VOLUME 9

NUMBER 1

DECEMBER

1996

A NOVEL TRANSFECTION REAGENT

Richard W. Wagner, Ph.D.
Director, Cell Biology
Gilead Sciences

The development of antisense technology as a research tool relies on the use of potent agents, efficient delivery of oligonucleotides (ONs) into cells, and the utilization of many controls in experiments[1]. C-5 propyne phosphorothioate ONs are improved antisense agents that show potent gene inhibition in cell culture when delivery techniques are utilized[2]. C-5 propyne phosphorothioate ONs have superior binding capabilities compared to ONs lacking the base modification and cause the selective reduction of protein and RNA levels in cells treated with the agents.

C-5 propyne phosphorothioate ONs are not readily permeable to cells in culture. ONs which show potent activity in microinjection experiments have undetectable activity when incubated with cells in culture medium at high concentration. Cell delivery methods, such as microinjection, electroporation, and cationic lipid formulation techniques, work well for delivering the ONs to cells. Of these methods, cationic lipid formulations (cytofectins) are the most versatile and allow the use of nanomolar concentrations of ONs in cell media for observing antisense effects.

Cytofectin Reagents

Several limitations previously surrounded the generalized use of cytofectin agents for delivering antisense ONs to cells. Most of the agents that are available suffer from one of the following shortcomings:

(i) toxicity,
(ii) inability to transfect in the presence of serum,
(iii) lack of efficiency for transfecting ONs into cells, and
(iv) inability to transfect a wide range of cell types.

Recently, a novel cationic lipid formulation, termed GS 2888 cytofectin, was described which showed superior ON delivery properties compared to commercially available agents[3]. This agent has been useful for delivering high quantities of ONs to cells in the presence of serum in the cell medium. The use of serum in experiments mitigates much of the toxicity observed with cytofectin delivery. This agent has the following advantages compared to other cytofectin agents:

(i) it delivers both plasmid DNA and ONs to cells in the presence of 10% serum in cell media,
(ii) it delivers at least 20-fold more ON to cells than other lipids used at their optimal concentrations,
(iii) it transfects a wide variety of cell types, and
(iv) its use requires 4-10 fold less concentration compared to other cytofectin agents.

Using GS 2888 cytofectin with C-5 propyne antisense ONs, gene inhibition can be achieved using low nanomolar concentrations of

(Continued on Page 4)

New Protected Monomers

3'-Labelling

New Oxidation Procedure

Cyclic Oligos

Recent Additions

PROTECTING GROUPS FOR DNA, RNA AND 2'-OMe-RNA MONOMERS

RNA Monomers

The use of acetyl (Ac) protecting groups on cytosine residues allows deprotection with a variety of strong organic bases instead of or in combination with ammonia. This is a consequence of the fact that the hydrolysis of the acetyl group occurs virtually instantly and the competing transamination reaction, described in Figure 2 using benzoyl (Bz) as an example, with the organic amine is eliminated.[1] This process which is in routine use for DNA synthesis has now become popular for RNA synthesis.[2] In the deprotection procedure using a 50:50 aqueous methylamine : ammonium hydroxide mix (AMA), the oligonucleotide made with the Ac-C monomer is cleaved and deprotected at 65° for 10 minutes. This occurs regardless (within reason) of the nature of the protecting groups on the A and G monomers. Glen Research is now offering A and G RNA monomers with benzoyl (Bz) and dimethylformamidine protecting groups, respectively. These monomers are offered in addition to our phenoxyacetyl (PAC) protected A and G monomers which are more versatile and allow a variety of very mild deprotection schemes to be used.

DNA and 2'-OMe-RNA Monomers

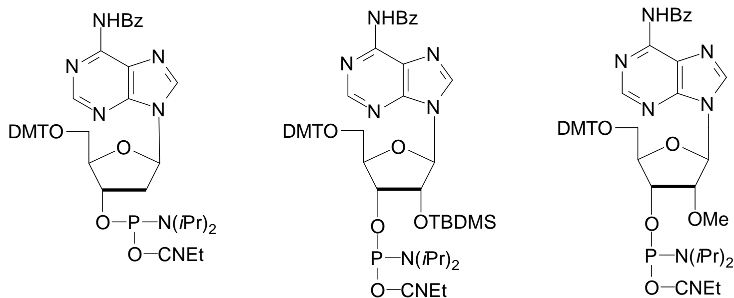
The updated set of base protecting groups we have chosen for RNA monomers (Bz-A, Ac-C and dmf-G) is already available for DNA and 2'-OMe-RNA monomers. For those researchers making chimeric oligos, this makes the deprotection more straightforward.

Methyl Phosphonamidites

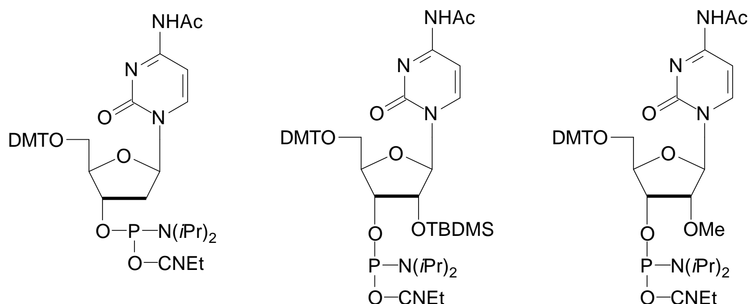
The methyl phosphonate backbone may be used to induce nuclease resistance into oligonucleotides prepared for antisense research. Because the backbone is very base labile, these oligos are traditionally deprotected with ethylene diamine in ethanol. In cooperation with Beckman Instruments, we can now offer Ac-dC methyl phosphonamidite. Using

(Continued on Page 3)

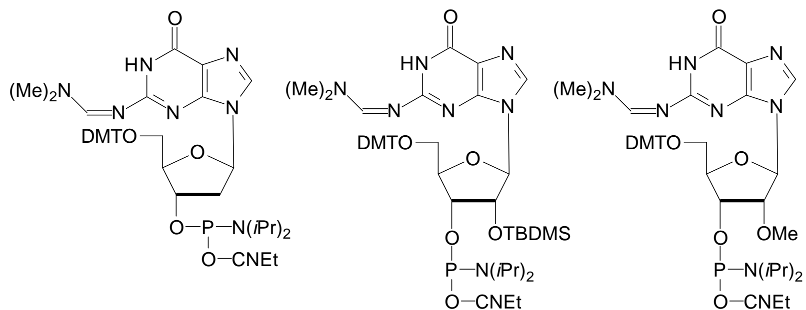
FIGURE 1: MONOMER STRUCTURES



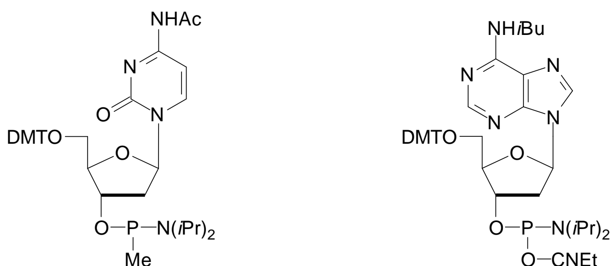
Bz-A CE Phosphoramidites



Ac-C CE Phosphoramidites



dmf-G CE Phosphoramidites



Ac-dC-Me Phosphonamidite **_i_Bu-dA-CE Phosphoramidite**

(Continued from Page 2)

