

# The Glen Report

2 2 8 2 5 D A V I S D R I V E      S T E R L I N G , V I R G I N I A      2 0 1 6 4



**GLEN RESEARCH**

**VOLUME 15**

**NUMBER 1**

**MARCH 2002**

**I N S I D E**

**NEW DYES, QUENCHERS**

**3'-PT-AMINO-MODIFIER**

**PYRROLO-dC**

**NOVEL MONOMERS**

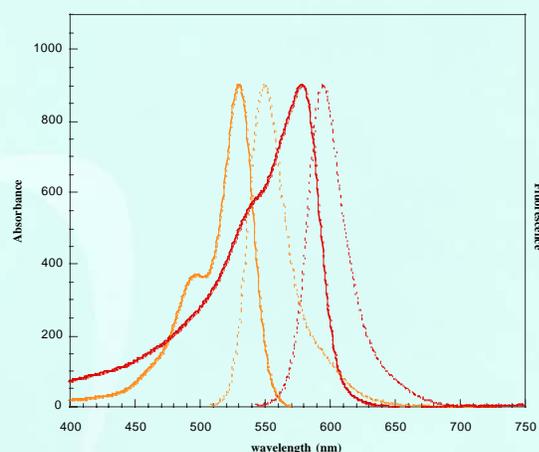
**CARBOXY-MODIFIER**

## REDMOND RED™ AND YAKIMA YELLOW™ DYES, AND ECLIPSE™ NON-FLUORESCENT QUENCHER

**G**len Research is happy to confirm our agreement with Epoch Biosciences, Inc., announced on February 6, 2002, to distribute several of Epoch's proprietary products designed for the synthesis of novel DNA probes. Initially we will provide products based on Epoch's Redmond Red™ and Yakima Yellow™ fluorophores and Eclipse™ non-fluorescent quencher, which will be described in detail in this article. We will also supply PPG, a modified nucleoside, which is covered in the article on Page 8 of this newsletter. It is a pleasure for us to expand our relationship with Epoch by helping to provide broad access for these compounds to research markets worldwide.

As part of the preamble to this article, it is instructive to quote from the Epoch press release, which positions these products perfectly. They note that the products covered by this agreement are as follows:

"all are innovative components for DNA probes, the workhorses of genetic analysis. Probes hybridize or bind to target DNA and then provide a signal so that the target can be detected. Many probes also carry a quencher that masks the signal until the binding has occurred. Redmond Red and Yakima Yellow are fluorescent dye tags that can be incorporated into synthetic DNA molecules to allow probe detection on a variety of platforms. The Eclipse Quencher is a new non-fluorescent quencher that allows DNA detection probes to be used for real time PCR applications such as measuring gene expression or detecting single nucleotide polymorphisms (SNPs). The PPG modified base is the first in a series of modified bases that Epoch has developed that allow DNA probes to be designed for regions of genomes that cannot be interrogated by probes containing normal nucleotide bases."



*Epoch Fluorophores and Quencher*

The use of fluorescent tags as an alternative to radiolabels in DNA probes and primers has blossomed over the years. Fluorescence is safely measured with inexpensive instrumentation and it is very straightforward to multiplex assays for exceptionally high throughput. Molecular beacon and fluorescence resonance energy transfer (FRET) probes can be used in assays, which can be carried out in closed tube formats with less sample handling at higher throughput. These probes are also suitable for use in techniques that include amplification of the target DNA.

Molecular beacon and FRET probes require efficient quenching until the probe is hybridized to the target. Molecular beacon probes are hairpin structures wherein the fluorescence is quenched by the proximity of the fluorophore to the quencher molecule. When the probe hybridizes to the target, it becomes linear, quenching is disrupted, and the probe fluoresces ready for detection. In FRET assays, when the probe is hybridized to the target, it is

*(Continued on Page 2)*

(Continued from Front Page)

digested by nuclease activity in the polymerase being used for amplification of target copies. The fluorophore, released from the target and separated from the quencher, is now highly fluorescent and ready for detection. Fluorophore/quencher pairs can be chosen based on spectral properties – the emission of the fluorophore should overlap the absorption of the quencher. The quencher may absorb its partner's fluorescence and emit the fluorescence at a new wavelength or, in the case of a non-fluorescent quencher, as heat.

The new Epoch products offer two new fluorescent dyes, available immediately as phosphoramidites and supports, as shown in Figure 1. Their absorbance and emission characteristics are shown in Figure 2. Yakima Yellow has an absorption maximum at 530 nm and emission maximum at 549 nm, while Redmond Red's absorption and emission maxima are at 579 nm and 595 nm, respectively.

Over the years, the dabcyI molecule has proved to be an excellent universal non-fluorescent (dark) quencher for molecular beacon probes. In addition, dabcyI is a very stable molecule and synthesis of doubly-labelled probes containing a dabcyI quencher is quite straightforward. The mechanism of quenching relies on the close proximity of the fluorophore to the dabcyI group, generally called static quenching, which is independent of spectral overlap between fluorophore and quencher. However in FRET probes, dabcyI's ability to act as a dark quencher is limited by its absorption spectrum to use with dyes emitting at 400 – 550 nm. Other fluorescent quenchers can be used to cover a broad spectrum of dyes, but synthesis of the doubly-labelled probes is made much more difficult by the lack of stability of some of the potential candidates to the conditions of oligo deprotection. These probes are best prepared in a two-step process requiring post-synthesis conjugation of the second dye, usually with purification problems.

The Eclipse Quencher from Epoch solves most of the problems inherent in the synthesis of molecular beacon and FRET probes. The Eclipse molecule is highly stable and can be used safely in all common oligo deprotection schemes. The absorption

