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Viewpoint

Robert Letsinger and the Evolution of Oligonucleotide Synthesis

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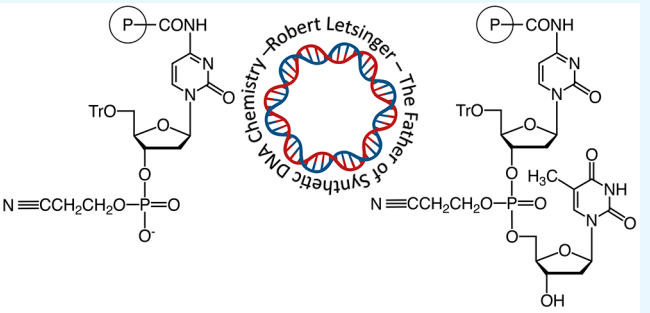
ABSTRACT: This retrospective summarizes a presentation given at the symposium "Bob Letsinger, PhD−100 Years of History" on September 28, 2021 as part of the 17th annual meeting of the Oligonucleotide Therapeutics Society (OTS). In it I look back at my encounters with Robert Lewis Letsinger (1921−2014) while at Northwestern University as Assistant Professor between 1995 and 2000.



1. INTRODUCTION

To mark the 100th anniversary of Robert L. Letsinger's birth in July, 1921, a virtual symposium titled "Bob Letsinger, PhD−100 Years of History" was held on the afternoon of Tuesday, September 28, 2021.[1] It was organized by Muthiah Manoharan, Alnylam Pharmaceuticals (Cambridge, MA), and Masad J. Damha, McGill University (Montreal, Quebec, Canada), as part of the annual meeting by the Oligonucleotide Therapeutics Society (OTS) and brought together a number of Bob's former students and collaborators (Figure 1) to celebrate the life and research of the father of synthetic DNA chemistry.[2]

It was an honor to pay tribute to Bob Letsinger, who was a member of the chemistry faculty at Northwestern University from 1946 until 1991, when he retired as Emeritus Professor. He continued his research collaborations and publication activity until 2008. I probably got to know Bob somewhat later than most of the symposium speakers, namely in the mid-90s when I was Assistant Professor in the Department of Molecular Pharmacology and Biological Chemistry at Northwestern University Medical School in downtown Chicago. Bob's office was in the Chemistry Department of the College of Arts and Science in Evanston, but the two campuses, Evanston and Chicago that is, were quite disconnected at the time. In fact, many on the Evanston campus hardly ever ventured to the Chicago campus and the same was true the other way around; there was no direct bus connection operated by the University. However, as I was working on nucleic acid structure, function, and activity, it was a must to visit Bob, who had pioneered DNA synthesis and contributed to the field in countless other ways. And that marked the beginning of many meetings with Bob.

2. THE EVOLUTION OF OLIGONUCLEOTIDE SYNTHESIS

Figure 2 provides an overview of key steps on the way to the approval of oligonucleotide therapeutics: blue−structure, green−concepts, gray−synthesis, purple−modifications, and orange−initially approved drugs, including antisense, splice site shifting, aptamer, and siRNA oligos.[3] I have circled Bob's pioneering contributions, starting with the solid phase approach, the phosphotriester method, and the P(III) phosphite−triester method that was then refined by Caruthers et al. to the modern phosphoramidite technology. Other participants in the symposium emphasized the importance of Bob's many contributions to the development of automated DNA synthesis.[4] However, this slide also depicts events in the evolution of chemically modified nucleic acids, and I would like to highlight some of Bob's innovative contributions to oligonucleotide analogs and conjugates.

3. VERSATILE FUNCTIONS OF SYNTHETIC OLIGONUCLEOTIDES

Many years ago, Bob used an illustration to depict different classes of oligonucleotides in one of his talks (Figure 3). It divides oligonucleotides into three categories: native oligos that

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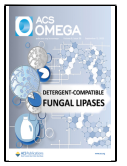

Figure 1. Zoom picture of symposium speakers. Top row: Muthiah Manoharan (Alnylam Pharmaceuticals), Martin Egli (Vanderbilt University), Masad J. Damha (McGill University). Middle row: Marvin H. Caruthers (University of Colorado), Paul S. Miller (Johns Hopkins University), Margaret E. Schott (Northwestern University). Bottom row: Sergei M. Gryaznov (MAIA Biotechnology), Reed Letsinger (son). Missing: Chad A. Mirkin (NWU) and Kevin K. Ogilvie (former President of Acadia University and Senator for Annapolis Valley—Hants, Nova Scotia, Canada). Photo credit: Martin Egli.