Bz-dC methyl phosphonamidite and deprotecting with ethylene diamine, the level of transamination has been measured to be of the order of 16%. Using the more labile isobutyryl (*i*Bu) protecting group, the level of transamination is much reduced at around 4%. However, using the Ac-dC monomer with ethylene diamine, the level of transamination is lowered to an undetectable level.[3]

Photochemical Cleavage Reactions

Photolabile supports are becoming popular for the preparation of 3'-modified oligonucleotides which can be released into solution with all protecting groups intact (see Page 6). Aromatic protecting groups like benzoyl absorb some of the photochemical energy during cleavage and, therefore, should be avoided.[4,5] We now offer isobutyryl protected dA (*i*Bu-dA) monomer to be used in conjunction with Ac-dC and dmf-dG. These monomers provide a set of protecting groups with the appropriate photochemical characteristics.

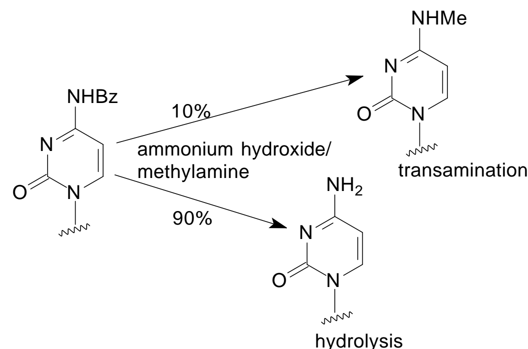
Gas Phase Deprotection

One of the most significant advances in deprotection has been described recently.[6] In this novel procedure, anhydrous ammonia gas is used to effect cleavage and deprotection safely and conveniently in parallel on as many columns as will fit in a reactor. Since no water is present, the fully-deprotected oligonucleotides remain adsorbed to the column matrix, guaranteeing no cross-contamination. The oligonucleotides can then be eluted with water and desalted or further purified, if desired. Using PAC-protected monomers, the cleavage and deprotection processes can be completed in 36 minutes.

References:

(1) M.P. Reddy, N.B. Hanna, and F. Farooqui, *Tetrahedron Lett.*, 1994, **35**, 4311-4314.
(2) M.P. Reddy, F. Farooqui, and N.B. Hanna, *Tetrahedron Lett.*, 1995, **36**, 8929-8932.
(3) M.P. Reddy, F. Farooqui, and N.B. Hanna, *Tetrahedron Lett.*, 1996, **37**, 8691-8694.
(4) D.J. Yoo and M.M. Greenberg, *J. Org. Chem.*, 1995, **60**, 3358-3364.
(5) D.L. McMinn and M.M. Greenberg, *Tetrahedron*, 1996, **52**, 3827-3840.
(6) J.H. Boal, A. Wilk, N. Harindranath, E.E. Max, T. Kempe, and S.L. Beaucage, *Nucleic Acids Res.*, 1996, **24**, 3115-3117.

FIGURE 2: TRANSAMINATION REACTION



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
RNA Monomers and Supports			
Bz-A-CE Phosphoramidite	10-3003-02	0.25g	65.00
	10-3003-05	0.5g	125.00
	10-3003-10	1.0g	225.00
Ac-C-CE Phosphoramidite	10-3015-02	0.25g	65.00
	10-3015-05	0.5g	125.00
	10-3015-10	1.0g	225.00
dmf-G-CE Phosphoramidite	10-3029-02	0.25g	65.00
	10-3029-05	0.5g	125.00
	10-3029-10	1.0g	225.00
U-CE Phosphoramidite	10-3030-02	0.25g	65.00
	10-3030-05	0.5g	125.00
	10-3030-10	1.0g	225.00
Bz-A-RNA 500	20-3303-02	0.25g	95.00
1 µmole columns	20-3403-41	Pack of 4	100.00
0.2 µmole columns	20-3403-42	Pack of 4	75.00
10 µmole column (TWIST Format)	20-3403-13	Pack of 1	225.00
15 µmole column (TWIST Format)	20-3403-14	Pack of 1	300.00
dmf-G-RNA 500	20-3329-02	0.25g	95.00
1 µmole columns	20-3429-41	Pack of 4	100.00
0.2 µmole columns	20-3429-42	Pack of 4	75.00
10 µmole column (TWIST Format)	20-3429-13	Pack of 1	225.00
15 µmole column (TWIST Format)	20-3429-14	Pack of 1	300.00
Methyl Phosphonamidite			
Ac-dC-Me Phosphonamidite	10-1115-02	0.25g	50.00
	10-1115-05	0.5g	100.00
dA Monomer for Photocleavage Experiments			
iBu-dA-CE Phosphoramidite	10-1009-02	0.25g	25.00
	10-1009-05	0.5g	50.00
	10-1009-10	1.0g	100.00

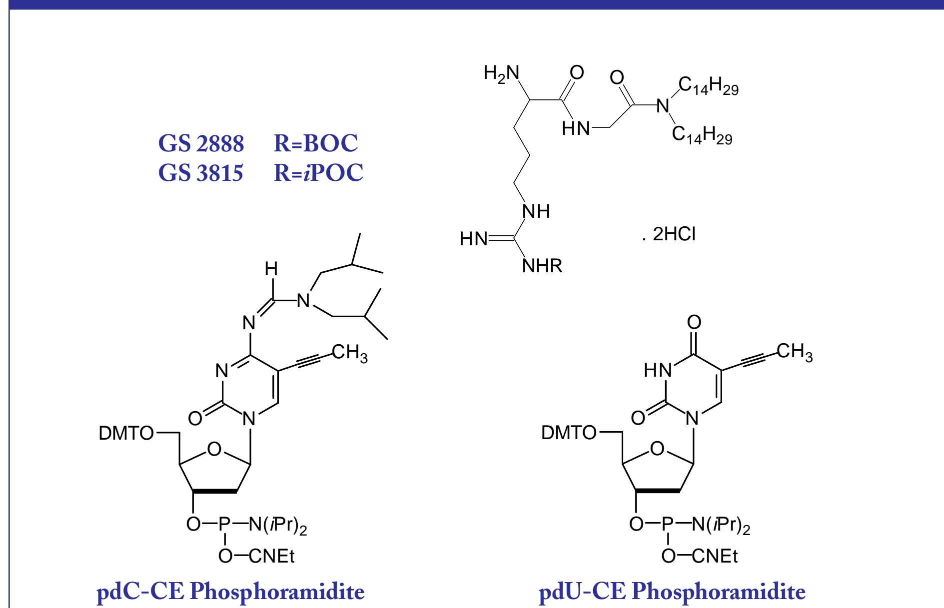
(Continued from Page 1)

ON. Inhibition has been described for several genes including cdc2 kinase[4], cyclin B1[4], luciferase[3,5], and p27kipl[6,7]. In each case, gene selective antisense inhibition was observed when compared to mismatched control ONs. These agents have been shown to be versatile tools for elucidating gene function, an area of increasing interest in the wake of the genome sequencing project.