FIGURE 1: STRUCTURES OF EPOCH DYES AND QUENCHER

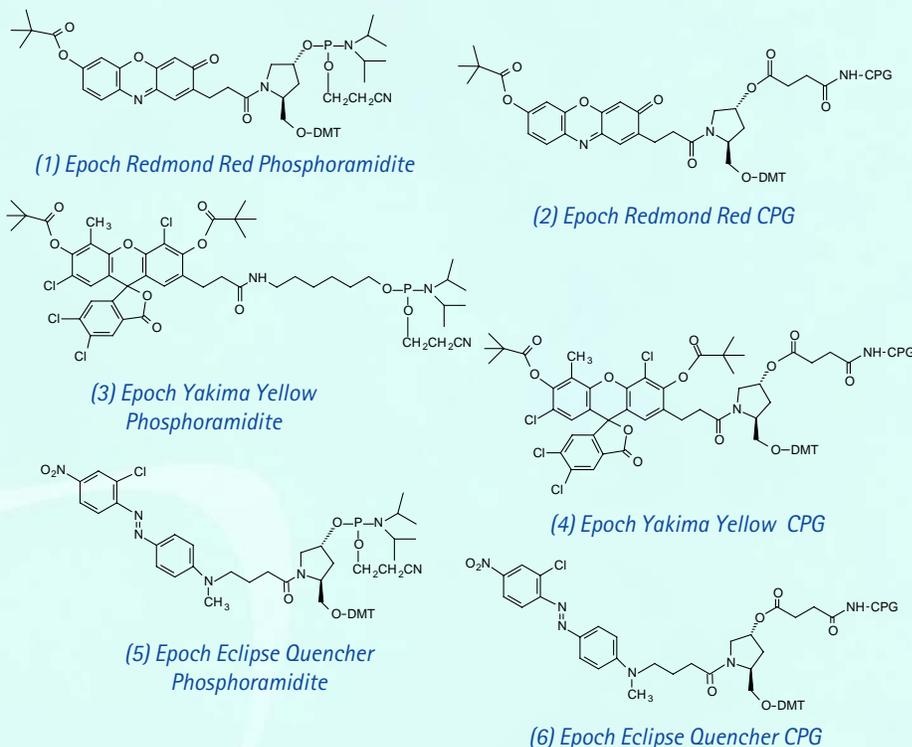
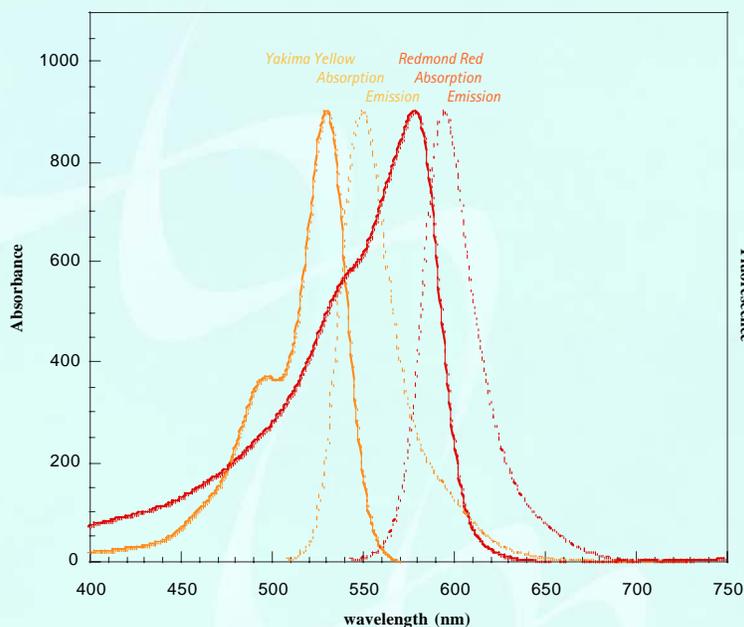


FIGURE 2: ABSORPTION AND EMISSION SPECTRA OF REDMOND RED AND YAKIMA YELLOW



|               | $\lambda_{max}$ Abs | $\lambda_{max}$ Em | Extinc. at max                     | QY   |
|---------------|---------------------|--------------------|------------------------------------|------|
| Yakima Yellow | 530.5 nm            | 549 nm             | 84,000                             | 0.96 |
| Redmond Red   | 579 nm              | 595 nm             | 52,300 (pH 7.1)<br>74,000 (pH 9.1) | 0.84 |

Measured in 0.1 M sodium phosphate. As indicated, Redmond Red is pH sensitive. Yakima Yellow is not and has the same extinction coefficient at pH 7 as pH 9.

The QYs (quantum yield of fluorescence) were determined in 0.1 M Tris-HCl pH 7.4 relative to reference dyes using the dye-labeled T12 oligo. For Yakima Yellow, the reference dye was carboxy tetramethylrhodamine in methanol. For Redmond Red, the reference dye was sulforhodamine 101 in EtOH.

maximum for Eclipse Quencher is at 522 nm, compared to 479 nm for dabcy1. In addition, the structure of the Eclipse Quencher (Figure 1) is substantially more electron deficient than that of dabcy1 and this leads to better quenching over a wider range of dyes, especially those with emission maxima at longer wavelength (red shifted) such as Redmond Red and Cy5. A simple experiment, using a molecular beacon probe, showed that quenching of the fluorescence of Cy5 was 53% more efficient with Eclipse Quencher than with dabcy1. Epoch researchers conducted a comparison<sup>1</sup> of the quenching ability of the Eclipse Quencher relative to dabcy1 in an enzymatic digestion assay designed to simulate the efficiency of quenching of FRET probes. Over the range of fluorophores from FAM (520 nm) to Cy5 (670 nm), Eclipse Quencher outperformed dabcy1 in a measurement of signal to background ratio (signal, defined as fluorescence after digestion, divided by background, defined as initial fluorescence). The improvement was most marked for the higher wavelength dyes. In addition, with an absorption range from 390 nm to 625 nm (Figure 3), the Eclipse Quencher is capable of effective performance in a wide range of colored FRET probes.

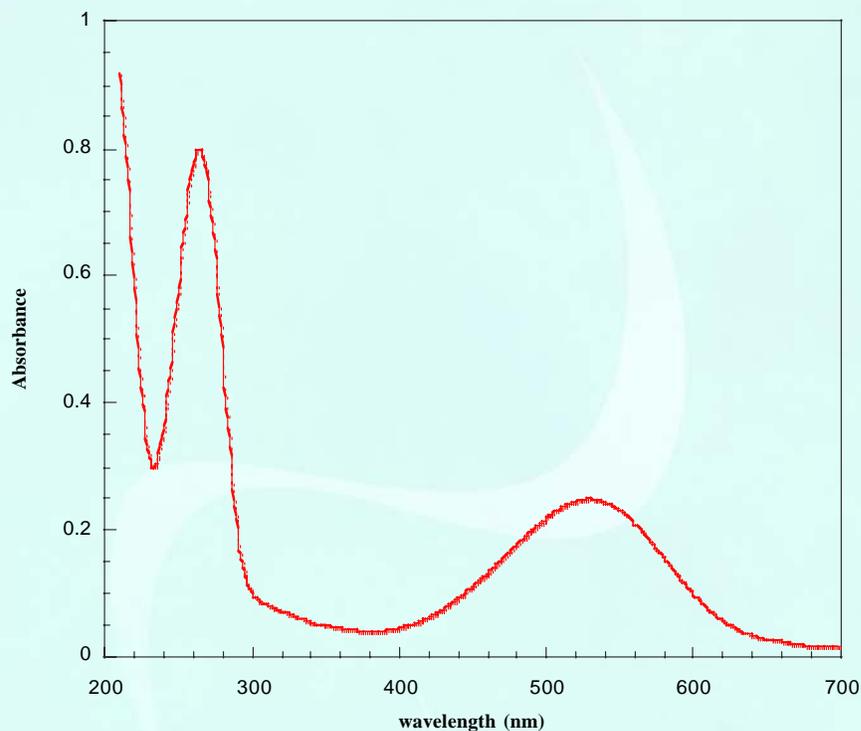
As is normal these days, the use of these products is restricted and users should heed the following qualification statement:

"These Products are for research purposes only, and may not be used for commercial, clinical, diagnostic or any other use. The Products are subject to proprietary rights of Epoch Biosciences, Inc. and are made and sold under license from Epoch Biosciences, Inc. There is no implied license for commercial use with respect to the Products and a license must be obtained directly from Epoch Biosciences, Inc. with respect to any proposed commercial use of the Products. "Commercial use" includes but is not limited to the sale, lease, license or other transfer of the Products or any material derived or produced from them, the sale, lease, license or other grant of rights to use the Products or any material derived or produced from them, or the use of the Products to perform services for a fee for third parties (including contract research)." Redmond Red, Yakima Yellow, and Eclipse are trademarks of Epoch Biosciences, Inc.

#### Reference:

(1) E.A. Lukhtanov, M. Metcalf, and M.W. Reed, *American Biotechnology Laboratory*, September, 2001.

