# The Glen Report

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GLEN RESEARCH

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INSIDE



## NEW PRODUCT - 1-ETHYNYL-dSPACER CE PHOSPHORAMIDITE

### INTRODUCTION

Efficient post synthesis modification of DNA and RNA offers the capability to attach a diverse set of labels that would not otherwise be accessible by standard phosphoramidite oligonucleotide synthesis. Through copper (I) alkyne-azide cycloaddition (CuAAC), a variety of azide labels can be efficiently attached to alkyne-modified DNA and RNA oligonucleotides.[1,2] As shown in Figure 1, our alkyne modifiers include internal nucleobase modifiers, 5'-modifiers, 3'-modifier supports, and serinol linkers.

### 1-ETHYNYL-dSPACER

In this report, we introduce 1-Ethynyl-dSpacer CE Phosphoramidite, (1) in Figure 2, that can be used in any position within an oligonucleotide while still retaining the high efficiency of click chemistry.

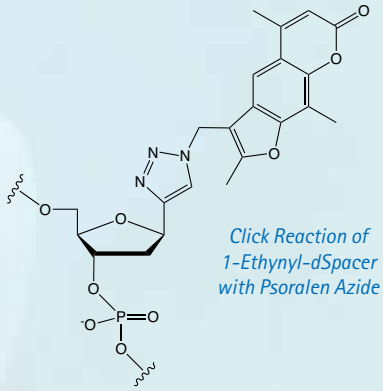
The click reaction of this modifier with an azide forms a 1,2,3-triazol-4-yl pseudo-nucleobase structure, as shown in Figure 2. These pseudo-nucleobases can act as fleximers that have the potential to moderate duplex stability and enzymatic activities.[3,4] In recent work, the 1,2,3-triazolyl modified DNA was evaluated for duplex stability in dsDNA and in triplex forming oligonucleotides (TFOs).[5,6] Several modifications retained duplex or triplex stability compared to the unmodified oligonucleotides and had the potential to function as universal bases.

### SYNTHESIS AND DEPROTECTION

The coupling efficiency of 1-ethynyl-dSpacer was optimal under the standard conditions used for regular DNA monomers.

Deprotection was evaluated for compatibility with the following conditions using a simple T6 oligo with a single addition of 1-ethynyl-dSpacer at the 5' terminus.

**REACTION OF 1-ETHYNYL-dSPACER WITH PSORALEN AZIDE**



*Click Reaction of 1-Ethynyl-dSpacer with Psoralen Azide*

- Ammonium hydroxide, 55°C overnight
- Ammonium hydroxide, 2 hours, RT
- AMA, 65°C 10 minutes
- Potassium carbonate in methanol, 4 hours, RT
- Ammonium Hydroxide, 5 minutes, RT (control)

No degradation was observed under these deprotection conditions.

Each deprotected sample was clicked with HEX azide to evaluate the possibility of any degradation by hydration occurring during deprotection. None was observed as all oligos labelled efficiently.

To evaluate for compatibility with standard synthesis reagents, oligos were exposed to reagents for an equivalent of a 50-mer exposure. The oligos showed broad compatibility with standard reagents. All oligos were clicked with HEX azide after exposure to confirm there was no degradation caused by exposure to the reagents.

The modifier is efficiently incorporated into oligonucleotides by standard phosphoramidite chemistry, is stable to common deprotection conditions, and is compatible with Glen-Pak™ purification and produces very pure oligonucleotides.

*(Continued on Page 2)*

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The best deprotection results were obtained with AMA for 10 minutes at 65°C.

## CLICK EVALUATION

The resulting modified oligonucleotide clicked efficiently using standard click chemistry with THPTA and a selection of azides (Figure 3). The high efficiency of the click reaction with a variety of azides is illustrated in Figure 4.

Interestingly, the efficiency of the cycloaddition does not appear to be significantly affected by the azide tag. In a simple experiment, a library of azides was evaluated for its efficiency to click to a 1-ethynyl-dSpacer labelled oligonucleotide in a single reaction. Generally, a minimum of 4 equivalents of azide maximizes labelling while minimizing the reaction time. In this experiment, the total azide concentration was slightly over 1 equivalent. All expected modifications were observed in the library by HPLC and UV/Vis spectroscopy.

## DUPLEX STABILITY

1-Ethynyl-dSpacer was incorporated into the T7 forward primer and clicked with several azides. The oligos were evaluated for their effect on duplex stability. The 1-ethynyl-dSpacer modification exhibits similar duplex stability to the standard dSpacer (10-1914) and destabilizes the duplex when internally incorporated. Upon cycloaddition, the duplex stability is moderated by the resulting structure of the modification. Simple 1,2,3-triazoles were destabilizing, as were modifications that incorporated TEG linkers (6-FAM-TEG and Amino-TEG). Modifications that incorporated *aromatic* functional groups restored duplex stability to varying degrees with coumarin restoring stability by 11°C and psoralen by 7°C. The results are shown in Table 1. Examples of the melting curves are shown in Figure 5.

## CONCLUSION

1-Ethynyl-dSpacer CE Phosphoramidite is a new tool in the click chemistry toolbox with the potential for simple access to a diverse set of modifications. As a dSpacer, the 1-ethynyl-dSpacer modifier has a similar profile to the current dSpacer, exhibiting the same stability to synthesis

