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NEW UNIVERSAL AND DEGENERATE BASES

INTRODUCTION

Universal bases must exhibit the ability to replace any of the four normal bases without significantly affecting either melting behavior of duplexes or the normal activities of the modified oligonucleotide. The effect of universal bases contained in oligonucleotide primers used for sequencing or PCR must be minimal. Modified sequencing primers must generate normal sequencing ladders and PCR primers must lead to the correct amplified product. In circumstances where this can not be achieved, we must turn to degenerate bases for help. Degenerate bases code as two or more but not all of the normal bases. Primer multiplicity is eliminated using universal bases and reduced using degenerate bases.

Universal Bases 3-Nitropyrrole and 5-Nitroindole

In late 1994, Glen Research began offering, under license from the University of Michigan, 3-nitropyrrole-CE phosphoramidite which was designed by researchers at Purdue University and the University of Michigan as a universal base.[1,2] The strategy behind the development of 3-nitropyrrole is elegantly simple. Duplexes containing 3-nitropyrrole are stabilized by stacking interactions rather than by hydrogen bonding, thereby removing any bias for an individual complementary base. Nevertheless, duplexes containing 3-nitropyrrole at one or more positions are significantly destabilized relative to the fully complementary duplex.

A subsequent report[3] described the preparation of nitroindole-CE phosphoramidites and their use as universal bases. The researchers compared 4-, 5- and 6-nitroindole with 3-nitropyrrole as universal bases. Like 3-nitropyrrole, all three nitroindole isomers acted indiscriminately towards the four natural bases. Furthermore, based on duplex melting experiments, 5-nitroindole was determined to be the most effective of the nitroindole isomers and to be superior to 3-nitropyrrole. The order of duplex stability was found to be 5-nitroindole > 4-nitroindole > 6-nitroindole > 3-nitropyrrole.

Degenerate Bases P and K

Some primer/template systems may be unable to tolerate the level of destabilization caused by these universal bases. Such systems may then be candidates for the use of primers containing P and K degenerate bases, where P ≡ C/T mix and K ≡ A/G mix.

UNIVERSAL BASES

Melting Behavior

The effect of substituting sites in primers with universal bases can be simply assessed using thermal dissociation experiments. It was found[1,3] that substitution with 3-nitropyrrole and 5-nitroindole towards the termini of oligonucleotides was less destabilizing than substitution towards the center. This may confirm that the universal bases stabilize the duplex by

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acting as intercalators. Also, oligonucleotides were destabilized less if the universal bases were grouped together rather than dispersed through the oligonucleotide. With multiple insertions, 5-nitroindole was shown to be the least destabilizing of the universal bases. Indeed, six insertions of 5-nitroindole into an oligonucleotide was found[3] to be more stable than three insertions of 3-nitropyrrole based on stacking enthalpy measurements. Thermal stability studies are clearly important in validating the usefulness of any base as a universal base. However, its performance in the real world of oligonucleotides for use in sequencing and PCR primers is also critical.

Universal Bases in Primers - 3-Nitropyrrole in Sequencing and PCR

The behavior of 3-nitropyrrole in experiments using a specific primer/target system was initially reported.[1] In dideoxy sequencing experiments, oligonucleotides containing 3-nitropyrrole substitutions were compared to the complementary sequence and to sequences prepared with A,C,G,T mix (N) and 2'-deoxyInosine (dI) which are the most common substitution strategies. The sequence containing 3-nitropyrrole at the third position of four codons gave an unambiguous sequencing ladder. In contrast, the sequencing ladder obtained from the identical sequence containing dI was only partially readable, while that obtained using N (a 256 fold degenerate mixture of primers) was unreadable. Acceptable sequencing ladders were also obtained when one, two and even three codons adjacent to the 3'-terminus were completely replaced by 3-nitropyrrole. It is assumed that the 2 correct bases left at the 3'-terminus in these experiments were insufficient to maintain a normal duplex at 37° and so the 3-nitropyrrole bases must contribute to correct duplex formation. Interestingly, an oligonucleotide containing 3-nitropyrrole at the 3'-terminus gave a readable sequencing ladder whereas a mismatch at the 3'-terminus did not. This result indicates that 3-nitropyrrole is an effective substrate for the polymerase enzyme rather than simply blocking chain extension. The performance of PCR primers containing 3-nitropyrrole was studied briefly and the results showed promise for this universal base.