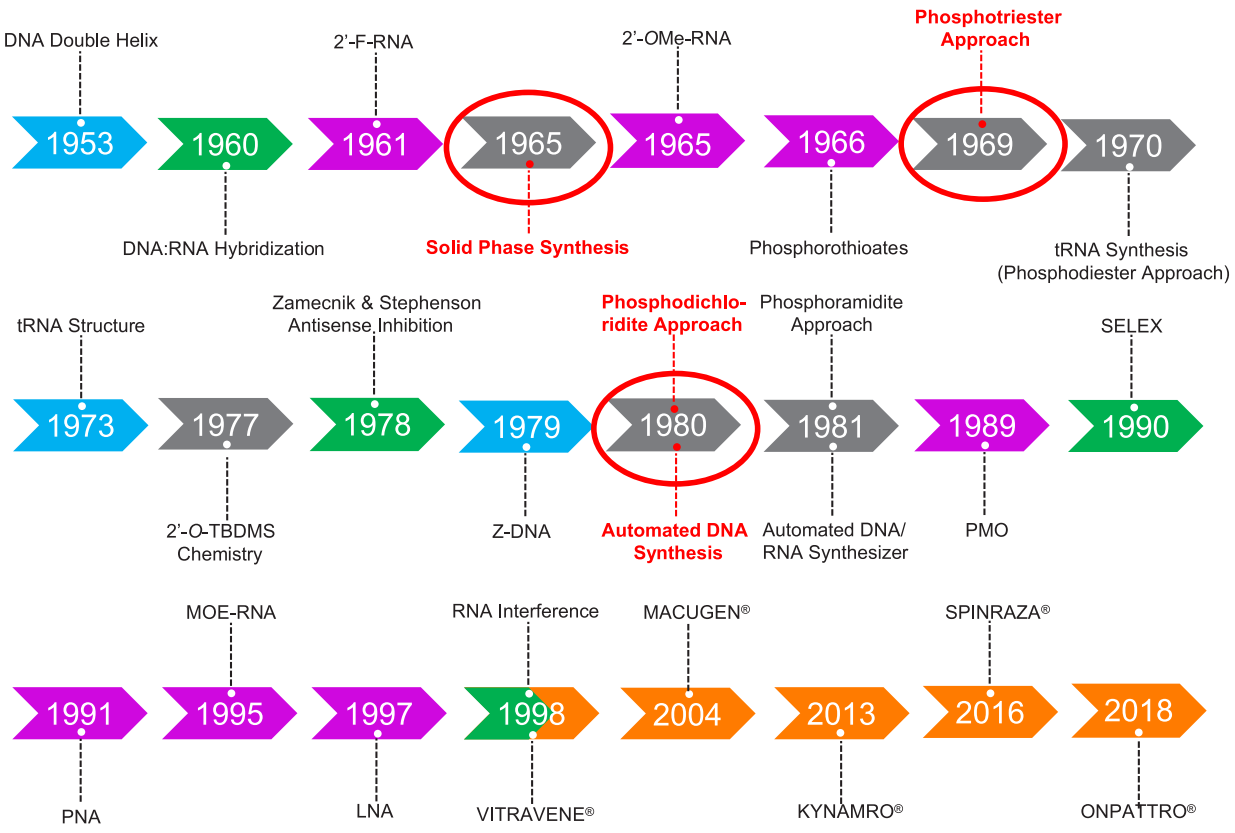

Figure 2. Key steps in the development of oligonucleotide synthesis with selected contributions by Bob circled in red and a (very) brief history of chemically modified nucleic acids and the FDA approval of oligonucleotide therapeutics. See also: https://www.trilinkbiotech.com/a-short-history-of-oligonucleotide-synthesis. Adapted with permission from Egli, M.; Manoharan, M. Chemistry, Structure and Function of Approved Oligonucleotide Therapeutics. *Nucleic Acids Res.* **2023**, *51*, 2529−2573. Copyright 2023 Oxford University Press.[3]

constitute indispensable tools in molecular biology, protein synthesis, PCR, sequencing, and so forth (left); chemically modified oligos for drug discovery and development and other investigations, for example, those directed at nucleic acid etiology, origin of life studies, etc. (center); oligonucleotide conjugates that play important roles in genomics, diagnostics, nanotechnology, and so forth (right). On the left, in Figure 3, I have inserted an offshoot of modified oligonucleotides, so-called xeno nucleic acids or XNAs that are at the heart of synthetic biology and genetics. Over the years, I have modified this scheme somewhat, but Bob's early overview of synthetic oligos and their applications has stood the test of time. I will now discuss some examples from Bob's work that focus on the

