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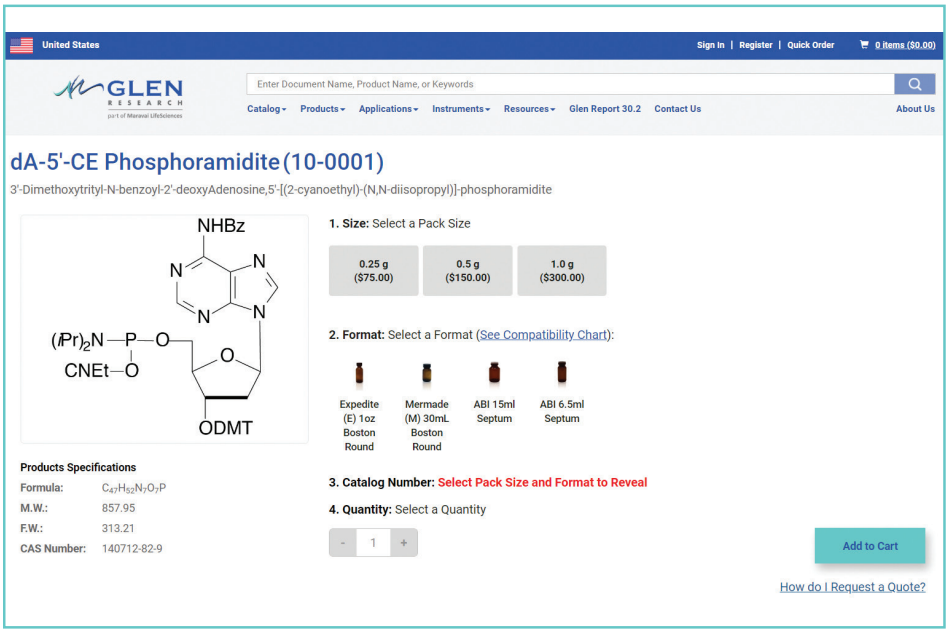

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Continued on Page 2

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Continued from Front Page

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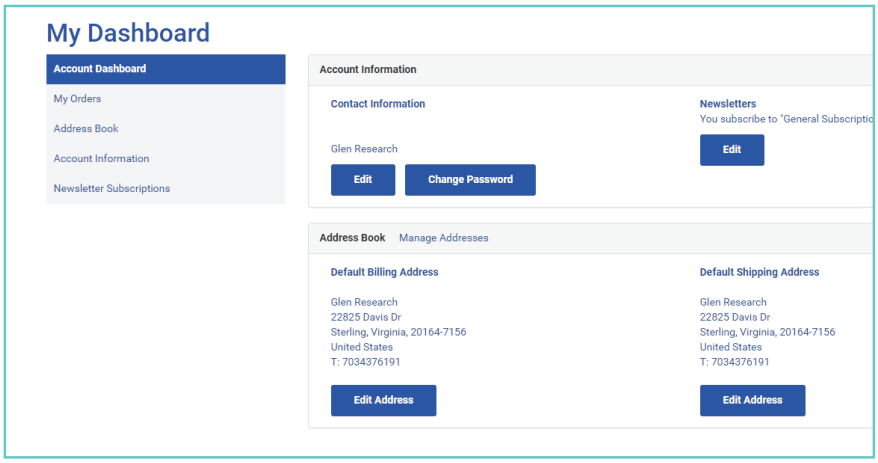

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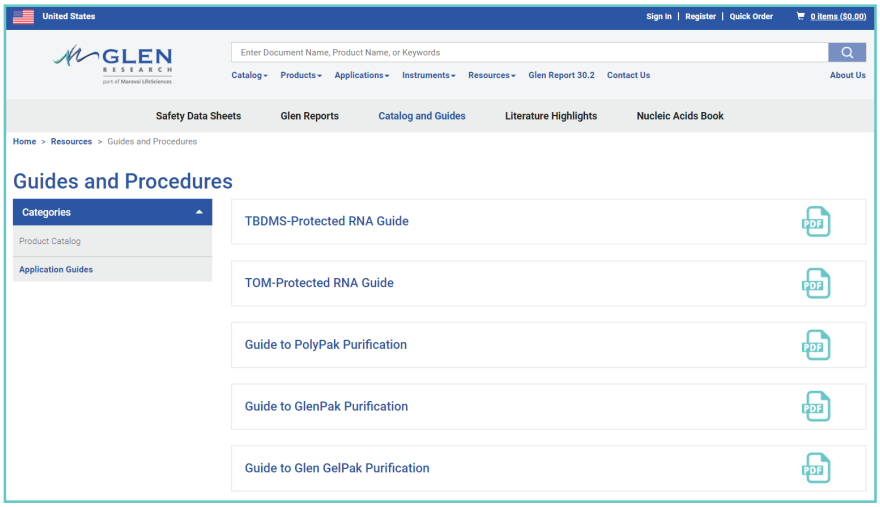

5-Bromo- and 5-Iodo-Pyrimidine Nucleosides; Efficient Oligo to Protein Photo-Cross-linkers (Part 1)

Author: Dick Keys, Ph.D.

This Glen Report is Part One of 2; Part 2 will be released in a subsequent report.

Introduction

In a recent Glen Report, we described the ultrafast photoreactive nucleosides ^{CNV}K and ^{CNV}D for DNA or RNA interstrand cross-linking. Alternatively, the photo-cross-linking reactions between modified nucleosides in DNA and RNA oligonucleotides with electron-rich amino-acid side-chains of proteins have proven broadly useful in elucidating the interaction of nucleic acids with specific binding proteins, and has seen an expanded interest in studying photo-cross-linking of nucleic acids to ribonucleoproteins. In an earlier Glen Research literature review of oligonucleotide crosslinking, the halogenated nucleosides received brief mention compared with other approaches; yet 5-halogenated pyrimidine nucleosides are especially useful in site-specific cross-linking of oligonucleotides to specific protein binding sites.

In this report we will describe improved methods for the photo-cross-linking of single- and double-stranded oligonucleotides containing 5-halogenated pyrimidines with corresponding oligo binding proteins. DNA and RNA oligonucleotides containing these modified nucleosides are readily synthesized via standard machine synthesis using phosphoramidite monomers (Figure 1). Through examples, we hope to improve recognition of the special utility of 5-bromo- and 5-Iodo-pyrimidine nucleosides in synthetic DNA and RNA oligonucleotides by providing background on a) the photolysis reaction mechanisms and their dependence on UV wavelength, b) the steric impact of 5-halogenated uridine containing oligos on binding affinity, and c) conditions used to obtain high efficiency photo-cross-linking to oligo binding protein. Hopefully data presented here can dispel a misplaced notion that low cross-linking yields are obtained when using these compounds, and that their highly specific photo-cross-linking reactions will lead to additional research utilization.