Of further interest is the ability of GS 2888 cytofectin to deliver high concentrations of plasmids to cells. This agent should be of interest to those wishing to use a general transfection agent for transient expression of proteins in cells and for making stable transformants that express a protein of interest.

Using older technology, antisense techniques proved to be problematic and irreproducible in many laboratories. The development of more potent oligonucleotides and improved delivery agents have resulted in a more robust and reliable technology. Researchers still need to screen several ONs to find ones that work (typically at least 5 or 6 for 15 nucleotide long C-5 propyne phosphorothioate ONs), and researchers should be prepared to utilize many controls in their studies. However, the advances described in this report, that are soon to be made available to Glen Research customers, should establish antisense ON technology as a powerful technique for elucidating gene function.

FIGURE 1: STRUCTURES OF GS 3815 and C-5 PROPYNYL PYRIMIDINES



References:

1. Wagner, R. W. 1994. Gene inhibition using antisense oligodeoxynucleotides. *Nature* **372:**333-5.
2. Wagner, R. W., Matteucci, M. D., Lewis, J. G., Gutierrez, A. J., Moulds, C. and Froehler, B. C. 1993. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* **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. 1996. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl. Acad. U. S. A.* **93:**3176-3181.
4. Flanagan, W. M., Su, L. and Wagner, R. W. 1996. Elucidation of cell cycle gene function using C-5 propyne antisense oligonucleotides. *Nature Biotechnology* **14:**1141-1145.
5. Flanagan, W. M., Kothavale A. and Wagner, R. W. 1996. Effects of oligonucleotide length, mismatches and mRNA levels on C-5 propyne-modified antisense potency. *Nucleic Acids Research* **24:**2936-2941.
6. Coats, S., Flanagan, W. M., Nourse, J. and Roberts, J. M. 1996. Requirement of P27kip1 for restriction point control of the fibroblast cell cycle. *Science* **272:**877-880.
7. St. Croix, B., Florenes, V. A., Rak, J. W., Flanagan, M., Bhattacharya, N., Slingerland, J. M. and Kerbel, R. S. 1996. Impact of the cyclin dependent kinase irihibitor P27kip1 on adhesion-dependent resistance of tumor cells to anticancer agents. *Nature Medicine* **2:**1204-1210.

Note added by Glen Research

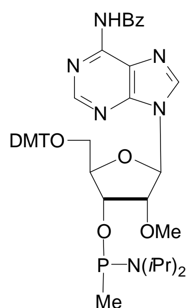
GS 3815 is Gilead's preferred cytofectin since it has better stability in solution during use. In GS 3815 the butyloxycarbonyl (BOC) group of GS 2888 is replaced with isopropyloxy-carbonyl (*i*POC).

ORDERING INFORMATION

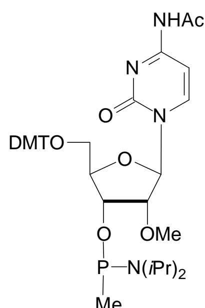
Item	Catalog No.	Pack	Price($)
pdC-CE Phosphoramidite	10-1014-90	100 µmole	85.00
	10-1014-02	0.25g	245.00
	10-1014-05	0.5g	490.00
pdC-lcaa-CPG 500	20-2014-01	0.1g	75.00
1 µmole columns	20-2114-41	Pack of 4	250.00
0.2 µmole columns	20-2114-42	Pack of 4	150.00
pdU-CE Phosphoramidite	10-1054-90	100 µmole	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	70-3815-XX	Inquire or check web site.	

A GALLERY OF RECENT ADDITIONS

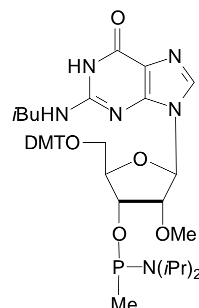
FIGURE 1: STRUCTURES OF RECENTLY ADDED PRODUCTS



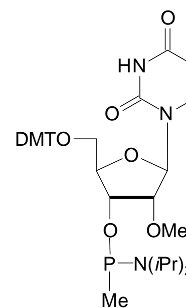
2'-OMe-A-Me Phosphonamidite



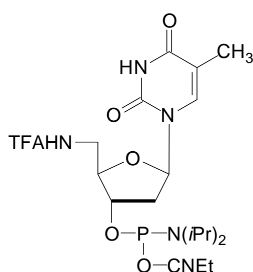
2'-OMe-C-Me Phosphonamidite



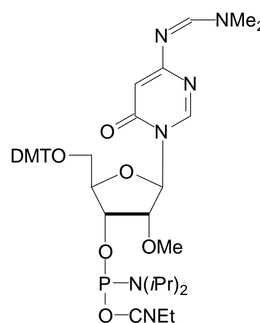
2'-OMe-G-Me Phosphonamidite



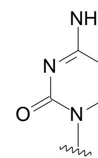
2'-OMe-U-Me Phosphonamidite



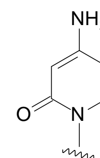
5'-Amino-dT-CE Phosphoramidite



2'-OMe-3-deaza-5-aza-C-CE Phosphoramidite



C



3-Deaza-5-aza-C

In no particular order and for no reason other than the fact that they exist, we introduce some new and interesting compounds.

2'-OMe-RNA Methyl Phosphonamidites

We bring the useful features of uncharged backbone linkages to the field of 2'-OMe-RNA.

5'-Amino-dT

Maybe you would like to attach a label directly to the 5' end of a sequence, or maybe you would like to attach a peptide or PNA sequence to an oligo. If so, then this might be the answer you have been looking for 5'-amino, 5'-deoxy-Thymidine CE phosphoramidite.