Initial results in sequencing experiments indicate that 3-nitropyrrole seems to be performing very well. However, PCR experiments using primers with several insertions at the

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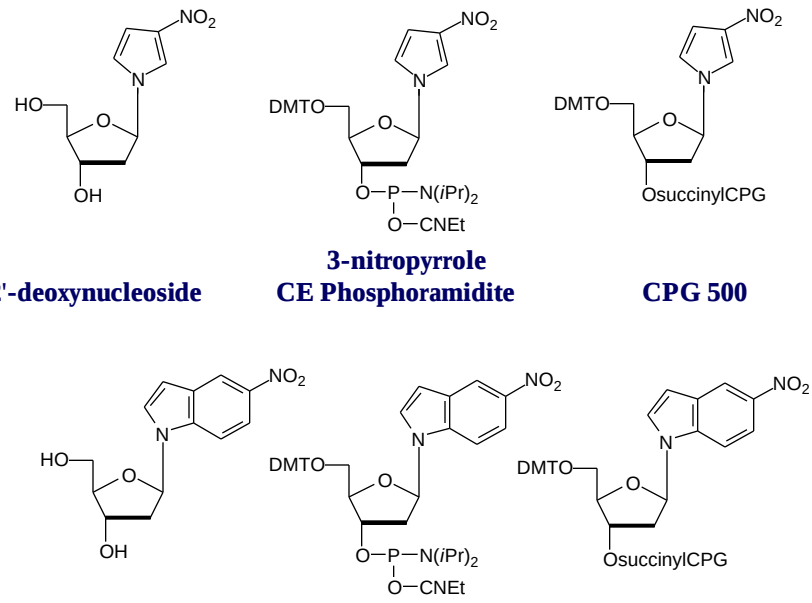
FIGURE 1: STRUCTURES OF UNIVERSAL BASES



2'-deoxynucleoside **3-nitropyrrole CE Phosphoramidite** **CPG 500**

2'-deoxynucleoside **5-nitroindole CE Phosphoramidite** **CPG 500**

TABLE 1: TENTATIVE RULES FOR THE DESIGN OF PRIMERS CONTAINING UNIVERSAL BASES

1.	Substitution of universal bases is less destabilizing towards the termini of oligonucleotides than towards the center.
2.	Grouped substitutions are more easily tolerated than spaced, i.e., contiguous rather than codon third substitutions.
3.	More than 2 - 3 codon third substitutions in a 20mer may not reliably yield a sequencing ladder or a PCR product.
4.	More than 3 contiguous substitutions in a primer may give reduced PCR products or an incorrect sequencing ladder.
5.	3'-Substitutions may lead to incorrect PCR amplification or failure to give a proper sequencing ladder.

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third position of several codons have been problematical. Presumably, problems occur when the melting temperature of the duplex falls too low.

Universal Bases in Primers - 5-Nitroindole in Sequencing and PCR

5-Nitroindole, due to its better stabilization properties, may generate improved performance in these difficult situations.[3] A further publication[4] from Dan Brown's group at the Medical Research Council in Cambridge, England describes a series of experiments which lead to conclusions which are in close agreement with our customer feedback. This report describes a stringent primer/template system used to evaluate the ability of duplexes containing universal bases 3-nitropyrrole and 5-nitroindole to prime DNA synthesis in both PCR and sequencing environments.

In the system described, sequencing experiments were less spectacular than previously described.[1,2] Only primers containing one or two substitutions at codon third positions gave readable ladders while those containing four to six substitutions failed to prime. Primers modified with up to four contiguous substitutions of 5-nitroindole led to readable ladders but only two 3-nitropyrrole substitutions were tolerated. For the template used, three contiguous substitutions of universal bases, two bases from the 3'-terminus of the primer, did not give readable ladders. Also in contrast to the previous work with 3-nitropyrrole, a primer with 5-nitroindole at the 3'-terminus did not give rise to a sequencing ladder, indicating that variations can occur among primers and templates.