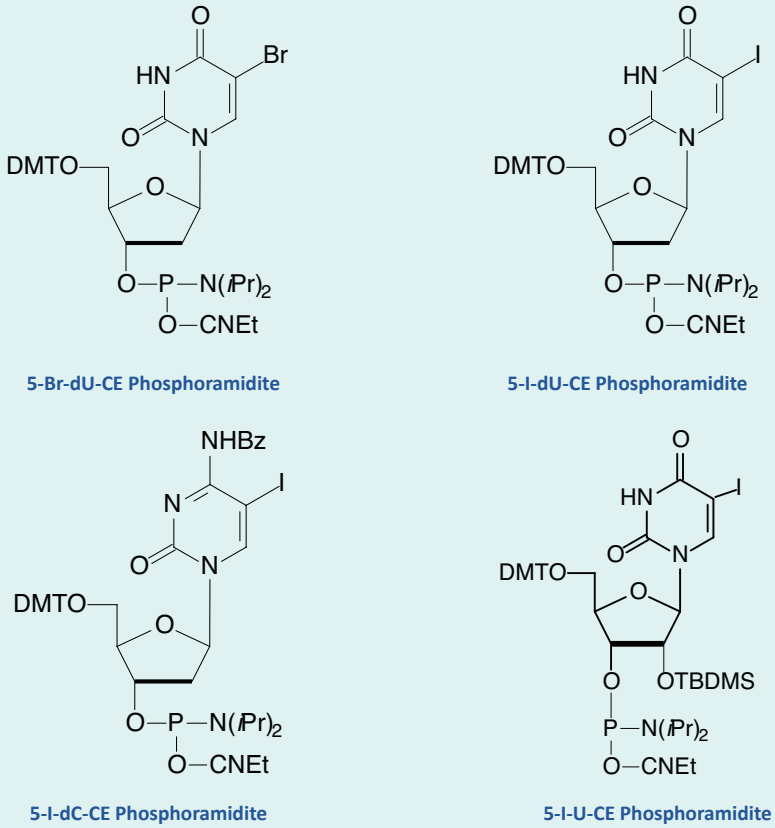

Figure 1. 5-halogen-modified nucleoside CE phosphoramidites

Long-time readers may recall that 5-halogen substituted deoxyuridine (dU) has been useful in solving oligonucleotide protein complex structures using X-Ray crystallography. In addition, 5-Bromo-dU (as triphosphate) incorporated into nascent transcripts has found application in cell cycle investigations where antibodies to 5-Br-dU are used for immunostaining of nuclear structures. Similarly, synthetic oligonucleotides containing 5-Br-dU are also useful as in situ hybridization probes, providing a route towards highly specific immunostaining of mRNA.

The use of short wavelength UV_{254nm} cross-linking (UVC) of native nucleic acids has utility in discovery research, but in some applications is limited by an associated propensity for non-specific covalent cross-links between double stranded nucleic acids and non-specific coupling to numerous nearby amino acid residues in nucleic acid-protein complexes. The concurrent use of sensitizers in photochemical cross-linking expanded the utility of photoreactions by providing higher cross-linking efficiency and has been shown to increase specificity by facilitating use of longer wavelength UV radiation (UVB),

affording fewer non-specific nucleic acid interstrand cross-links and strand breaks. For example, in the case of psoralens as photosensitizers, interstrand photo-cross-linking and photo-reversal of crosslinks facilitate precise mapping of folded RNA substructures.

Early photo-cross-linking studies with Br-dU modified oligos used short wavelength UV_{254nm}, and results were confounded due to photo-activated side reactions (see below). However, the Tad Koch lab at the University of Colorado, Boulder demonstrated that lower energy UV irradiation (UVB or UVA) could be used to minimize protein and nucleic acid side reactions and promote high-yield photo-cross-linking of halogen-nucleoside containing oligo with protein when complexed to the target protein. In model studies of the photo-cross-linking reactions, irradiation wavelength dependence and its relationship to the formation of different products were studied, and two major routes to these differing products were elucidated.

While UV_{254nm} results in homolytic C-Br bond cleavage and generation of a highly reactive uridyl radical, the formation of highly specific cross-linking was proposed to occur using lower energy (308nm) excitation initially yielding a n,π* singlet state. Through intersystem crossing, the molecule converts to a lower energy triplet state that can oxidize a nearby electron-rich peptide moiety such that the resulting uridyl radical ion will couple to an adjacent electron-rich peptide functional group, substituting it for the bromine (Figure 2. Jablonski diagram for photoactivation of the 5-Bromo-Uracil chromophore. Reprinted from "Photochemical coupling of 5-bromouracil (BU) to a peptide linkage. A model for BU-DNA protein photocrosslinking," T.M. Dietz, R.J. von Trebra, B.J. Swanson, and T.H. Koch, 1987, Journal of the American Chemical Society, 109 (6), 1793-1797. Copyright 1987 American Chemical Society.).

Much has been learned since that early work. In designing photo-cross-linking studies, one should note that the photocoupling reaction can be compromised by using excess laser power since this can cause secondary cross-link breakage as well as protein photodamage as was shown using phage R17 coat protein.[8] In addition, intrastrand crosslinks can form between 5-Br-dU or 5-Br-dC and adjacent 5'- or 3'-dG and-dA residues.[9] Yields of intrastrand crosslinks are much higher when the neighboring nucleoside is a dG versus when it is a dA, and Br-dC forms these cross-links much more efficiently than 5-Br-dU. Recently 300 nm UV (UVB) was used to study single strand breaks and intrastrand crosslinks in double stranded oligo containing 5-Br-dC or 5-Br-dU.[10] These studies indicate that Br-dC is not the better choice when designing oligo to protein photo-cross-linking studies. In addition, minimization of undesirable strand cleavage and intrastrand cross-linking side reactions can be impacted by the position of the halogen containing nucleoside in the oligo sequence (e.g. adjacent to dG), and by the wavelength of UV used. Despite the potential limitations, high efficiency photo-cross-linking using these molecules continues to be a valuable technique.

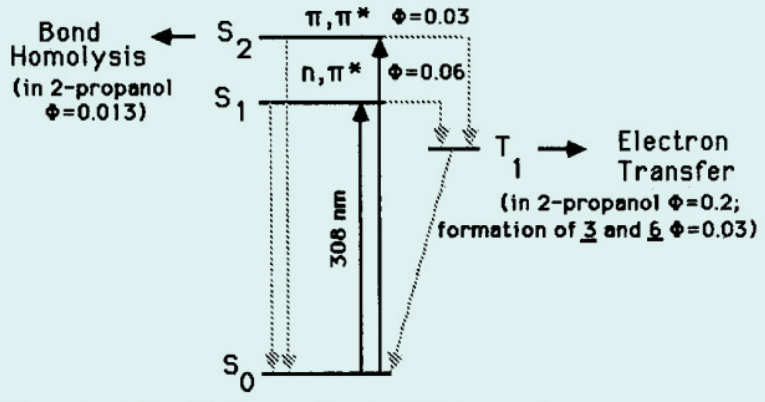

Figure 2. Jablonski diagram for photoactivation of the 5-Bromo-Uracil chromophore. Reprinted from "Photochemical coupling of 5-bromouracil (BU) to a peptide linkage. A model for BU-DNA protein photocrosslinking," T.M. Dietz, R.J. von Trebra, B.J. Swanson, and T.H. Koch, 1987, Journal of the American Chemical Society, 109 (6), 1793-1797. Copyright 1987 American Chemical Society.

Today, we increasingly understand that folded RNA structures in concert with linear sequence in RNA-protein complexes are involved in the control of gene expression. As a result, researchers continue to seek better understanding of the molecular details of these regulatory ribonucleoprotein complexes, including the specific location of the interacting oligo and protein partners in the complex.[11] In this report we highlight a few discovery applications that include DNA- and RNA-protein cross-linking.

Cross-linking BrdU modified DNA to Protein

Nuclear factor NF BA1

Studying NF BA1, a nuclear factor that binds to the promoter region of human apoB gene, Kardassis prepared a synthetic DNA oligonucleotide modified with five 5-Br-dU residues replacing T and corresponding to the coding sequence (-80 to-63) of the apoB gene (Figure 3. apoB Promoter

binding motif oligo, modified with 5-Br-dU). The 5-Br-dU oligo was radiolabeled and annealed to its complementary oligo forming the double stranded sequence that binds NF BA1[12] protein. The double stranded DNA was incubated with purified NF BA1 protein alone or in the presence of single stranded oligo sequences either fully homologous, with mismatches, or fully non-homologous to the coding sequence.
The specificity of the photo-cross-linking reaction was demonstrated by PAGE analysis (Figure 4. Cross-linking of ApoB Gene to NF BA1). The presence of photo-cross-linked 67 kD radiolabeled protein adduct was abolished by including in the photo-cross-linking reaction either BA1 or CIII-B oligonucleotides that bind NF BA1 (see corresponding gel lanes). Photo-cross-linking was highly sequence specific, as shown by including BM2 or CIII-C oligos in the cross-linking reaction mixture. BM2 is a 3-base mismatched analog of BM1 oligo, while CIII-C is a non-homologous promoter sequence just downstream of the BA1 promoter sequence. As the gel lanes demonstrate, the presence of either the partially complementary oligo BM2 or non-homologous CIII-C oligo do not interfere with cross-linking of brominated double stranded BA1 DNA oligo to NF-BA1 protein to form the 67kD covalent adduct.