**FIGURE 3: ABSORPTION SPECTRUM OF 3'-ECLIPSE-LABELLED dT12 OLIGO**



## ORDERING INFORMATION

| Item                                    | Catalog No. | Pack      | Price(\$) |
|---|-------------|-----------|-----------|
| Epoch Redmond Red™ Phosphoramidite      | 10-5920-95  | 50 µm     | 205.00    |
|   | 10-5920-90  | 100 µm    | 410.00    |
|   | 10-5920-02  | 0.25g     | 995.00    |
| Epoch Yakima Yellow™ Phosphoramidite    | 10-5921-95  | 50 µm     | 205.00    |
|   | 10-5921-90  | 100 µm    | 410.00    |
|   | 10-5921-02  | 0.25g     | 995.00    |
| Epoch Eclipse™ Quencher Phosphoramidite | 10-5925-95  | 50 µm     | 225.00    |
|   | 10-5925-90  | 100 µm    | 450.00    |
|   | 10-5925-02  | 0.25g     | 1125.00   |
| Epoch Redmond Red™ CPG                  | 20-5920-01  | 0.1g      | 180.00    |
|   | 20-5920-10  | 1.0g      | 1500.00   |
|   | 20-5920-41  | Pack of 4 | 300.00    |
|   | 20-5920-42  | Pack of 4 | 150.00    |
|   | 20-5920-13  | Pack of 1 | 750.00    |
|   | 20-5920-14  | Pack of 1 | 1125.00   |
| Epoch Yakima Yellow™ CPG                | 20-5921-01  | 0.1g      | 180.00    |
|   | 20-5921-10  | 1.0g      | 1500.00   |
|   | 20-5921-41  | Pack of 4 | 300.00    |
|   | 20-5921-42  | Pack of 4 | 150.00    |
|   | 20-5921-13  | Pack of 1 | 750.00    |
|   | 20-5921-14  | Pack of 1 | 1125.00   |
| Epoch Eclipse™ Quencher CPG             | 20-5925-01  | 0.1g      | 230.00    |
|   | 20-5925-10  | 1.0g      | 1925.00   |
|   | 20-5925-41  | Pack of 4 | 350.00    |
|   | 20-5925-42  | Pack of 4 | 175.00    |
|   | 20-5925-13  | Pack of 1 | 995.00    |
|   | 20-5925-14  | Pack of 1 | 1495.00   |

## COMPARISON OF DEPROTECTION METHODS FOR 3'-PT-AMINO-MODIFIER CPG

A few months ago, we introduced the new 3'-Amino-Modifier supports, shown in Figure 1, (1) and (2). In this alternative approach<sup>1</sup>, the nitrogen destined to become the 3'-amino group is included in a phthalimide (PT) group which is attached to the support through an amide group on the aromatic ring. This simple linkage is very stable to all conditions of oligonucleotide synthesis and contains no chiral center. In the last Glen Report<sup>2</sup>, we compared yields and purity of crude oligonucleotides produced with 3'-Amino-Modifier C7 CPG (3) and 3'-PT-Amino-Modifier C6 CPG (2). The results indicated that the yield of product from the 3'-PT-Amino-Modifier C6 CPG is about 20% lower if deprotected with ammonium hydroxide. However, the purity of amino-modified product is significantly higher due to the absence of the acetyl capped product.

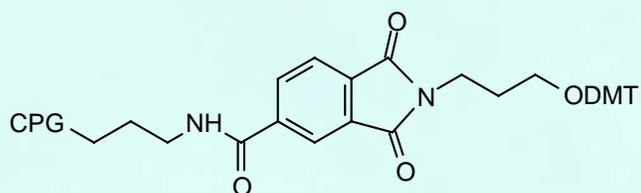
One of our main concerns about the PT-Amino supports was the efficiency of the cleavage reaction from the support by ammonium hydroxide, which led to the lower yield of crude oligonucleotide. Also, we felt it was necessary to compare the cleavage efficiencies of the PT-Amino-Modifier supports under a variety of other, commonly used oligonucleotide deprotection conditions, since a wide range of conditions has had to be developed for the deprotection of modified oligonucleotides. For these supports to become universally accepted, they have to be capable of use with the diverse group of modified bases and dyes we offer that has various sensitivities to basic conditions.

A series of experiments was set up to examine the products from 3'-PT-Amino-Modifier C3 and C6 CPG after deprotection by a variety of popular techniques. As an experimental control, the 3'-amino-modified oligonucleotides were compared to a thymidyl 20mer prepared from a standard, ester-linked, thymidine CPG.

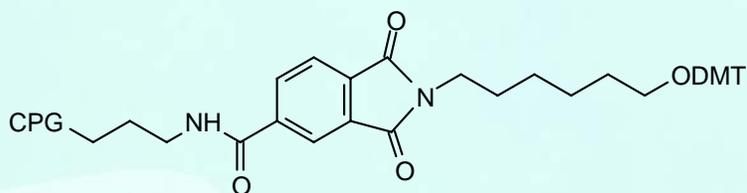
### Results

The experiments were carried out by synthesizing a thymidyl 20mer on a 15 umole scale on each of the following supports: 3'-PT-Amino-Modifier C3 and C6 CPG, and T-CPG. The initial dimethoxytrityl solutions were collected so that the exact

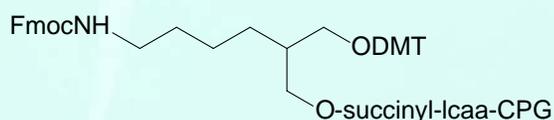
FIGURE 1: STRUCTURES OF 3'-AMINO-MODIFIERS



(1) 3'-PT-Amino-Modifier C3 CPG



(2) 3'-PT-Amino-Modifier C6 CPG



(3) 3'-Amino-Modifier C7 CPG

TABLE 1: COUPLING EFFICIENCIES OF TEST OLIGONUCLEOTIDES

| Support                         | Coupling Efficiency |
|---------------------------------|---------------------|
| Thymidine (1)                   | 98.8%               |
| Thymidine (2)                   | 98.7%               |
| 3'-PT-Amino-Modifier C3 CPG (1) | 97.4%               |
| 3'-PT-Amino-Modifier C3 CPG (2) | 97.8%               |
| 3'-PT-Amino-Modifier C6 CPG (1) | 96.9%               |
| 3'-PT-Amino-Modifier C3 CPG (2) | 97.8%               |

starting scale could be determined, as well as the coupling efficiencies, shown in Table 1.

After synthesis, the supports were dried and carefully weighed into 1 μmole scale aliquots in screw cap vials. Aliquots were then subjected, in duplicate, to the deprotection conditions shown in Table 2. The product was carefully isolated and the yield determined by absorbance at 260 nm. Table 2 shows the results of these deprotection experiments, averaged and normalized to account for differences in actual amount deprotected and for differences in coupling efficiencies. The results are given as ratios to the highest

yielding deprotection of the thymidine support, which was the potassium carbonate in methanol method.

The products were analyzed by PAGE for purity. The products all looked exceptional with little n-1 evident for any of the syntheses. No evidence of any partially deprotected species, shown in Figure 2, was observed by mass spectral analyses.

### Discussion

The results confirmed that the PT-Amino-Modifier supports do not fully cleave using ammonium hydroxide, although the differences of 10% to 20% seem hardly

worth considering given the benefits of having no additional chiral center and the absence of N-acetylated products.<sup>2</sup> In general, the various ammonium hydroxide conditions all yielded equal amounts of amino labeled product, which was 80-90% of the yield from the thymidine support, after taking into account the slightly lower coupling efficiencies.

Since neither of the potential partially protected impurities shown in Figure 2 was observed, we can assume that the two phthalimidyl amide bonds cleave well before the linkage to the support. The aromatic amide link apparently does not cleave under these conditions, or some of those side products would be observed in the crude product mix.