2'-OMe-3-deaza-5-aza-C

This 3-deaza-5-aza-C (Reverse C) derivative has the potential to mimic in oligonucleotides 5-azacytidine, a DNA methylase inhibitor. Its ability to bind as a C will likely be diminished.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
2'-OMe-A-Me Phosphonamidite	10-3300-90	100 µmole	150.00
	10-3300-02	0.25g	400.00
	10-3300-05	0.5g	800.00
2'-OMe-C-Me Phosphonamidite	10-3315-90	100 µmole	150.00
	10-3315-02	0.25g	400.00
	10-3315-05	0.5g	800.00
2'-OMe-G-Me Phosphonamidite	10-3320-90	100 µmole	150.00
	10-3320-02	0.25g	400.00
	10-3320-05	0.5g	800.00
2'-OMe-U-Me Phosphonamidite	10-3330-90	100 µmole	150.00
	10-3330-02	0.25g	400.00
	10-3330-05	0.5g	800.00
5'-Amino-dT-CE Phosphoramidite	10-1930-90	100 µmole	150.00
	10-1930-02	0.25g	400.00
2'-OMe-3-deaza-5-aza-C-CE Phosphoramidite	10-3116-95	50 µmole	177.50
	10-3116-90	100 µmole	355.00
	10-3116-02	0.25g	975.00

3'-LABELLING - DABCYL CPG, PHOTOLABILE 3'-AMINO-CPG

Dabcyl CPG

The hybridization of an oligonucleotide to its complementary strand is one of the most specific molecular recognition events. Consequently, labelled oligonucleotides have become incredibly powerful tools as diagnostic probes for the determination of gene function as well as for the detection of diseases. One drawback in the process is the fact that non-hybridized probe which would lead to background signal must be removed prior to the detection event. A recent article describes[1] the general structure of probes which have the potential to allow real-time measurement. The authors describe these probes as molecular beacons.

A molecular beacon probe has its natural fluorescence quenched in solution unless it is hybridized to the target sequence. Consequently, the design of a molecular beacon requires a fluorophore to be at one terminus and a quencher molecule to be at the other, with both molecules being separated from the oligonucleotide by a hydrocarbon spacer. The probe sequence is in the center of the molecule and the bases towards both termini are self-complementary and about 5 - 8 nucleotides in length. The lengths of the probe sequences are chosen to maximize the separation of the fluorophore and quencher molecules when the probe is hybridized to the target as illustrated in Figure 1. This occurs when the probe sequence is 15, 25 or 35 nucleotides in length, where the open arms of the molecular beacon are arrayed in a trans configuration.

A number of fluorophore quencher sets could be easily postulated but the chemical synthesis of these doubly-labelled oligonucleotides may be arduous. The synthesis can be accomplished by conjugating an amine-specific fluorophore and a thiol-specific quencher (or *vice versa*) with the suitably modified oligonucleotide. A simpler approach would involve the use of a universal quencher support for 3'-terminal labelling along with a 5'-fluorophore phosphoramidite. We are offering the dabcyl support (1) as a potential basis for a universal quencher.

FIGURE 1: MOLECULAR BEACON

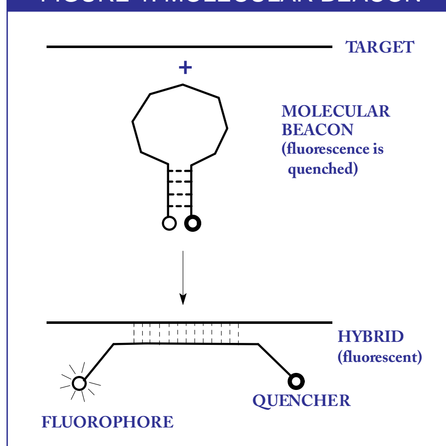
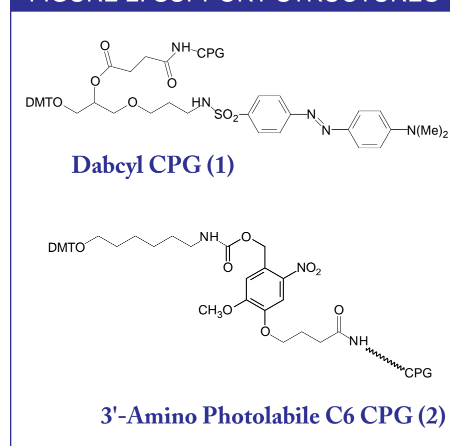


FIGURE 2: SUPPORT STRUCTURES



3'-Amino Photolabile CPG

In late 1995, Glen Research introduced the first commercial photo-cleavable support. This product, 3'-Carboxylate Photolabile C6 CPG, is designed[2] predominantly for the synthesis of fully protected oligonucleotide 3'-carboxylic acids. However, it can also be used without photolysis to yield fully deprotected oligonucleotide 3'-carboxylic acids. It is sold under license from Colorado State University. Typically these photolysis reactions are carried out in a photochemical reactor set at the optimum wavelength. However, these photocleavage reactions may also be carried out on a long wavelength (365nm) transilluminator of the type used in most biology labs.

Now, a similar strategy[3] allows 3'-alkylamino oligonucleotides to be prepared using 3'-Amino Photolabile C6 CPG (2). After conventional synthesis, oligonucleotides can be cleaved from the support photochemically to release the fully protected 3'-amino oligonucleotides into solution. Conjugation of a hapten to the amino group can then be carried out in organic solution. This avoids the need for the vast excess of hapten which is used routinely in conjugation reactions with fully deprotected oligonucleotides in aqueous solution, typically at pH 9. It also makes removal of excess hapten much more facile.

References:

(1) S. Tyagi and F.R. Kramer, *Nature Biotechnology*, 1996, **14**, 303-308.
(2) D.J. Yoo and M.M. Greenberg, *J. Org. Chem.*, 1995, **60**, 3358-3364.
(3) D.L. McMinn and M.M. Greenberg, *Tetrahedron*, 1996, **52**, 3827-3840.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
3'-Dabcyl CPG 500	20-5911-01	0.1g	120.00
	20-5911-10	1.0g	975.00
1 µmole columns	20-5911-41	Pack of 4	200.00
0.2 µmole columns	20-5911-42	Pack of 4	120.00
3'-Carboxylate Photolabile C6 CPG 500	20-4090-01	0.1g	120.00
	20-4090-10	1.0g	975.00
1 µmole columns	20-4090-41	Pack of 4	200.00
0.2 µmole columns	20-4090-42	Pack of 4	120.00
3'-Amino Photolabile C6 CPG 500	20-4091-01	0.1g	120.00
	20-4091-10	1.0g	975.00
1 µmole columns	20-4091-41	Pack of 4	200.00
0.2 µmole columns	20-4091-42	Pack of 4	120.00

PROPERTIES OF OLIGONUCLEOTIDES CONTAINING THE BASES P AND K

Early in 1995, Glen Research was happy to introduce as nucleoside phosphoramidites the two bases P and K (Figure 1) which have been proposed as degenerate bases mimicking a C/T and an A/G mix, respectively.[1] They may replace the natural DNA bases with little destabilization, and it has been demonstrated[2] that they can be substituted independently into oligonucleotide primers for the polymerase chain reaction. Now, the properties of the degenerate nucleosides dP and dK in templates and primers have been determined in much more detail.[3]

Template properties

To examine the behavior of the degenerate bases when copied by *Thermus aquaticus (Taq)* DNA polymerase, two oligonucleotide templates were synthesized (Figure 2). One template contained six dP residues, and the other six dK residues. Each template was amplified using two flanking primers. The PCR products from each template were pooled and cloned. Sixteen clones from each pool were sequenced. The dP nucleotide was copied as if it were either T or dC, with T predominating by a small margin (ratio T:C was 1.5:1). The dK nucleotide was copied as if it were dG or dA, but showed a more marked preference for directing the incorporation of thymidine (ratio of A:G was 7: 1).