Similar utility has been shown for other 5-Br-dU modified DNA oligonucleotides where point contacts have been

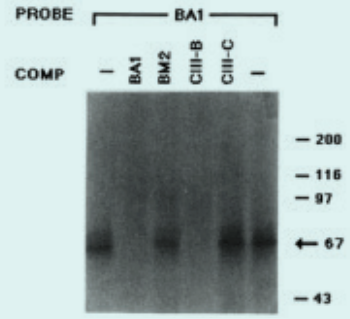

Figure 4. Cross-linking of ApoB Gene to NF BA1

established by photo-cross-linking to associated proteins. [13,14]

Escherichia coli lac Repressor
Similar photo-cross-linking of a 5-Br-dU containing duplex DNA to associated proteins included the mapping of point contacts at the oligo-protein interface. The E. coli lac Repressor protein, a 150-kDa tetramer of identical subunits having two operator binding sites and two inducer sites, has been extensively studied. Investigations in several labs revealed the double-stranded operator sequence region through which lac Repressor protein recognizes its specific operator sequence and, when bound, RNA polymerase transcription of the lactose metabolic genes is inhibited.[15] The inducer molecule allolactose binds to sites on lac Repressor which serve to induce transcription of the genes regulated by lac Repressor; in the study below the allolactose mimic isopropyl-β-D-thiogalactoside (IPTG) is used as inducer.

5′-GCG-CCC-(5-Br-dU)(5-Br-dU)(5-Br-dU)-GGA-CC(5-Br-dU)-(5-Br-dU)TT-3′

Figure 3. apoB Promoter binding motif oligo, modified with 5-Br-dU

Top strand	5′-TGT TGT GTG GAA TTG TGA GCG GAT AAC AAT TTC ACA CAG G-3′
Bottom strand	3′-ACA ACA CAC CTT AAC ACT CGC CTA TTG TTA AAG TGT GTC C-5′
	-10 +1 +10 +20 +30

Figure 5. Double Stranded Operator DNA for lac Repressor

Thus, Wick and Matthews annealed two synthetic complementary 40-mer oligos to create the double stranded DNA operator sequence that binds to the lac Repressor protein (Figure 5).
To identify points of interaction of the operator sequence in its protein complex, a homologous series of synthetic double stranded bromodeoxyuridine-substituted Operator DNA mimics were prepared with 5-Br-dU replacing single thymine residues and their binding affinity and photo-cross-linking with lac Repressor protein were studied.

Thus, a series of double stranded operator DNA mimics were prepared, nine in which each singly 5-Br-dU substituted top strand oligo was annealed to its unmodified complementary bottom strand oligo, and similarly another nine in which each singly 5-Br-dU substituted bottom strand oligo was annealed with its unmodified top strand. In addition to these singly 5-Br-dU substituted operator mimics, two heavily 5-Br-dU modified control operator oligos were prepared, one, T_{per}/B, has 9 central thymidine positions substituted with 5-Br-dU and an unmodified bottom strand (see Figure 5). The other, denoted T/B_{per}, contains a bottom strand with all T residues replaced with 5-Br-dU and an unmodified upper strand. To detect reactions using denaturing PAGE, oligos were end-labeled using ^{32}P-ATP with T4 polynucleotide kinase and the reaction products were visualized by autoradiography.

In this early study a UV_{254nm} source was used for cross-linking and so intrastrand bond scission is a significant side reaction. PAGE analysis of control experiments in the absence of lac repressor protein showed that 90 second UV irradiation of the highly 5-Br-dU substituted operators yields an array of shorter-mer fragments

corresponding to cleavage of labeled strands at each substituted position. Thus, T_{per}/B yielded T strand fragments at-5, -3, +3, +4, +6, +14, +20, +21, +22 and B_{per}/T yielded strand +1, +2, +8, +13, +15, +16, +18, +19, +24.

The eighteen singly 5-Br-dU modified Operator duplexes were then used to study duplex binding affinity to lac Repressor. The Tx modified mimics (top strand modified) had dissociation constants, K_d, in the range 4.1 to 8.0 x 10^{-10} M and the nine Bx mimics K_d were in the range 4.0 to 8.3 x 10^{-10} M. Essentially in all singly modified analogs affinity was little changed from the completely unmodified operator sequence (K_d = 6.2 x 10^{-10} M). These results confirm that substituting bromine for a methyl group on the pyrimidine ring has little steric influence on binding affinity, in accord with expectation, since the van der Waals radius of bromine (1.95 Å) is quite similar to a methyl group (1.99 Å).

Impact of Transcription activator isopropyl-β-D-thiogalactoside (IPTG): Each of these 18 5-Br-dU operator mimics was photo-cross-linked in the presence and absence of IPTG; as a transcription activator it was thought that IPTG binding to the tetrameric protein may affect concurrent binding of the double stranded operator sequence and, as a result, influence the specificity of any cross-linking reactions. Indeed, it was observed that cross-linking specificity was profoundly impacted by the presence of IPTG. In its absence some amount of cross-linking was observed using all the duplexes with their varied 5-Br-dU substitution positions. However, with IPTG present, photo-cross-linking was much more specific, occurring essentially at predominantly 5 positions of substitution, revealing the presence of five specific points of close contact between the operator and lac Repressor (Figure 6).

Thus, singly 5-Br-dU containing DNA oligonucleotides confirmed that halogen substitution for a methyl group has a minimal impact on duplex affinity for the lac repressor binding site. In addition, these modified operators revealed the identity of the specific points of contact between the operator duplexes and the lac Repressor, and also showed the impact of transcription activator IPTG in focusing crosslinking to a small subset of sites within the binding pocket.

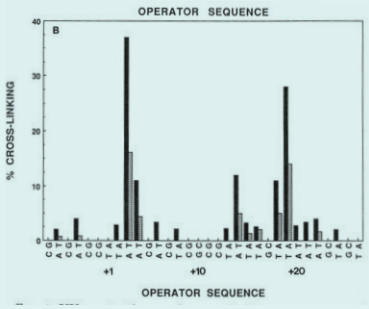

Figure 6. Cross-linking Operator to lac Repressor Protein. Solid bars-specific cross-link formation; Shaded bars-protein to DNA cross-linking in presence of IPTG inducer.

Cross-linking 5-Iodouridine and 5-Bromouridine modified RNA to Protein

Photo-cross-linking of a short hairpin RNA fragment to bacteriophage R17 coat protein was studied and the efficiency and specificity benefits of using lower energy UV irradiation of the 5-I-U nucleoside were demonstrated. Bacteriophage R17 replicase gene translation is repressed when a small hairpin RNA binds to the bacteriophage R17 coat protein. Earlier workers used homologous synthetic RNA variants to establish the binding site size and critical RNA sequence parameters and found a 21-nucleotide fragment of R17 RNA binds to phage coat protein with similar affinity to the natural sequence.[16] In photo-cross-linking studies in Tad Koch's lab,[17] Gott and coworkers prepared R17 RNA hairpins by *in vitro* transcription from synthetic DNA templates to contain 5-bromo-U substituted for various uridine residues.[18] From studying photo-cross-linking of the varied 5-Br-U containing analogs to coat protein, it was clear that bromouridine in the hairpin loop was implicated, and while some of the other analogs were capable of cross-linking, these cross-linkings were strongly dependent on the position of 5-Br-U in the fragment.

Willis and co-workers[19] synthesized by *in vitro* transcription (IVT) native and singly substituted 5-Br-U and 5-I-U RNA variants of the close-analog 19-nucleotide R17 RNA hairpin and studied their binding affinity and photo-cross-linking to purified coat protein, using a nitrocellulose filter method and alpha ^{32}P-C labeled R17 RNA (Figure 7).