**FIGURE 1: STRUCTURES OF GLEN RESEARCH ALKYNE MODIFIERS**

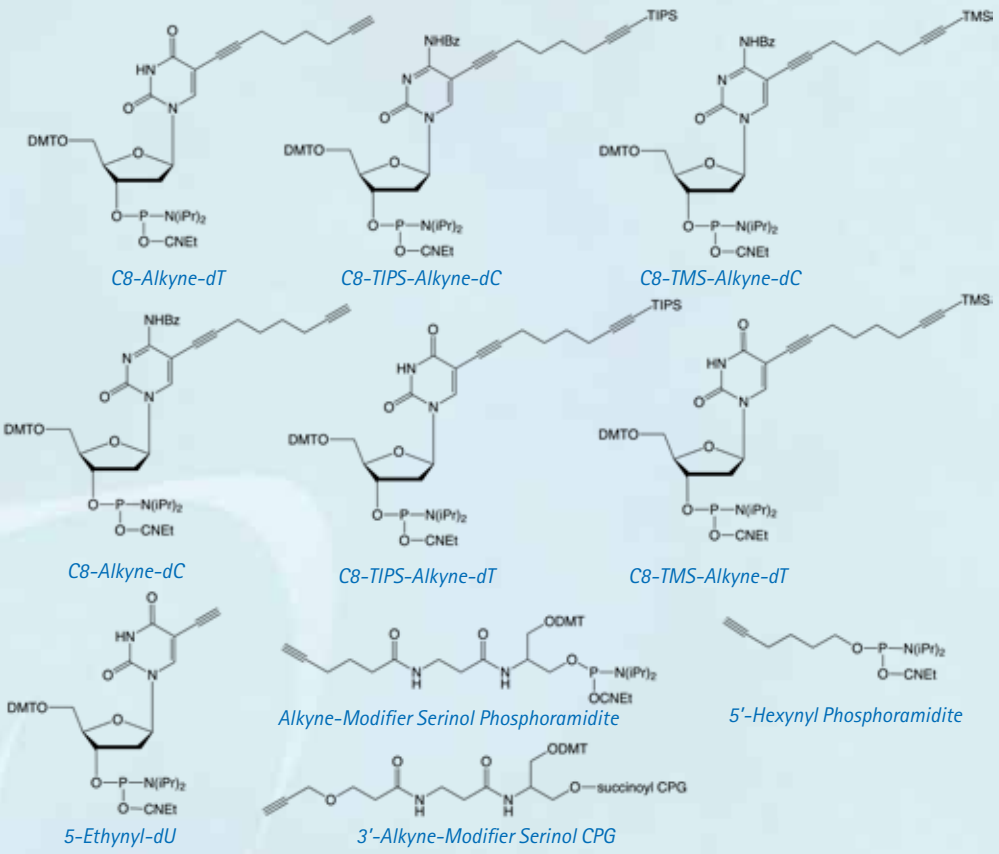



**FIGURE 2: 1-ETHYNYL-dSPACER AND ITS CLICK REACTION WITH AZIDES**

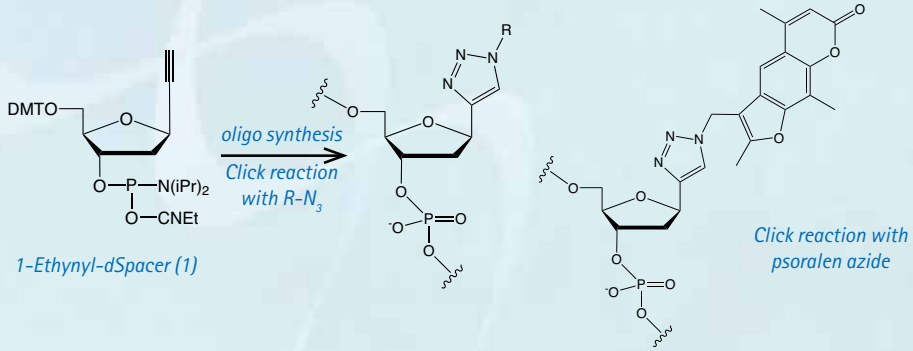
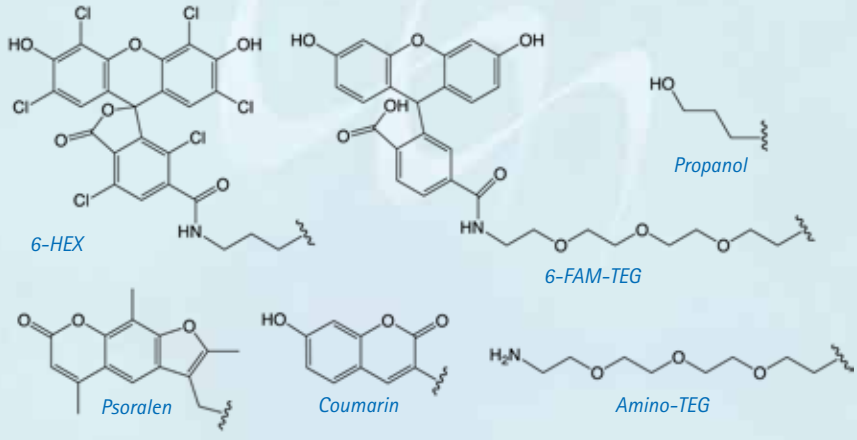



**FIGURE 3: AZIDES USED IN THIS STUDY**



and deprotection with a similar effect in DNA duplexes. As an alkyne modifier, 1-ethynyl-dSpacer combines the flexibility of an internal or terminal modifier with the high efficiency of click chemistry. Additionally, 1-ethynyl-dSpacer is a unique modifier which generates a substituted 1,2,3-triazole pseudo-nucleobase after click chemistry. These modified pseudo-nucleobases have the potential for use in research as universal bases or hybridization probes.

**TABLE 1: DUPLEX STABILITY**

| *Modification* | *Tm* |
|---|---|
| 1-Ethynyl-dSpacer | 40°C |
| 6-HEX | 40°C |
| 6-FAM-TEG | 40°C |
| Psoralen | 47°C |
| Coumarin | 51°C |
| Propanol | 43°C |
| Amino-TEG | 43°C |
| dSpacer (10-1914) | 40°C |
| Control, X=A | 55°C |

T7 forward primer and complement
5'-TAA TAC G**X**C TCA CTA TAG GG - 3'
5'-CCC TAT AGT GAG **T**CG TAT TA - 3'

## SUMMARY

- 1-Ethynyl-dSpacer is stable to standard synthesis conditions.
- No changes needed to standard coupling.
- The product is stable to standard deprotection conditions.
- The product can be used as an internal modification and a terminal modification.
- The product is stable to CuAAC and clicks efficiently.
- The product has similar duplex stability to dSpacer.
- 1-Ethynyl-dSpacer lowers the stability of the duplex by the same amount as dSpacer.
- Upon clicking, the duplex stability can be modified based on the resulting structure of the incorporation.
- The product has been evaluated with the following azides:
  6-HEX Azide, 6-FAM-TEG Azide, Psoralen Azide, Coumarin Azide, Cyanine 7 Azide, Azido propanol, Amino-TEG Azide

**FIGURE 4: SAMPLE RP HPLC OF MODIFIED OLIGOS**

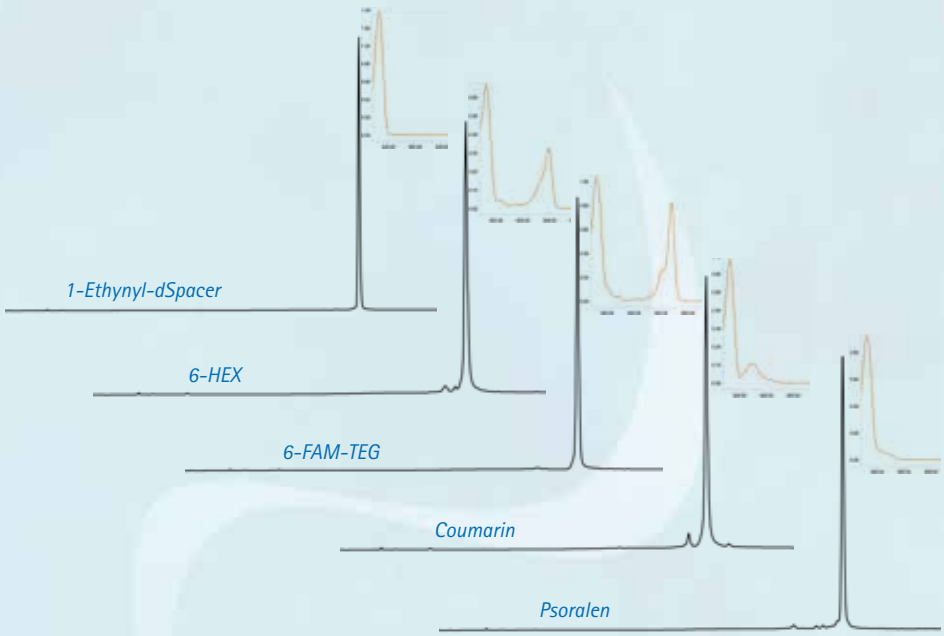
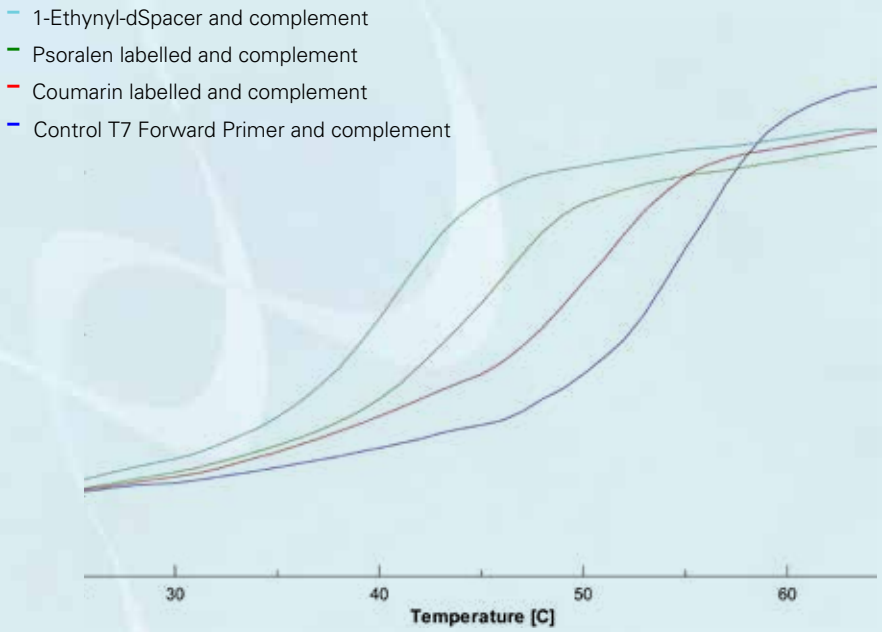



**FIGURE 5: MELTING CURVES USING T7 FORWARD PRIMER AND COMPLEMENT**





*References:*

1. V.V. Rostovtsev, L.G. Green, V.V. Fokin, and K.B. Sharpless, *Angew Chem Int Ed*, 2002, **41**, 2596-2599.
2. Q. Wang, et al., *Journal of the American Chemical Society*, 2003, **125**, 3192-3193.
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5. M. Nakahara, *et al.*, *Bioorganic & Medicinal Chemistry Letters*, 2009, **19**, 3316-3319.
6. Y. Hari, *et al.*, *Bioorganic & Medicinal Chemistry*, 2011, **19**, 1162-1166.