Using substituted PCR primers, it was found that up to three contiguous 3-nitropyrrole substitutions and up to four 5-nitroindole substitutions were tolerated, as long as the substitutions were not adjacent to the 3'-terminus. Further substitution might be acceptable if the annealing temperature of the PCR experiment was lowered to accommodate the lower melting temperature of the duplex. Using primers containing substitutions at codon third positions, only two substitutions were tolerated for normal amplification. When four or six codon third positions were substituted by 5-nitroindole, a PCR product could be observed but in low yield. In this same system, a sequence containing six dI substitutions was an effective PCR primer.

Universal Conclusions

Some aspects of the use of universal bases in sequencing and PCR experiments have been clarified. The original report covering 3-nitropyrrole generated dramatic results which served to indicate the promise of this approach. The subsequent report about 5-nitroindole which offered at least equivalent results with less duplex destabilization, served to accentuate this interest. However, it was clear from our customers' feedback that promise does not translate into successful experiments in a wide variety of primers and templates. We have attempted to formulate some simple rules for the use of these universal bases, as shown in Table 1. With added customer feedback, perhaps these can be refined into more general rules. It is clear that there is no "universal" universal base as yet.

Differing circumstances currently might dictate the use of either 3-nitropyrrole or 5-nitroindole or dI as the universal base. Clearly, the use of N at degenerate sites can play a significant role, especially in situations where the degree of degeneracy is kept low. The degenerate bases P and K, described in the next section, will clearly help since their use, even in combination, will significantly decrease primer multiplicity.

DEGENERATE BASES

The results described in the preceeding sections indicate that the search for the perfect universal base is not over. dI has functioned relatively well in its role as a universal base but its hybridization properties are not ideal and, when incorporated into PCR primers, it has been reported to code primarily as G.[5] 3-Nitropyrrole and 5-nitroindole are certainly significant additions to the group of universal bases but their destabilizing effect on duplexes makes them suitable for use in PCR primers with only a few substitution sites. Fully

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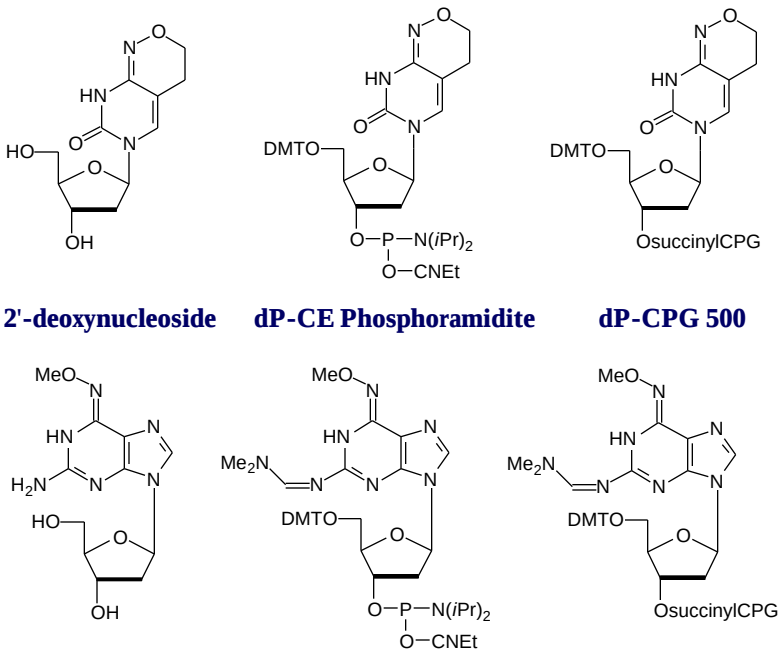
FIGURE 2: STRUCTURES OF P AND K MONOMERS AND SUPPORTS



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degenerate sites may be formed in an oligonucleotide using an A/C/G/T mix but the complexity of the mixture of oligonucleotides formed in this way obviously rises with each insertion and limits the usefulness of this technique.

The modified bases shown in Figure 2, designated P and K, show considerable promise as degenerate bases. The pyrimidine derivative P, when introduced into oligonucleotides, base pairs with either A or G[6], while the purine derivative K base pairs with either C or T.[7] This is made possible by the ability of P and K to form both amino and imino tautomers, as shown in Figure 3. Oligonucleotides containing one or more P substitutions were found[5] to form duplexes of stability equivalent to the parent sequence and exhibited sharp transitions on melting. Substitution with one or more K residues led to duplexes of reduced but still effective stability.