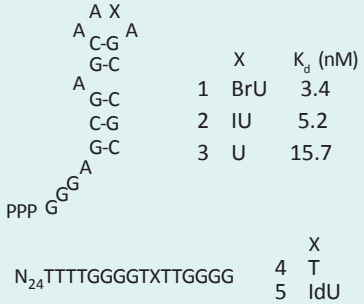

Figure 7. Structures of the bacteriophage R17 RNA Hairpins 1, 2 and 3

Interestingly, the dissociation constants, K_d, for the iodo- and bromo-modified RNAs bound to coat protein revealed 3- and 5-fold *stronger* binding affinity to the coat protein, respectively, than the native sequence. Thus, Willis et al. found 5-Br-U and 5-I-U labeled R17 RNA hairpins were well-tolerated within the binding site of R17 coat protein; this strong binding occurs despite that the van der Waals radii of bromine (1.95 Å) and iodine (2.15 Å) are considerably larger than the hydrogen

at ring position 5 of uracil (1.2 Å).[20] It was noted that 5-Br-U cross-linking yields were low, and the researchers observed that photoactivation using a low-pressure mercury lamp UV_{254nm} versus monochromatic UV_{308nm} from a xenon chloride excimer laser resulted in different cross-linking results; using longer wavelength UV higher photo-cross-linking yields were obtained, with less protein damage and less RNA strand scission. High energy photo-cross-linking appears to result in C-Br bond homolysis forming a highly reactive uridyl and Br radical pair, while the lower energy irradiation generates an alternative excited state[6].

Thus, using the 308 nm XeCl excimer laser, the 5-Br-U containing R17 RNA hairpin 1 (5-Br-U-R17 RNA1) achieved a maximal 40% coupling yield with R17 coat protein, while the 5-I-U-R17 RNA2 achieved 80% coupling yield in 5 minutes of irradiation.

The researcher's interest in further reducing photo-generated side products prompted the use of longer wavelength light to activate and cross-link the 5-I-U nucleoside modified R17 hairpin analog. The time course of photo-cross-linking using 325 nm helium cadmium (HeCd) laser irradiation of 5-I-U-RNA 2 to the R17 coat protein is shown in Figure 8. SDS PAGE analysis revealed up to 94% coupling yields with negligible amounts of side products even upon extended irradiation.

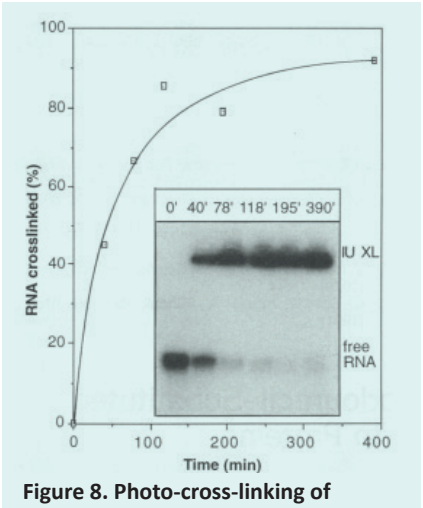

Figure 8. Photo-cross-linking of 5-I-U RNA to R17 coat protein with monochromatic emission at 325nm.

To establish the identity of the cross-linked amino acid in the RNA-coat protein adduct, a 308 nm XeCl laser irradiated cross-linking reaction was carried out using the radiolabeled 5-Br-U RNA and coat protein. The mixture was ethanol precipitated and the redissolved pellet was trypsin digested. The mixture was purified by DEAE adsorption to remove free RNA and was subjected to a salt step gradient to remove peptides. Then RNA and tryptic adducts were eluted in 0.6 M NaCl, ethanol precipitated and purified by denaturing 20% PAGE followed by electroblotting onto a PVDF membrane. The RNA-peptide adduct was then sequenced via automated Edman degradation directly off the membrane, revealing that a single tyrosine residue, Tyr85, had been covalently cross-linked to the uridine residue.

In summary, this work showed that even the replacement of uridine by the larger 5-Br-U and 5-I-U in R17 RNA did not significantly perturb binding affinity for phage coat protein; surprisingly the already strong RNA-R17 coat protein binding was enhanced in the halogen modified R17 RNA hairpins. In addition, both the bromine and iodine singly halogen substituted RNAs were rapidly photo-cross-linked to coat protein with high efficiency using monochromatic laser irradiation at 308 nm or 325 nm, respectively; a single tyrosine residue in R17 coat protein was found to be cross-linked. Photo-cross-linking studies using longer wavelength UV irradiation resulted in reduced side-reactions and are more specific for 5-halogen-uridine containing RNA and DNA cross-links.

Low energy UV photo-cross-linking can be achieved using quite simple equipment. Dietz *et al.*[21] demonstrated similar specific cross-linking results to those achieved with the laser by excitation instead using the 313 nm emission from a high-pressure mercury lamp selected with a monochromator at 310 nm with a band pass of 20 nm. Gott and coworkers used a medium wavelength transilluminator for some experiments. Even simpler methods to obtain useful photo-cross-linking conditions are available, as described by Xue and Nicholson.[22]

Photo-cross-linking in Aptamer Discovery and Characterization

RNA Aptamer Development

Several labs have recognized the utility of photochemical cross-linking methods to better understand aptamer interaction with targets. An early application of photo-cross-linking to aptamer development by Jensen and coworkers used *in vitro* selection to direct the covalent attachment of high-affinity RNA ligands, aptamers containing 5-I-U, to human immunodeficiency virus type 1 Rev protein.[23]

A feature of the Jensen lab study was the application of SELEX methodology to include 5-iodouridine triphosphate (5-I-UTP) instead of UTP in the phage T7 RNA polymerase transcription reaction mixtures during evolution of the aptamers. The resulting high affinity RNA aptamers were used for efficient photo-cross-linking of the aptamer to HIV-1 Rev protein and, although this work used transcription of 5-iodouridine triphosphates (as opposed to synthetic oligos from phosphoramidite synthesis), the value of photo-cross-linking methodology in elucidating aptamer interaction with target protein was clearly shown.

DNA Aptamer development

The utility of directly using 5-Br-dU or 5-I-dU as a randomization monomer amidite in SELEX is complicated by the base pairing ambiguity of these two monomers. Still, there is another use for these nucleoside phosphoramidites later in aptamer studies by substituting for T residues in an already optimized aptamer. For example, Mallikaratchy et al. used SELEX to evolve aptamers from live cells.[24] Using Ramos cells, a Burkitt's lymphoma cell line, an aptamer, TD05, was discovered that recognizes with high affinity (K_d 0.75 nM) a membrane bound heavy mu chain of IgM5 (IGHM), this latter a protein component of a B-cell receptor complex in these cells.[25] Then, using the fluorescent FITC-labeled TD05 aptamer, a cell binding assay was established by fluorescence activated cell sorting (FACS). Using this assay, specific binding of the aptamer to a yet unidentified target within cells could be demonstrated by competition with unlabeled TD05.

The researchers then prepared a series of 5-I-dU modified TD05 aptamers (5-iodo-2'-deoxyUridine is called 5dUI in the paper) using machine DNA synthesis where 5-I-dU was substituted for thymine, including fully substituted (all T replaced) and various partially substituted TD05 analogs. Fully substituted 5-I-dU TD05 did not bind to cells, however less-substituted variants did bind strongly; an optimally labeled 5-I-dU TD05 aptamer analog was identified that contained four 5-I-dU residues (Figure 9). An analogous variant was then synthesized by solid phase synthesis that also incorporated a 3'-cleavable disulfide linked biotin tail to capture and identify the protein target.

