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Introducing ribo-tC^{O}

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The bright fluorescent tricyclic cytosine analogues tC (Figure 1) and tC^{O} that we have developed and that Glen Research has successfully supplied since 2009, stand out among fluorescent bases due to their virtually unquenched fluorescence inside single- or double-stranded DNA.[1-3] Until recently the family of tricyclic cytosines had only been studied[1-3] and used[4-12] in DNA contexts and, importantly, introduced as possible donors of the first DNA base analogue FRET-pair with tC$_{nitro}$ (Figure 2, Page 2).[4]

However, in 2017, we also reported on the synthesis of the tC^{O} ribonucleoside (Figure 2c) and its incorporation into a range of RNA sequences, where it was shown to be a very potent and useful fluorophore also in this context.[13] Therefore, Glen Research in cooperation with ModyBase HB has decided to offer this useful fluorescent ribonucleoside analogue.

Fluorescent base analogues for RNA are limited in number compared to their DNA counterparts. To facilitate the application of such analogues, characterization of their structural and dynamics behavior in RNA compared to the corresponding natural nucleoside is important. Moreover, characterization of their photophysical properties as an effect on various surrounding RNA

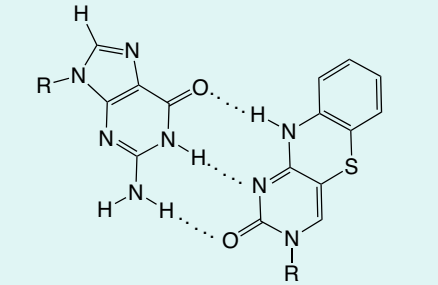

Figure 1. Guanine base paired with tC

bases is vital. However, such thorough characterizations inside RNA are rare. Therefore, when we reported on the first synthesis and RNA-incorporation of the tC^{O}-ribonucleoside and characterized its base-mimicking and fluorescence properties in RNA in 2017 this was one of the first such thorough investigations.[13] The tC^{O}-ribonucleoside, like its deoxy-counterpart, can be selectively excited with a peak around 365 nm well outside the nucleobase region and has a broad emission band with a peak, slightly dependent on sequence context, at approximately 455 nm (see Figure 3, Page 2 and Table 1, Page 2).

As in DNA, tC^{O} displays high quantum yields inside RNA duplexes (<Φ_F>=0.22) that are almost unaffected by neighboring RNA bases (Table 1). This results in an average brightness of 1900

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Introducing ribo-tC^{O}, a Fluorescent Ribonucleoside Analogue (cont.)

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$M^{-1}cm^{-1}$, which is significantly higher than previously reported fluorescent bases inside RNA.[13]

The average fluorescence lifetime of tC^{O} inside RNA duplexes with different neighboring bases is 4.3 ns (see Table 1 for details) and in general two lifetimes are required to fit the exponential decays.[13] The fluorescence properties in single-stranded RNA are characterized by a slight increase in average quantum yield (<Φ_F>=0.24) compared to the corresponding duplex RNAs, with a broader distribution and somewhat shorter average lifetimes (Table 1).[13] Importantly, using circular dichroism (CD) we also found that the tC^{O}-modified RNA duplexes form regular A-helices and in UV-melting experiments the stability of the duplexes was found to be only slightly higher than that of the corresponding natural RNA (<ΔTm>=+2.3°C).[13]

Glen Research is therefore pleased to introduce the ribonucleoside of tC^{O} (Ribo-tC°-CE Phosphoramidite, Figure 4) and believes that its properties make it a highly interesting and useful bright internal RNA label for a wide range of spectroscopy and microscopy experiments and also as an excellent analogue of cytosine in RNA.

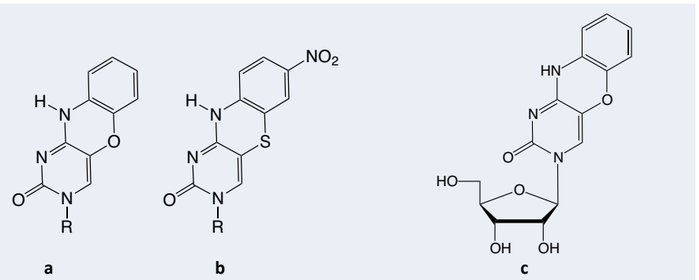

Figure 2. Structures of tricyclic cytosine analogues tC^{O} (a) tC$_{nitro}$ (b) and the tC^{O} ribonucleoside (c).

Sequence name[a]	single-stranded RNA $\lambda_{Abs,max}$ [nm]	single-stranded RNA $\lambda_{Em,max}$ [nm]	single-stranded RNA Φ_F[b]	single-stranded RNA <τ> [ns][c]	double-stranded RNA $\lambda_{Abs,max}$ [nm]	double-stranded RNA $\lambda_{Em,max}$ [nm]	double-stranded RNA Φ_F[b]	double-stranded RNA <τ> [ns][c]
tC^{O} monomer	359	457	0.30	3.4				
UGGU	367	455	0.31	4.7	373	453	0.22	4.5
UUGU	364	456	0.21	3.2	368	452	0.20	3.8
UGUU	364	458	0.19	2.8	373	460	0.22	4.5
UAAU	368	458	0.34	5.4	371	457	0.23	4.3
UAUU	363	457	0.22	3.3	370	459	0.23	4.2
UCCU	368	453	0.33	4.9	371	452	0.25	4.7
GUUU	364	457	0.17	2.6	370	456	0.22	4.2
GAUU	364	457	0.22	3.3	373	459	0.22	4.3
UUUG	362	458	0.21	3.1	371	456	0.23	4.1

Table 1. Photophysical properties of the tC^{O} monomer ribonucleoside and tC^{O} in various RNA sequence surroundings.
Measurements were performed in PBS buffer (100 mM Na+, pH 7.5).
a. Sequences are named after the bases flanking tC^{O} in the sequence 5'- CACX$_2$X$_1$tC^{O}Y$_1$Y$_2$CC - 3'.
b. Fluorescence quantum yields are measured relative to the quantum yield of the potassium salt of the tC^{O}-monomer in water (ΦF = 0.30).[3]
c. Amplitude-weighted mean fluorescence lifetime, <τ> = Saiti/Sa

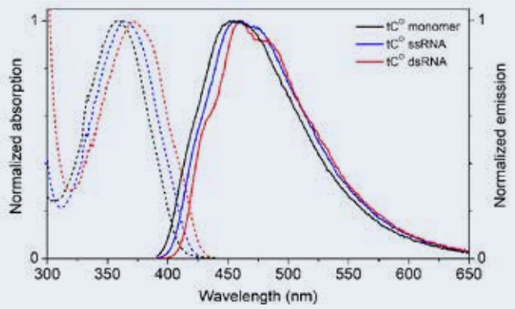

Figure 3. Normalized absorption and emission of tCO as a monomer ribonucleoside and inside single- and double-stranded RNA.