Glen Research is now offering the CE phosphoramidite of P which is equivalent to a C/T mix and of K which is equivalent to an A/G mix. The structures of the CE phosphoramidites are shown in Figure 2. A P/K mix to be equivalent to an N (A/C/G/T mix) is also offered, as are the corresponding supports.

References:

(1) R. Nichols, P.C. Andrews, P. Zhang, and D.E. Bergstrom, *Nature*, 1994, **369**, 492-493.

(2) D.E. Bergstrom, P. Zhang, P.H. Toma, P.C. Andrews, and R. Nichols, *J. Am. Chem. Soc.*, 1995, **117**, 1201-1209.

(3) D. Loakes and D.M. Brown, *Nucleic Acids Res.*, 1994, **22**, 4039-4043.

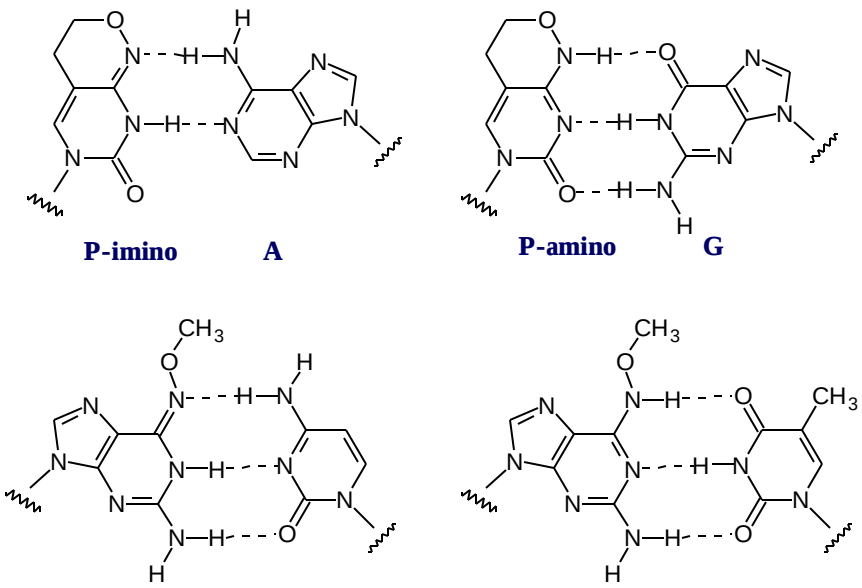
(4) D. Loakes, D.M. Brown, S. Linde, and F. Hill, *Nucleic Acids Res.*, 1995, In press.

(5) P. Kong Thoo Lin and D.M. Brown, *Nucleic Acids Res.*, 1992, **20**, 5149-5152.

(6) P. Kong Thoo Lin and D.M. Brown, *Nucleic Acids Res.*, 1989, **17**, 10383.

(7) D.M. Brown and P. Kong Thoo Lin, *Carbohydrate Research*, 1991, **216**, 129-139.

FIGURE 3: P AND K BASE PAIRS



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
3-Nitropyrrole-CE Phosphoramidite	10-1043-90	100 µmole	125.00
	10-1043-02	0.25g	325.00
3-Nitropyrrole-CPG	20-2043-01	0.1g	60.00
1 µmole columns	20-2143-41	Pack of 4	200.00
0.2 µmole columns	20-2143-42	Pack of 4	120.00
5-Nitroindole-CE Phosphoramidite	10-1044-90	100 µmole	125.00
	10-1044-02	0.25g	325.00
5-Nitroindole-CPG	20-2044-01	0.1g	60.00
1 µmole columns	20-2144-41	Pack of 4	200.00
0.2 µmole columns	20-2144-42	Pack of 4	120.00
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

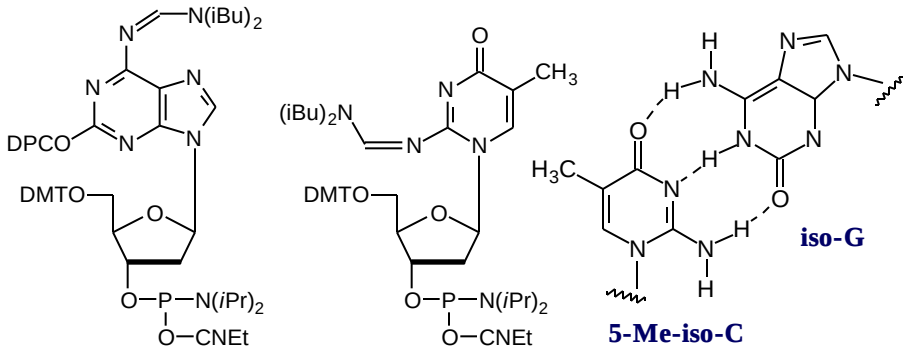
5-METHYL-ISOCYTIDINE / ISOGUANOSINE BASE PAIR