## ORDERING INFORMATION

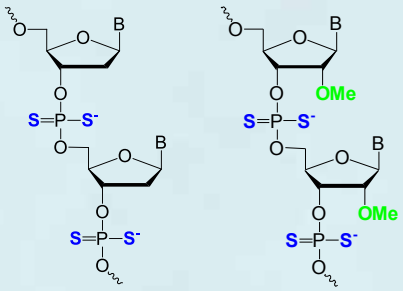
| *Item* | *Catalog No.* | *Pack* | *Price($)* |
|---|---|---|---|
| 1-Ethynyl-dSpacer CE Phosphoramidite | 10-1910-95 | 50 µmole | 180.00 |
| | 10-1910-90 | 100 µmole | 340.00 |
| | 10-1910-02 | 0.25g | 1250.00 |

## INTRODUCING 2'-OMe-THIOPHOSPHORAMIDITES

*Xianbin Yang*
*AM Biotechnologies*

Advances in phosphorodithioate oligodeoxynucleotide (Figure 1: DNA-PS2 or DS2) chemistry, biochemical and biological studies have shown very interesting and potentially useful results. These include observations that DNA-PS2 or DS2 activates RNase H *in vitro*, strongly inhibits human immunodeficiency virus (HIV) reverse transcriptase, induces B-cell proliferation and differentiation, is completely resistant to hydrolysis by various nucleases, and binds to protein targets in the form of dithiophosphate aptamers (thioaptamers) (reviewed by Dr. Yang (1)). All these studies encouraged us to introduce the DNA-thiophosphoramidites to synthesize a DNA-PS2 or DS2 (Figure 1) several years ago.

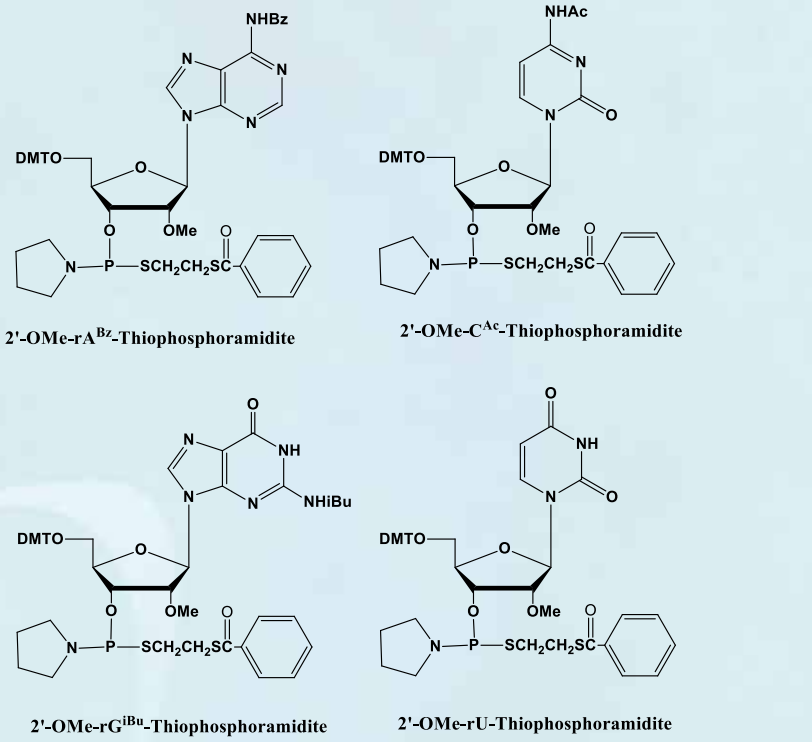
**FIGURE 1: PHOSPHORODITHIOATE LINKAGE**




DS2 (DNA-PS2) MS2 (2'-OMe-PS2)

Recently, siRNAs containing PS2 modifications were shown to exhibit favorable properties for therapeutic applications (2-4). Combining the PS2 with the 2'-O-methyl ribose modification (2'-OMe-PS2 or MS2 in Figure 1) afforded increased loading of modified siRNA duplexes into the RNA induced silencing complex (RISC) as well as enhanced gene-silencing antitumor activity (5). Interestingly, the former property can be attributed to MS2 modification at two residues adjacent to the 3'-TT overhang in the sense siRNA strand. This conclusion is supported by higher association based on *in vitro* pull-down assays, and tighter intracellular binding between MS2-siRNA and Ago2 protein, relative to the corresponding RNA and RNA-PS2. Very recently we have found that the use of MS2 substitution on a single nucleotide of 2'-OMe-RNA aptamer targeting VEGF-165 dramatically improves target binding affinity by approximately a thousand fold (from nanomolar to picomolar). In addition, the MS2-modified RNA aptamers demonstrate improved biochemical and biophysical characteristics (N. Dinuka Abeydeera *et al.* submitted to Nature communication). Moreover the X-ray crystal structures demonstrated that single PS2 or MS2 modified RNAs have virtually identical conformations with native RNA duplexes (6). In particular PS2 or MS2 modification does not trigger any changes in the ribose conformation and the backbone torsion angles (6). The structures of the PS2 and MS2 monomers are shown in Figure 4 on Page 5.

**FIGURE 2: THE STRUCTURES OF 2'-OMe-THIOPHOSPHORAMIDITES**



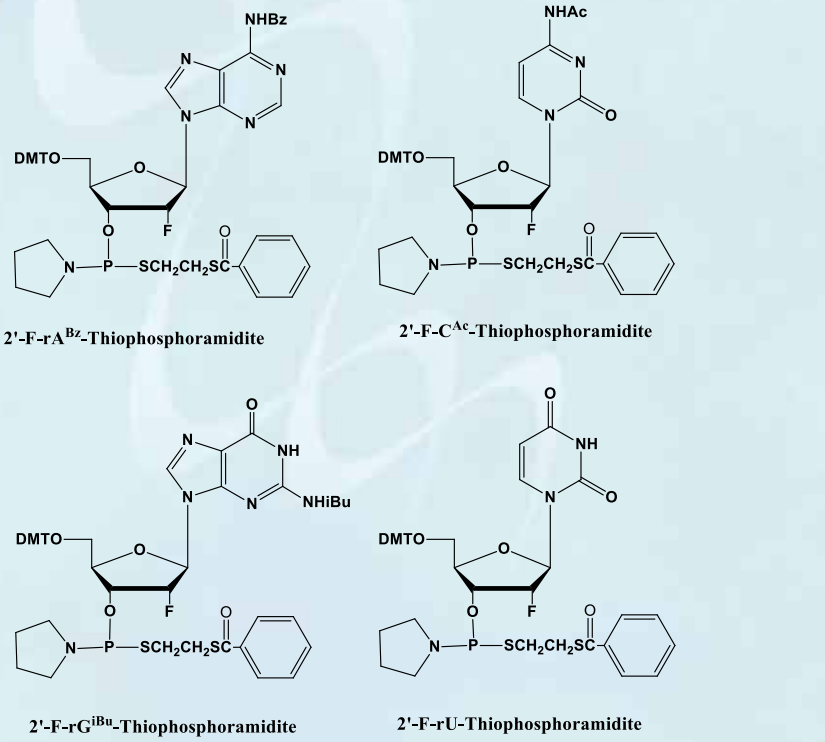

2'-OMe-rA$^{Bz}$-Thiophosphoramidite

2'-OMe-C$^{Ac}$-Thiophosphoramidite

2'-OMe-rG$^{iBu}$-Thiophosphoramidite

2'-OMe-rU-Thiophosphoramidite

**FIGURE 3: THE STRUCTURES OF 2'-F-THIOPHOSPHORAMIDITES**




2'-F-rA$^{Bz}$-Thiophosphoramidite

2'-F-C$^{Ac}$-Thiophosphoramidite

2'-F-rG$^{iBu}$-Thiophosphoramidite

2'-F-rU-Thiophosphoramidite

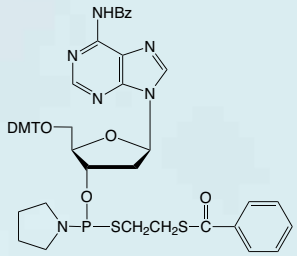
2'-Deoxy-2'-fluoro-RNA (2'-F-RNA) has been popular in ribozymes, siRNA, and anti-miRNA research. As a substitute for 2'-F-RNA phosphoromonothioate, we will make the four 2'-F-thiophosphoramidites available soon (Figure 3). In this way, we hope to open up a full spectrum of research applications for phosphorodithioates (PS2).

At this time, Glen Research is pleased to offer the 2'-OMe Thiophosphoramidites as shown in Figure 4.