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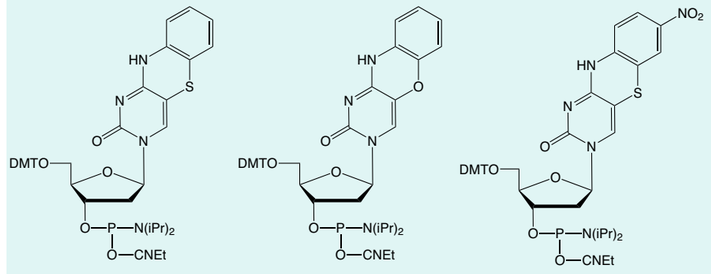
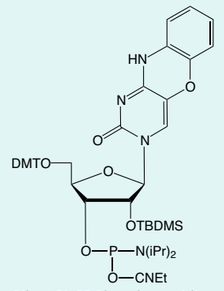


tC-CE Phosphoramidite | tC°-CE Phosphoramidite | tC$_{nitro}$-CE Phosphoramidite



Ribo-tC°-CE Phosphoramidite

Figure 4. Structures of tricyclic cytosine analogue phosphoramidites.

Item	Catalog No.	Pack	Price ($)
tC-CE Phosphoramidite	10-1516-95	50 µmole	250.00
	10-1516-90	100 µmole	490.00
	10-1516-02	0.25g	1460.00
tC°-CE Phosphoramidite	10-1517-95	50 µmole	250.00
	10-1517-90	100 µmole	490.00
	10-1517-02	0.25g	1460.00
tC$_{nitro}$-CE Phosphoramidite	10-1518-95	50 µmole	265.00
	10-1518-90	100 µmole	520.00
	10-1518-02	0.25g	1460.00
Ribo-tC°-CE Phosphoramidite	10-3517-95	50 µmole	245.00
	10-3517-90	100 µmole	470.00
	10-3517-02	0.25g	1195.00

DBCO-Serinol Phosphoramidite

Technical Snippets

Question: Why have we been unable to make a 145mer with multiple 5-carboxy-dC additions?

Discussion:

Our customer was attempting to synthesize a 145mer oligo with multiple additions of 5-carboxy-dC (caC) interspersed throughout. The coupling efficiency based on the instrument trityl monitor's readings was excellent but no full length product was later revealed. *After checking that the recommended deprotection procedure was followed correctly, we set out to mimic the synthesis of a 100mer by conducting the synthesis of a mixed base 12mer oligo with three additions of caC. The support was split into four portions and treated as follows:*

- *3 hr at RT with 3% TCA in DCM (deblock)*
- *30 minutes at RT with 0.02 M Iodine oxidizer solution*
- *30 minutes at RT with standard Cap A/B*
- *No treatment which served as a control*

These were then deprotected overnight at RT using 0.4 M NaOH MeOH/water 4:1 (v/v) and then desalted and purified on GlenPak™ cartridges. Analysis of the four oligos by ESI MS showed that three were good but the fourth, treated with excess acetic anhydride capping, showed the presence of an addional hydroxyl group at around the 25% level. This suggested that the caC base was activated by capping, allowing a Michael addition of a hydroxyl ion on deprotection.

Resolution:

We suggested remaking the oligo with capping using UniCap (10-4410-xx), a phosphoramidite-based capping reagent. Our customer reported that the oligo synthesis was then successful.

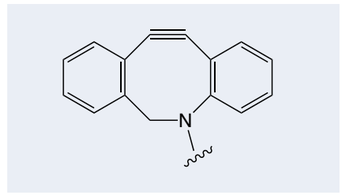


Figure 1. Dibenzocyclooctyl (DBCO) structure

Introduction

Cyclooctyne is the smallest cyclic octyne that can be isolated. Because of the severe deformation of the alkyne from its desired linear geometry, cyclooctynes are highly reactive towards azides without the need for copper catalysis. Copper-free Click Chemistry has some advantages over copper (I) catalyzed [3+2] azide-alkyne cycloaddition (CuAAC). The dibenzocyclooctyne group (DBCO) allows Click Chemistry to be done in the absence of copper, which may have a deleterious effect in biological systems. Also, DBCO is bioorthogonal *in vivo* and will not react with the plethora of other active groups in biological systems, such as hydroxyl, amine, etc. DBCO is especially useful in situations where users are looking for a simple alternative to CuAAC.

Copper-Free Click

From the variety of cyclooctyne-based copper-free click reagents so far described, we chose to offer compounds based on the dibenzocyclooctyl (DBCO) structure, shown in Figure 1.[1-3] DBCO products exhibit the following desirable properties:

- Simple to use.
- Stable in solution on the synthesizer
- Stable to ammonium hydroxide and AMA
- Excellent click performance in 17 hours or less at room temperature

For 5'-modification, we chose to use 5'-DBCO-TEG Phosphoramidite (1) in Figure 2, in which the very hydrophobic DBCO moiety is separated from the phosphoramidite and subsequent oligo with a triethyleneglycol (TEG) spacer. We also chose to offer a soluble DBCO-sulfo-NHS ester sodium salt (2) in Figure 2 for post-synthesis conjugation reactions with amino-modified oligonucleotides and proteins.

In addition to these DBCO-based products, we also offer DBCO-dT-CE Phosphoramidite (3) in Figure 2 for inserting a DBCO group at any position within the oligonucleotide. This type of dT analogue has proved to be popular in the past since the tag is projected into the major groove of duplex DNA where it does not disrupt the DNA duplex, while being readily accessible for further reaction.

For increased versatility, we now introduce a further DBCO phosphoramidite – DBCO-Serinol Phosphoramidite (4) in Figure 2. Using our proprietary serinol backbone as a non-nucleosidic spacer allows the DBCO group to be placed at any location within a sequence with multiple additions clearly possible.

Synthesis and Deprotection

In a recent article,[4] we noted the sensitivity of DBCO to multiple cycles of iodine oxidation. We now recommend that synthesis of oligos containing DBCO be completed using 0.5 M CSO oxidizer after the DBCO addition. Acceptable results can be achieved with iodine oxidation if DBCO is subjected to no more than 8-10 cycles after addition.