While attempts are being made to simplify the genetic code by the use of universal and degenerate bases, it remains a topic of more than academic interest to extend the genetic code by the addition of new base pairs. A non-standard base pair which has received considerable attention is that formed between isocytosine (iso-C) and isoguanine (iso-G). Indeed, isoguanosine is a naturally occurring ribonucleoside known as crotonoside. A standard Watson and Crick base pair is formed between iso-C and iso-G, but the hydrogen bonding pattern, shown in Figure 1, is quite different from the natural base pairs A-T and C-G.[1] (The 5-methyl analogue, as shown in Figure 1, was chosen as the synthetic target due to the reported[2] instability of 2'-deoxyisocytidine caused by deamination during oligonucleotide synthesis or deprotection.)

Since 5-methyl-2'-deoxyisocytidine (5-Me-isodC) and 2'-deoxyisoguanosine (isodG) are both very susceptible to cleavage of the glycosidic bond under acidic conditions, we chose formamidine protecting groups for both. Therefore, 5-Me-isodC was protected at the N2 position and isodG at the N6 position with diisobutyl formamidine. The O2 position of isodG was protected with diphenylcarbamoyl (DPC) which is labile under standard ammonium hydroxide deprotection conditions. [A recent publication[2] describes the use of a 4-nitrophenyl protecting group for the O2 position but this requires an additional deprotection step (20% DBU/ acetonitrile) prior to the ammonium hydroxide deprotection.] The structures of the two CE phosphoramidite monomers are shown in Figure 1.

Even with the use of formamidine protecting groups designed to stabilize the glycosidic linkages, oligonucleotides containing these modified bases should be prepared using dichloroacetic acid (DCA) rather than trichloroacetic acid (TCA) in the deblocking mix. Most synthesizers already use DCA in the deblocking mix. The exception is Applied Biosystems synthesizers and an alternative deblocking mix containing DCA is available from Glen Research. Base composition analyses was carried out on two 17mers containing three 5-Me-isodC and three isodG residues, respectively. The results, shown in Figure 2, confirm that these labile deoxy-nucleosides are still capable of producing accurate oligonucleotides with minimal degradation. Digestion and HPLC conditions are available on request.

FIGURE 1: 5-Me-isoC / iso-G MONOMERS AND BASE PAIR



isodG **5-Me-isodC**

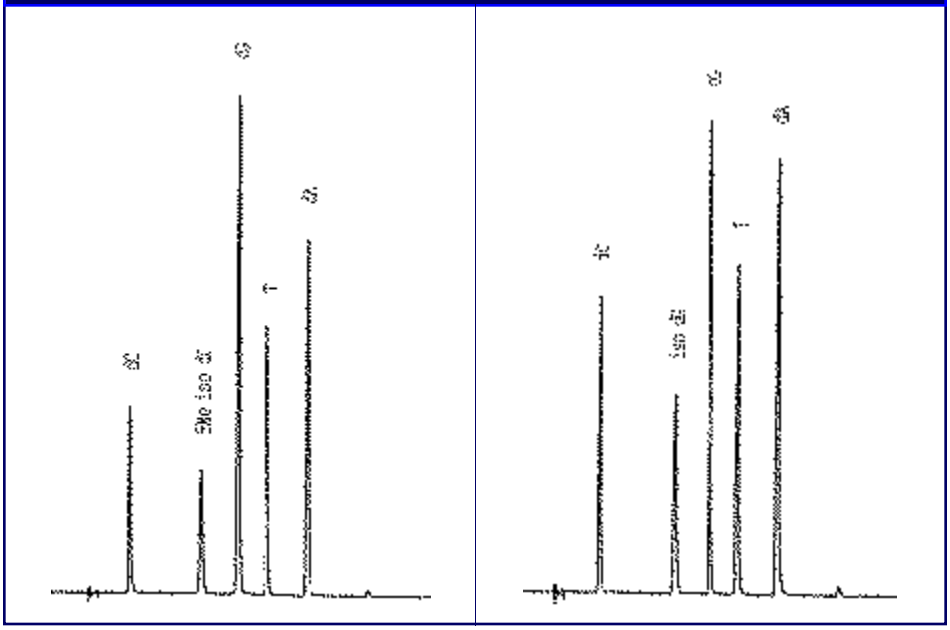
CE Phosphoramidites

References:

(1) C.Y. Switzer, S.E. Moroney, and S.A. Benner, *Biochemistry*, 1993, **32**, 10489-10496.

(2) T. Horn, C.A. Chang, and M.L. Collins, *Tetrahedron Lett.*, 1995, **36**, 2033-2036.

FIGURE 2: BASE COMPOSITION ANALYSIS CONTAINING 5-Me-isodC and isodG



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5-Me-isodC-CE Phosphoramidite	10-1067-95	50 µmole	125.00
	10-1067-90	100 µmole	250.00
	10-1067-02	0.25g	675.00
isodG-CE Phosphoramidite	10-1077-95	50 µmole	225.00
	10-1077-90	100 µmole	450.00
	10-1077-02	0.25g	975.00

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

2'-OMe-Ac-C, 2'-OMe-5-Me-C and 2'-OMe-I

UltraFAST DNA deprotection[1] has revolutionized the downstream processing of oligodeoxynucleotides by cutting the cleavage and deprotection steps to less than 15 minutes. Glen Research has extended this utility to 2'-OMe-RNA with the introduction of the acetyl-protected 2'-OMe-Cytidine-CE Phosphoramidite. While using this monomer, all of the advantages of speed of deprotection is brought to 2'-OMe-RNA and chimeric oligos containing 2'-deoxynucleotides and 2'-OMe-ribonucleotides.

Glen Research has also introduced the 5-methyl analogue of protected 2'-OMe-Cytidine which also has acetyl protection and is compatible with UltraFAST deprotection. Also added to our line is 2'-OMe-Inosine-CE Phosphoramidite. Oligonucleotides containing 2'-OMe-5-Me-C and 2'-OMe-I would be of interest to researchers involved in triplex and antisense studies using 2'-OMe-RNA.

Ac-C

Glen Research now has available acetyl protected Cytidine for routine RNA synthesis. Check our World Wide Web site for a new protocol for fast deprotection of RNA oligos.

Dmf-dG

Glen Research is happy to introduce 2'-deoxyGuanosine protected with a dimethylformamidine (dmf) group. We have used dmf protection on our 2'-OMe-G monomer for many years and the addition of dmf-dG will allow researchers to prepare 2'-OMe-RNA/DNA hybrid molecules[2] with identical protecting groups. This monomer would also be suitable for use in oligos which require mild deprotection.

2-Amino-dA

Glen Research has reintroduced the 2,6-diaminopurine monomer (2-amino-

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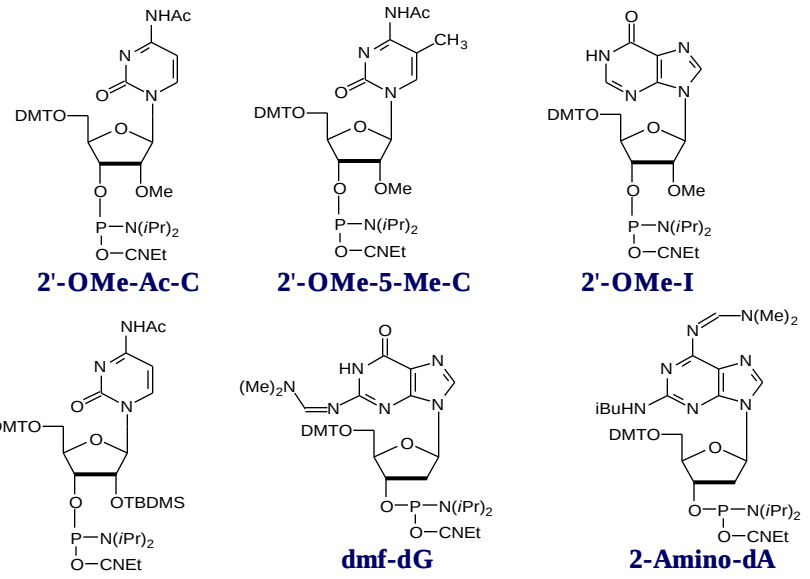
FIGURE 1: STRUCTURES OF PROTECTED MONOMERS