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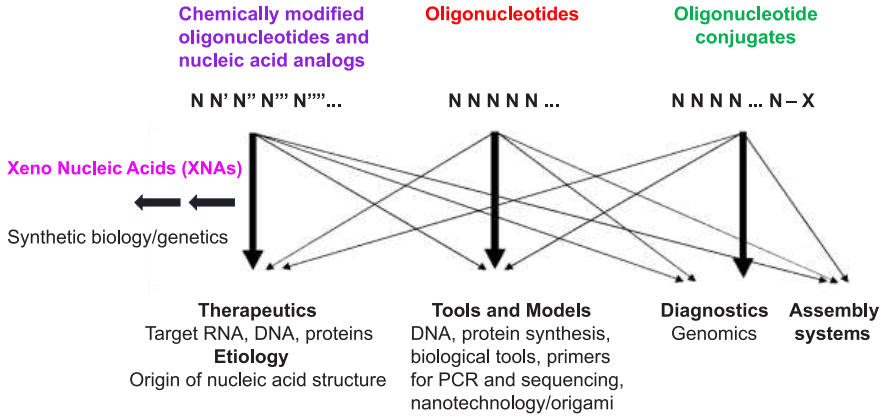

Figure 3. Classification of synthetic oligonucleotides.

modified and conjugated oligonucleotide areas that I was involved in.

4. DISCOVERY AND DEVELOPMENT OF NUCLEIC ACID THERAPEUTICS

Before discussing Bob's work, I would like to remind readers of some of the issues that researchers in the oligonucleotide field at the time considered key to the discovery and development of nucleic acid therapeutics. The graphic shown in Figure 4 is

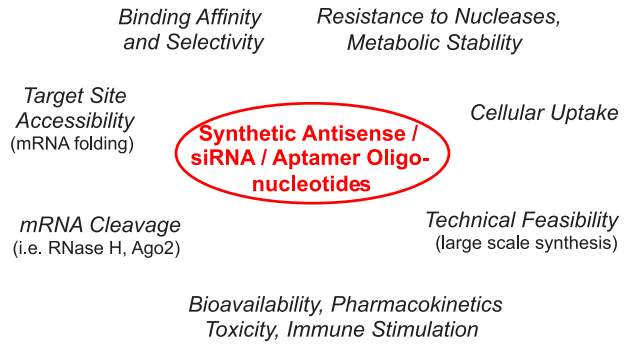

Figure 4. Key issues in the discovery and development of nucleic acid therapeutics.

adapted from an article published by the group headed by Heinz Moser at Ciba Central Research Laboratories in Basel, Switzerland, 25 years ago.[5] As you can see, they focused on binding affinity, nuclease resistance, uptake, technical feasibility, RNA folding, and target accessibility, eliciting key enzymes such as RNase H, pharmacokinetics and -dynamics, as well as toxicity and immune stimulation. As far as binding affinity and pairing selectivity were concerned, concepts such as conformational preorganization and underlying stereoelectronic effects received particular attention. Let me use an example of a DNA analog that was being investigated in the context of an etiology of nucleic acid structure in the research group of Albert Eschenmoser as part of efforts that were initiated over 30 years ago.

5. HOMO-DNA PAIRING

Homo-DNA is 6′ → 4′ linked oligo-2′,3′-dideoxyglucopyranosyl nucleic acid (Figure 5a) compared to the 5′ → 3′ linked DNA (Figure 5b).[6] Homo-DNA does not cross-pair with DNA or RNA or other analogs but displays stable self-pairing. The melting temperature T_m of a homo-DNA dodecamer duplex of

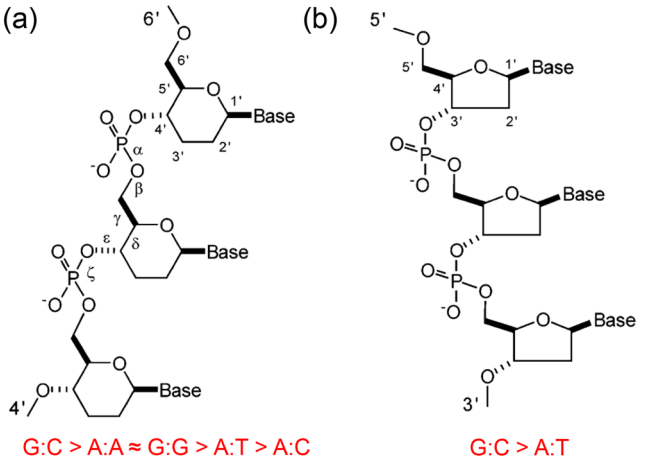

Figure 5. Structures of (a) homo-DNA and (b) DNA and base pairing priorities in the two systems.