A coupling time of 12 minutes was found to be optimal for DBCO-serinol (4). It was found that DBCO-modified oligos were stable to deprotection with ammonium hydroxide for 2 hours at 65°C or overnight at room temperature, which would allow the use of regular phosphoramidites, including dmf-dG but not ibu-dG. Deprotection with AMA for 2 hours at room temperature showed only slight degradation of the cyclooctyne, making the modification compatible with ibu-dG if Ac-dC is used. DBCO-modified oligos are also compatible with UltraMild deprotection conditions.

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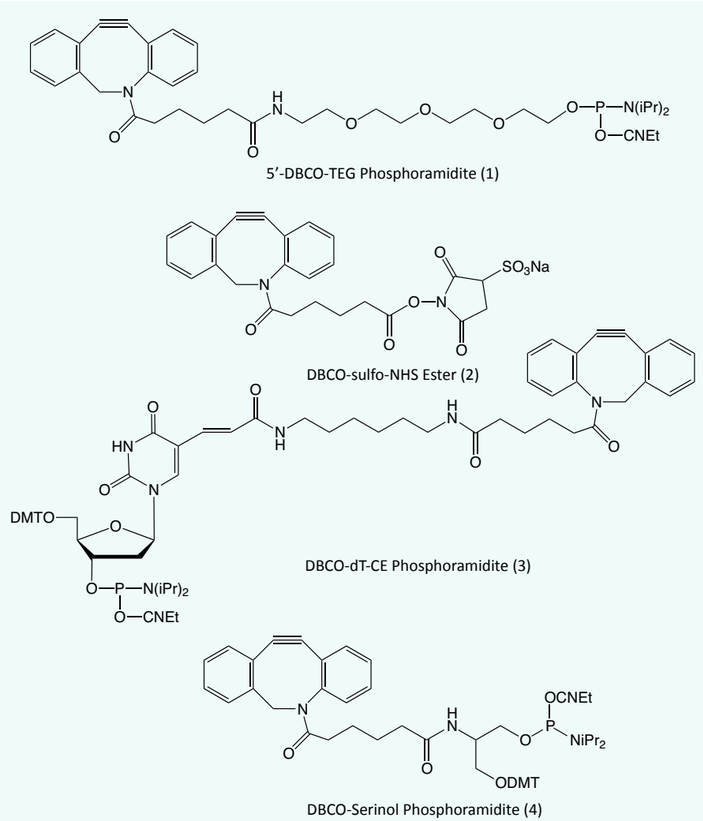

Figure 2. Structures of DBCO Products

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
DBCO-sulfo-NHS Ester	50-1941-23	5.2mg	60.00
(Dissolve 5.2mg in 60µL water or DMSO)	50-1941-24	52mg	300.00
DBCO-dT-CE Phosphoramidite	10-1539-95	50 µmole	250.00
	10-1539-90	100 µmole	485.00
	10-1539-02	0.25g	975.00
DBCO-Serinol Phosphoramidite	10-1998-95	50 µmole	180.00
	10-1998-90	100 µmole	340.00
	10-1998-02	0.25g	895.00

Significant Improvement of CRISPR Specificity with 2’-OMe-PACE Modifications

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Introduction

Over the last 30 years there has been a vigorous search for modifications that enhance or enable nucleic acid therapeutics and diagnostics. The concept is that chemical modifications placed in specific positions can enhance the features of oligonucleotides and therefore impart therapeutic activity, while at the same time enhance the bioavailability through stabilization to nucleases and increased cellular penetration. The results of this search have essentially yielded two categories of chemical modifications showing wide utility:

- phosphorus modifications and
- modification at the 2’-position on the ribose sugar.

Modifications at the 2’-position can affect the binding characteristics to other nucleic acids and inhibit endonucleases but have limited effect on exonucleases, cellular penetration or bioavailability. Phosphorus modifications are useful to inhibit both endonucleases and exonucleases and have also been shown to increase bioavailability through association with serum proteins and enhanced cellular penetration.

Phosphonoacetate (PACE) Modification and Nuclease Resistance

One of the most recent phosphorus modifications to be developed is the phosphonoacetate (or thiophosphonoacetate) modification (PACE) which was reported first by

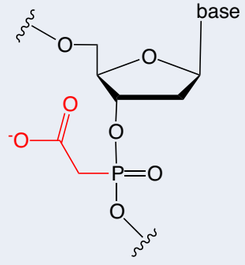

Figure 1. PACE Linkage

Dellinger ***et al.*** in 2003.[1] The PACE modification (Figure 1) contains a carbon-phosphorus bond and, like methylphosphonates, results in an internucleotide bond that is almost impervious to nucleases.

The ability of PACE modified oligonucleotides to impart nuclease resistance while retaining full or enhanced biological activity has been demonstrated.[2] But unlike methylphosphonates, PACE backbone modifications retain the crucial nucleic acid recognition element of a negative charge on the backbone as a result of the carboxylate functional group. This negative charge, in addition to being an important recognition element for nucleic acid binding and function, makes the modification easy to incorporate into standard DNA or RNA chemical synthesis protocols and gives the resulting modified oligonucleotide typical solubility in aqueous solutions. PACE modified oligonucleotides allow efficient unassisted cellular uptake and significant miRNA inhibition effects without the need for transfecting agents.[3] These interesting and easy modifications have been mostly overlooked based upon the timing of their discovery and a two-decade focus on increasing duplex binding affinity.

Effect of PACE Modification on Duplex Stability

In the initial publications on PACE modified oligonucleotides, the duplex stability experiments showed decreased T_m values for PACE modifications.[4] In the case of DNA-DNA duplex experiments, the observed ΔT_m change per linkage modification was -0.3 °C for the phosphonoacetate and -0.5 °C for the thiophosphonoacetate. When RNA was the complementary strand, the duplexes formed A-like DNA-RNA heteroduplexes also having decreased T_m values compared to natural DNA-RNA duplexes. The measured ΔT_m per linkage modification were -1.3 and -1.8 °C for the phosphonoacetate and thiophosphonoacetate, respectively.

In 2012, the synthesis and biological evaluation of 2’-O-methyl-phosphonoacetate and thiophosphonoacetate RNA oligomers were also reported.[3] According to the authors, the first two modifications incorporated, one at each end of the oligomer, increased the T_m by 0.9 °C for an 18-mer, but building in more modifications decreased the T_m by ~-1 °C per modification for phosphonoacetates and ~-0.75 °C per modification for thiophosphonoacetates. Later work[5] using a 20mer RNA annealed to a DNA target strand gave a ΔT_m of -1.0-1.1 °C per incorporation on average, whether the 2’-OMe PACE modifications were consecutively or non-consecutively inserted relative to unmodified RNA. As the thiophosphonoacetate, probes with consecutive incorporations were slightly more destabilizing with a ΔT_m -1.3 °C compared to a ΔT_m of -1.1 °C when the 2’-O-methyl thiophosphonoacetates were inserted non-consecutively within the sequence.