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
2'-OMe-Ac-C-CE Phosphoramidite	10-3115-90	100 µmole	50.00
	10-3115-02	0.25g	125.00
	10-3115-05	0.5g	250.00
	10-3115-10	1.0g	500.00
2'-OMe-Ac-C-RNA 500	20-3615-02	0.25g	95.00
1 µmole columns	20-3715-41	Pack of 4	100.00
0.2 µmole columns	20-3715-42	Pack of 4	75.00
2'-OMe-I-CE Phosphoramidite	10-3140-90	100 µmole	150.00
	10-3140-02	0.25g	360.00
2'-OMe-5-Me-C-CE Phosphoramidite	10-3160-90	100 µmole	150.00
	10-3160-02	0.25g	360.00
Ac-C-CE Phosphoramidite	10-3015-02	0.25g	75.00
	10-3015-05	0.5g	150.00
	10-3015-10	1.0g	275.00
Ac-C-RNA 500	20-3315-02	0.25g	95.00
1 µmole columns	20-3415-41	Pack of 4	100.00
0.2 µmole columns	20-3415-42	Pack of 4	75.00
dmf-dG-CE Phosphoramidite	10-1029-02	0.25g	12.50
	10-1029-05	0.5g	25.00
	10-1029-10	1.0g	50.00
dmf-dG-CPG 500	20-2029-02	0.25g	40.00
1 µmole columns	20-2129-41	Pack of 4	60.00
0.2 µmole columns	20-2129-42	Pack of 4	60.00
2-Amino-dA-CE Phosphoramidite	10-1005-95	50 µmole	177.50
	10-1005-90	100 µmole	355.00
	10-1005-02	0.25g	975.00

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dA). Oligos containing this base require 7 days at 55° for deprotection in ammonia alone, but can be deprotected using AMA at 55° for 17 hours. Acetyl protected dC monomers are of course required to avoid base modification of dC residues during deprotection with AMA.

References:

(1) M.P. Reddy, N.B. Hanna, and F. Farooqui, *Tetrahedron Lett.*, 1994, **35**, 4311-4314.

(2) B.P. Monia, et al., *J. Biol. Chem.*, 1993, **268**, 14514-14522.

3'-TERMINATORS

Some sequencing strategies as well as PCR probes require the 3'-terminus of an oligonucleotide to be blocked from allowing polymerase extension. This may be achieved by modifying the 3'-terminus with a phosphate group, a phosphate ester, or using an inverted 3'-3' linkage. However, side reactions during deprotection of the oligonucleotide or enzymatic impurities may free the 3'-hydroxyl group to a small extent. So far, the 3'-propyl phosphate formed using 3'-Spacer C3 CPG has proved to be the simplest and most effective non-nucleosidic blocker of the 3'-terminus.

2',3'-Dideoxynucleosides

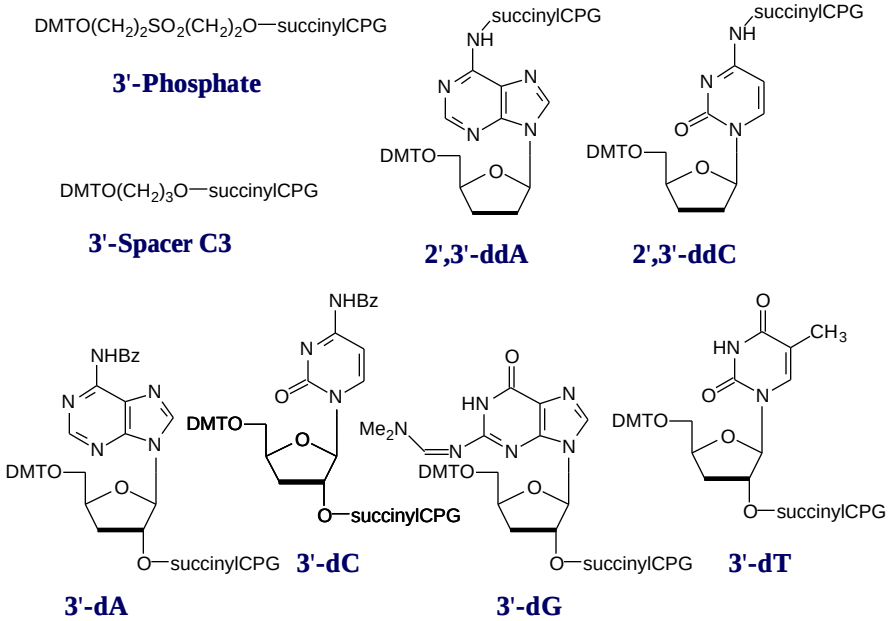
The surest way to guarantee blocking the 3'-terminus is using a 2',3'-dideoxynucleoside support. Unfortunately, only ddA and ddC are amenable to attachment to the support through the exocyclic amino group. Both of these supports are now available.