sequence CGCGAATTCGCG is 30° higher than that of the corresponding DNA, and the increased stability is based on the entropic term. This is not surprising considering that hexose sugars are more-or-less locked in the chair conformation. However, it is particularly noteworthy that the pairing priorities change owing to the switch from 2′-deoxyribose to 2′,3′-dideoxyglucopyranose in the backbone. Thus, homo-DNA exhibits stable G:G and A:A base pairing of the reverse-Hoogsteen type that is favorable relative to A:T pairing (Figure 5). This is an important lesson and demonstrates that the Watson–Crick pairing priorities are a function of the nature of the sugar in the backbone.

6. HOMO-DNA CONFORMATION

Another lesson from the study of homo-DNA that is clearly of relevance for the natural DNA and RNA counterparts emerges from an analysis of the most likely conformation of its sugar–phosphate backbone that takes into account the following criteria: the conformations around all torsions should be staggered, 1,3- and 1,5-repulsions need to be avoided, and the conformations around the phosphodiester bonds are consistent with the anomeric effect, i.e., overlaps between O3′ and O5′ lone pairs (O4′ and O6′ in the case of homo-DNA) and the antibonding orbitals of the adjacent P–O bonds. Remarkably, this leaves only one repetitive conformation out of a total of 486 (Figure 6a)! A flip of the γ angle from *+sc* to *ap* affords a slightly

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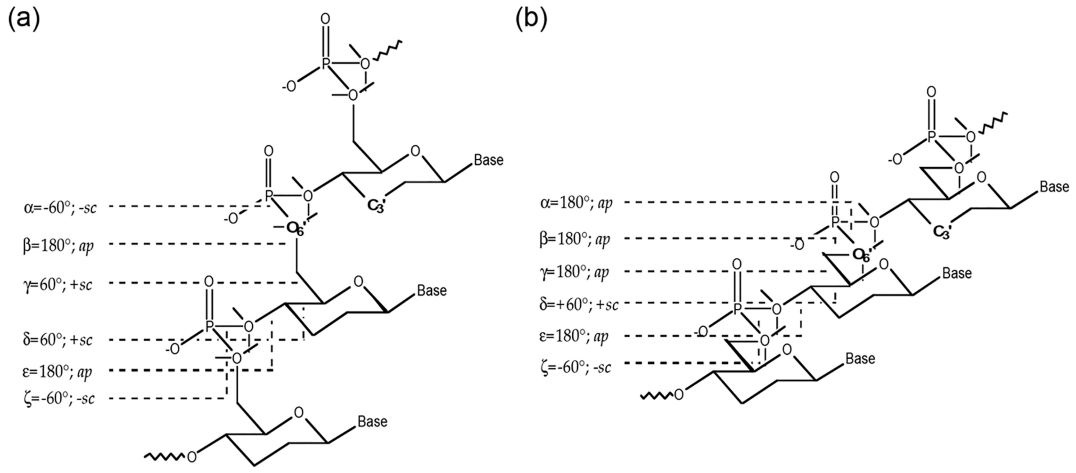

Figure 6. Conformational analysis of homo-DNA and the proposed (a) *sc*/*sc* and (b) *ap*/*sc* (α/ζ) backbone models. For the crystal structure of homo-DNA, please see ref 9.