At the time the PACE modifications were introduced, there was a generally held belief that higher duplex stability could provide higher activity, and therefore PACE modifications were not considered as viable candidates for oligonucleotide therapeutics. That view began to change when researchers in the field of siRNA discovered that in certain circumstances decreasing duplex binding affinity decreased off-target activity, and increased on-target activity.[6] As a result, a more modern view is that benefits can be obtained from modifications that either decrease or increase binding affinity. Such modifications can be used to enhance activity and increase specificity, thereby increasing the overall potency and decreasing the toxicity of oligonucleotide therapeutics. Most recently, it has become obvious that oligonucleotide therapeutics that are optimized to resist nucleases and target specific organs are having greater success in clinical trials.[7]

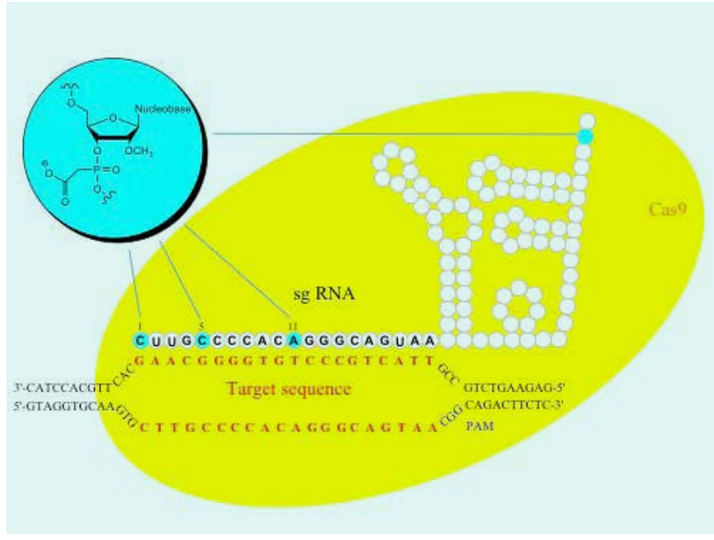

Figure 2. 2’-OMe-PACE (inset) modified sgRNA lead to decreased off-target cutting of Cas9 *in vitro.*

2’-OMe-PACE and CRISPR Gene Editing

PACE modifications have enjoyed a resurgence in interest as applied to the field of CRISPR gene editing. In an initial publication, it was shown that single guide RNAs (sgRNA) provided significantly higher activity in cells when 2’-O-methylthiophosphonoacetates were incorporated on the ends of the guide RNA to protect against cellular nucleases.[8]

In subsequent studies, 2’-OMe PACE modified single guide RNAs were also shown to significantly increase on-target specificity of the CRISPR-Cas9 (***Streptococcus pyogenes***) DNA cleavage in eukaryotic cells. In a recent paper, the incorporation of 2’-OMe PACE modified nucleotides in the 20-nucleotide guide region of the single guide RNA was shown (Figure 2) to decrease off-target cutting by over an order of magnitude while in most cases increasing the overall on-target efficiency as compared to unmodified single guide RNA.[5]

The authors utilized deep sequencing technology to evaluate off-target insertions and deletions (in/dels) at the known sites but also surveyed a library of 960 predicted potential off-target sites which had less than 6 mismatches to their initial intended target site. The authors observed a number of important results. The first was that, in the CRISPR-Cas9 system, the 2’-OMe PACE modification increased the sequence specificity and decreased the off-target cleavage. In addition, the 2’-OMe PACE modification also led to an overall increase of homology directed repair (HDR) at the desired site for gene editing. Finally, the authors found that the 2’-OMe PACE modification at positions 5 or 11 in the guide

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Use of 2’-OMe-PACE Monomers During Oligo Synthesis

Improvement of CRISPR Specificity continued from Page 7

sequence gave the highest increase in sequence specificity across multiple gene targets, suggesting that this modification can be used ubiquitously in CRISPR systems to increase site-specific gene editing. The necessity for a high degree of sequence specificity in the field of Gene Editing is obvious; no one wants to create more mutations in the genome while trying to fix a specific mutation.[9] However, it brings to light that increasing sequence specificity through chemical modifications is possible and will require a next generation of chemical modifications that can easily be incorporated into oligonucleotides using phosphoramidite chemistry.

Conclusion

Probably, the most remarkable characteristic of short oligonucleotides is their ability to differentially recognize and read the linear sequence of another oligonucleotide. No other biological molecule evolved this extraordinary ability. All hybridization based applications in modern biology utilizing chemically synthesized DNA and RNA rely upon this code selective binding phenomenon. This includes PCR, Sequencing, Probe Diagnostics, Array Hybridization, Antisense, siRNA, DNA barcoding, DNA storage, and many more.

We often take for granted that DNA and RNA have the unique ability to distinguish matched from mis-matched sequences, but there are limits. As you change the length, base composition and add modifications to an oligonucleotide, you change its ability to distinguish match from mis-match. In the early days of antisense therapeutics, there was a theory that “DNA Drugs” could be treated just like small molecule therapeutics and assumed they functioned as if the formation of a duplex to a target mRNA was analogous to a receptor/ligand binding event. That assumption led to the idea that tighter binding meant “better” activity. The result was a two decade-long search for “tighter binding” DNA and RNA modifications. Unfortunately, recognizing and preferentially binding to a target DNA or RNA sequence, and not binding to other similar sequences is a more complex event and may require less-tight binding modifications to achieve high target specificity, as shown in the recent CRISPR publication.[5]

As our knowledge of nucleic acid therapeutics has evolved it is clear that many early failures were the result of underestimating the need for a high level of nuclease resistance to allow for full therapeutic effect. As the field continues to evolve, highly nuclease resistant modifications, like PACE, may play a broader role in modification patterns used for therapeutics.

Glen Research is the exclusive provider of PACE modified monomers.

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7. R.A. Haraszti***, et al.***, ***Nucleic Acids Research,*** 2017, **45**, 7581-7592.
8. A. Hendel***, et al.***, ***Nat Biotechnol,*** 2015, **33**, 985-989.
9. H. O’Geen, A.S. Yu, and D.J. Segal, ***Curr Opin Chem Biol,*** 2015, **29**, 72-8.

Use of 2’-OMe-PACE Monomers

The structures of the DNA and 2’-OMe-RNA PACE monomers are shown in Figure 3.

Optimal Cycle

We recommend using DCI as an activator (30-3150-XX) and a 15 minute coupling time.