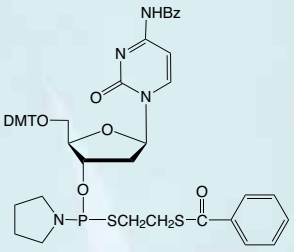
## References:


1. Xianbin Yang* and Ellen Mierzejewski. *Synthesis of nucleoside and oligonucleoside dithiophosphates. New J. Chem.* **34**(5): 805-819 (2010).
2. Xianbin Yang*, Malgorzata Sierant, Magdalena Janicka, Lukasz Peczek, Carlos Martinez, Tom Hassell, Na Li, Xin Li, Tianzhi Wang, and Barbara Nawrot. *Gene silencing activity of siRNA molecules containing phosphorodithioate substitutions. ACS Chem. Biol.* **7** (7), 1214–1220 (2012).
3. Malgorzata Sierant, Xianbin Yang, Magdalena Janicka, Na Li, Carlos Martinez, Tom, Hassell and Barbara Nawrot. *SiRNA with phosphorodithioate modification.* Collection Symposium Series, 12:135-139(2011), XVth Symposium on Chemistry of Nucleic Acid Compounds, Cesky Krumlov, Czeck Republic, June 5-10, 2011.
4. Malgorzata Sierant, Xianbin Yang and Barbara Nawrot. *SiRNA analogs containing phosphorodithioate substitutions.* Phosphorus, Sulfur, and Silicon. **188**: 427-436 (2013).
5. Sherry Y. Wu, Xianbin Yang* Kshipra M. Gharpure, Hiroto Hatakeyama, Martin Egli, Michael H. McGuire, Archana S. Nagaraja, Takahito M. Miyake, Rajesha Rupaimoole, Chad V. Pecot, Morgan Taylor, Sunila Pradeep, Malgorzata Sierant, Cristian Rodriguez-Aguayo, Hyun J. Choi, Rebecca A. Previs, Guillermo N. Armaiz-Pena, Li Huang, Carlos Martinez, Tom Hassell, Cristina Ivan, Vasudha Sehgal, Richa Singhania, Hee-Dong Han, Chang Su, Ji Hoon Kim, Heather J. Dalton, Chandra Kowali, Khandan Keyomarsi, Nigel A.J. McMillan, Willem W. Overwijk, Jinsong Liu, Ju-Seog Lee, Keith A. Baggerly, Gabriel Lopez-Berestein, Prahlad T. Ram, Barbara Nawrot, and Anil K. Sood*. *2'-OMe-phosphorodithioate modified siRNAs show increased loading into the RISC complex and enhanced anti-tumour activity. Nat. Commun.* **5**(12), 3459-3462 (2014).
6. Pardeep Pallan, Xianbin Yang*, Malgorzata Sierant, Nuwan Abeydeera, Tom Hassell, Carlos Martinez, Magdalena Janicka, Barbara Nawrot and Martin Egli*. *Crystal structure, stability and Ago2 affinity of phosphorodithioate-modified RNAs. RSC Adv.* DOI: 10.1039/c4ra10986d. **4**(110), 64901-64904 (2014).

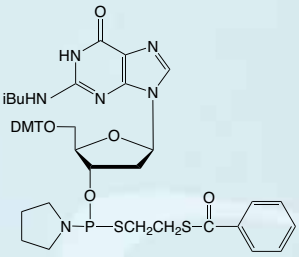

***FIGURE 4:* THE STRUCTURES OF THIOPHOSPHORAMIDITES**



*dA-Thiophosphoramidite*



*dC-Thiophosphoramidite*



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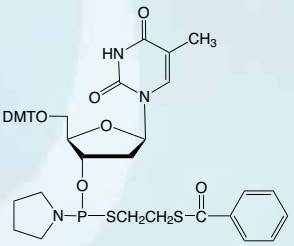
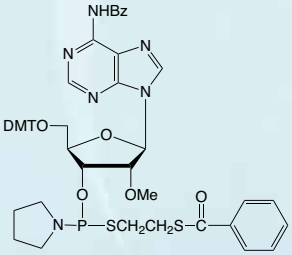
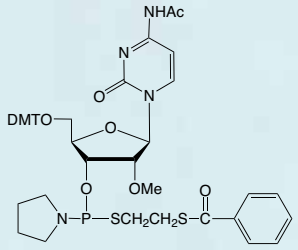


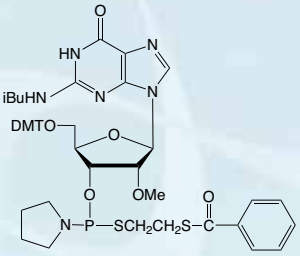
*dT-Thiophosphoramidite*



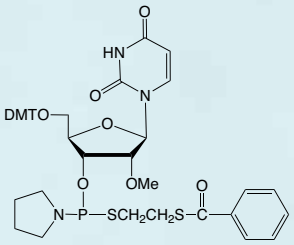
*2'-OMe-A-Thiophosphoramidite*



*2'-OMe-C-Thiophosphoramidite*



*2'-OMe-G-Thiophosphoramidite*



*2'-OMe-U-Thiophosphoramidite*

## ORDERING INFORMATION

| Item | Catalog No. | Pack | Price($) |
|---|---|---|---|
| dA-Thiophosphoramidite | 10-1700-90 | 100 µmole | 175.00 |
| | 10-1700-02 | 0.25g | 420.00 |
| dC-Thiophosphoramidite | 10-1710-90 | 100 µmole | 175.00 |
| | 10-1710-02 | 0.25g | 420.00 |
| dG-Thiophosphoramidite | 10-1720-90 | 100 µmole | 175.00 |
| | 10-1720-02 | 0.25g | 420.00 |
| dT-Thiophosphoramidite | 10-1730-90 | 100 µmole | 175.00 |
| | 10-1730-02 | 0.25g | 420.00 |
| 2'-OMe-A-Thiophosphoramidite | 10-3170-90 | 100 µmole | 300.00 |
| | 10-3170-02 | 0.25g | 720.00 |
| 2'-OMe-C-Thiophosphoramidite | 10-3171-90 | 100 µmole | 300.00 |
| | 10-3171-02 | 0.25g | 720.00 |
| 2'-OMe-G-Thiophosphoramidite | 10-3172-90 | 100 µmole | 300.00 |
| | 10-3172-02 | 0.25g | 720.00 |
| 2'-OMe-U-Thiophosphoramidite | 10-3173-90 | 100 µmole | 300.00 |
| | 10-3173-02 | 0.25g | 720.00 |

## NEW PRODUCT – PAC-2-AMINO-dA

### *INTRODUCTION*

As we have noted in the past, the simplest approach to the design of high affinity primers and probes would be to substitute A sites with 2-amino-A, since the 2-amino-A-T base pair is equivalent in strength to the G-T base pair (Figure 1). 2-Amino-A also destabilizes A-G wobble mismatches, thus increasing specificity.

In a manner analogous to dA, 2-amino-dA is very susceptible to depurination during the acidic deblocking step of DNA synthesis. From previous development work, we know that acyl protecting groups at N6 seem to favor depurination while formamidine protecting groups seem to suppress depurination during detritylation. In 1998, we introduced the 2-amino-dA monomer (1), which appears to solve all of the earlier problems: deprotection is fast and effective; and it is stabilized to depurination during synthesis. However, the highly nucleophilic character of this bis formamidine protected diaminopurine leads to nucleophilic attack on the C-3' of the sugar moiety during oxidation with iodine, resulting in strand scission. This problem can be reduced by employing non aqueous oxidation conditions, as described in Glen Report 23.2, page 11. Nevertheless, we concluded that it was time to consider a 2-Amino-dA monomer with optimized protection.

We defined the following criteria for the "ideal" 2-Amino-dA monomer:

- Stable during oligonucleotide synthesis
- Stable during oxidation (see above)
- Stable during detritylation (depurination)
- Labile towards final deprotection conditions (NH3, AMA, MeNH2)
- The nucleobase protecting groups should be cleavable under mild conditions, e.g., compatible with 2-thio-dT containing sequences.

From previous development work, it is well known that protecting groups at the N2 position are **much** less labile than identical protecting groups at the N6 position of 2,6-diaminopurine. Therefore, two different protecting groups for these positions were called for. We also rejected the use of two acyl protecting groups since the N6-acyl group would destabilize the glycosidic bond, potentially leading to depurination during detritylation. Consequently, a formamidine protecting group at the N6 position is clearly indicated and dibutylformamidine is a good choice at the N6 position since it suppresses depurination and is easily removed from that position during deprotection. A formamidine at the N2 position causes some strand scission during iodine oxidation, therefore the N2 position should be protected by an acyl group. In order to achieve deprotection of the N2 position under mild conditions, a labile acyl group like Pac or Methoxyacetyl (Mac) should be chosen.

These criteria led us to choose the Pac-Protected 2-Amino-dA and we prepared the phosphoramidite (2) for evaluation.