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Synthesis and properties of oligonucleotides containing aminodeoxythymidine units

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ABSTRACT

Procedures are described for synthesis *via* solid support methodology of oligonucleotide analogues derived in part from 3′-amino-3′-deoxythymidine or 5′-amino-5′-deoxythymidine. Oligothymidylate decamers terminated with a 3′-amino group or containing a 3′-NHP(O)(O⁻)O-5′ internucleoside link are found to form unusually stable complexes with poly(dA), poly(A), and oligo(dA). For related derivatives of 5′-amino-5′-deoxythymidine enhancement is less or absent, and in the case of multiple substitution destabilization of the heteroduplex may be observed. That the effect of the 3′-amino group is general for oligonucleotide derivatives is indicated by enhanced Tm values for heteroduplex complexes of the mixed-base oligomer, d(TATTCAGTCAT(NH$_2$)), and the methyl phosphonate derivatives, TmTmTmTmTmTm-TmTmTmT(NH$_2$) and d(TmAmTmTmCmAmGmTm-CmAmT(NH$_2$)).

polymerase[17,18]; however, they differ in hydrolytic stability in that the P-N bonds are unusually sensitive to aqueous acids[1,9,18] and are unusually resistant to some restriction enzymes.[11–13]

In spite of the extensive work reported on these analogues, no procedures have been described for the solid phase synthesis of oligomers derived from 3′-aminonucleosides and no direct data have been available on the effects of terminal amino groups and internucleoside phosphoramidate links (3′-OP(O)(OR)NH-5′ and 3′-NHP(O)(OR)O-5′) on binding of the modified oligomers to complementary polynucleotide strands. We describe in this paper (a) some useful modifications in methods for incorporating 5′-aminonucleosides into oligonucleotides, (b) a convenient procedure for synthesizing oligomers containing 3′N,5′O phosphoramidate links on a solid support, (c) an extension of this chemistry to enable synthesis of oligomers terminated with a 3′-amino group, and (d) a survey of the binding properties of oligomers containing the aminonucleoside units.

Figure 7. Publication by Gryaznov and Letsinger in *Nucleic Acids Research* describing the synthesis and properties of oligonucleotides containing aminodeoxythymidine units. Adapted with permission from Gryzanov, S. M.; Letsinger, R. L. Synthesis and Properties of Oligonucleotides Containing Aminodeoxythymidine Units. *Nucleic Acids Res.* **1992**, *20*, 3403−3409. Copyright 1992 Oxford University Press.

more extended backbone (Figure 6b), and this is also found in RNA or A-form DNA.[7] However, all other conformations would essentially preclude pairing, "bite themselves in the tail", and so forth. So, first-principles, conformationally speaking, and diamond-lattice sectors[8] essentially help rationalize or predict the B- and A-form DNA conformations. Still: is the anomeric effect really that important?—DNA 5′- and 3′-oxygen feature two lone pairs, and either one or the other lone pair can overlap with the antibonding orbital of the bridging P−O bond.

7. REPLACEMENT OF THE DNA 3′- OR 5′-BRIDGING OXYGEN WITH AN AMINO GROUP

Enter phosphoramidate DNA with either the 3′- or 5′-bridging oxygen replaced by an amino group, as first reported in a paper by Sergei Gryaznov and Bob in *Nucleic Acids Research* in 1992 (Figure 7). That may seem a small change, but of course the amino group introduces an H-bond donor into the DNA backbone that is completely devoid of donors in the parent state. Interestingly, the two analogs showed opposite behaviors in terms of pairing stability: the N3′ modification had a strongly stabilizing influence, but that effect was absent with a single N5′ modification and multiple N5′ substitutions were destabilizing.

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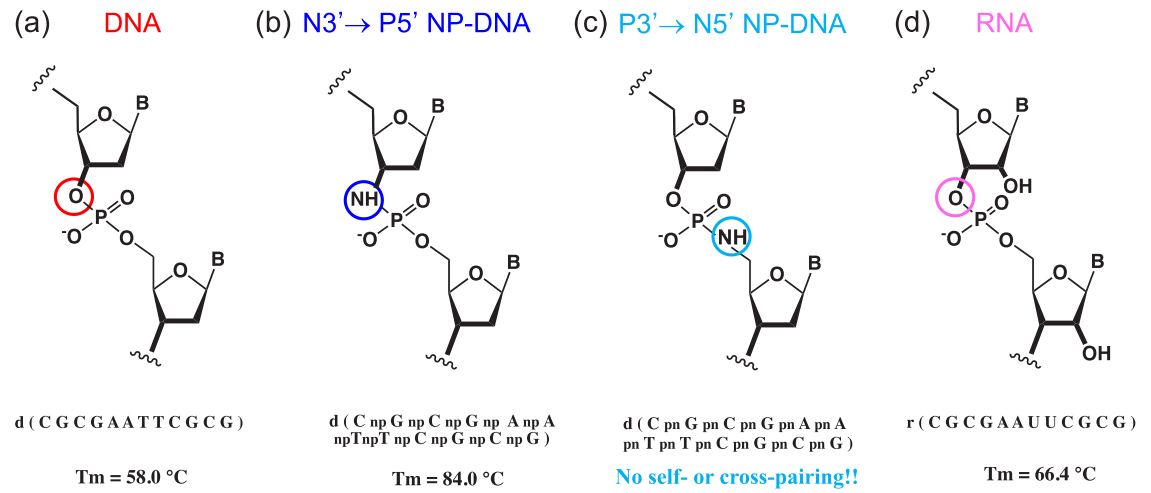