We were pleasantly surprised that the UltraFast system using AMA yielded higher results than with the T-CPG. The fact that the longer deprotection gave even better results lends credence to the data. The 10-minute deprotection with AMA is an excellent choice for the PT-Amino-Modifier support. It is convenient to prepare and well suited for today's high throughput requirements.

Unfortunately, but not too surprisingly, UltraMild deprotection conditions with potassium carbonate in methanol yielded considerably less product and should not be considered for use with the PT-Amino-Modifier supports.

In conclusion, we found that with one exception, potassium carbonate in methanol, the PT-Amino-Modifier supports can be deprotected with any of the common deprotection conditions with good results.

We are happy to acknowledge that this work was carried out by our colleagues at TriLink BioTechnologies, Inc. We thank Rick Hogrefe, Paul Imperial and David Colms for expanding our knowledge of the PT-Amino-Modifier supports.

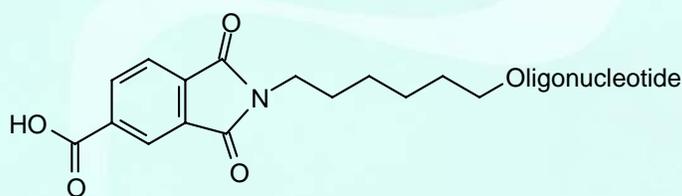
#### References:

- (1) C.R. Petrie, M.W. Reed, A.D. Adams, and R.B. Meyer, Jr., *Bioconjugate Chemistry*, 1992, **3**, 85-7.
- (2) *Glen Report*, 2001, **14.1**, 5.

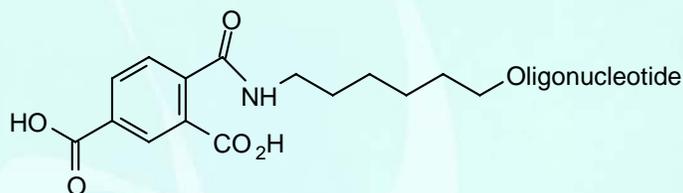
**TABLE 2: DEPROTECTION CONDITIONS AND RESULTS**

| Method  | Temp | Time    | PT-C3 | PT-C6 | T-20 Std |
|---|------|---------|-------|-------|----------|
| NH <sub>4</sub> OH                              | RT   | 24 hrs  | 0.92  | 0.92  | 0.99     |
| NH <sub>4</sub> OH                              | RT   | 48 hrs  | 0.80  | 0.87  | 1.00     |
| NH <sub>4</sub> OH                              | 55°C | 15 hrs  | 0.83  | 0.82  | 0.97     |
| NH <sub>4</sub> OH                              | 65°C | 4 hrs   | 0.72  | 0.79  | 0.96     |
| 3/1 NH <sub>4</sub> OH/EtOH                     | RT   | 48 hrs  | 0.87  | 0.91  | 0.95     |
| 1/1 MeNH <sub>2</sub> /NH <sub>4</sub> OH (AMA) | 55°C | 10 min. | 1.04  | 1.11  | 0.90     |
| 1/1 MeNH <sub>2</sub> /NH <sub>4</sub> OH (AMA) | 55°C | 15 hrs  | 1.13  | 1.18  | 0.98     |
| 0.4 M NaOH in 4/1 MeOH/H <sub>2</sub> O         | RT   | 15 hrs  | 0.97  | 1.00  | 0.90     |
| K <sub>2</sub> CO <sub>3</sub> in MeOH          | RT   | 15 hrs  | 0.15  | 0.13  | 1.00     |

**FIGURE 2: STRUCTURES OF POTENTIAL IMPURITIES**



*Potential impurity formed by hydrolysis of the amide linkage to the support.*



*Further hydrolysis would yield this potential di-carboxylic acid impurity. Neither impurity was observed in oligonucleotide products.*

## ORDERING INFORMATION

| Item                        | Catalog No. | Pack      | Price(\$) |
|-----------------------------|-------------|-----------|-----------|
| 3'-PT-Amino-Modifier C3 CPG | 20-2954-01  | 0.1g      | 95.00     |
|                             | 20-2954-10  | 1.0g      | 675.00    |
| 1 μmole columns             | 20-2954-41  | Pack of 4 | 140.00    |
| 0.2 μmole columns           | 20-2954-42  | Pack of 4 | 85.00     |
| 10 μmole column (ABI)       | 20-2954-13  | Pack of 1 | 250.00    |
| 15 μmole column (Expedite)  | 20-2954-14  | Pack of 1 | 375.00    |
| 3'-PT-Amino-Modifier C6 CPG | 20-2956-01  | 0.1g      | 95.00     |
|                             | 20-2956-10  | 1.0g      | 675.00    |
| 1 μmole columns             | 20-2956-41  | Pack of 4 | 140.00    |
| 0.2 μmole columns           | 20-2956-42  | Pack of 4 | 85.00     |
| 10 μmole column (ABI)       | 20-2956-13  | Pack of 1 | 250.00    |
| 15 μmole column (Expedite)  | 20-2956-14  | Pack of 1 | 375.00    |

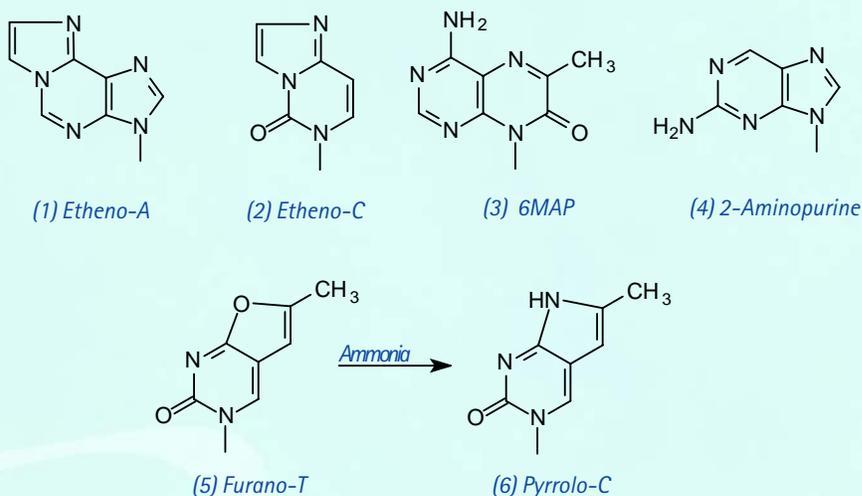
## PYRROLO-C - A NOVEL FLUORESCENT NUCLEOSIDE

Researchers have long been searching for fluorescent nucleosides to probe DNA structure. Fluorescent nucleoside bases would also be useful in the analysis of DNA-protein interactions.