As with methyl phosphonates, the 2’-OMe-PACE modification is degraded by N-methylimidazole during capping and is susceptible to cleavage during aqueous oxidation using iodine. For this reason, we recommend using Unicap (40-4410-XX), a phosphoramidite-based capping reagent, and 0.5 M CSO (40-4632-XX), a non-aqueous oxidizer, for best results.

Following coupling of the 2’-OMe PACE monomer, cap using Unicap with a regular coupling time and then oxidize using the 0.5 M CSO for 3 minutes.

Alternative Cycle

Alternatively, a 33 minute coupling time using 0.45 M tetrazole, oxidation using low-water iodine (40-4032-XX) followed by capping with 6.5% DMAP as Cap B will give acceptable results.

Deprotection

Pre-treat the synthesis column with 1.5% DBU in anhydrous acetonitrile for 60 minutes at room temperature to remove 1,1-dimethyl-2-cyanoethyl protecting groups. Rinse the column with ACN, dry under with argon and complete deprotection with 40% Methylamine for 2 hours at room temperature.

Purification and Analysis of 2’-OMe-PACE oligos

Using the optimized synthesis conditions listed above, a simple oligo was synthesized for ease and accuracy of analysis:
5’-TXT TXT TXT TTT-3’, where X is 2’-OMe U-PACE.

After the two step deprotection, the oligonucleotide was GlenPak™ purified by standard methods and analyzed by RP HPLC using a Waters X-Bridge column. The resulting chromatogram (Figure 4, Page 10) shows partially resolved diastereomers and a very clean oligo.

However, when the oligo was analyzed by ESI MS, the deconvolved spectrum showed multiple peaks that corresponded to decarboxylation events and the loss of CO_2 (-44 Da) totaling approximately 20% relative to the target mass (Figure 5A, Page 11). This is despite the fact there was no indication of the presence of methyl phosphonate impurities in the HPLC chromatogram. (Note, the small +303 Da peak is a trivial impurity due to incomplete removal of the 5’-DMT during GlenPak™ purification).

Upon review of the mass spectrum prior to deconvolution, an interesting observation was made - only the highly charged ions (-5 or higher) showed the presence of peaks corresponding to decarboxylation, suggesting these peaks were an artifact of the ESI MS. To test this hypothesis, the same sample was re-run using a lower voltage and the deconvolved spectrum showed an amazing improvement. The peaks corresponding to decarboxylation dropped to a mere 3.5% with the largest impurity being a +53 Da

Continued on Page 10

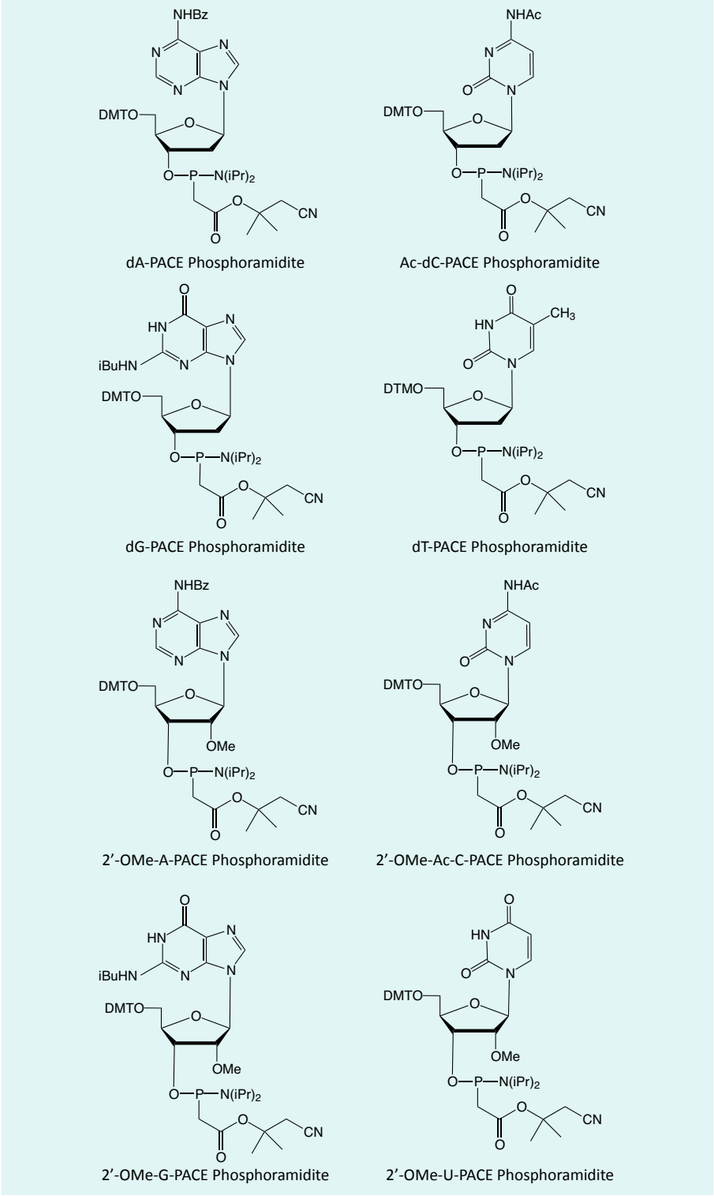


Figure 3. Structures of DNA and 2’-OMe-RNA PACE Monomers

Use of 2’-OMe-PACE Monomers During Oligo Synthesis (cont.)

Continued from Page 9

cyanoethylation peak resulting from the dT CEP monomers incorporated in the oligo (Figure 5B, Page 11).

Note that this cyanoethylation can be avoided by modifying the procedure to remove the cyanoethyl protecting groups by first treating the support for 2-3 minutes with 1.5% DBU in acetonitrile, expelling the solution to waste, and then completing the 60 minute elimination using a fresh solution of 1.5% DBU.

Catalog information for the PACE monomers is shown on Page 11.

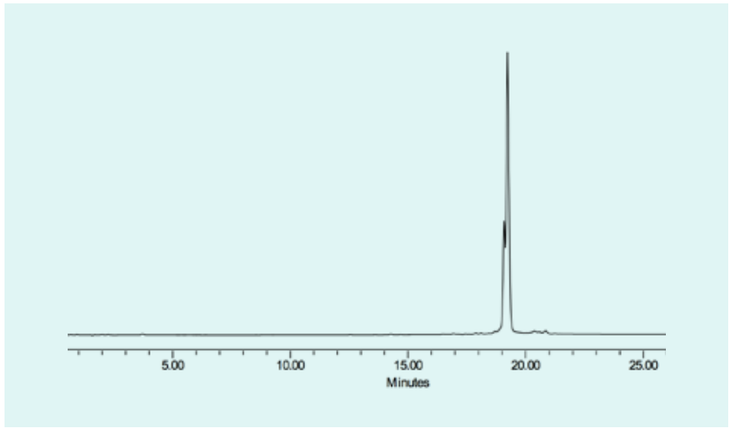


Figure 4. 5’-TXT TXT TXT TTT-3’, where X is 2’-OMe-U-PACE, deprotected as described and analyzed by RP HPLC using a Waters X-Bridge RP HPLC column.