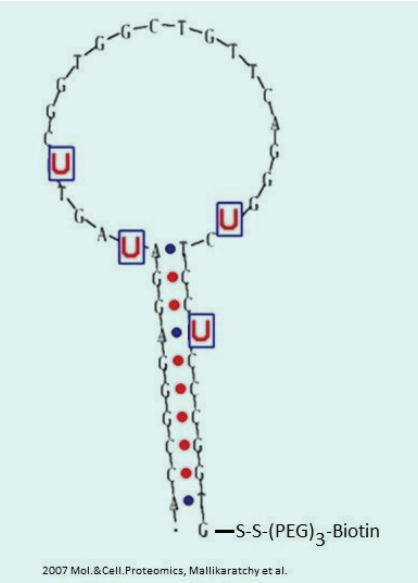

Figure 9. Modified aptamer with photoactive 5-I-dU linked to biotin via a disulfide bond. U, 5-I-dU; S, sulfur; PEG, polyethylene glycol linker

To identify the aptamer target, the 5'-^{32}P radiolabeled modified aptamer was photo-cross-linked to its target within cells using nanopulsed XeCl excimer laser (308 nm) irradiation. The resulting cells were lysed/homogenized, and solids were pelleted to yield crude membrane protein extract; this was detergent solubilized and separated from insoluble cell debris. The soluble fraction was incubated with streptavidin magnetic beads, washed, and the cross-linked protein mixture was released from beads by disulfide bond cleavage; mass spectrometry was used to identify four candidate protein targets among what remained a complex mixture also containing nuclear proteins. Further experiments demonstrated IGHM was a TD05 target. This effort led to subsequent studies in which the TD05 aptamer was prepared covalently coupled to light-activated photosensitizer molecule chlorin e6 (Figure 10) and, using the conjugate, these workers demonstrated specific killing of cancer model Ramos cells.[26]

In summary, SELEX optimized DNA and RNA aptamers have been modified with 5-iodouracil nucleoside residues substituted at one or more T or U residues, respectively, resulting in 5-iodouracil-modified aptamers with retention of strong target binding affinity. The specific, efficient photo-cross-linking within a complex milieu of live cells has facilitated identification of a membrane bound receptor specific to these Burkitt's lymphoma cells and led to development of a cancer cell-specific aptamer-drug conjugate.

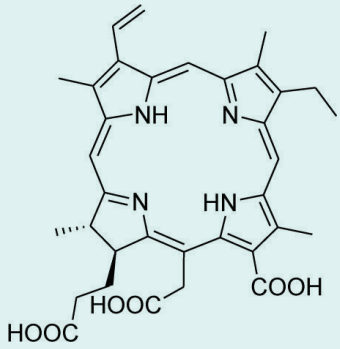

Figure 10. Chlorin e6, Light Activated Photosensitizer

Cross-linked Adduct Structures

The photo-cross-linking regiospecificity of these methods as applied to many DNA and RNA-protein complexes has been used to reveal molecular details of complex formation. Today, an arsenal of halogen modified deoxyribonucleosides and ribonucleosides are activated by long-wavelength UV to yield photoadducts, as shown in Figure 11 below, written generally to include deoxyribo- and ribonucleotides. Glen Research carries many of the halogen containing phosphoramidites needed for chemical synthesis of photo-cross-linkable oligonucleotides. Because these nucleosides interact mainly with the electron rich amino acids phenylalanine, tyrosine, tryptophan and histidine, researchers may encounter nucleic acid-protein complexes in which a photoactivatable probe's protein binding site may not have a suitably located

nearby amino acid side chain with which to efficiently photo-cross-link. Even so, taking advantage of oligo design flexibility a great many examples have proven successful and instructive.

Although not the main focus of this report, it should be mentioned that thio-analogs of nucleosides have also been used as photo-cross-linking probes of nucleic acid interaction with proteins.[27] This includes the DNA analogs 4-thio-dT, 4-thio-dU, 6-thio-dG and the RNA analog 6-thio-G. Possible advantages of using the thio derivatives for photo-cross-linking is their similarity to the natural structures and the availability of additional protein interfaces for cross-linking due to the additional photoactive analogs thioG and thiodG. Typically, thio-analogs are photoactivated at 340 nm wavelength and so, similar to irradiation of 5-Br-uracil and 5-I-uracil containing nucleosides, will result in low non-specific side reactions.

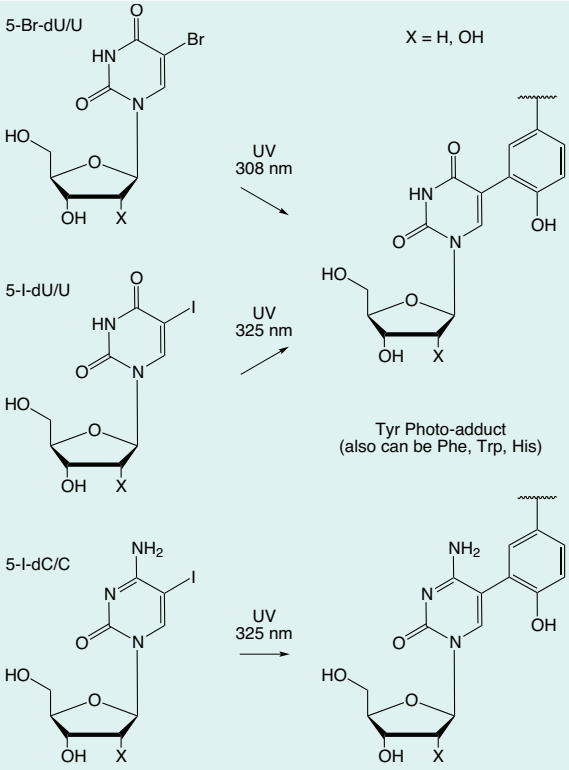

Figure 11. Photoactivatable DNA and RNA Nucleoside Analogs and Adducts

Conclusions

A long history of photo-cross-linking using 5-bromo and 5-iodopyrimidine nucleoside analogs in synthetic DNA and RNA continues aiding researchers to understand the intimate interactions of nucleic acids with partner proteins involved in cellular control and signaling pathways. Researchers have found new applications of these molecules in aptamer design and characterization, including development of drug candidates through cell-based SELEX methods in the discovery of membrane bound receptors for potential use in targeting cancers. The use of these efficient photo-cross-linkers seems well positioned to lead to other research insights among the growing network of ribonucleoproteins and RNP complexes.

The size change resulting from bromine and iodine substitution on position 5 of the uracil ring has a minimal impact on DNA oligo binding to its receptor protein binding site. Surprisingly, halogen substitution in RNA, despite the increased size of bromine and iodine over hydrogen, did not reduce binding affinity in the examples shown. Rapid and highly specific photo-cross-linking of 5-Br-dU/5-Br-U and 5-I-dU/5-I-U residues in single and double stranded DNA or RNA oligonucleotides in association with cognate binding proteins can be achieved using longer wavelength UV irradiation (laser or bandpass filtered) centered at 308 nm and 325 nm, respectively.

Glen Research's line of molecules includes the halogenated-deoxycytidine and-deoxyuridine phosphoramidites and bromodeoxyuridine CPG support. For RNA constructs, the bromo- and iodo-uridines are available as TBDMS protected phosphoramidites and also as the bromo-uridine, 2'-O-methyl phosphoramidite. In general, mild room temperature deprotection is required. See use instructions for dissolution, coupling and deprotection conditions for each monomer.

In Part 2 of this series, we will describe elegant studies of the RNA interference pathway demonstrating how photo-cross-linking of 5-halogen-Uracil containing oligos have been used to unravel the mechanistic details of the multiple step, multiple component ribonucleoprotein RISC complex.

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3. Abbreviations: 5-Br-dU, 5-bromo-2'-deoxyuridine; 5-I-dU, 5-iodo-2'-deoxyuridine; 5-Br-U, 5-bromouridine; 5-I-U, 5-iodouridine; 5-Br-dC, 5-bromo-2'-deoxycytidine; 5-I-dC, 5-iodocytidine; FITC, fluorescein isothiocyanate; PAGE,

5-Bromo- and 5-Iodo-Pyrimidine Nucleosides; Efficient Oligo to Protein Photo-Cross-linkers (Part 1) (cont.)

polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactoside; UVA, 315-400nm wavelength; UVB, 280-315nm wavelength; UVC, 100-280nm wavelength.