**FIGURE 1: HYDROGEN BONDING PATTERNS FOR REGULAR AND MODIFIED BASES**

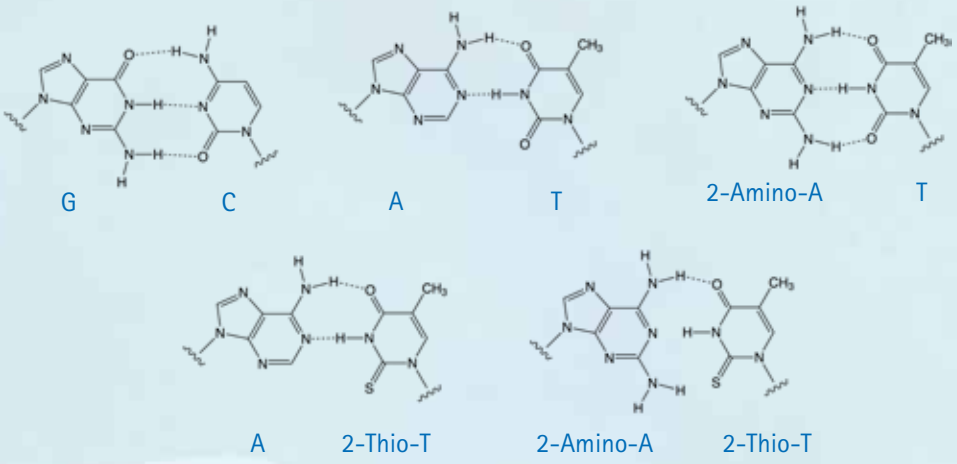
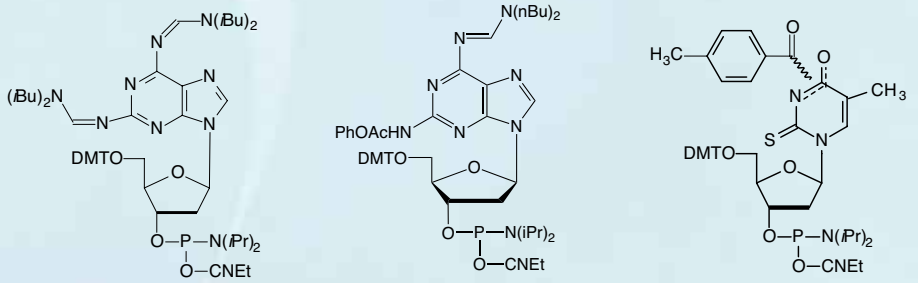



**FIGURE 2: STRUCTURES OF MONOMERS DESCRIBED IN THIS ARTICLE**




*2-Amino-dA (1)* *Pac-2-Amino-dA (2)* *2-Thio-dT (3)*

### *SYNTHESIS AND DEPROTECTION*

It was quickly confirmed that Pac-2-Amino-dA coupled as well as regular DNA monomers under identical coupling conditions with no change in coupling time. It was also gratifying to find that iodine oxidation had no effect on the coupling efficiency as confirmed by HPLC analysis of the resulting oligo. So no chain scission was observed using regular iodine oxidation.

Deprotection of oligos containing Pac-2-Amino-dA was analyzed using AMA and ammonium hydroxide. The results are shown below. We found that complete deprotection required about the same time as oligos containing dmf-dG.

| *Deprotection Solution* | *Temperature* | *Time* |
|---|---|---|
| AMA | 65 °C | 20 min |
| NH4OH | 65 °C | 4 hr |
| NH4OH | 55 °C | 8-17 hr |
| NH4OH | room temp | 24-36 hr |

So far so good, but how would the new product perform in the test we carried out a few years ago with our existing monomer? In that test, we added 2-Amino-dA six times in a mixed base 16mer. The results are shown in Figure 3, where the full length oligo in the crude mixture was only 13% using iodine oxidation but was raised to 60% using non aqueous oxidation with CSO. As noted above, with an electron-donating

formamidine protecting group on the N2, the N3 becomes nucleophilic and can attack the C3' causing strand scission and a lower yield of full-length oligo. Also shown in Figure 3 is the chromatogram of the same oligo sequence with six additions of Pac-2-Amino-dA using standard coupling and deprotected with ammonium hydroxide. In this case, the yield of full length oligo was raised to over 95%.

## DUPLEX STABILITY STUDY

With the optimal system in place for the preparation of oligos containing multiple additions of 2-Amino-dA, we carried out an experiment to determine if the increase in melting temperature inherent in the 2-Amino-A-T base pair is cumulative as the substitution of A by 2-Amino-A increases. The following oligos were prepared and their melting behavior examined.

The sequences were:

Reg AT fwd: 5'-TCT GAA GCT GTT ACT CCG-3'
Reg AT rev: 5'-CGG AGT AAC AGC TTC AGA-3'
**A** 1 Sub: 5'-CGG AGT AAC **A**GC TTC AGA-3'
**A** 2 Sub: 5'-CGG **A**GT AAC **A**GC TTC AGA-3'
**A** 3 Sub: 5'-CGG **A**GT AAC **A**GC TTC **A**GA-3'
**A** 5 Sub: 5'-CGG **A**GT **AA**C **A**GC TTC **A**GA-3'

The resulting melting plot is shown in Figure 4. The color code is:

| | | Tm |
|---|---|---|
| Blue: | Reg AT bases fwd/rev | 59.9 °C |
| Green: | **A** 1 Substitution | 60.6 °C |
| Red: | **A** 2 Substitutions | 62.0 °C |
| Teal: | **A** 3 Substitutions | 62.9 °C |
| Yellow: | **A** 5 Substitutions | 65.0 °C |

where **A** is 2-Amino-dA.

The resulting melting curves shown in Figure 4 and summarized above indicate that the increase in melting temperature inherent in the 2-Amino-A-T base pair is indeed cumulative. An increase in Tm of 3.0° was observed with three substitutions fairly evenly spaced throughout our test oligo to give an average of 1.0° per insertion relative to the control. When a further two substitutions were made less optimally at adjacent sites in our test oligo, the increase in Tm was 5.1° relative to the control, with the average still at 1.0° per insertion.

Our simple experiment demonstrates that the increase in melting temperature by replacing the A-T base pair with the 2-Amino-A-T base pair is cumulative over five additions in an 18mer.

***FIGURE 3:*** **RP HPLC OF CRUDE 16MER MIXED BASE OLIGO WITH SIX 2-AMINO-dA ADDITIONS**

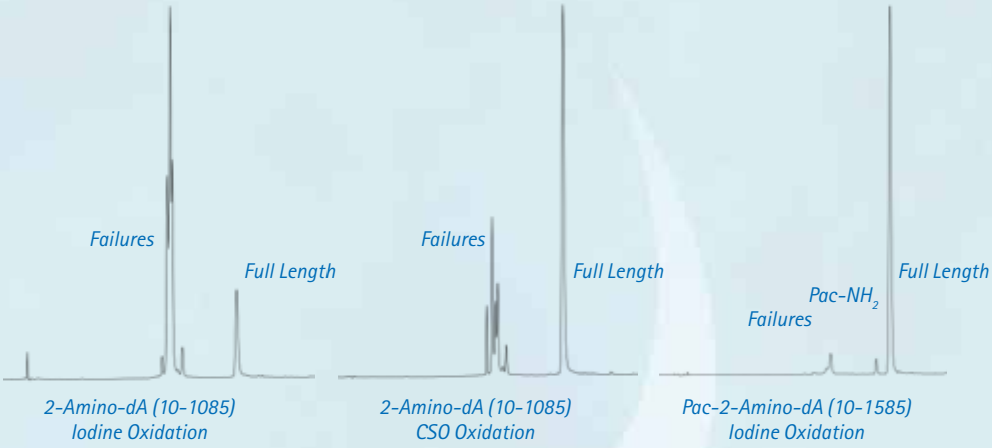
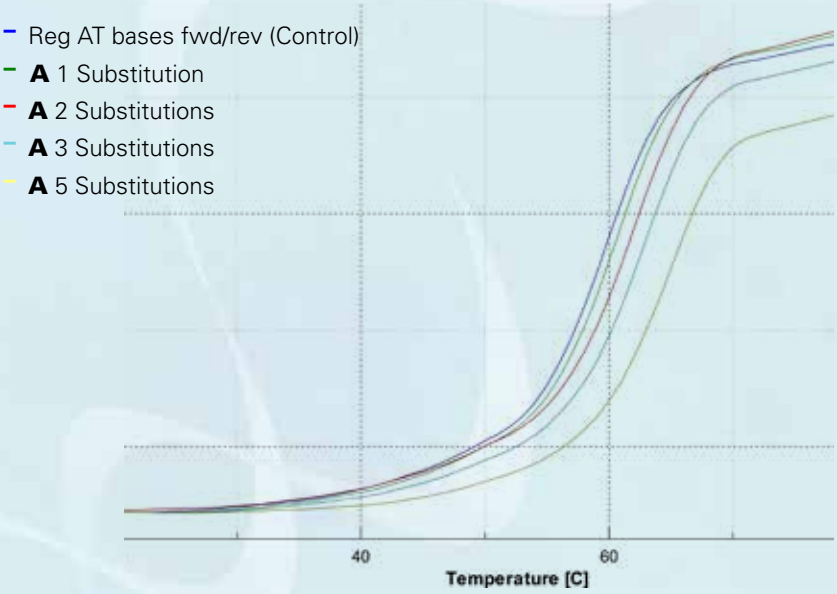



***FIGURE 4:*** **MELTING CURVES OF OLIGOS CONTAINING 0-5 2-AMINO-dA SUBSTITUTIONS**




The runs were performed in degassed 0.1 M Tris-HCl, pH 7.4 with the oligos at 1.25 µM concentration, using a Jasco V-530 fitted with a Peltier temperature control unit.