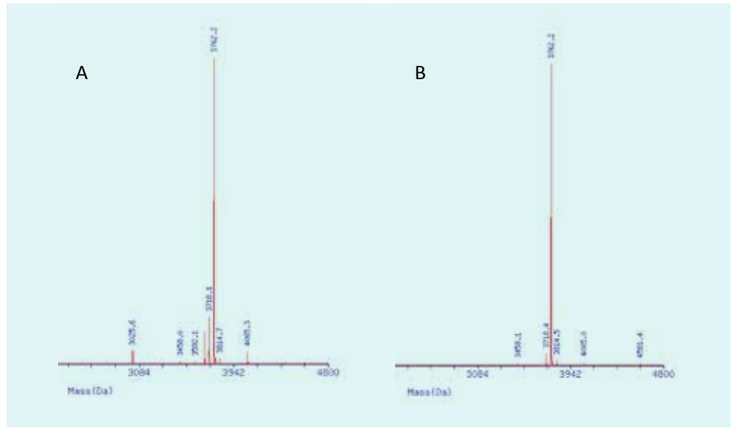


Figure 5A. ESI MS of 5’-TXT TXT TXT TTT-3’ from Figure 4.
Figure 5B. ESI MS of 5’-TXT TXT TXT TTT-3’ repeated at lower voltage.

Item	Catalog No.	Pack	Price ($)
dA-PACE Phosphoramidite	10-1140-02	0.25g	100.00
	10-1140-05	0.5g	200.00
	10-1140-10	1.0g	400.00
Ac-dC-PACE Phosphoramidite	10-1150-02	0.25g	100.00
	10-1150-05	0.5g	200.00
	10-1150-10	1.0g	400.00
dG-PACE Phosphoramidite	10-1160-02	0.25g	100.00
	10-1160-05	0.5g	200.00
	10-1160-10	1.0g	400.00
dT-PACE Phosphoramidite	10-1170-02	0.25g	100.00
	10-1170-05	0.5g	200.00
	10-1170-10	1.0g	400.00
2’-OMe-A-PACE Phosphoramidite	10-3150-02	0.25g	110.00
	10-3150-05	0.5g	220.00
	10-3150-10	1.0g	440.00
2’-OMe-Ac-C-PACE Phosphoramidite	10-3151-02	0.25g	110.00
	10-3151-05	0.5g	220.00
	10-3151-10	1.0g	440.00
2’-OMe-G-PACE Phosphoramidite	10-3152-02	0.25g	110.00
	10-3152-05	0.5g	220.00
	10-3152-10	1.0g	440.00
2’-OMe-U-PACE Phosphoramidite	10-3153-02	0.25g	110.00
	10-3153-05	0.5g	220.00
	10-3153-10	1.0g	440.00

Literature Highlight

RNA Control by Photoreversible Acylation

Willem A. Velema , Anna M. Kietrys, and Eric T. Kool*
Department of Chemistry, Stanford University, Stanford, California 94305, United States

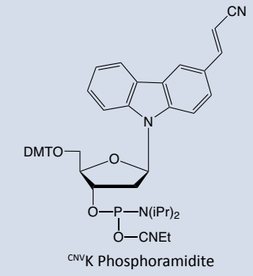
J. Am. Chem. Soc., **2018**, *140* (10), pp 3491–3495

Abstract: External photocontrol over RNA function has emerged as a useful tool for studying nucleic acid biology. Most current methods rely on fully synthetic nucleic acids with photocaged nucleobases, limiting application to relatively short synthetic RNAs. Here we report a method to gain photocontrol over RNA by postsynthetic acylation of 2’-hydroxyls with photoprotecting groups. One-step introduction of these groups efficiently blocks hybridization, which is restored after light exposure. Polyacylation (termed cloaking) enables control over a hammerhead ribozyme, illustrating optical control of RNA catalytic function. Use of the new approach on a transcribed 237 nt RNA aptamer demonstrates the utility of this method to switch on RNA folding in a cellular context, and underlines the potential for application in biological studies.

Lab Talk

Coming Soon

We are happy to announce that Glen Research and Maravai LifeSciences have completed an agreement with Nicca Chemical to begin supplying ^{CNV}K Phosphoramidite to the research market worldwide, with the exception of Japan. You may remember that ^{CNV}K is one of the most effective DNA interstrand crosslinkers so far described.



^{CNV}K Phosphoramidite

We are delighted that ^{CNV}K Phosphoramidite will be available in late spring. Please watch out for the announcement!

Methylene Blue II - A Unique Dye

Methylene Blue, which belongs to the phenothiazine family of dyes, is a unique dye with a variety of useful properties. Despite its high extinction coefficient in the visible region (81,000 L/mol.cm), it is weakly fluorescent due to its high rate of intersystem crossing from the S_1 excited state to the T_1 triplet state. This property makes it an excellent photosensitizer, and it has been used extensively to produce highly reactive singlet oxygen. In DNA, singlet oxygen leads to the oxidation of guanosine, resulting in the formation of 8-oxo-dG. Methylene Blue was subsequently used to determine that DNA polymerase eta (Pol η) was responsible for bypassing this lesion during replication.[1]

Another interesting property of methylene blue is its ability to both intercalate in duplex DNA, preferring G:C over T:A base pairs and its ability to act as an electrochemical redox probe.[2] Exploiting this fact, Pheeny and Barton tethered methylene blue to a single-stranded probe attached to a gold surface to interrogate target oligonucleotides. They were able to detect a single mismatch in the probe-target duplex due to the reduced efficiency of charge transport through a DNA duplex containing a mismatch base pair.[3]

In a comprehensive study of redox-active reporters for electrochemical biosensors, it was found that methylene blue was unmatched in performance.[4]

In an earlier Glen Report,[5] we introduced Methylene Blue C3 Phosphoramidite (1). Unfortunately, this product proved to have quite limited stability and has been discontinued. As an additional option, we introduced Methylene Blue NHS Ester (2) to allow researchers to label amino-modified oligonucleotides with this interesting dye.