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Item	Catalog No.	Pack	Price ($)
5-Br-dC-CE Phosphoramidite	10-1080-90	100 µmole	60.00
	10-1080-02	0.25 g	160.00
5-I-dC-CE Phosphoramidite	10-1081-90	100 µmole	135.00
	10-1081-02	0.25 g	355.00
5-Br-dU-CE Phosphoramidite	10-1090-90	100 µmole	60.00
	10-1090-02	0.25 g	160.00
5-I-dU-CE Phosphoramidite	10-1091-90	100 µmole	60.00
	10-1091-02	0.25 g	160.00
5-Br-dU-CPG	20-2090-01	0.1 g	50.00
1.0 µmole columns	20-2090-41	Pack of 4	200.00
0.2 µmole columns	20-2090-42	Pack of 4	120.00
Br-U-CE Phosphoramidite	10-3090-95	50 µmole	98.00
	10-3090-90	100 µmole	195.00
	10-3090-02	0.25 g	475.00
5-I-U-CE Phosphoramidite	10-3091-95	50 µmole	98.00
	10-3091-90	100 µmole	195.00
	10-3091-02	0.25 g	475.00
2'-OMe-5-Br-U-CE Phosphoramidite	10-3190-90	100 µmole	240.00
	10-3190-02	0.25 g	675.00
4-Thio-dT-CE Phosphoramidite	10-1034-95	50 µmole	165.00
	10-1034-90	100 µmole	295.00
	10-1034-02	0.25 g	675.00
4-Thio-dU-CE Phosphoramidite	10-1052-95	50 µmole	165.00
	10-1052-90	100 µmole	295.00
	10-1052-02	0.25 g	675.00
4-Thio-U-TOM-CE Phosphoramidite	10-3052-95	50 µmole	212.50
	10-3052-90	100 µmole	425.00
	10-3052-02	0.25 g	975.00
6-Thio-dG-CE Phosphoramidite	10-1072-95	50 µmole	177.50
	10-1072-90	100 µmole	355.00
	10-1072-02	0.25 g	975.00

New Product - dmf-dG-5'-CPG

Oligonucleotide synthesis typically proceeds from the 3' to 5' direction, mostly because the phosphoramidites for this direction are straight forward to synthesize. These reagents allow the synthesis of oligonucleotides for a majority of the required applications. However, there are a few scenarios where 5' to 3' synthesis reagents are necessary (Figures 1 and 2).
One of these scenarios is the generation of 3' to 3' linkages, particularly as a 3'-cap for nuclease resistance. Natural oligonucleotides are highly susceptible to nucleases, and the addition of an inverted nucleotide at the 3'-end dramatically increases resistance toward 3'-exonucleases.[1] The most common method of introducing such a 3'-cap is to begin the synthesis with dT-5'-CPG (20-0302) and then carrying out the rest of the synthesis as one usually would in the 3' to 5' direction. The resulting oligonucleotide would have a 3' inverted dT, and it should be noted that such a 3'-cap would also prevent polymerase extension.

A second scenario is the synthesis of oligonucleotides containing modifications at the 3' terminus that would not be possible otherwise.[2] Examples of such modifications include certain dideoxy nucleotides and phosphoramidite reagents that are only available as terminal labels.

A third scenario is the synthesis of attached oligonucleotides that can be extended with polymerases. This type of synthesis arrangement is the key to single cell RNAseq methods such as Drop-seq[3] that have been developed in recent years. In Drop-seq, the synthesis begins with mono-sized microparticles containing linkers that are stable to deprotection conditions thus ensuring the oligo remains attached to the bead (Figure 3). Briefly, reverse direction DNA synthesis is performed to synthesize

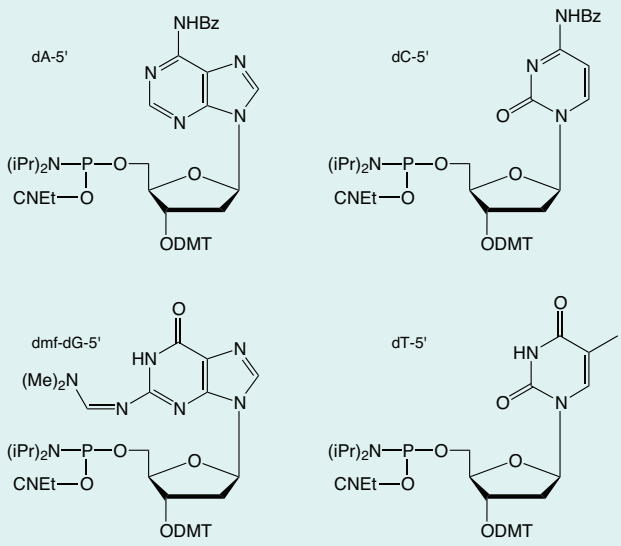

Figure 1. Reverse DNA Phosphoramidites

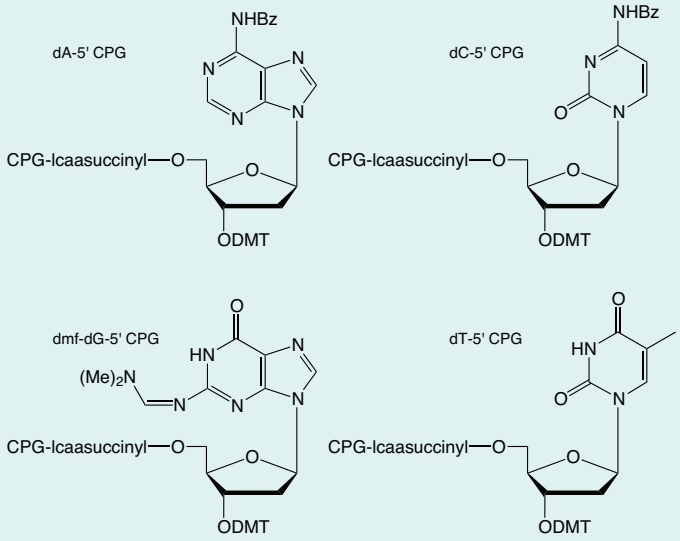

Figure 2. Reverse DNA CPG Supports

relatively long oligonucleotides that contain several functional regions, one of which is a stretch of T's. In a microfluidics environment, the poly T region of the oligonucleotide attached to one individual bead will hybridize to/capture the poly A tails of mRNA from one individual cell. A reverse transcriptase can then append a cDNA copy of the mRNA to the bead bound oligo. Each DNA sequence contains 2 barcode regions, one to differentiate one bead from another, and the other to differentiate one strand from another on the same bead. As a result, the DNA on these beads can be amplified by PCR for analysis by next generation sequencing to quantify individual mRNA sequences at the cellular level.

Use of dmf-dG-5'-CPG

For many years now, Glen Research has been providing reverse DNA synthesis reagents to the research community. All these reagents have protecting

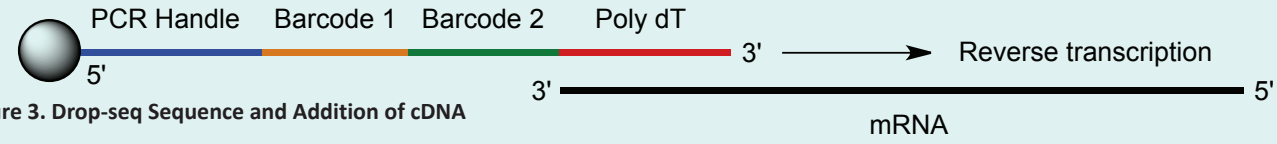

Figure 3. Drop-seq Sequence and Addition of cDNA

groups and attachment points between the 5’ and 3’ positions that have been reversed. Everything else is identical; the protection groups are similar and the synthesizer protocols are the same.

Early on, Glen Research offered ibu-dG-5’-CE Phosphoramidite and ibu-dG-5’-CPG; however, several years ago, we began offering the dmf-protected version of the phosphoramidite in place of the ibu-protected version while leaving the CPG offering unchanged. Many customers who are performing reverse direction synthesis may not require the dG-CPG, such as Drop-seq researchers, but for those who require the dG-CPG as well, they are forced to use more harsh conditions necessary for removal of the ibu protecting group. As listed in the Glen Research Deprotection Guide, dmf-dG is significantly faster to deprotect when using standard deprotection conditions (Table 1). Due to this mismatch in protecting groups, Glen Research has added dmf-dG-5’-CPG to its catalog.