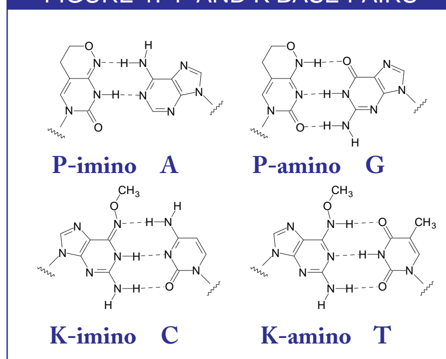
P & K in Sequencing primers

Oligomers containing either a mixture of **P** and **K** or 2'-deoxyinosine (dI), which is often used as a "universal" nucleoside[4], were compared as sequencing primers on three plasmids containing homologous but not identical sequences. The **P/K** primer proved to be more effective, priming T7 DNA polymerase specifically from each of three related sites; the primer containing six dI residues primed well on only two of the templates and not at all on the third.

P & K in PCR primers

To test primers containing **P/K** mixtures (which is equivalent to a universal base) and to compare with the

FIGURE 1: P AND K BASE PAIRS



equivalent primers containing dI in PCR, a second pair of primers, again containing six modified bases was synthesized. The primers direct synthesis towards the priming site used for sequencing; the pair of modified primers should amplify a 185 base-pair product from each of the three plasmids. The **P/K** pair of primers amplified the correct product from each of the templates using 50 picomoles and 100 picomoles of each primer per reaction. Even at 200 picomoles of each primer, the dI pair of primers only amplified from two of the templates; no product was obtained using 50 picomoles.

Conclusion

The degenerate bases P and K are copied as either pyrimidine (C or T) or either purine (A or G), respectively. They can be used together in oligomers to prime DNA synthesis in PCR and in sequencing reactions. In a direct comparison, they were more effective in primers than dI.

We thank David Loakes, MRC Cambridge, for allowing us to abstract part of the information he presented at the Nucleosides and Nucleotides Round Table in September, 1996.

FIGURE 2: TEMPLATES AND PRIMERS USED FOR DETERMINING TEMPLATE PROPERTIES

5' GAATTTTGACCTTCTTAAGCTT (Where X = P or K)

5' GAATTTTGACCTTCTTAAGCTTGC**X**GG**X**GA**XX**TC**X**AGT**X**CAACCCTGGGCCCATGTTACGT 3'

GGACCCGGGTACAATGCA 5'

References:

(1) P. Kong Thoo Lin. and D.M. Brown, *Nucleic Acids Res.*, 1989, **17**, 10373-10383.
(2) P. Kong Thoo Lin. and D.M. Brown, *Nucleic Acids Res.*, 1992, **20**, 5149-5152.
(3) F. Hill, D.M. Loakes, and D.M. Brown, *Nucleosides and Nucleotides Round Table*, 1996, La Jolla, CA.
(4) E. Ohtsuka, S. Matsuki, M. Ikehara, Y. Takahashi, and K. Matsubara, *J. Biol. Chem.*, 1985, **260**, 2605-2608.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
dP-CE Phosphoramidite	10-1047-90	100 µmole	195.00
	10-1047-02	0.25g	595.00
dP-CPG 500	20-2047-01	0.1g	125.00
1 µmole columns	20-2147-41	Pack of 4	250.00
0.2 µmole columns	20-2147-42	Pack of 4	150.00
dK-CE Phosphoramidite	10-1048-90	100 µmole	195.00
	10-1048-02	0.25g	595.00
dK-CPG 500	20-2048-01	0.1g	125.00
1 µmole columns	20-2148-41	Pack of 4	250.00
0.2 µmole columns	20-2148-42	Pack of 4	150.00
dP+dK-CE Phosphoramidite	10-1049-90	100 µmole	195.00
	10-1049-02	0.25g	595.00
dP+dK-CPG 500	20-2049-01	0.1g	125.00
1 µmole columns	20-2149-41	Pack of 4	250.00
0.2 µmole columns	20-2149-42	Pack of 4	150.00

NON-AQUEOUS OXIDATION WITH 10-CAMPHORSULFONYL-OXAZIRIDINE

Some phosphoramidites have been found to be unstable to oxidation with iodine containing solutions, regardless of iodine concentration. This is most noticeable in the production of oligonucleotides containing 7-deaza-dG. These oligos undergo damage during the oxidation step following coupling of the 7-deaza-dG monomer. This is evidenced in a discoloration of the support after iodine oxidation of the newly formed 7-deaza-dG phosphite diester linkage. Subsequent oxidation steps do not cause any further damage but the maximum number of 7-deaza-dG residues which can be effectively included in an oligo prepared this way is still only two. Additionally, both ion-exchange (IEX) and reverse phase (RP) HPLC analysis of oligonucleotides containing 7-deaza-dG show major failure peaks corresponding to oligonucleotides truncated at 7-deaza-dG sites (Figure 2). For these reasons, an alternate oxidizer for synthesis of oligos containing 7-deaza-dG would be desirable.

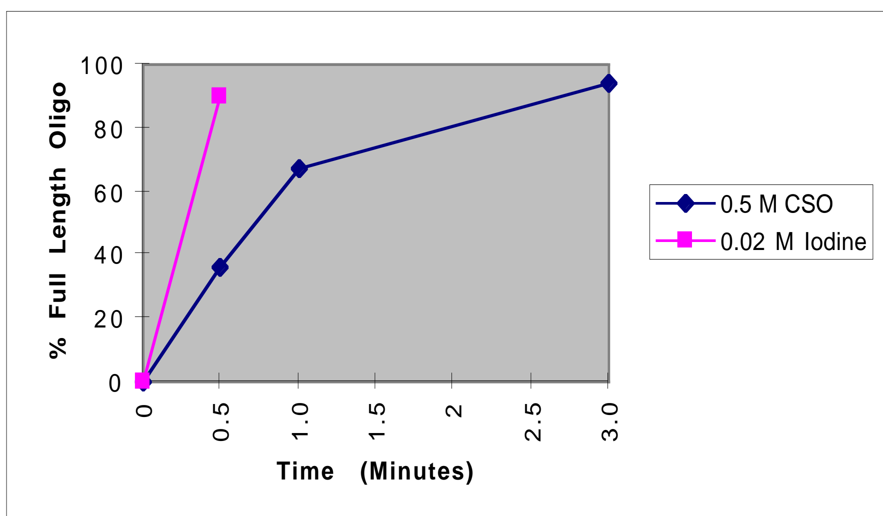
Non-Aqueous Oxidation

One non-aqueous oxidizer which has been used for both DNA[1] as well as RNA[2] synthesis is *t*-butyl hydroperoxide. Peroxides, however, are unstable compounds and extended exposure to them has been reported to result in degradation of oligonucleotides.[3] Enantiomers of (10-camphorsulfonyl)-oxaziridine have been used in the mild enantioselective oxidation of phosphites and other P(III) compounds.[4] More recently they have been investigated for the enantioselective oxidation of methyl phosphonamidates.[5] We, therefore, decided to evaluate them as oxidizers for oligonucleotide synthesis using phosphoramidite chemistry.