With the encouragement and technical expertise of Carole Chaix and her colleagues at the University of Lyon, we decided to prepare an alternative structure that seemed to have a much superior stability profile - Methylene Blue II Phosphoramidite (3). Fortunately, this structure did indeed prove more stable and we are now able to offer again a Methylene Blue Phosphoramidite.

We recommend a 3 minute coupling for Methylene Blue II Phosphoramidite. In addition, UltraMild monomers and capping must be used to allow deprotection with 0.05M potassium carbonate in methanol (Catalog No. 60-4600-30).

The UV/Visible spectrum of an MB labelled oligo is shown in Figure 2.

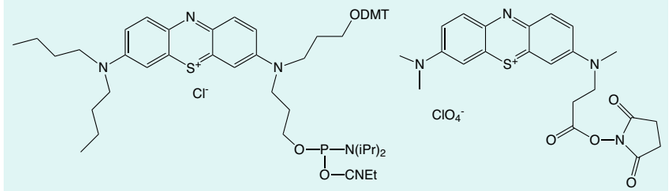
We are happy to provide this unique electrochemical reporter, Methylene Blue II Phosphoramidite, in collaboration with Carole Chaix from the University of Lyon.

Methylene Blue II is covered under patent applications FR12 51739 and PCT/FR2013/050356 and is sold under license from the University of Lyon.

Item	Catalog No.	Pack	Price ($)
Methylene Blue NHS Ester	50-1960-23	5.4mg	540.00
(Dissolve 5.4mg in 60µL of DMSO)			
Methylene Blue II Phosphoramidite	10-5961-95	50 µmole	310.00
	10-5961-90	100 µmole	595.00
	10-5961-02	0.25g	1500.00

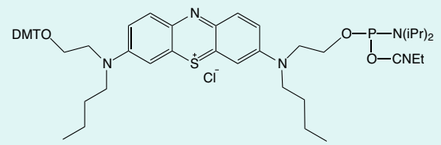
References

1. D.H. Lee, and G.P. Pfeifer, ***Mutat Res,*** 2008, **641**, 19-26.
2. C.G. Pheeney, and J.K. Barton, ***Langmuir,*** 2012, **28**, 7063-70.
3. E. Tuite, and B. Norden, ***J. Amer. Chem. Soc.,*** 1994, **116**, 7548-7556.
4. D. Kang, F. Ricci, R.J. White, and K.W. Plaxco, ***Anal Chem,*** 2016, **88**, 10452-10458.
5. ***The Glen Report,*** 2013, **25.2**, 1-2.



Methylene Blue C3 Phosphoramidite (1)

Methylene Blue NHS Ester (2)



Methylene Blue II Phosphoramidite (3)

Figure 1: Structures of Methylene Blue Products

Figure 2: UV/VIS Spectrum of an oligo labeled with Methylene Blue

Technical Snippets

Question: Why would my synthesis of oligo-dI be disastrous as determined by trityl yield?

Discussion:
We have never encountered someone who has had problems with the synthesis of oligo-dI. How fast did you see the trityl drop? Was it stepwise from the beginning (x % drop every cycle for instance) or did you see the first few trityls looking just fine and then see a quick drop to nothing? How many bases did you add in the synthesis before you saw the issue?
"The trityl was a slow decrease until maybe around base 12-15. We had good color for probably the first 10 or so, with most of the color change happening between bases 10-15. At that point most everything was clear."
We have seen some bases that are sensitive to successive cycles do much better with a low water oxidizer. We suggest trying one more time using the dI you have on the machine and 0.5M CSO instead of the standard Iodine based oxidizer (with a 3 minute oxidation time).
"We were able to run the poly dI with CSO oxidation and found that the synthesis worked well."

Resolution:
Inosine is somewhat susceptible to damage by iodine during oxidation. If there are >6 incorporations of inosine within a sequence, use 0.5M CSO in anhydrous acetonitrile (40-4632-xx) with a 3 minute oxidation time for best results.

Technical Brief – 5’-Phosphorylation of RNA

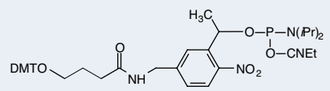
The presence of a 5’ phosphate on RNA oligonucleotides is critical for a variety of biological functions. It is a required structural component for RIG-1 to recognize single-stranded viral RNA and turn on the innate immune response.[1]

Similarly, 5’-phosphorylation of siRNA and piRNA is also required for the RNAi machinery to properly load and suppress gene translation (in the case of double-stranded siRNA) and protect germline cells from genetic damage due to transposons (in the case of single-stranded piRNA).[2]

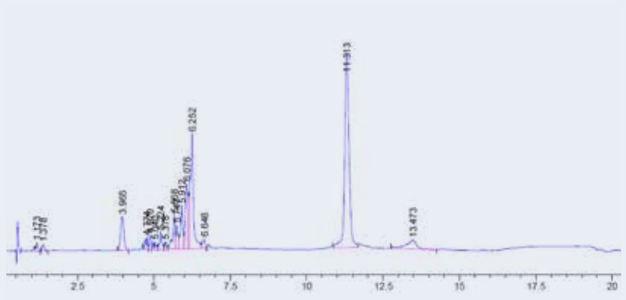
For more practical applications, 5’-phosphorylated RNA allows long RNA oligonucleotides to be synthesized through enzymatic ligation of shorter, more easily purified RNA oligonucleotides.[3]

The efficient production of pure, 5’-phosphorylated RNA oligonucleotides by chemical synthesis has been hampered by the inability to purify these oligonucleotides by reverse phase cartridges or HPLC. While there are a number of options for the DMT-On purification of DNA,[4] they all require treatment with base following the removal of the DMT which leads to both strand scission and 2’-3’ migration of phosphodiester linkages within the RNA.

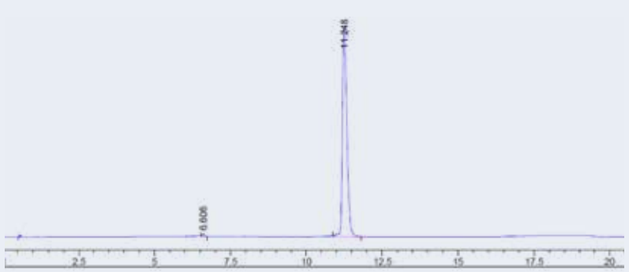
However, a researcher at Horizon Discovery/Dharmacon, Dr. Shoeb Khan, approached us with an elegant solution to this problem using an existing product of ours - the photocleavable PC Spacer. He suggested that by coupling the PC Spacer to the 5’ terminus of the RNA during synthesis, the RNA oligo could be cleaved and deprotected while maintaining the 5’ trityl on the PC Spacer which then could be used as a reverse-phase handle to purify the full-length oligo from the failures. After purification, the entire PC Spacer is removed by simple irradiation with long UV light to reveal a pure, 5’ phosphorylated RNA oligo. We were happy to provide Dr. Khan with reagents for testing his procedure, which worked remarkably well. Dr. Khan has generously shared his results and protocol below.