3'-Deoxynucleosides

In situations where it is necessary to have a selection of all four bases available, it is possible to use the 3'-deoxynucleoside supports as 3'-terminators. Although the 2'-hydroxyl group is still present in the final oligonucleotides, it is not a substrate for at least the routinely used polymerases. All four 3'-deoxynucleoside supports will shortly be available, along with their phosphoramidite counterparts.

3'-TERMINATORS

FIGURE 2: STRUCTURES OF 3'-TERMINATORS



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
3'-Phosphate CPG 500	20-2900-01	0.1g	85.00
	20-2900-10	1.0g	600.00
1 µmole columns	20-2900-41	Pack of 4	125.00
0.2 µmole columns	20-2900-42	Pack of 4	75.00
3'-Spacer C3 CPG 500	20-2913-01	0.1g	85.00
	20-2913-10	1.0g	600.00
1 µmole columns	20-2913-41	Pack of 4	125.00
0.2 µmole columns	20-2913-42	Pack of 4	75.00
2',3'-ddA-CPG 500	20-2007-01	0.1g	300.00
1 µmole columns	20-2107-41	Pack of 4	600.00
0.2 µmole columns	20-2107-42	Pack of 4	200.00
2',3'-ddC-CPG 500	20-2017-01	0.1g	300.00
1 µmole columns	20-2117-41	Pack of 4	600.00
0.2 µmole columns	20-2117-42	Pack of 4	200.00
3'-dA-CPG 500	20-2004-01	0.1g	300.00
1 µmole columns	20-2104-41	Pack of 4	600.00
0.2 µmole columns	20-2104-42	Pack of 4	200.00
3'-dC-CPG 500	20-2064-01	0.1g	300.00
1 µmole columns	20-2164-41	Pack of 4	600.00
0.2 µmole columns	20-2164-42	Pack of 4	200.00
3'-dG-CPG 500	20-2074-01	0.1g	300.00
1 µmole columns	20-2174-41	Pack of 4	600.00
0.2 µmole columns	20-2174-42	Pack of 4	200.00
3'-dT-CPG 500	20-2084-01	0.1g	300.00
1 µmole columns	20-2184-41	Pack of 4	600.00
0.2 µmole columns	20-2184-42	Pack of 4	200.00

Poly-Pak™ II CARTRIDGES FOR THE 1 µMOLE SYNTHESIS SCALE

Poly-Pak™ Cartridges from Glen Research were introduced for the purification of synthetic oligonucleotides on a small scale using the DMT-on purification strategy. The original cartridges have a capacity of up to 0.3 µmoles and are popular for the purification of oligonucleotides prepared on the 40 nmole and 0.2 µmole scales. However, 1 µmole syntheses have to be split among three of the original cartridges for optimal yield.

Now, we are happy to introduce Poly-Pak II cartridges for the purification of oligonucleotides on a synthesis scale of up to 1 µmole. (We will, of course, continue to offer the original Poly-Pak cartridges.) Using a Poly-Pak II cartridge, virtually all of the DMT-on product from a 1 µmole synthesis is absorbed during the loading process.

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Poly-Pak is a Trademark of Glen Research Corporation.

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Item	Catalog No.	Pack	Price($)
Poly-Pak II Cartridge	60-3100-01	each	12.00
	60-3100-10	Pack of 10	120.00

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