Figure 8. Thermal stabilities of dodecamer duplexes with (a) DNA, (b) N3′ → P5′ phosphoramidate DNA, (c) P3′ → N5′ phosphoramidate DNA, and (d) RNA backbones. The T_m values were measured in 10 mM Tris·HCl pH 7.0 and 150 mM NaCl.

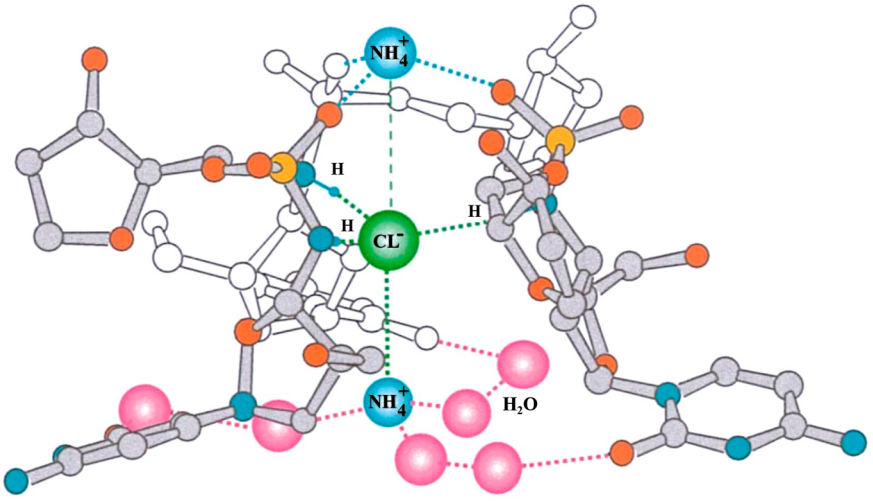

Figure 9. Ion coordination in the crystal structure of the N3′ → P5′ duplex [d(CnpGnpCnpGnpAnpAnpTnpTnpCnpGnpCnpG)]$_2$ established the orientations of the 3′-amino group lone pair and hydrogen. Adapted with permission from Egli, M; Gryaznov, S. M. Synthetic Oligonucleotides as RNA Mimetics: 2′-Modified RNAs and N3′ → P5′ Phosphoramidates. *Cell. Mol. Life Sci.* **2000**, *57*, 1440−1456. Copyright 2000 Springer Nature.[13]

Initially, this is quite surprising as one would probably not expect a regiospecific effect, although the N3′ modification alters the gauche effect with O4′ in the sugar of the so-called N3′ → P5′ phosphoramidate DNA.

8. N3′ → P5′ PHOSPHORAMIDATE DNA, AN RNA MIMIC

Sergei Gryzanov subsequently pursued the synthesis and characterization of fully modified phosphoramidate DNAs, and these have fascinating properties (Figure 8). For example, N3′ → P5′ phosphoramidate DNA (Figure 8b) exceeds by far the stability of either DNA (Figure 8a) or RNA (Figure 8d).[10] In the case shown, the T_m values were measured for the so-called Dickerson−Drew dodecamer sequence. N3′ → P5′ phosphoramidate DNA also cross-pairs with both DNA and RNA. It is actually an RNA mimic, and it was found that HIV-1 TAR RNA, the transactivation response element RNA, or HIV-1 RRE RNA, the Rev-response element RNA, produced as all-phosphoramidate DNAs of the same respective sequence, acts as a decoy for the Tat and Rev binding proteins, respectively, and they do so despite the complete absence of ribose 2′-hydroxyl groups.[11]

However, it is clear that the 2′-deoxyribose of N3′ → P5′ phosphoramidate DNA adopts an RNA-like C3′-*endo* sugar pucker. Conversely, P3′ → N5′ phosphoramidate DNA (Figure 8c) shows no self-pairing and does not pair with either DNA or RNA. How can we explain all these observations, and what does it tell us about the importance of the anomeric effect in the phosphodiester backbone?