Comparison of CSO with Iodine

A 0.5M solution of (1S)-(+)-(10-camphorsulfonyl)oxaziridine in acetonitrile (0.5 M CSO) was evaluated as an oxidizing solution. Oxidation time course was evaluated by synthesizing a test oligo using different oxidizing times and analyzing the crude oligo by both IEX and RP HPLC to determine the percent full-length product.

FIGURE 1: TIME COURSE OF OXIDATION



Oligo T_6 synthesized using either 0.02M iodine oxidizing solution with a 30 sec. wait step or 0.5M CSO in acetonitrile with indicated oxidation wait. The percent full-length product was determined by RP HPLC of the crude DMT-on oligo.

To test the efficiency of the oxidation, a test oligonucleotide $d(A_4C_3G_5T_5)$, was synthesized using either 0.02 M iodine oxidizer or 0.5 M CSO. Following synthesis the crude DMT-on oligonucleotides were analyzed for purity by RP HPLC. The oligos were then purified on a Poly-Pak cartridge and their purity again assayed by RP HPLC. Aliquots of the Poly-Pak purified oligos were then enzymatically digested and the resultant nucleosides analyzed by RP HPLC using a gradient of methanol in 50 mM potassium phosphate (pH 4.0).

Oxidation time course experiments indicated complete oxidation with a 3 minute oxidation wait step, as shown in Figure 1. Shorter oxidation times resulted in an accumulation of shorter truncated fragments when assayed by both IEX and RP HPLC analysis of the crude test oligos. This occurs because incomplete oxidation of phosphorous P(III) to P(V) leaves a population of oligos containing terminal phosphite triester linkages. These linkages are not stable to the acid detritylation step resulting in an accumulation of truncated failures.

When the oligo synthesized using the 0.5 M CSO oxidizing solution with a 3 minute oxidation wait step was compared to the 0.02M iodine control oligo, it was found to be of equivalent or better purity when analyzed by both RP and IEX HPLC. Enzymatic degradation of the product oligonucleotides followed by base composition analysis indicated no modification of bases by CSO.

CSO and 7-deaza-dG

Additionally, an oligonucleotide where three of the dG bases were substituted with 7-deaza-dG was synthesized using 0.5 M CSO as the oxidizer. RP HPLC of the DMT-on oligonucleotide exhibited the profile of a normal oligonucleotide (Figure 3). Following Poly-Pak purification, the product oligonucleotide was digested enzymatically. Base composition analysis by HPLC revealed the expected nucleosides with no sign of side product formation (Figure 4). The peak corresponding to 7-deaza-dG was confirmed by UV spectrum and coelution with an authentic standard.

Conclusion

It was found that a 0.5 M solution of (1S)-(+)-(10-camphorsulfonyl)-

(Continued on Page 9)

(Continued from Page 8)

oxaziridine in acetonitrile (0.5M CSO) with a 3 minute oxidation wait step was an effective oxidizer for DNA synthesis. A mixed base oligo synthesized using 0.5M CSO was indistinguishable by HPLC analysis from the same oligo synthesized using 0.02M iodine oxidizer. Additionally, oxidation using 0.5M CSO resulted in no distinguishable modification of the bases as determined by base composition analysis of the enzyme digested oligos.

When 0.5M CSO was used for the oxidation in the synthesis of an oligo containing multiple 7-deaza-dG residues, no evidence of damage to the oligo was detected when the crude oligo was analyzed by RP HPLC. Successful incorporation of 7-deaza-dG was verified by base composition analysis of the enzyme digested oligo. The peak corresponding to 7-deaza-dG in the enzyme digested sample, co-migrated with a nucleoside standard of 7-deaza-dG and had an identical UV spectrum.

References:

(1) Y. Hayakawa, M. Uchiyama, and R. Noyori, *Tetrahedron Lett.*, 1986, **27**, 4191-4194.

(2) B. Sproat, et al., *Nucleosides and Nucleotides*, 1995, **14**, 255-273.

(3) R.I. Hogrefe, Genta Inc., *Personal Commun.*

(4) I. Ugi, et al., *Nucleosides and Nucleotides*, 1988, **7**, 605-608.

(5) R.I. Hogrefe, Genta Inc., *Personal Commun.*

FIGURE 2: 7-DEAZA-dG + IODINE

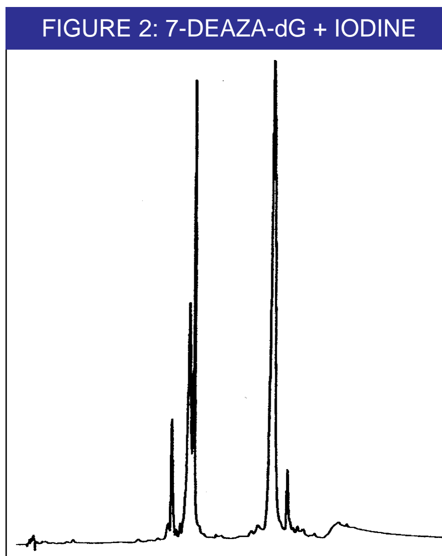


FIGURE 3: 7-DEAZA-dG + CSO

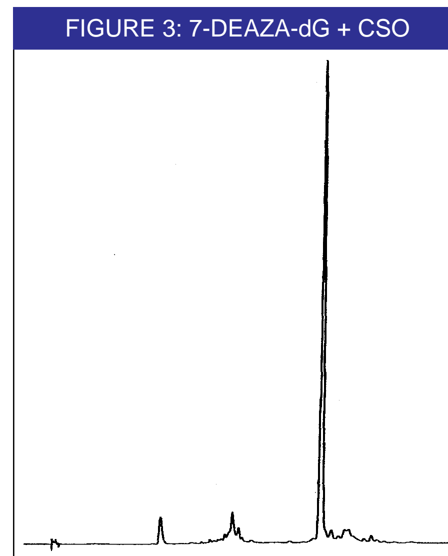
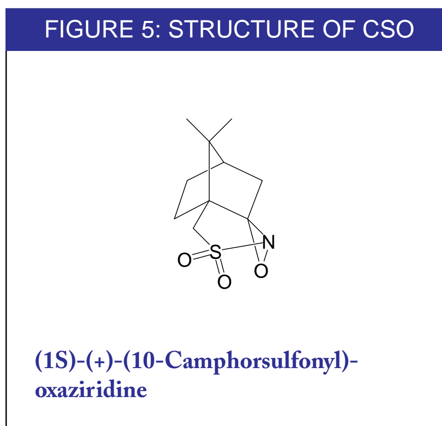
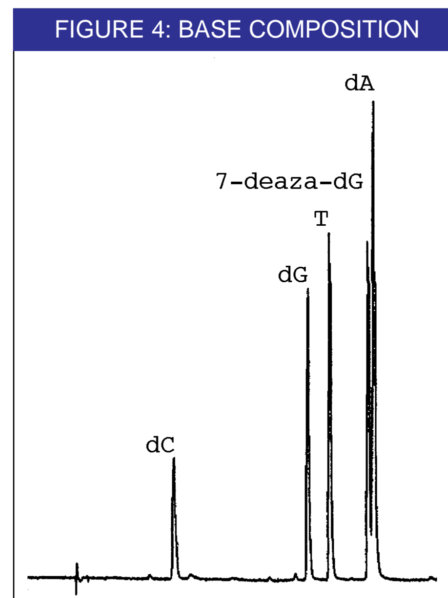