## SBC OLIGOS

Selective Binding Complementary (SBC) oligonucleotides[1] are able to form stable, sequence specific hybrids with complementary unmodified DNA or RNA but are unable to form stable hybrids with each other. Oligos in which A has been replaced with 2-amino-A and T with 2-thio-T represent an excellent example of SBC oligos. While 2-amino-A forms a very stable base pair with T containing three hydrogen bonds, the stability of the base pair with 2-thio-T is greatly diminished. The steric interactions between the 2-thio group of thymidine and the 2-amino group of adenine tilt the bases relative to each other yielding a base pair that effectively contains only a single hydrogen bond. However, 2-thio-T base pairs perfectly well with A, as shown in Figure 1. SBC 20mers annealed against a DNA 20mer target exhibited Tm values 10° higher than the corresponding DNA-DNA hybrid, whereas the SBC-SBC hybrid yielded Tm values up to 30° lower.[1]

2-Thio-dT (3) is best deprotected under mild conditions using ammonium hydroxide at room temperature. Pac-2-amino-dA (2) is also efficiently deprotected in ammonium hydroxide and so is compatible with 2-thio-

dT. But is this combination of Pac-2-amino-dA and 2-thio-dT capable of producing SBC oligos? The following experiment with SBC oligos was carried out.

The sequences were:

SBC fwd: 5′-TCT G**A**A GC**T** GTT **A**C**T** CCG-3′
SBC rev: 5′-CGG **A**G**T** AAC **A**GC T**T**C AGA-3′
Reg AT fwd: 5′-TCT GAA GCT GTT ACT CCG-3′
Reg AT rev: 5′-CGG AGT AAC AGC TTC AGA-3′

where **A** and **T** are 2-Amino-dA and 2-Thio-dT, respectively.

The oligos were deprotected in ammonium hydroxide at room temperature for 24 hours and purified on Glen-Pak™ cartridges.

Figure 5 shows the melting plot which has been smoothed and normalized to 0.386 AU at 260 nm at 20 °C. The color code is:

| | | Tm |
|---|---|---|
| Blue: | SBC fwd/SBC rev | 46.0 °C |
| Teal: | Reg AT bases fwd/rev | 59.0 °C |
| Green: | SBC fwd/AT bases rev | 64.0 °C |
| Red: | AT bases fwd/SBC rev | 64.5 °C |

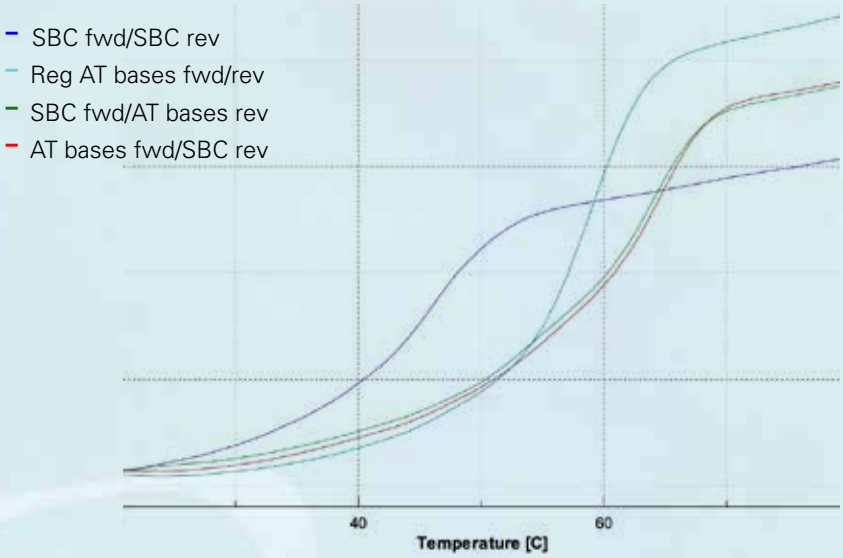
In our experiment, the SBC-SBC hybrid yielded a Tm 13° lower than the control standard oligo hybrid, while the SBC-regular hybrids had Tm 5° higher than the control oligo. This experiment confirms that our Pac-2-amino-dA monomer allows relatively straightforward production of SBC oligos.

## CONCLUSION

We are happy to offer an alternative monomer for the synthesis of oligos containing 2-Amino-dA. Our goals of designing a monomer that is fully compatible with regular oligo synthesis and deprotection have been achieved, as evidenced by the simple preparation of oligos with multiple 2-Amino-dA residues, as well as the preparation of SBC oligos. We thank Stefan Pitsch of Pitsch Nucleic Acids for his help in the design and production of Pac-2-Amino-dA-CE Phosphoramidite.

*Reference:*
1. I.V. Kutyavin, *et al.*, *Biochemistry,* 1996, **35**, 11170-11176.

***FIGURE 5:*** **MELTING CURVES USING SBC OLIGOS AND CONTROLS**




The runs were performed in degassed 0.1 M Tris-HCl, pH 7.4 with the oligos at 1.25 µM concentration using a Jasco V-530 fitted with a Peltier temperature control unit.

## ORDERING INFORMATION

| *Item* | *Catalog No.* | *Pack* | *Price($)* |
|---|---|---|---|
| 2-Amino-dA-CE Phosphoramidite | 10-1085-95 | 50 µmole | 70.00 |
| (2,6-diaminopurine) | 10-1085-90 | 100 µmole | 125.00 |
| | 10-1085-02 | 0.25g | 250.00 |
| Pac-2-Amino-dA-CE Phosphoramidite | 10-1585-95 | 50 µmole | 70.00 |
| (2,6-diaminopurine) | 10-1585-90 | 100 µmole | 125.00 |
| | 10-1585-02 | 0.25g | 250.00 |
| 2-Thio-dT-CE Phosphoramidite | 10-1036-95 | 50 µmole | 165.00 |
| | 10-1036-90 | 100 µmole | 295.00 |
| | 10-1036-02 | 0.25g | 675.00 |

## NEW PRODUCT - 1-METHYL-PSEUDOURIDINE

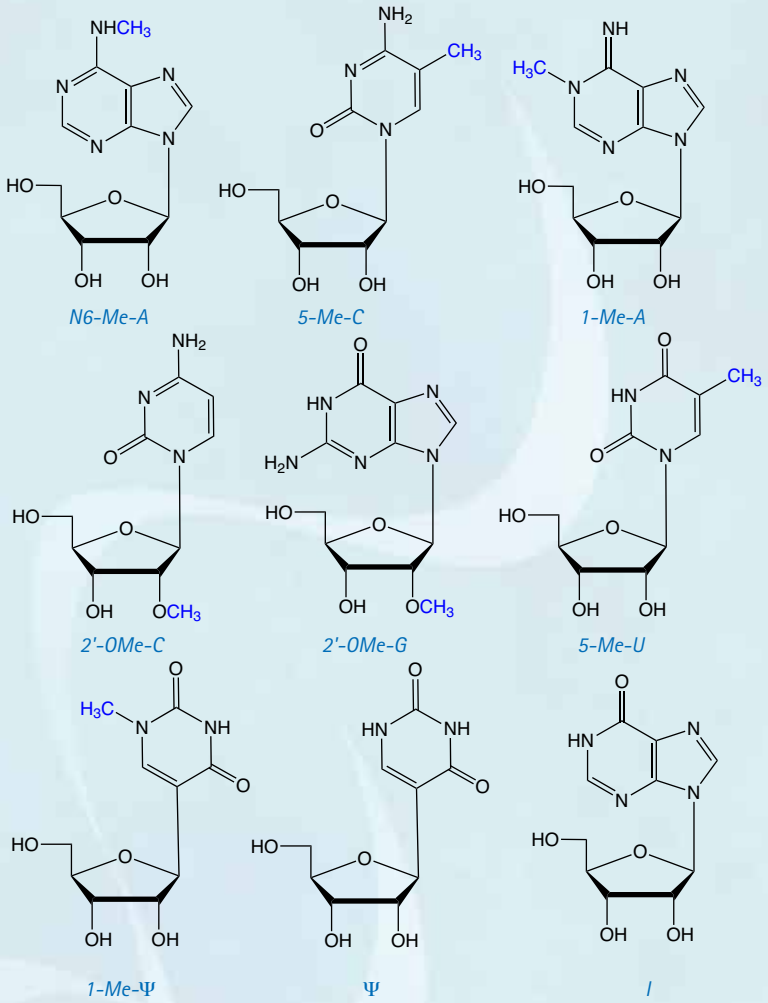
RNA methylation occurs in a large selection of RNA nucleosides and this post transcriptional modification of RNA, carried out by a variety of RNA methyltransferases, appears in a wide variety of RNA species - including tRNA, but also mRNA, miRNA and RNA viruses. The most common methylated ribonucleoside in eukaryotes is N6-methyladenosine (m6A). In an earlier Glen Report 26.2, page 1, Qing Dai and Chuan He of the University of Chicago discussed the recent finding that the behavior of m6A offers an example of reversible RNA methylation with a significant role as an epigenetic modification.[1] In this article, we will focus on the role of methylated ribonucleosides in tRNA and mRNA.