9. CRYSTAL STRUCTURE OF AN N3′ → P5′ PHOSPHORAMIDATE DNA DODECAMER DUPLEX

In the crystal lattice of a fully modified N3′ → P5′ phosphoramidate DNA dodecamer (Figure 8b) that was crystallized in the presence of ammonium chloride, ammonium cations and chloride anions are arranged between three adjacent amidate duplexes.[12] Thus, individual ammonium ions bridge three neighboring phosphate groups, and individual chloride ions bridge three neighboring 3′-amino groups (Figure 9). The latter observation informs us of the relative orientations of the amino group lone pair and hydrogen atom in the backbone of N3′ → P5′ phosphoramidate DNA.

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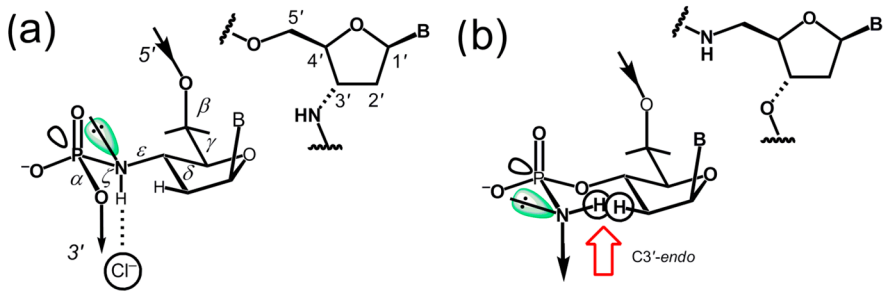

Figure 10. Crystal structure of (a) N3′ → P5′ phosphoramidate-DNA helped rationalize its stable self-pairing and cross-pairing with and mimicking of RNA as well as the inability of (b) P3′ → N5′ phosphoramidate-DNA to self-pair and cross-pair with RNA. Nitrogen lone pair and P−O5′ antibonding orbital are indicated by green and black lobes, respectively, and a red arrow indicates a steric conflict. Adapted with permission from Tereshko, V.; Gryaznov, S.; Egli, M. Consequences of Replacing the DNA 3′-Oxygen by an Amino Group: High-Resolution Crystal Structure of a Fully Modified N3′ → P5′ Phosphoramidate DNA Dodecamer Duplex. *J. Am. Chem. Soc.* **1998**, *120*, 269−283. Copyright 1998 American Chemical Society.[12]

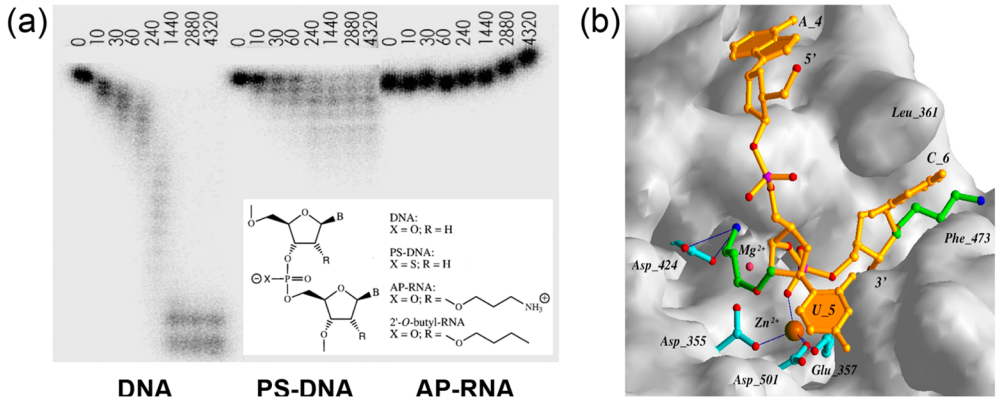

Figure 11. Origins of AP-RNA's resistance to degradation by SVPD. (a) Gel images depicting time courses (in min) of the degradation of DNA, PS-DNA and AP-RNA. (b) Crystal structure of the complex between *E. coli* DNA Pol I Klenow 3′-exonuclease and an oligo-2′-deoxynucleotide carrying three AP-RNA residues at its 3′-terminal end.[14]

10. STRUCTURAL AND STEREOELECTRONIC INSIGHTS FROM N3′ → P5′ PHOSPHORAMIDATE DNA: BACKBONE ANOMERIC EFFECT DOMINATES SUGAR GAUCHE EFFECT