FIGURE 5: STRUCTURE OF CSO



(1S)-(+)-(10-Camphorsulfonyl)-oxaziridine

FIGURE 4: BASE COMPOSITION



HPLC conditions used for the chromatograms on this page are available on request.

FIGURE 6: CSO OXIDATION PROCEDURE

Materials:

- (1S)-(+)-(10-camphorsulfonyl)oxaziridine (Aldrich # 34,535-0)
- Anhydrous acetonitrile
- Disposable syringe (10-30 mL)
- Solvent resistant syringe filter (0.22-0.45 µ)

Procedure:

- Dissolve (1S)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) in anhydrous acetonitrile (8.72 mL/g). This can be done using the disposable syringe in the same way as for dissolving amidites.
- When the CSO is completely dissolved, take it up into the syringe, attach the syringe filter and filter into a bottle that fits onto the appropriate port on the synthesizer.
- Modify the sulfurization cycle on the synthesizer to include a 3 minute wait step subsequent to delivery of the CSO oxidizer solution.
- Synthesize the oligo using CSO oxidation at each step in the synthesis. All other conditions are the same.
- Cleave and deprotect the oligo using standard conditions.

The synthesis cycle used for the experiments using CSO oxidation was a modified sulfurization cycle on an ABI 392 synthesizer with a 3 minute oxidation wait step. The oxidizing solution can either be delivered from the standard oxidizer port (bottle 15) or, in this case, from the cleave reservoir (bottle 10). The oxidation step occurred prior to the capping step as in phosphorothioate synthesis. Presumably any synthesizer with a sulfurizing cycle can be used if the oxidation wait step is ≥ 3 minutes.

CYCLIC OLIGONUCLEOTIDES

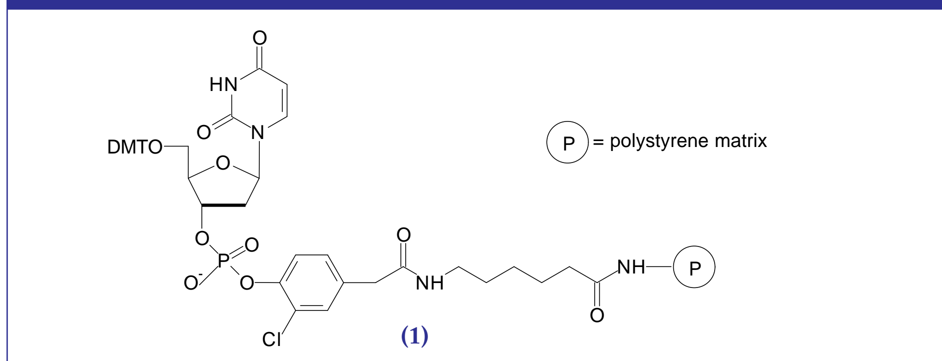
Among the many ways to stabilize oligonucleotides to exonuclease enzymes is the formation of cyclic oligonucleotides in which no terminus is left for the digestion process to commence. Cyclic oligonucleotides would clearly be of interest to antisense researchers for this property alone. In addition, properly designed cyclic structures can be used to form interesting duplex structures similar to the dumbbells and hairpin loops formed in viruses. A variety of triplex structures with the third strand without and within the cyclic oligonucleotide could also be envisaged. There are clearly many reasons to study cyclic oligonucleotides and several ways to prepare them. An excellent review[1] of cyclic oligonucleotides was published earlier this year.

Preparation of Cyclic Oligonucleotides

There is no universal procedure, as yet, for the preparation of cyclic oligonucleotides, regardless of length. Short oligonucleotide circles have been prepared by chemical cyclization of protected oligonucleotides in solution[2] or on solid phase.[3] Since the likelihood of the ends meeting successfully decreases with oligonucleotide length, this method is most suitable for short sequences. For long sequences, a DNA template is used to line up the ends which are then joined chemically or ligated enzymatically.[4,5] Therefore until now, medium-sized cyclic oligonucleotides (10- to 30-mers) have been the most difficult to prepare.

A group from the University of Barcelona has reported a simple, general and effective method for the synthesis of small- to medium-sized cyclic oligodeoxyribonucleotides. The elongation of the oligonucleotide chain is carried out on a novel support (1), Figure 1, using standard phosphoramidite chemistry. The cyclization reaction is then also carried out on the solid support using a phosphotriester coupling procedure. These steps are detailed in Figure 2. On final deprotection with ammonium hydroxide, virtually the only product which comes into solution is the desired cyclic oligonucleotide for reasons depicted in Figure 3.

FIGURE 1: STRUCTURE OF CYCLIC OLIGO SUPPORT dT



Support Preparation

3-Chloro-4-hydroxyphenylacetic acid is used to anchor the oligo-nucleotide chain to the solid matrix. The reaction between its 2,4,5-trichloro-phenyl ester derivative and T-CE Phosphoramidite in the presence of tetrazole, followed by oxidation, provides the nucleotide-linker which is anchored to an amino-derivatized resin. The cyanoethyl protecting group is removed to afford the support (1). This unusual nucleotide support is offered by Glen Research under license from the University of Barcelona. Initially, we will offer only the T support since the synthesis of the cyclic oligonucleotide can commence from any T residue in the circle.

Cyclic Oligonucleotide Synthesis

From this T support, chain elongation takes place by standard phosphoramidite chemistry. After the chain assembly, removal of the 5'-DMT group allows the regioselective condensation of the 5'-terminal OH and the 3'-phosphate using 1-mesitylene-sulfonyl-3-nitro-1,2,4-triazole (O.1M in pyridine, 12-24 hours) as the cyclization reagent. Finally, the cyclic oligonucleotide is obtained after the product is cleaved from the solid support with TMG syn-pyridine-2-aldoximate (O.1M in dioxane/water, 8-16 hours) and the nucleobases are deprotected with ammonium hydroxide. After gel-filtration the crude cyclic oligonucleotides are analyzed, purified and characterized by usual procedures.

