repair these lesions. The study of the resulting lesions and the enzymes responsible for their repair is currently a major area of scientific research.[5] One group of compounds of particular interest are the 5,6-dihydro pyrimidines. They are a naturally occurring class of compounds that are structural components of alanine transfer RNA. Dihydrouracil is also a major base damage product formed by exposure of cytosine in DNA to ionizing radiation under anoxic conditions.

A major difficulty in the study of damaged nucleobases is their inherent instability. This has made it difficult to incorporate them into synthetic oligonucleotides for model studies since they must be stable to the conditions used for oligonucleotide synthesis, cleavage and deprotection. This makes it necessary to do most experiments using preparations of DNA which are then exposed to various agents followed by isolation and characterization of the damaged products. However, the use of our UltraMILD monomers allows strongly basic hydrolytic conditions to be avoided during cleavage and deprotection, opening the possibility that oligos incorporating some damaged bases might be synthesized. Oligonucleotides synthesized using 5,6-dihydro-dU (5) or 5,6-dihydro-dT (6) and UltraMILD monomers can be cleaved using either concentrated ammonium hydroxide or 50 mM potassium carbonate in anhydrous methanol. Complete cleavage and deprotection can be accomplished at room temperature in 2-4 hours without damaging either the dihydro-dU or dihydro-dT bases.

2'-5' Linked Oligonucleotides

Cellular DNA and RNA are made up of ribo- and 2'-deoxyribonucleic acids linked together via 3'-5' phosphodiester linkages and by far comprise the bulk of polynucleic acids found in cells. Much less common are oligonucleotides which have 2'-5' linkages. However, a unique feature of 2'-5' linked oligonucleotides is their ability to bind selectively to complementary RNA.[6, 7] These features

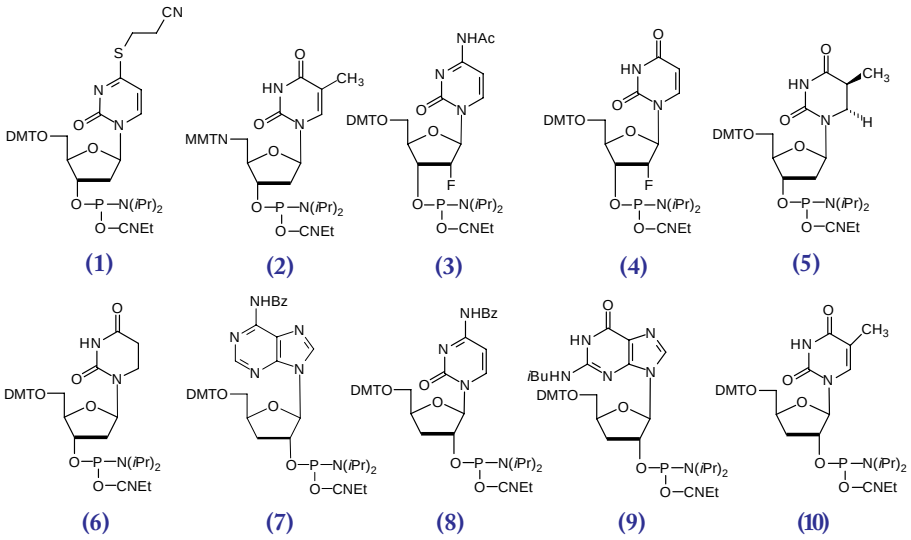
5,6-DIHYDRO-PYRIMIDINES, 2'-PHOSPHORAMIDITES

suggest a number of interesting uses for 2'-5' linked oligos such as their use as RNA specific probes or in antisense oligos.

Recently, chimeric oligos have been synthesized using 3'-deoxy-2'-phosphoramidites and 2'-deoxy-3'-phosphoramidites.[3] Using these amidites the authors synthesized phosphorothioate oligos with 2'-5' linkages and chimeras with 2'-5' linked ends and 3'-5' linked central regions. They found that 2'-5' phosphorothioate oligos: 1) bind selectively to complementary RNA with the same affinity as phosphodiester oligos; 2) exhibit much nonspecific binding to cellular proteins; 3) do not activate RNase H. In experiments with Chinese hamster ovary cells transfected with human 5a-reductase-II (5aR-II), chimeric antisense oligos complementary to the 5' untranslated region of 5aR-II, containing seven 3'-5' linkages in the center, were effective in inhibiting 5aR-II protein in a dose dependent manner. The same oligos with 2'-5' linkages only were ineffective in inhibiting 5aR -II protein synthesis.