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Table 1. Deprotection of dG

dG Protection	Temperature	Time
ibu-dG	RT	36h
	55°C	16h
	65°C	8h
dmf-dG	RT	16h
	55°C	4h
	65°C	2h

Item	Catalog No.	Pack	Price ($)
dA-5'-CE Phosphoramidite	10-0001-02	0.25 g	75.00
	10-0001-05	0.5 g	150.00
	10-0001-10	1.0 g	300.00
dC-5'-CE Phosphoramidite	10-0101-02	0.25 g	75.00
	10-0101-05	0.5 g	150.00
	10-0101-10	1.0 g	300.00
dmf-dG-5'-CE Phosphoramidite	10-9201-02	0.25 g	75.00
	10-9201-05	0.5 g	150.00
	10-9201-10	1.0 g	300.00
dT-5'-CE Phosphoramidite	10-0301-02	0.25 g	75.00
	10-0301-05	0.5 g	150.00
	10-0301-10	1.0 g	300.00
dA-5'-CPG	20-0002-01	0.1 g	50.00
	20-0002-10	1.0 g	375.00
1 µmole columns	20-0012-41	Pack of 4	100.00
0.2 µmole colums	20-0012-42	Pack of 4	75.00
10 µmole column (ABI)	20-0012-13	Pack of 1	225.00
15 µmole column (Expedite)	20-0012-14	Pack of 1	300.00
dC-5'-CPG	20-0102-01	0.1 g	50.00
	20-0102-10	1.0 g	375.00
1 µmole columns	20-0112-41	Pack of 4	100.00
0.2 µmole colums	20-0112-42	Pack of 4	75.00
10 µmole column (ABI)	20-0112-13	Pack of 1	225.00
15 µmole column (Expedite)	20-0112-14	Pack of 1	300.00
dmf-dG-5'-CPG	20-9202-01	0.1 g	50.00
	20-9202-10	1.0 g	375.00
1 µmole columns	20-9212-41	Pack of 4	100.00
0.2 µmole colums	20-9212-42	Pack of 4	75.00
10 µmole column (ABI)	20-9212-13	Pack of 1	225.00
15 µmole column (Expedite)	20-9212-14	Pack of 1	300.00
dT-5'-CPG	20-0302-01	0.1 g	50.00
	20-0302-10	1.0 g	375.00
1 µmole columns	20-0312-41	Pack of 4	100.00
0.2 µmole colums	20-0312-42	Pack of 4	75.00
10 µmole column (ABI)	20-0312-13	Pack of 1	225.00
15 µmole column (Expedite)	20-0312-14	Pack of 1	300.00

New Product - Methacrylate C6 Phosphoramidite

Polyacrylamide gels have long since been essential tools in nucleic acid and peptide laboratories. The flexibility in preparing polyacrylamide gels allows unparalleled control of resolution through a broad range of sizes with a large loading capacity for the purification of nucleic acids. New applications for polyacrylamide gels continue to emerge.

The copolymerization of acrylamide and bis-acrylamide crosslinker to form polyacrylamide gels is a vinyl addition polymerization reaction typically initiated by ammonium persulfate and tetramethylethylenediamine (TEMED). Ammonium persulfate acts as the free radical source and TEMED as a free radical catalyst. The persulfate free radicals react with acrylamide-containing monomers, converting them into additional free radicals, as well as polymerizing with unreacted acrylamide monomers to form the gel matrix. Including a 5'-methacrylate-labeled oligonucleotide in the reaction mixture covalently incorporates the oligonucleotide into the gel matrix.

Methacrylate C6 Phosphoramidite (Figure 1) is a modifier that can be used to attach methacrylate to an oligonucleotide using conventional phosphoramidite chemistry and subsequently incorporated into a polymer (Figure 2). Methacrylate-modified oligonucleotides have been used to generate oligonucleotide-labeled hydrogels, microspheres, microarrays, and functionalized polymeric surfaces for use in purification, hybridization detection assays, affinity capture, biosensors, and sequencing.
In the first hybridization proof-of-concept experiments, methacrylate-labeled oligonucleotides were polymerized onto an acrylic-functionalized slide.[1] Subsequent hybridization with asymmetric fluorescently-labeled PCR products confirmed the utility of acrylamide-labeled oligonucleotides in detection assays. This initial research on methacrylate-labeled oligonucleotides also confirmed that methacrylate labels are stable to standard PCR conditions and once polymerized, form polymers with high thermal stability, high density, and low non-specific absorption.[1]

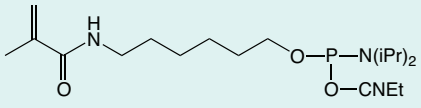

Figure 1. Methacrylate C6 polymerization

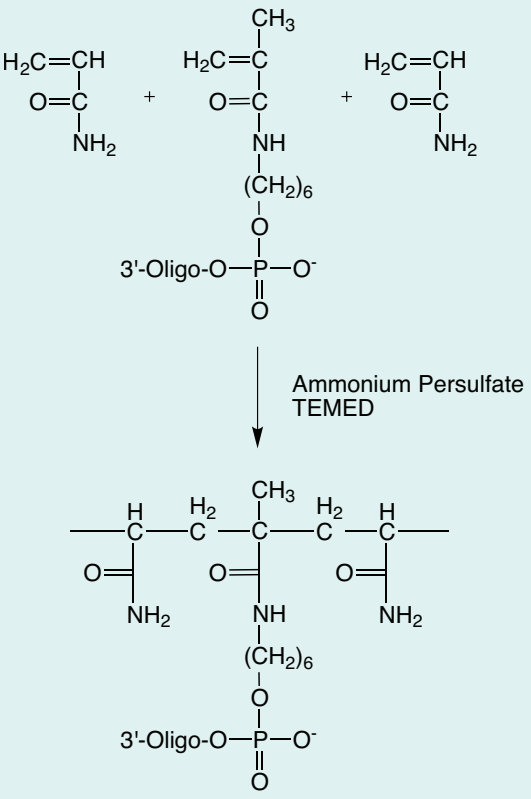

Figure 2. Incorporation of a methacrylate-labelled oligonucleotide into a polymer matrix

More recently, methacrylate-labeled oligonucleotides have been used in aptamer-based hydrogels, molecular imprinting, and single molecule inexpensive FISH (smiFISH) techniques.[2-4] Aptamer-based hydrogels use the selectivity of aptamers to bind their targets for detection using the physical properties of gels, as well as for electrochemical detection, and colorimetric detection. In one example, ochratoxin aptamers were incorporated into a hydrogel resulting in high selectivity for ochratoxin and a detection limit of 0.51 ppb in food products.[5]

Molecular imprinting uses biomolecules, such as antibodies, proteins, and glycoproteins, as templates to create molecularly imprinted polymers (MIPs).[3]

New Product - Methacrylate C6 Phosphoramidite (cont.)

Biomolecules are mixed with the pre-polymer, polymerized in place, and subsequently removed to create a recognition cavity. Incorporating aptamers as the biomolecule in MIPs takes advantage of the binding affinity and selectivity of aptamers. As such, aptamer-based MIPs can enhance the sensitivity of MIPs down to femtomolar concentrations as shown for thrombin MIPs.[3]

In smFISH, a precursor to smiFISH, gene-specific primary fluorescent probes are used to localize individual mRNA within cells. By comparison, smiFISH probes are composed of primary and secondary probes. Primary probes are unlabeled and gene specific probes. Secondary probes are fluorescently labeled (FLAPs) and bind the FLAP sequence on the primary probes. When the secondary probe also contains a methacrylate label, smiFISH probes can be used in Expansion Microscopy (ExM).[4,6,7] ExM is the *in situ* polymerization of labeled molecules followed by hydration of the matrix to induce expansion of the biological structure, retaining the 3D orientation of the original biological structure.[6,7] Combining smiFISH with ExM provides a 2-fold increase in signal-to-noise ratio and the ability to resolve overlapping transcripts.[4]

In each of these techniques, methacrylate-labeled oligonucleotides are incorporated into a gel matrix and the canonical properties of the oligonucleotides provide specificity and functionality within the application. Glen Research is pleased to offer Methacrylate C6 Phosphoramidite for the synthesis of methacrylate-labeled oligonucleotides.

Item	Catalog No.	Pack	Price ($)
Methacrylate C6 Phosphoramidite	10-1891-90	100 µmole	110.00
	10-1891-02	0.25 g	650.00

Use of Methacrylate C6 Modifier

Methacrylate C6 Modifier is compatible with standard coupling times and deprotection conditions. This modifier does not contain a DMT protecting group and cannot be purified with DMT-ON purification techniques. However, the purification step can typically be omitted since only the oligonucleotides containing the methacrylate modification will be polymerized into the gel matrix. Unlabeled oligos are easily washed away after the polymerization step is completed.