Using these strategies, cyclic oligonucleotides within the range of 2- to 30-mer have been prepared, containing all the nucleobases and without sequence restrictions. Yields of crude products depend on the size of the cyclic structure (up to 50% for the smallest cycles, 10% or less for the largest ones). However, highly pure crude cyclic oligonucleotides are obtained (typically >90% by HPLC). This fact illustrates the power of this solid-phase synthetic method, since during the cleavage step the non-cyclized product and other impurities remain anchored to the support through a phosphate diester bond, whereas the phosphate triester-linked cyclic molecule is removed from the resin, as illustrated in Figure 3.

We thank Professor Enrique Pedroso, University of Barcelona for allowing us to abstract part of the

(Continued on Page 11)

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
dT Nucleotide PS	26-2630-01	0.1g	120.00
(for cyclic oligo synthesis)	26-2630-10	1.0g	995.00
1 μmole columns	26-2630-41	Pack of 4	200.00
0.2 μmole columns	26-2630-42	Pack of 4	120.00

FIGURE 2: PROCESS FOR THE PREPARATION OF CYCLIC OLIGONUCLEOTIDES

P = polystyrene matrix

B = Bz-A, Bz-C, ibu-G, T

(1)

1. n+1 cycles
2. remove 5'-DMT

phosphotriester coupling with MSNT to cyclize oligo

1. Cleavage from the support with aldoximate
2. Deprotect bases with ammonia

B = A,C,G, T

(Continued from Page 10)

information he presented at the Nucleosides and Nucleotides Round Table in September, 1996.

References:

(1) E.T. Kool, *Annual Review of Biophysics and Biomolecular Structure*, 1996, **25**, 1-28 and references cited therein.

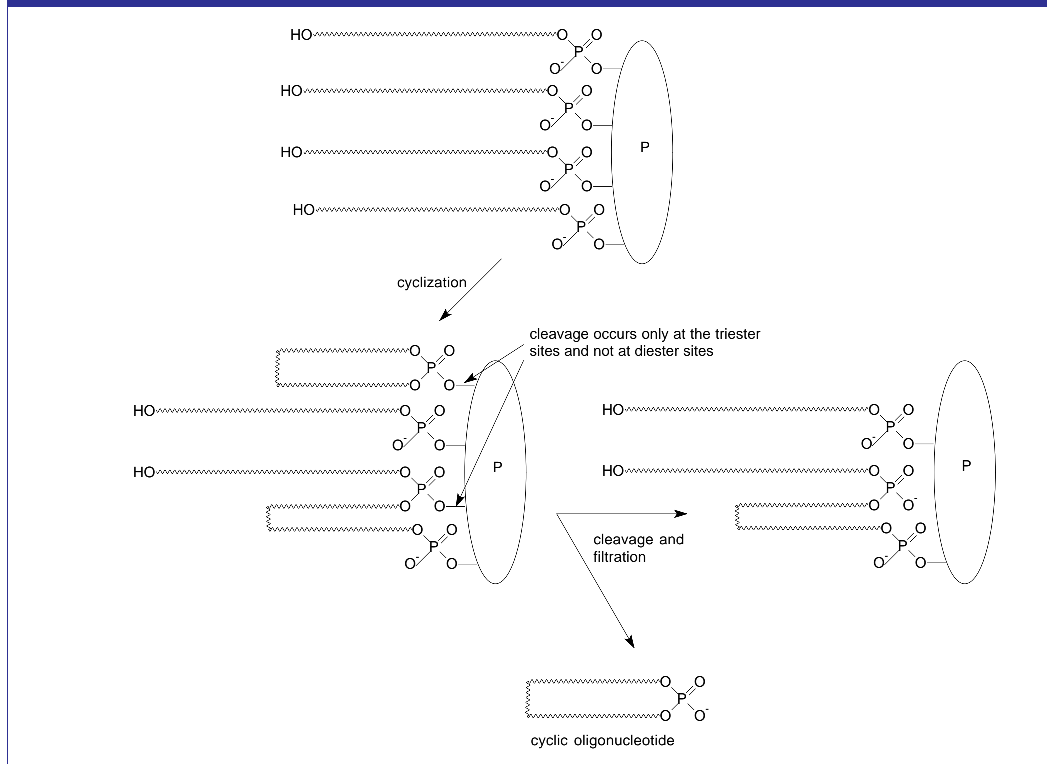
(2) M.V. Rao and C.B. Reese, *Nucleic Acids Res.,* 1989,**17**, 8221-8239.

(3) L. De Napoli, A. Messere, D. Montesarchio, G. Piccialli, C. Santacroce, and G.M. Bonora, *Nucleosides and Nucleotides,* 1993,**12**, 21-30.

(4) G.W. Ashley and D.M. Kushlan, *Biochemistry,* 1991**, 30**, 2927-2933.

(5) G. Prakash and E.T. Kool, *J. Amer. Chem. Soc.,* 1992,**114**, 3523-3527.

FIGURE 3: ONLY CYCLIC OLIGOS ARE RELEASED INTO SOLUTION



DNA SYNTHESIS COLUMNS

LOW VOLUME POLYSTYRENE

DNA synthesis has traditionally been quite wasteful of monomer due to significant dead volume in the synthesis columns. This problem has now been alleviated by the introduction of low volume columns for Applied Biosystems' instruments. For optimal flow characteristics, polystyrene is preferred as the support in these columns. We have chosen the industry-standard "SNAP" design for these columns which are available in 40 nmole and 200 nmole sizes. With reduced volumes and the optimized cycles from Applied Biosystems, these columns will lead to more cost-effective use of small packs of expensive monomers like the phosphoramidites of unusual bases, and modification and labelling reagents.

TWIST 10 AND 15 MICROMOLE

TWIST™ columns are now available in 10 and 15 micromole sizes suitable for Applied Biosystems and PerSeptive synthesizers, respectively. These larger columns can be opened as easily as the original small-scale TWIST columns.

TWIST is a trademark of Glen Research Corporation.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
dA LV-PS 40	26-2100-45	Pack of 4	40.00
dC LV-PS 40	26-2110-45	Pack of 4	40.00
dG LV-PS 40	26-2120-45	Pack of 4	40.00
dT LV-PS 40	26-2130-45	Pack of 4	40.00
dA LV-PS 200	26-2100-42	Pack of 4	40.00
dC LV-PS 200	26-2110-42	Pack of 4	40.00
dG LV-PS 200	26-2120-42	Pack of 4	40.00
dT LV-PS 200	26-2130-42	Pack of 4	40.00
Empty 10/15 µmole TWIST Columns	20-0040-00	10	300.00

GLEN RESEARCH ON THE WORLD WIDE WEB

Visit Glen Research at http://www.glenres.com. Find what's new, obtain catalog numbers, and access technical support information.