Etheno-A (1) and etheno-C (2)<sup>1</sup> are two readily accessible fluorescent structures but these molecules are both non-hybridizing. Indeed, etheno-dA is a mutagenic nucleoside formed by the action of vinyl chloride on DNA. Other notable fluorescent base analogues are the pteridine nucleoside analogues actively being investigated by Pfeleiderer, Hawkins and co-workers. Although very challenging from a synthetic standpoint, these analogues hold great promise because of their substantial fluorescence, as well as their potential to hybridize specifically with natural bases. The most promising analogue described to date<sup>2</sup> is the adenosine analogue (3) but guanosine and other analogues have also been intensively investigated.<sup>3-5</sup> 2-Aminopurine (4) is a mildly fluorescent base, which has found significant use in probing DNA structures. It is especially useful in that it is capable of hybridizing with T (Figure 4) in Watson-Crick geometry.<sup>6</sup>

Two years ago, in our constant search for nucleosides with interesting fluorescence properties, we introduced furano-dT (7). We had no reason to believe that this molecule would exhibit any base pairing properties and we expected it to act like etheno-dA in that regard. Still, there is always a need for a novel fluorescent nucleoside to aid in DNA structural analysis, so we launched it as an interesting alternative fluorescent base. Before long, it became apparent that there was more to this product than we initially observed. We were aware that this base was not very stable to deprotection conditions, although normal ammonium hydroxide treatment seemed to cause no problems. However, other techniques using potassium carbonate or sodium hydroxide caused decomposition of the base, thereby losing its fluorescent properties. The next indication we had that there were problems inherent with this structure came when we examined mass spectrometric data on oligonucleotides containing multiple additions of furano-dT. Consistently, the molecular weights were 1 Dalton less per addition of furano-dT than expected. When

FIGURE 1: STRUCTURES OF FLUORESCENT BASES



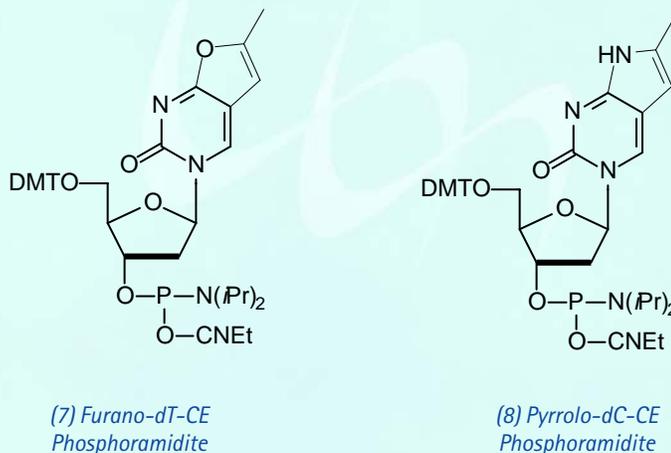
we checked melting behavior of oligonucleotides containing furano-dT, the answer quickly became clear. Melting curves generated using oligos containing "furano-dT" with complementary strands containing A, C, G or T opposite the "furano-dT" sites clearly revealed the solution. "Furano-dT" did not hydrogen bond to A, C, or T, but formed a perfect base pair with G. Mass spec and melting data were trying to tell us that furano-dT had been transformed in the ammonium hydroxide cleavage and deprotection to the equivalent amino compound, which we have chosen to call pyrrolo-dC (6) (Figure 1).

A literature search quickly generated further evidence that furano-dT is not stable to

ammonia, which converts it to the equivalent pyrrolo-pyrimidine, pyrrolo-dC. Interestingly, researchers at Epoch<sup>7</sup> who synthesized a pyrrolo-dC analogue were examining potential base pairs for selectively binding complementary (SBC) oligonucleotides. They did not note the fluorescent properties of the pyrrolo-pyrimidine ring system. However, they did observe that their version of this ring system, different from pyrrolo-dC only in lacking the methyl group on the pyrrolo ring, formed a mismatch with G and was quite unstable to deprotection conditions.

In the meantime, one group had already applied "furano-dT" to their research endeavors. We were able to alert them to

FIGURE 2: STRUCTURES OF FURANO-dT AND PYRROLO-dC



the transformation to pyrrolo-dC in ammonia. In their publication<sup>8</sup>, they made use of the fact that the fluorescence intensity of pyrrolo-dC is sensitive to its environment and can be used to provide direct information on local melting of the DNA. This example makes use of the fact that the fluorescence is quenched in duplex DNA relative to its fluorescence in single-stranded DNA.

In response to the instability of furano-dT to base, we have discontinued the phosphoramidite monomer (7) from our catalog (although it is still available to any researchers who are continuing research started with it). We now introduce Pyrrolo-dC CE Phosphoramidite (8) and look forward to this monomer having a long, stable existence as a Glen Research specialty, while allowing researchers to probe DNA structure and activity using a fluorescent base that forms a perfect match to G on the complementary strand.

Figure 3 shows the normalized excitation and emission spectra of Pyrrolo-dC in a single-stranded oligonucleotide. The fluorescence is, of course, partially quenched in double-stranded oligonucleotides.

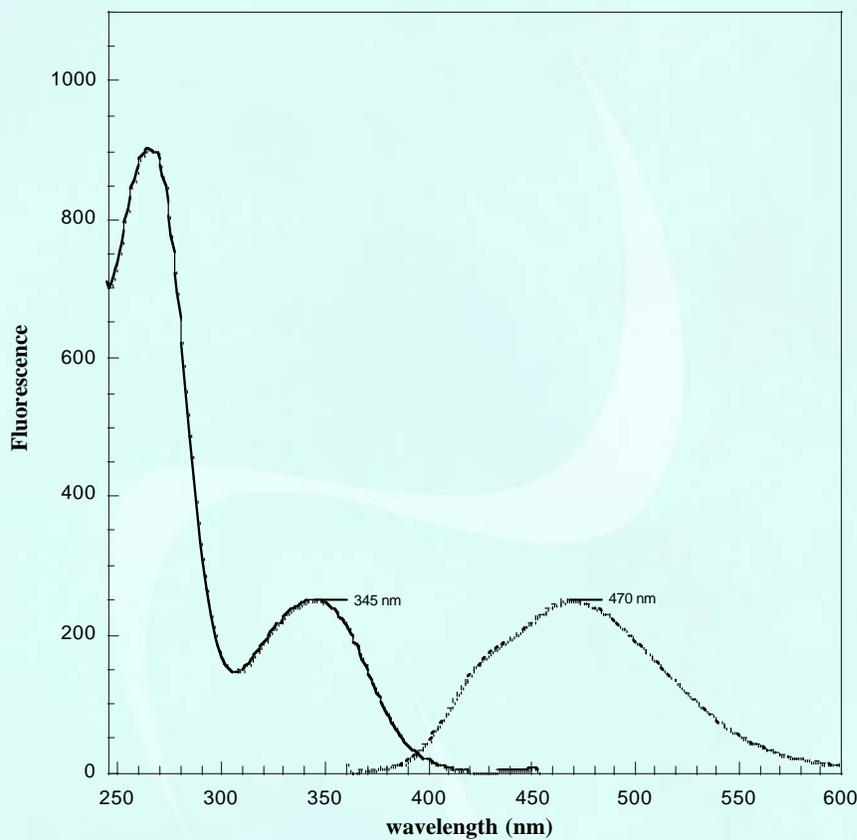
In the meantime, we continue to carry out experiments designed to investigate the properties of pyrrolo-dC and we will report on these in detail in the coming months.

Pyrrolo-dC is a joint development of Glen Research and Berry & Associates.

#### References:

- (1) S.C. Srivastava, S.K. Raza, and R. Misra, *Nucleic Acids Res*, 1994, **22**, 1296-304.
- (2) M.E. Hawkins, W. Pfeleiderer, O. Jungmann, and F.M. Balis, *Anal Biochem*, 2001, **298**, 231-240.
- (3) M.E. Hawkins, W. Pfeleiderer, F.M. Balis, D. Porter, and J.R. Knutson, *Anal Biochem*, 1997, **244**, 86-95.
- (4) S.L. Driscoll, M.E. Hawkins, F.M. Balis, W. Pfeleiderer, and W.R. Laws, *Biophys J*, 1997, **73**, 3277-86.
- (5) R. Charubala, et al., *Nucleos Nucleot*, 1997, **16**, 1369-1378.
- (6) J.M. Jean and K.B. Hall, *Proc Natl Acad Sci U S A*, 2001, **98**, 37-41.
- (7) J. Woo, R.B. Meyer, Jr., and H.B. Gamper, *Nucleic Acids Res*, 1996, **24**, 2470-5.
- (8) C. Liu and C.T. Martin, *J Mol Biol*, 2001, **308**, 465-75.