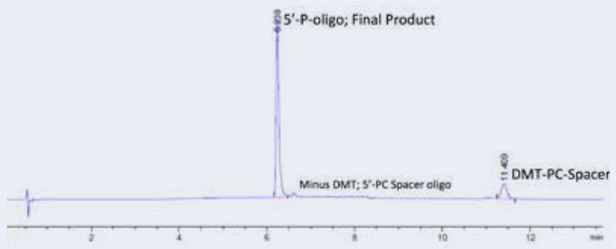
PC Spacer Phosphoramidite



PC Spacer-77mer RNA DMT-ON Crude



PC Spacer-77mer RNA DMT-ON Purified

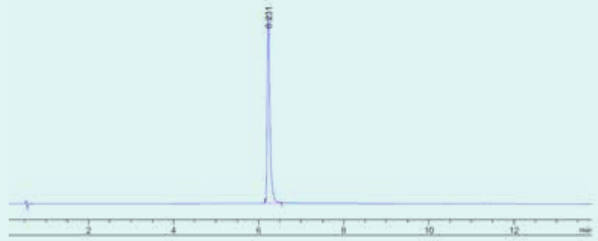


5-Phosphate 77mer RNA - UV Treatment for 20 minutes

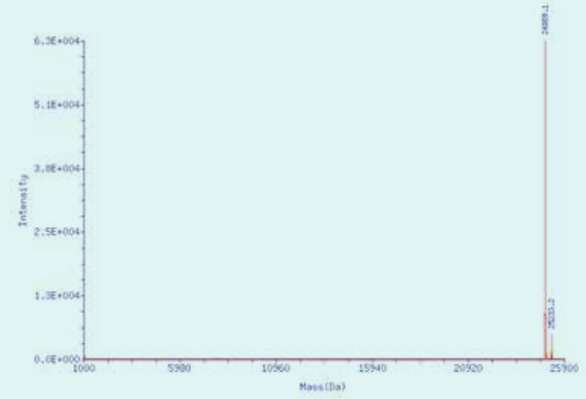
Methods

- 5’-Phosphate 77 bases RNA was synthesized on an Akta Oligopilot synthesizer.
- RNA synthesis was carried out using 2’-TBDMS chemistry.
- DMT-ON method selected.
- Cleavage & deprotection by AMA treatment, 20 min at 65°C.
- 2’-Deprotection by TEA:3HF for 2hr at 65 °C.
- Purified by preparative Waters RP-HPLC.
- Removal of PC-spacer by UV at 365nm for 20min, then additional 20min.
- The oligo was then ethanol precipitated to remove the remnants of the tritylated PC Spacer and analyzed.

We are happy to supply detailed information about Dr. Khan’s method on request. On a smaller synthesis scale, we recommend the use of GlenPak™ RNA cartridge purification as an alternative to HPLC.



5-Phosphate 77mer RNA - UV Treatment for additional 20 minutes



5-Phosphate 77mer RNA - ESI MS

Item	Catalog No.	Pack	Price ($)
PC Spacer Phosphoramidite	10-4913-90	100 µmole	135.00
	10-4913-02	0.25g	395.00

References

1. A. Pichlmair, *et al.*, *Science,* 2006, **314**, 997-1001.
2. M. Ha, and V.N. Kim, *Nat Rev Mol Cell Biol,* 2014, **15**, 509-24.
3. G.C. Walker, O.C. Uhlenbeck, E. Bedows, and R.I. Gumport, *Proc Natl Acad Sci U S A,* 1975, **72**, 122-6.
4. *The Glen Report,* 2011, **23**, 10-11.

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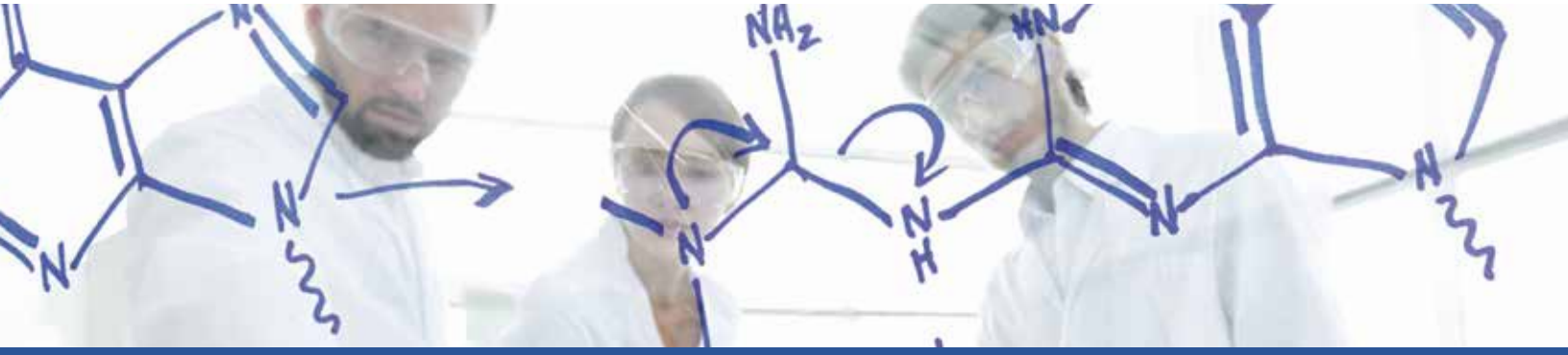

The Glen Report Then and Now

The Glen Report mirrors the advances in oligonucleotide synthesis and modification.

In 1988, Glen Research published The Glen Report, Volume 1, Number 1. We launched simple 5'-amino-modifiers and chemical phosphorylation reagents. The company then offered around 20 products. Fast forward 30 years and Glen Research now offers close to 600 products and The Glen Report now publishes articles from world renowned researchers, as well as work from our own labs. The current publication describes 5'-phosphorylation of long RNA sequences, regulation of off-target effects of CRISPR Cas9, and a fluorescent RNA nucleoside, among the articles. Who would have guessed that 30 years ago? Glen Research is now part of Maravai LifeSciences and the company and its employees are looking forward to a further 30 years of innovation and excellence.

glenresearch.com