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New Product - 2'-F-5-Me-U-ANA

2'-F-arabinonucleic acid (2'-F-ANA) oligonucleotides are cousins to the widely used 2'-F-RNA, with the only difference being an inverted stereocenter at the 2'-carbon. Like 2'-F-RNA, 2'-F-ANA has high base pairing specificity and enhanced hydrolytic and nuclease stability. Glen Research began offering 2'-F-ANA versions of A, C, G and U in 2010 (Figure 1), and since then, this line of products has proven to be popular. Over the years, there have been numerous publications highlighting exciting research using these reagents.

With the goal of enhancing the pharmacological properties of G-quadruplexes, members of the Phan Lab conducted a systematic investigation to understand the effects of several sugar modifications on G-quadruplex structure and stability.[1] One of these modifications was 2'-F-ANA. They synthesized a total of twenty singly 2'-F-ANA-modified variants for two types of G-quadruplexes, a (4+0) parallel and a (3+1) hybrid. Using a combination of ^{1}H-NMR, UV absorption and CD, they found that 2'-F-ANA substitutions in anti positions were well tolerated whereas substitutions in syn positions were destabilizing.

In another investigation, Holliger and coworkers investigated several different types of catalysts from non-DNA/RNA backbones such as 2'-F-ANA in the context of the origin of life.[2] All their studies involved the use of *in vitro* selection (SELEX) and previously engineered polymerases.[3] Using a self-cleavage strategy, they were able to isolate RNA-cleaving enzymes from an initial random library of 10^{14} different 2'-F-ANA oligonucleotides after 13-17 rounds of selection. The most active enzyme catalyst was able to cleave RNA in a site- and sequence-specific manner with an observed rate constant of 0.058 min^{-1}. Like DNAzymes and ribozymes, this "FANAzyme" cleaves RNA resulting in the formation of a 5'-hydroxyl group and a 2',3'- cyclic phosphate. In addition to RNA cleavage, in separate experiments, they were also able to develop FANAzymes that performed the reverse reaction, RNA

ligation. The RNA ligase was able to join 3’-imidazolylphosphoryl-RNA to the 5’-OH of another RNA strand, also in a sequence specific manner. Finally, they were able to use a similar selection methodology to also discover a FANAzyme that performed 2’-F-ANA ligation. The ligases exhibited rate constants of 0.0002 and 0.038 min^{-1}, respectively.

Recently, members of the Chaput Lab developed their own RNA-cleaving 2’-F-ANA enzymes as biologically more stable therapeutic candidates.[4] Also using SELEX techniques, they were able to isolate superior RNA-cleaving FANAzymes. The most active catalyst was able to cleave RNA in a sequence-specific manner with a maximum rate constant of 0.2 min^{-1} (Figure 2). Unlike earlier FANAzymes, this catalyst follows Michaelis-Menten kinetics. The researchers confirmed that the enzyme could be re-engineered to target almost any desired RNA sequence by changing the substrate binding domains of the catalyst.

Use of 2’-F-5-Me-U-ANA

Although 2’-F-ANA are epimers of 2’-F-RNA, when 2’-F-ANA hybridizes with DNA, it adopts a more DNA-like B-type helix structure rather than an RNA-like A-type structure. Due to this, it should not come as a surprise that 2’-F-5-Me-U-ANA, which has the extra methyl group similar to thymidine, increases duplex stability relative to 2’-F-U-ANA. To give customers more control in the use of 2’-F-ANA, we are adding 2’-F-5-Me-U-ANA to our catalog (Figure 1). Like our other 2’-F-ANA phosphoramidites, 2’-F-5-Me-U-ANA can be dissolved in acetonitrile and coupled using a coupling time of six minutes using tetrazole as the activator. No changes to standard deprotection methods are required.

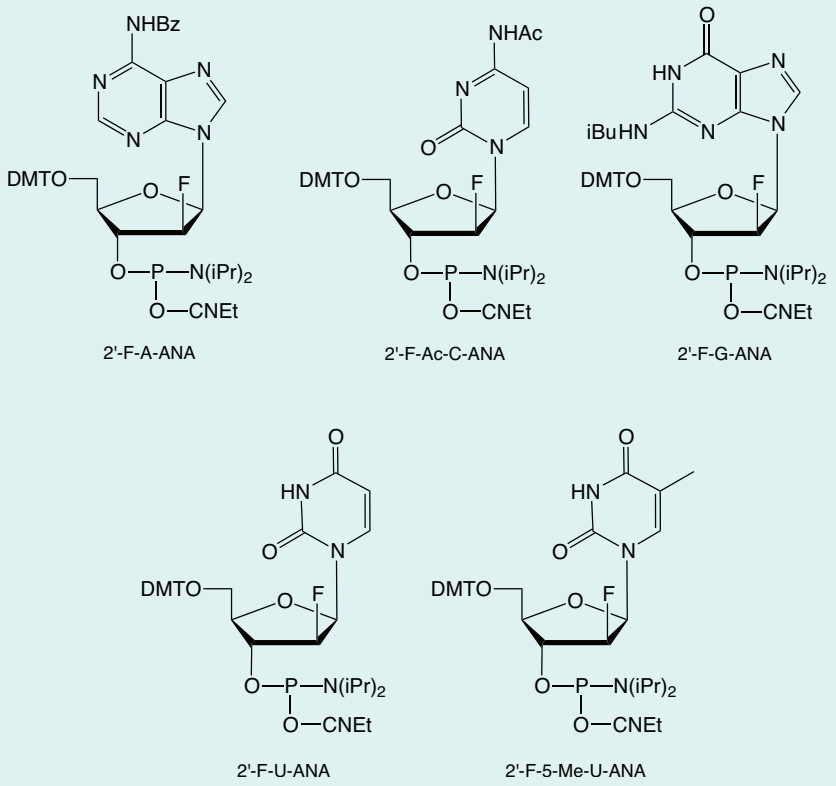

Figure 1. 2’-F-ANA Phosphoramidites

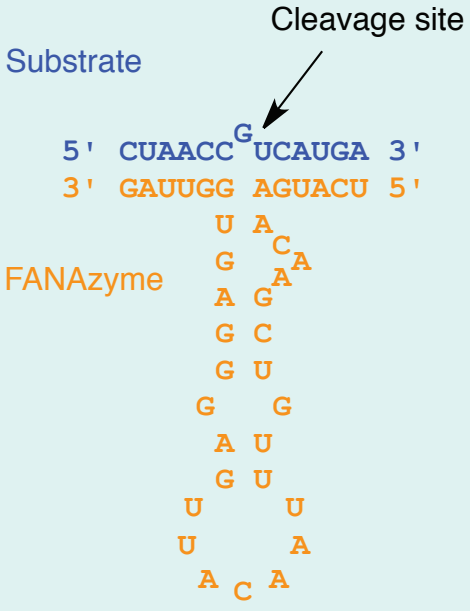

Figure 2. RNA-cleaving FANAzyme from the Chaput Lab

GLEN RESEARCH

part of Maravai LifeSciences

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PERMIT NO 536

Item	Catalog No.	Pack	Price ($)
2'-F-A-ANA-CE Phosphoramidite	10-3800-90	100 µmole	150.00
	10-3800-02	0.25 g	375.00
2'-F-Ac-C-ANA-CE Phosphoramidite	10-3815-02	0.25 g	200.00
	10-3815-05	0.5 g	400.00
2'-F-G-ANA-CE Phosphoramidite	10-3820-90	100 µmole	165.00
	10-3820-02	0.25 g	425.00
2'-F-U-ANA-CE Phosphoramidite	10-3830-02	0.25 g	125.00
	10-3830-05	0.5 g	250.00
2'-F-5-Me-U-ANA-CE Phosphoramidite	10-3850-02	0.25 g	250.00
	10-3850-05	0.5 g	500.00

References:

1. Z. Li, C.J. Lech, and A.T. Phan, Nucleic Acids Research, 2014, **42**, 4068-4079.
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3. V.B. Pinheiro, et al., Science, 2012, **336**, 341-4.
4. Y. Wang, A.K. Ngor, A. Nikoomanzar, and J.C. Chaput, Nat Commun, 2018, **9**, 5067.