A recent review[2] reveals that over 90 methylated nucleosides have been found in tRNA and that these play a significant role in the stabilization of tRNA structure, reinforcement of the codon-anticodon interaction, regulation of wobble base pairing, and prevention of frameshift errors. In addition, methylation appears to mark the tRNA as mature, preventing its degradation as well as directing localization within the cell.

Ever since it was discovered[3] that injecting mRNA directly into mouse skeletal muscle led to the expression of the encoded protein, there has been great interest in using mRNA as a therapeutic species. However, the cell's innate immune response, designed to detect the foreign RNA of viruses, can be activated after mRNA delivery, leading to the shutdown of protein translation and ultimately, cell death. It was found, however, that some nucleobase modifications can greatly enhance the properties of mRNA by reducing immunogenicity and increasing stability. Specifically, mRNA modified with pseudouridine (Ψ) alone, or in combination with 5-methylcytidine (5-Me-C), significantly increased translation levels. When Ψ was substituted with 1-methylpseudouridine (1-Me-Ψ), even higher levels of protein expression were observed.[4]

Further studies showed[5] that mRNA containing 1-Me-Ψ alone, or in combination with 5-Me-C, outperformed the equivalent Ψ and/or 5-Me-C mRNA by providing higher reporter gene expression upon transfection into cell lines and mice. It was shown that 1-Me-Ψ/5-Me-C modified mRNA resulted in reduced intracellular innate immunogenicity and improved cellular viability upon *in vitro* transfection.[5]

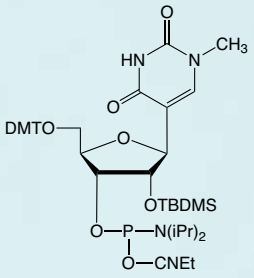
**FIGURE 1: STRUCTURES OF RIBONUCLEOSIDES SUPPORTED BY GLEN RESEARCH**




We take this opportunity to introduce 1-Me-Ψ to our catalog of RNA minor bases. 1-Me-Pseudouridine Phosphoramidite is fully compatible with standard RNA synthesis conditions. Similarly, with no protecting groups to be removed, oligo deprotection can be carried out under standard conditions, including our preferred deprotection with AMA at 65°C for 10 minutes.

All of the RNA minor bases shown in Figure 1 are available from Glen Research as phosphoramidites. In future, we will continue our introduction of minor bases involved in RNA epigenetics and methylated RNA in the hope and expectation that their availability as phosphoramidites will act as a catalyst to promote these exciting research activities.

**FIGURE 2: STRUCTURE OF 1-Me-PSEUDOURIDINE**




*1-Me-Pseudouridine Phosphoramidite*

### ORDERING INFORMATION

| Item | Catalog No. | Pack | Price($) |
|---|---|---|---|
| 1-Me-Pseudouridine Phosphoramidite | 10-3056-95 | 50 µmole | 420.00 |
| | 10-3056-90 | 100 µmole | 820.00 |
| | 10-3056-02 | 0.25g | 2300.00 |


*References:*

1. Y. Fu, D. Dominissini, G. Rechavi, and C. He, *Nat Rev Genet*, 2014, **15**, 293-306.
2. H. Hori, *Front Genet*, 2014, **5**, 144.
3. J.A. Wolff, *et al.*, *Science*, 1990, **247**, 1465-8.
4. D. Weissman, *Expert Rev Vaccines*, 2015, **14**, 265-81.
5. O. Andries, *et al.*, *J Control Release*, 2015, **217**, 337-44.

Oligonucleotides containing multiple labels have practical applications in a number of technologies including PCR, Fluorescence Resonance Energy Transfer (FRET), and Fluorescence *In Situ* Hybridization (FISH).

Dual-labelled oligo probes are commonly synthesized by phosphoramidite chemistry in a one synthesis-one oligo (OSOO) approach. This synthetic approach can often limit the diversity of fluorophores and quenchers available since they must be compatible with the synthesis and deprotection conditions. Common post-synthesis labelling techniques, including thiol, amine and carboxy labelling, often result in less than robust labelling efficiency for a variety of reasons, such as side reactions with functional groups and deprotection reagents.

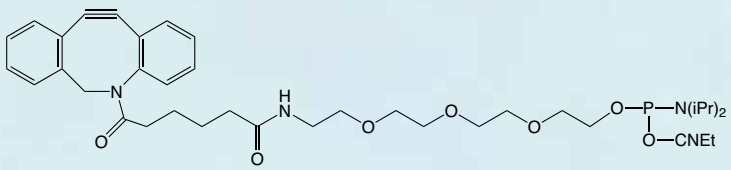
Click Chemistry is an alternative post-synthesis labelling technique that is robust, efficient and bioorthogonal. This technique avoids many of the side reactions with naturally occurring functional groups, and may offer better compatibility with standard deprotection techniques.

Glen Research supports two click techniques: copper (I) catalyzed azide-alkyne cycloaddition (CuAAC); and strained cyclooctyne cycloaddition with dibenzocyclooctyne (DBCO). Copper-assisted cycloadditions are efficient and, with the THPTA ligand, solution phase conjugation is complete in as little as 15 minutes. The reaction between DBCO and an azide is a simpler and equally efficient conjugation that avoids a copper catalyst.

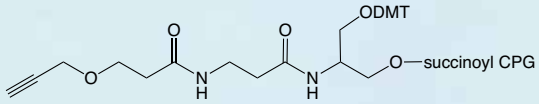
In this brief, we demonstrate the use of these two click techniques on a single oligo, 5'-DBCO – Oligo – Alkyne-3', to prepare a dual-labelled probe. The products used are shown in Figure 1. By design, the crude deprotected oligo is ideal for a simple Glen-Pak™ purification since DBCO is hydrophobic and well retained on a Glen-Pak™ cartridge. Failure sequences are eliminated at the load and wash steps, producing a high purity oligo for subsequent labelling.

The first conjugation step is to click an azide with DBCO, minus a copper catalyst. The next step clicks a second azide with the 3'- alkyne in the presence of the copper (I) catalyst. This two-step approach produces a dual-labelled oligonucleotide with a profile free of any dye degradation that can occur during deprotection, potentially simplifying HPLC purification. A chromatograms illustrating the procedure is shown in Figure 2.

**FIGURE 1: PRODUCTS USED FOR DUAL-LABELLING**

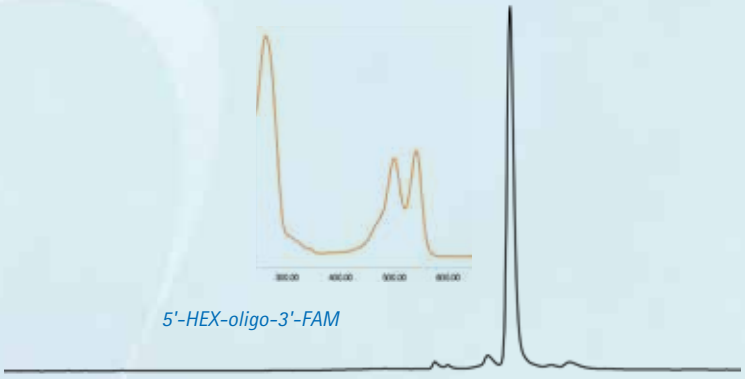



*5'-DBCO-TEG Phosphoramidite*




*3'-Alkyne-Modifier Serinol CPG*

**FIGURE 2: DUAL-LABELLING OLIGO SYNTHESIS USING RP HPLC ANALYSIS**



*5'-HEX-oligo-3'-FAM*

This orthogonal approach allows the incorporation of a diverse set of labels. Notably, one oligo synthesis, when paired with several fluorophore azides, can yield many dual-labelled oligos, resulting in a one synthesis-many oligos (OSMO) approach. This may be especially useful in designing FRET probes or double-labelled FISH probes since many pairs can be evaluated from one oligo synthesis.

Another benefit of this strategy is that the oligo can be further purified by IEX or RP-HPLC prior to labelling resulting in an exceptionally pure oligo. Purification can also be performed after each labelling step, further enhancing oligo purity, which may be important for highly sensitive assays. Additionally, the mild labelling conditions avoid any restriction on labels that could be incompatible with synthesis, deprotection and purification.

The complete protocol and further details of this Technical Brief can be found on our web site under Glen Report 27.2.