Glen Research now offers all of the 3'-deoxy-2'-phosphoramidites (7-10) for use in synthesizing 2'-5' oligonucleotides.

FIGURE 1: MONOMER STRUCTURES



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
4-Thio-dU-CE Phosphoramidite	10-1052-95	50 µmole	165.00
	10-1052-90	100 µmole	295.00
	10-1052-02	0.25g	675.00
5'-Amino-dT-CE Phosphoramidite	10-1932-90	100 µmole	150.00
	10-1932-02	0.25g	400.00
2'-F-Ac-C-CE Phosphoramidite	10-3415-90	100 µmole	165.00
	10-3415-02	0.25g	500.00
2'-F-U-CE Phosphoramidite	10-3430-90	100 µmole	165.00
	10-3430-02	0.25g	500.00
Dihydro-dT-CE Phosphoramidite	10-1530-90	100 µmole	195.00
	10-1530-02	0.25g	595.00
Dihydro-dU-CE Phosphoramidite	10-1550-90	100 µmole	195.00
	10-1550-02	0.25g	595.00
3'-dA-CE Phosphoramidite	10-1004-95	50 µmole	177.50
	10-1004-90	100 µmole	355.00
	10-1004-02	0.25g	975.00
3'-dC-CE Phosphoramidite	10-1064-95	50 µmole	177.50
	10-1064-90	100 µmole	355.00
	10-1064-02	0.25g	975.00
3'-dG-CE Phosphoramidite	10-1074-95	50 µmole	177.50
	10-1074-90	100 µmole	355.00
	10-1074-02	0.25g	975.00
3'-dT-CE Phosphoramidite	10-1084-95	50 µmole	177.50
	10-1084-90	100 µmole	355.00
	10-1084-02	0.25g	975.00

References:

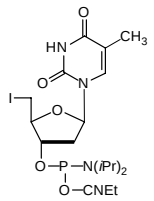
(1) T.T. Nikifirov and B.A. Connolly, *Tetrahedron Lett.*, 1991, **32**, 3851-3854.
(2) T.T. Nikiforov and B.A. Connolly, *Nucleic Acids Res.*, 1992, **20**, 1209-1214.
(3) R.S. Coleman and E.A. Kesicki, *J. Amer. Chem. Soc.*, 1994, **116**, 11636-11642.
(4) A.M. Kawasaki, et al., *J. Med. Chem.*, 1993, **36**, 831-841.
(5) L. Augeri, Y.M. Lee, A.B. Barton, and P.W. Doetsch, *Biochemistry*, 1997, **36**, 721-729.
(6) P.A. Giannaris and M.J. Damha, *Nucleic Acids Research*, 1993, **21**, 4742-4749.
(7) P. Bhan, A. Bhan, M.K. Hong, J.G. Hartwell, J.M. Saunders, and G.D. Hoke, *Nucleic Acids Res*, 1997, **25**, 3310-3317.

NON-ENZYMATIC LIGATION OF SINGLE-STRANDED AND DUPLEX DNA

Chemical alternatives to enzymatic procedures have the potential to lower cost, increase efficiency, and allow the use of unnatural bases. Recently, a new procedure for nonenzymatic ligation of oligonucleotides has been described.[1] In this procedure, the 5'-terminus of the oligonucleotide to be ligated is modified as a 5'-iodo group. The 3'-terminus to be ligated is modified as a 3'-thiophosphate, prepared by sulfurizing in the first cycle using 3'-phosphate CPG.

The authors have used this strategy to construct large linear and circular biologically active oligonucleotides. Oligonucleotides containing a 5'-iodo group were prepared conventionally with the exception that deprotection is carried out in ammonium hydroxide at room temperature for 24 hours. Under these conditions, degradation of the iodo group was less than 2%. Oligonucleotides can be purified by RP HPLC since the 5'-iodo group retards the movement of the oligonucleotide on reverse phase. Chemical ligation was achieved successfully using a short "splint" oligonucleotide complementary to the ends to bring them together, followed by the chemical ligation reaction. Yields varied from 44% for simple ligation to 20% for a two-step ligation and cyclization to form a large cyclic oligonucleotide from two smaller fragments.

FIGURE 1: 5'-I-dT



For Chemical Ligation

Reference:

(1) Y.Z. Xu and E.T. Kool, *Tetrahedron Lett.*, 1997, **38**, 5595-5598.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-I-dT-CE Phosphoramidite	10-1931-90	100 µmole	85.00
	10-1931-02	0.25g	295.00

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