**FIGURE 3: EXCITATION AND EMISSION SPECTRA OF PYRROLO-dC**



The sequence is: 5'-GCC TAA CTT CXG GAG ATG T-3', where X is Pyrrolo-dC.

The oligo is single-stranded in water.

The spectral properties of pyrrolo-dC are the following and the QY (quantum yield of fluorescence) was determined relative to quinine sulfate in 1.0 N H<sub>2</sub>SO<sub>4</sub>:

$\lambda$  maxima: 265 and 347 nm

E260: 4000 L/mol cm

E347: 3700 L/mol cm

hybridization state

single-stranded

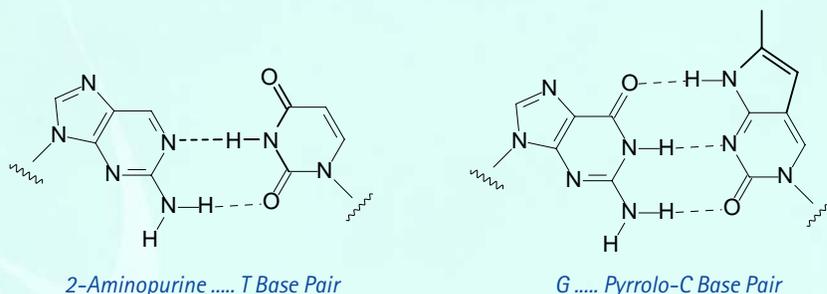
double-stranded

QY

0.07

0.02

**FIGURE 4: BASE PAIRS OF FLUORESCENT BASES**



#### ORDERING INFORMATION

| Item                          | Catalog No. | Pack        | Price(\$) |
|-------------------------------|-------------|-------------|-----------|
| Pyrrolo-dC-CE Phosphoramidite | 10-1017-95  | 50 $\mu$ m  | 110.00    |
|                               | 10-1017-90  | 100 $\mu$ m | 220.00    |
|                               | 10-1017-02  | 0.25g       | 675.00    |

What would a Glen Report be without a selection of new nucleoside analogues? Of course, GR 15.1 is no different!

7-deaza-8-aza-dG (PPG)

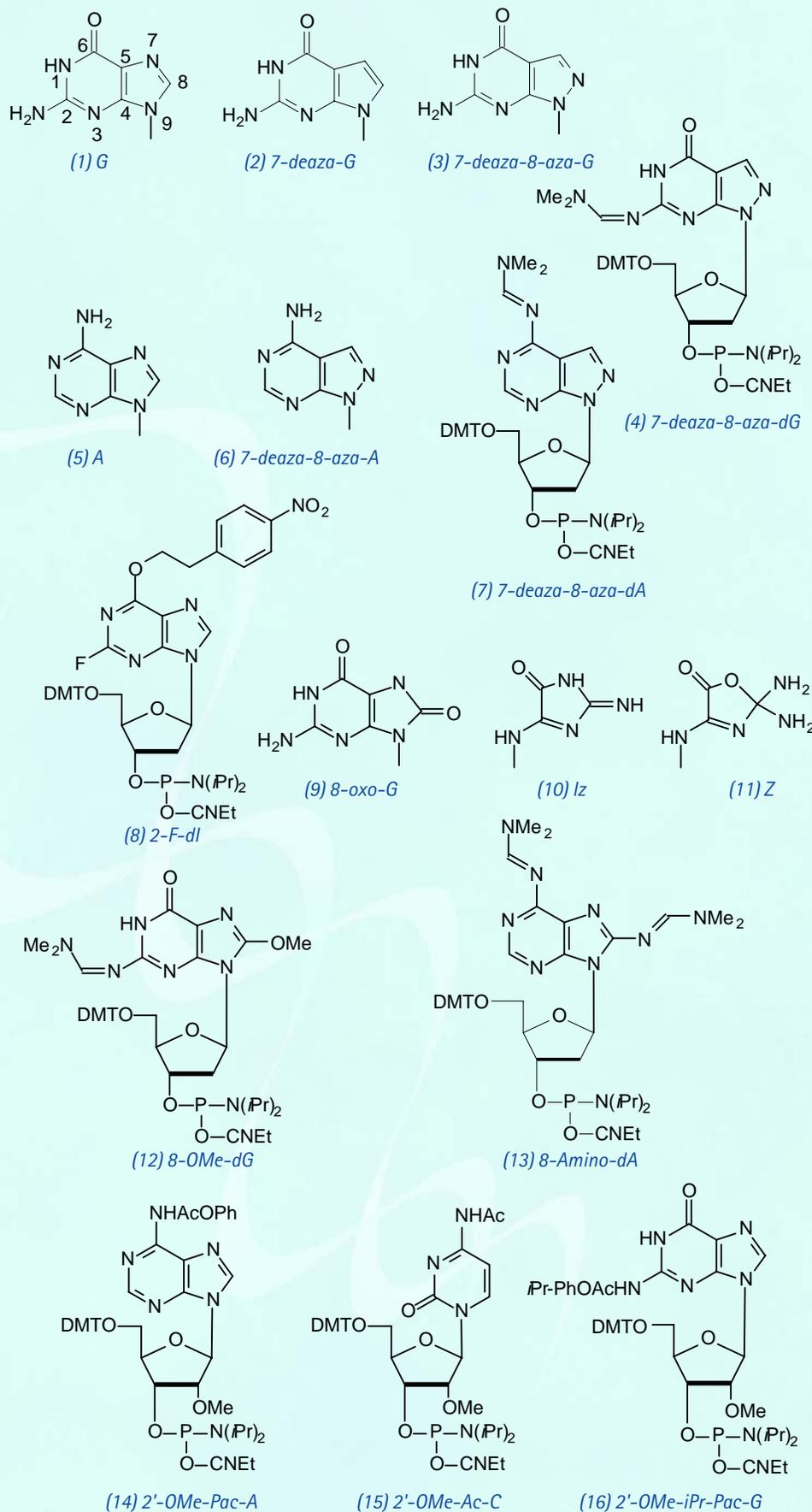
One of the perennial problems of DNA research occurs when analyzing DNA segments that are G-rich. Basically, DNA structure dictated by Watson-Crick base pairing is disrupted in G-rich segments because of their ability to create inter and intra strand hydrogen bonding. This aggregation causes enzymatic disruption so sequencing and PCR experiments become highly problematical. In probe experiments, these segments are not accessible due to this aggregation of G residues on the probe and/or target. The problem arises from extra hydrogen bonding forming at the N7 position of G (1).

Traditionally, 7-deaza-G (2) has been used to overcome these problems with some success. However, the 7-deaza-G - C base pair is destabilized relative to the G - C base pair by around 1° per insertion. Also, 7-deaza-G is relatively unstable to the iodine oxidation in the regular synthesis cycle and, if several insertions have to be made into an oligonucleotide, a non-iodine containing oxidizer must be used.

A solution to these problems may be found in the substitution of 7-deaza-8-aza-G (pyrazolo[3,4-d]pyrimidine) (PPG) (3) for G. This modification has been examined<sup>1</sup> and 7-substituted analogues were evaluated several years ago by Seela's group. In 7-deaza-8-aza-G, the N7 and C8 atoms of G are flipped (Figure 1), allowing the modified base to retain the same electron density as the guanine ring system. The 7-deaza-8-aza-G - C base pair was found to be stabilized relative to G - C by around 1° per insertion. This stability enhancement has led to interest in the use of PPG in diagnostic probe applications.