## ORDERING INFORMATION

| Item | Catalog No. | Pack | Price($) |
|---|---|---|---|
| 5'-DBCO-TEG Phosphoramidite | 10-1941-95 | 50 µmole | 125.00 |
| | 10-1941-90 | 100 µmole | 230.00 |
| | 10-1941-02 | 0.25g | 775.00 |
| 3'-Alkyne-Modifier Serinol CPG | 20-2992-01 | 0.1g | 105.00 |
| | 20-2992-10 | 1.0g | 800.00 |
| 0.2 µmole columns | 20-2992-42 | Pack of 4 | 100.00 |
| 1 µmole columns | 20-2992-41 | Pack of 4 | 175.00 |
| 10 µmole column (ABI) | 20-2992-13 | Pack of 1 | 260.00 |
| 15 µmole column (Expedite) | 20-2992-14 | Pack of 1 | 390.00 |

## INTRODUCTION

*by Lumidyne Technologies*
*New York, NY 10010*

Bright, long-lasting and non-phototoxic organic fluorophores are essential to the continued optimization of a diverse range of imaging applications. While many remarkable advances have been made towards this goal, all currently available technologies remain susceptible to undesirable photophysical phenomena, such as transient (blinking) and irreversible (photobleaching) transitions to dark states as well as related off-target effects including phototoxicities. Dark states arise from triplet state excursions, non-fluorescent electronic configurations from which the rate of return to the excitable ground state is often slow. Such tendencies compromise all fluorescence applications by unpredictably reducing the signal-to-noise ratio (SNR) as well as limiting the total duration of time over which information can be gathered.

The direct conjugation of small-molecule protective agents (PAs) has enabled significant improvements in the photon budget of cyanine-class organic fluorophores spanning the visible spectrum by reducing the lifetime of reactive triplet states through intra-molecular triplet quenching[1-5] (Fig. 1). These technologies can now be readily implemented as a general approach to increase the photon yields of a range of chemically and structurally diverse fluorophores by covalently linking PAs in proximity of the fluorogenic center[6]. Through a partnership with Lumidyne Technologies, Glen Research has created a novel PA-linked phosphoramidite using cyclooctatetraene (COT). This product represents one of many possible iterations demonstrating the utility of a PA in this context, thereby promising investigations that will accelerate the frontiers of nucleic acid imaging.

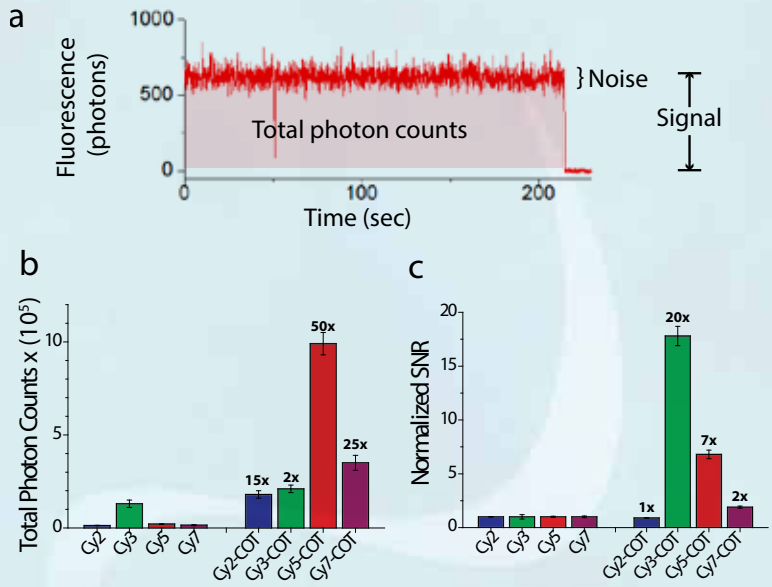
## COT SERINOL PHOSPHORAMIDITE

After the paper, *Enhanced photostability of cyanine fluorophores across the visible spectrum*, was published,[2] Glen Research recognized the impact that these photo-protective agents could have upon the research community by providing a triplet state quencher (TSQ) as a phosphoramidite. It would provide a modular way to improve the photostability (and therefore performance) of virtually any fluorescent dye by coupling a single TSQ vicinal to the fluorophore. This is because all fluorophores have a propensity to undergo intersystem crossing from an excited singlet state to a triplet state. This triplet state is long-lived because relaxation to the ground state is spin-forbidden as the excited state electron has the same spin as the electron in the ground state. In the excited triplet state, the fluorophore can react with dissolved oxygen to produce reactive and cytotoxic singlet oxygen as well as other reactive-oxygen species that lead to photobleaching of the dye. However, even in the absence of molecular oxygen, the fluorophore in the triplet state can undergo redox reactions with solvent and other biomolecules to produce radical cations or anions which again leads to photobleaching of the dye.

While there are a variety of photo-protective species to choose from,[1] we decided to use cyclooctatetraene. Cyclooctatetraene (COT) is compatible with DNA synthesis and deprotection conditions and is a well-known triplet state quencher. We chose to use our popular serinol linker to provide sufficient linker length and flexibility for the COT and the dye to interact, leading to efficient relaxation to the ground state from the triplet state. COT Serinol Phosphoramidite (1) is shown in Figure 2.

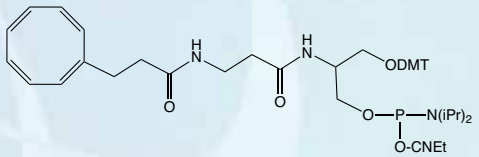
To confirm the photo-protective effects of the COT, the sequences 5'-(Cyanine 5)-(COT)-T12-3' and the control 5'-(Cyanine 5)-T12-3' were synthesized. After Glen-Pak™ purification, a fluorescence time course study was performed exciting at 640 nm and observing at 660 nm. The monotonic decrease in fluorescence is indicative of photobleaching of the cyanine 5 fluorophore. From our spectrofluorometric

***FIGURE 1***




(a) Representative single-molecule trace of a photostabilized fluorophore on a DNA oligonucleotide. (b and c) Comparison of total photon emission and data quality (as represented by the signal-to-noise ratio) of conventional and PA-conjugated cyanine dyes across the visible spectrum (adapted from reference 5).

***FIGURE 2:*** **STRUCTURE OF CYCLOOCTATETRAENE (COT) SERINOL PHOSPHORAMIDITE**




*(1) COT Serinol Phosphoramidite*

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20164



studies, it is clear that the presence of COT limited the amount of photobleaching of the cyanine dye.

## HANDLING AND USE

A three minute coupling time is recommended for the COT phosphoramidite and the product was found to be compatible with standard DNA deprotection conditions. We have observed an interesting side reaction can occur with COT-labelled oligos. If the oligo, while still bound to the solid support, is thoroughly dried and allowed to sit at room temperature for extended periods, some cleavage of the vicinal phosphodiester linkages can be observed upon deprotection. This side reaction can be completely avoided by deprotecting the oligo immediately after synthesis or storing the column at -20 °C until the deprotection is initiated. This reaction can occur to a lesser extent upon drying the deprotected oligo down.

We thank Roger Altman and Scott Blanchard of Lumidyne for their collaboration in the preparation of this article.

*References:*

1: Altman RB, Terry DS, Zhou Z, Zheng Q, Geggier P, Kolster RA, Zhao Y, Javitch JA, Warren JD, Blanchard SC. Cyanine fluorophore derivatives with enhanced photostability. *Nat Methods*. 2011 Nov 13;**9**(1):68-71.

2: Altman RB, Zheng Q, Zhou Z, Terry DS, Warren JD, Blanchard SC. Enhanced photostability of cyanine fluorophores across the visible spectrum. *Nat Methods*. 2012 Apr 27;***9***(5):428-9.

3: Zheng Q, Jockusch S, Zhou Z, Altman RB, Warren JD, Turro NJ, Blanchard SC. On the Mechanisms of Cyanine Fluorophore Photostabilization. *J Phys Chem Lett*. 2012 Aug 16;**3**(16):2200-2203

4: Zheng Q, Jockusch S, Zhou Z, Blanchard SC. The contribution of reactive oxygen species to the photobleaching of organic fluorophores. *Photochem Photobiol*. 2014 Mar-Apr;**90**(2):448-54.

5: Zheng Q, Juette MF, Jockusch S, Wasserman MR, Zhou Z, Altman RB, Blanchard SC. Ultra-stable organic fluorophores for single-molecule research. *Chem Soc Rev*.2014 Feb 21;**43**(4):1044-56.

6: Zheng Q, Jockusch S, Rodríguez-Calero GG, Zhou Z, Zhao H, Altman RB, Abruña HD, Blanchard SC. Intra-molecular Triplet Energy Transfer is a General Approach to Improve Organic Fluorophore Photostability. RSC *Photochem & Photobiol Sci* 2015 (in press).

## ORDERING INFORMATION

| *Item* | *Catalog No.* | *Pack* | *Price($)* |
|---|---|---|---|
| COT Serinol Phosphoramidite | 10-1996-95 | 50 µmole | 310.00 |
| | 10-1996-90 | 100 µmole | 600.00 |
| | 10-1996-02 | 0.25g | 1800.00 |