The 7-deaza-8-aza-dG-CE Phosphoramidite monomer (4) is a very stable structure and, therefore, requires no changes from the regular synthesis cycles and deprotection procedures. It is made available as part of our distribution agreement with Epoch

FIGURE 1: STRUCTURES OF THE MODIFIED BASES DESCRIBED



(Continued on Page 9)

Biosciences, Inc., discussed in detail on the Front Page of this issue.

### 7-Deaza-8-aza-dA

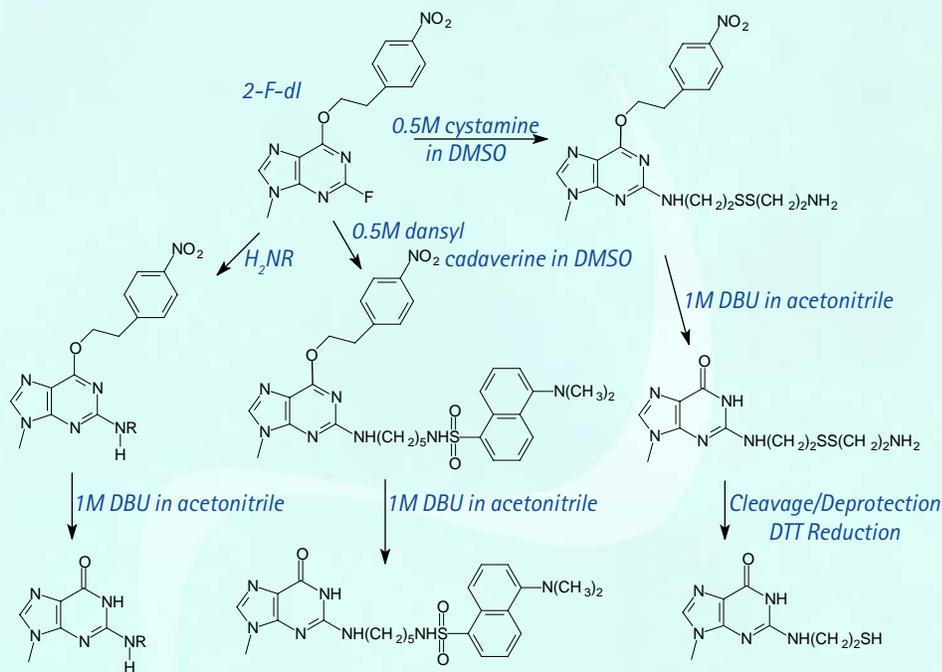
Similarly, the Adenine (5) analogue is 7-deaza-8-aza-A (6). Again, this molecule has been studied in depth over the years by Seela's group as the unsubstituted nucleoside<sup>2</sup>, but more recently with substituents on the 7-position. The melting behavior of 7-deaza-8-aza-A is similar to the G analogue in that the  $T_m$  of the 7-deaza-8-aza-A – T base pair is generally raised relative to the A – T base pair. The different electron density of the pyrazolo[3,4-d]pyrimidine ring system probably allows for better base stacking in a duplex.<sup>3</sup>

Again, the 7-deaza-8-aza-dA-CE Phosphoramidite monomer (7) is a stable structure and there is no need to change conditions during its use in oligonucleotide synthesis and deprotection.

### 2-F-dI

It has been a long time but we are at last adding to our list of Convertible Nucleosides, 2-F-dI-CE Phosphoramidite (8). 2-Fluoro-2'-deoxyinosine (2-F-dI) can be converted to 2-substituted dG derivatives by reaction with a primary amine, which displaces the fluorine atom.<sup>4,5</sup> The timing of the conversion step is a little tricky because small alkyl primary amines are capable of doing the conversion while also cleaving and deprotecting the oligonucleotide. For example, reaction with ethylamine would convert 2-F-dI to N2-ethyl-dG but would simultaneously cleave and deprotect the oligonucleotide. Although that may be interesting in its own right, we have chosen to focus on larger primary amines in our development work. For example, treatment of the oligonucleotide (while still fully protected on the synthesis column) with dansyl cadaverine converts the nucleoside to an N2-dansyl-dG derivative, as shown in Figure 2. Further conventional deprotection of the oligonucleotide leads to the final product. The product oligonucleotide now has a fluorescent tag which, when hybridized to the target strand, will project into the minor groove of the double-stranded duplex. In a further example, we

**FIGURE 2: CONVERSION OF 2-F-dI TO 2-AMINO DERIVATIVES**



used cystamine to convert the 2-F-dI to a product containing a thiol group at the N2 position (Figure 2). Once this converted oligo is hybridized to the target, the thiol is available for cross-linking to, for example, a protein binding to the minor groove. The thiol can also form a disulfide crosslink with a similarly modified G on the complementary strand.<sup>6</sup>

As with all convertible nucleosides, we caution that these reactions are not trivial and should be undertaken by researchers with a good background in chemistry and access to appropriate analytical techniques.

### 8-OMe-dG

Oxidative damage to G residues in biological systems leads to the formation of 8-oxo-G (9), the predominant product of G damage. 2-Aminoimidazolone (Iz, 10) and its hydrolysis product imidazolone (Z, 11) are also major oxidation products of G. Access to these two potential lesions is not possible during oligonucleotide synthesis because they are so base-labile. A suitable precursor, 8-methoxy-dG (8-OMe-dG, 12), to dlz has now been described.<sup>7</sup> We have, therefore, added the convertible nucleoside monomer 8-OMe-dG CE Phosphoramidite (12) to our series of products offered for researching DNA damage and repair.

Oligonucleotide synthesis and deprotection in the presence of 8-OMe-dG is straightforward. The conversion of 8-OMe-dG to dlz takes place by irradiation of the oligonucleotide (1 mM) in 50 mM sodium cacodylate buffer, pH 7, in the presence of riboflavin (50  $\mu$ M) for 2 minutes on a transilluminator (366 nm), under aerobic conditions at 4°C. Surprisingly for a photochemical reaction, the conversion is virtually quantitative. But the molecular gymnastics occurring are completely mind-blowing and we would invite reaction enthusiasts to review the proposed mechanism.

### 8-Amino-dA

8-Amino-2'-deoxyAdenosine has been substituted for 2'-deoxyAdenosine in oligonucleotides used in the studies of triple helix formation. It has been shown<sup>8</sup> that oligonucleotides containing this modified base form stable triple helices at neutral pH, whereas regular triple helical structures are normally observed under acidic conditions. We are happy to make available 8-Amino-dA-CE Phosphoramidite (13), which can be used for oligonucleotide synthesis and subsequent deprotection without need for modification of normal procedures.

(Continued on Page 10)

**UltraMild 2'-OMe-RNA**

The use of UltraMild monomers in oligonucleotide synthesis has allowed very sensitive dyes like TAMRA, HEX and Cy5 to be used virtually routinely. The DNA and RNA monomers are currently available and, by popular demand, we are adding the set of 2'-OMe-RNA monomers. In our rendering of this chemistry, we use as protecting groups phenoxyacetyl (Pac) for A, acetyl (Ac) for C, and isopropyl-phenoxyacetyl (iPr-Pac) for G. The structures of the UltraMild 2'-OMe-RNA monomers are shown in Figure 1, (14) – (16).

It has become clear<sup>9</sup> that acetic anhydride in the conventional capping mix can cause transamidation in situations where an amine protecting group is quite labile. This leads to acetyl protection on the amino group that may be slow to be removed. Consequently, if many dG residues are included in the oligonucleotide, we recommend the use of phenoxyacetic anhydride (Pac<sub>2</sub>O) in Cap A. This modification removes the possibility of exchange of the iPr-Pac protecting group on the dG with acetate from the acetic anhydride capping mix.

References are listed at the foot of column 3 on this page.

**ORDERING INFORMATION**

| Item  | Catalog No. | Pack   | Price(\$) |
|---|-------------|--------|-----------|
| 7-Deaza-8-aza-dG-CE Phosphoramidite (PPG)               | 10-1073-95  | 50 µm  | 197.50    |
|   | 10-1073-90  | 100 µm | 395.00    |
|   | 10-1073-02  | 0.25g  | 1095.00   |
| 7-Deaza-8-aza-dA-CE Phosphoramidite                     | 10-1083-95  | 50 µm  | 177.50    |
|   | 10-1083-90  | 100 µm | 355.00    |
|   | 10-1083-02  | 0.25g  | 975.00    |
| 2-F-dI-CE Phosphoramidite                               | 10-1082-95  | 50 µm  | 180.00    |
|   | 10-1082-90  | 100 µm | 360.00    |
|   | 10-1082-02  | 0.25g  | 975.00    |
| 8-OMe-dG-CE Phosphoramidite                             | 10-1075-95  | 50 µm  | 177.50    |
|   | 10-1075-90  | 100 µm | 355.00    |
|   | 10-1075-02  | 0.25g  | 975.00    |
| 8-Amino-dA-CE Phosphoramidite                           | 10-1086-95  | 50 µm  | 177.50    |
|   | 10-1086-90  | 100 µm | 355.00    |
|   | 10-1086-02  | 0.25g  | 975.00    |
| 2'-OMe-Pac-A-CE Phosphoramidite                         | 10-3601-90  | 100 µm | 25.00     |
|   | 10-3601-02  | 0.25g  | 62.50     |
|   | 10-3601-05  | 0.5g   | 125.00    |
|   | 10-3601-10  | 1.0g   | 250.00    |
| 2'-OMe-Ac-C-CE Phosphoramidite                          | 10-3115-90  | 100 µm | 20.00     |
|   | 10-3115-02  | 0.25g  | 50.00     |
|   | 10-3115-05  | 0.5g   | 100.00    |
|   | 10-3115-10  | 1.0g   | 200.00    |
| 2'-OMe-iPr-Pac-G-CE Phosphoramidite                     | 10-3621-90  | 100 µm | 25.00     |
|   | 10-3621-02  | 0.25g  | 62.50     |
|   | 10-3621-05  | 0.5g   | 125.00    |
|   | 10-3621-10  | 1.0g   | 250.00    |
| <b>Cap Mix A</b>  |             |        |           |
| THF/Pyridine/Pac <sub>2</sub> O<br>(Applied Biosystems) | 40-4210-52  | 200mL  | 120.00    |
|   | 40-4210-57  | 450mL  | 260.00    |
| THF/Pac <sub>2</sub> O<br>(Expedite)                    | 40-4212-52  | 200mL  | 115.00    |
|   | 40-4212-57  | 450mL  | 250.00    |

**A NOVEL ROUTE TO**

**M**ost conjugation schemes start with the amino or thiol group on the oligonucleotide and require that the molecule to be conjugated is first converted to either an active ester for amine reactions, such as a succinimide, or a thiol reactive maleimide. In either case, it is very costly to conduct a screen of a wide number of different conjugates. Also, it is not always possible to find a convenient starting material in order to prepare an activated ester. In some cases, the molecule is not even amenable to activation because of other properties that interfere with the chemistry. Another option is to react the oligonucleotide with either a heterobifunctional or homobifunctional linker. This broadens the applicability by allowing conjugation with other types of molecules but adds steps to the process and is often difficult to reproduce.

An alternative approach is to place the active ester on the oligonucleotide and conjugate with a molecule containing a primary amine. A wider range of unusual amines is readily available and generally more affordable. If the amine component is stable to the mild base used for deprotection, then it can be used with carboxy-modified oligonucleotides.

Glen Research is pleased to add 5'-Carboxy-Modifier-CE Phosphoramidite (Figure 2, Back Page) to our range of products for the

**References (from column 2 above):**

- (1) F. Seela and H. Driller, *Helvetica Chimica Acta*, 1988, **71**, 1191-1198.
- (2) F. Seela and K. Kaiser, *Helvetica Chimica Acta*, 1988, **71**, 1813-1823.
- (3) F. Seela and G. Becher, *Nucleic Acids Res.*, 2001, **29**, 2069-2078.
- (4) L.V. Nechev, I. Kozekov, C.M. Harris, and T.M. Harris, *Chem Res Toxicol*, 2001, **14**, 1506-1512.
- (5) A.R. Diaz, R. Eritja, and R.G. Garcia, *Nucleos Nucleot*, 1997, **16**, 2035-2051.
- (6) D.A. Erlanson, J.N.M. Glover, and G.L. Verdine, *J. Amer. Chem. Soc.*, 1997, **119**, 6927-6928.
- (7) H. Ikeda and I. Saito, *J. Amer. Chem. Soc.*, 1999, **121**, 10836-10837.
- (8) R.G. Garcia, E. Ferrer, M.J. Macias, R. Eritja, and M. Orozco, *Nucleic Acids Res.*, 1999, **27**, 1991-1998.
- (9) Q. Zhu, M.O. Delaney, and M.M. Greenberg, *Bioorg Medicinal Chem Letter*, 2001, **11**, 1105-1107.



(Continued from Page 11)

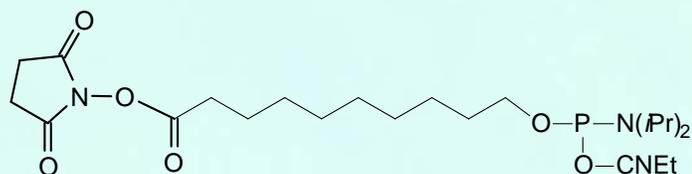
- b. Wash the support with acetonitrile and air dry it. Add the support to a vial containing 10 equivalents of the amine dissolved in 2 mL of dichloromethane or other appropriate solvent with 10% triethylamine. Leave the reaction at room temperature for 4 hours with continuous agitation. Decant the amine solution from the support and wash with further dichloromethane. Air dry the support.

Proceed to regular deprotection, keeping in mind the stability of the modified oligonucleotide to the deprotection conditions.

## 2. Preparation of Oligonucleotides with 5' Carboxylic Acid Linkers

- a. Couple the 5'-Carboxy-Modifier-CE Phosphoramidite to the oligonucleotide using any conventional cycle. Do NOT allow the synthesizer to proceed to normal cleavage with ammonium hydroxide.

FIGURE 2: STRUCTURE 5'-CARBOXY-MODIFIER C10



5'-Carboxy-Modifier C10

- b. Deprotect the oligonucleotide overnight at room temperature using 0.4 M methanolic sodium hydroxide (Methanol/Water 4:1)
- c. Isolate the oligonucleotide by first passing the reaction through the desalting process of your choice, and then purify as usual. The product may also be used crude after desalting.

## ORDERING INFORMATION

| Item                    | Catalog No. | Pack        | Price(\$) |
|-------------------------|-------------|-------------|-----------|
| 5'-Carboxy-Modifier C10 | 10-1935-90  | 100 $\mu$ m | 50.00     |
|                         | 10-1935-02  | 0.25g       | 200.00    |



**GLEN RESEARCH**

22825 DAVIS DRIVE  
STERLING, VIRGINIA  
20164