As per the crystal structure of N3′ → P5′ phosphoramidate DNA, the lone pair of the 3′-amino nitrogen indeed overlaps with the antibonding orbital of the P−O5′ bond, consistent with the anomeric effect (Figure 10a). Torsion angles α and ζ around phosphodiester bonds both adopt a gauche conformation, and the N−H bond has an axial orientation in this idealized structure of the backbone, with the sugar assuming a North C3′-*endo* pucker. Moving on to P3′ → N5′ phosphoramidate DNA shown in Figure 10b and attributing the same importance to the anomeric effect, we see that this would lead to a short contact between N−H and H2′ that cannot be relieved without altering backbone torsion angles. This informs us about at least one potential source of destabilization that appears to preclude stable self- and cross-pairing with this analog (Figure 8c). Certainly, phosphoramidate DNA is a fascinating modification that provided insight into fundamental aspects of nucleic acid structure and stability.

11. A ZWITTERIONIC RNA ANALOG

Another early foray by Bob into chemically modified nucleic acids concerned analogs with cationic backbones. In the case of the analog discussed here, 2′-*O*-(3-aminopropyl)-modified RNA (AP-RNA) that was studied at Ionis Pharmaceuticals, the positive charge is tethered to the sugar, thus creating a zwitterionic analog. As depicted in the gel image (Figure 11a), AP-RNA affords excellent protection against attack by a 3′-exonuclease, in this case snake venom phosphodiesterase or SVPD. By comparison, DNA and phosphorothioate DNA (PS-DNA) oligonucleotides get degraded more rapidly,[14] and the neutral 2′-*O*-butyl modification (the degradation data are not shown here) is dealt with swiftly by SVPD as well. In a subsequent study, we were interested in getting to the bottom of how AP-RNA can dodge degradation. Brautigam and Steitz had analyzed the different behaviors of stereopure *Sp*- and *Rp*-phosphorothioate DNA opposite *Escherichia coli* DNA polymerase I Klenow 3′-exonuclease before.[15] We proceeded to use that model system for our structural investigation with AP-RNA. We found in the crystal structure of the complex that the aminopropyl moiety reached into the active site of the enzyme and interfered with the binding of metal ion B, thereby choking activity. The small sphere indicates the former position of the Mg^{2+} that is now absent in the complex with AP-RNA (Figure 11B). In that figure panel, the salt bridge between asparate and aminopropyl is difficult to see.

12. MECHANISM OF 3′-EXONUCLEASE INHIBITION BY AP-RNA

A close-up, schematic version of the inhibition of the 3′-exonuclease by AP-RNA is shown in Figure 12. The blue aminopropyl moiety forms a salt bridge with Asp-424 and precludes the latter's role in stabilizing metal ion B. The

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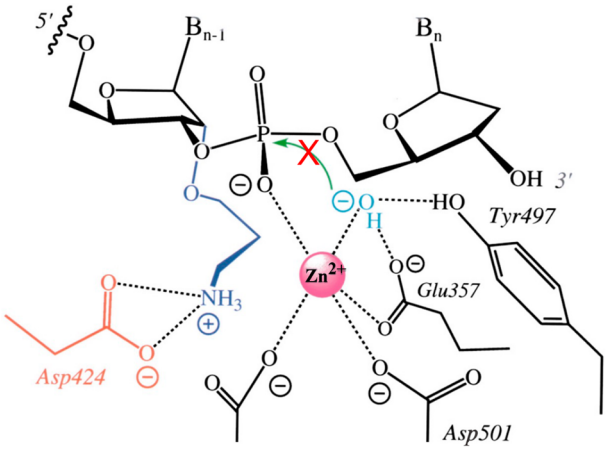

Figure 12. Mechanism of 3′-exonuclease inhibition by AP-RNA. The absence of a second metal ion due to formation of a salt bridge between Asp424 and the 2′-*O*-AP substituent prevents cleavage of the phosphodiester bond (red cross). Adapted with permission from Egli, M; Gryaznov, S. M. Synthetic Oligonucleotides as RNA Mimetics: 2′-Modified RNAs and N3′ → P5′ Phosphoramidates. *Cell. Mol. Life Sci.* **2000**, *57*, 1440−1456. Copyright 2000 Springer Nature.[13]

exonuclease relies on a dual metal ion mechanism and is stopped in its tracks by AP-RNA. Bob had agreed to handle the manuscript for *PNAS* and was instrumental in improving the manuscript. Specifically, one reviewer thought that the mechanism of inhibition we proposed was likely based on a structural artifact. However, we preferred to think more along the lines of *"what you see is what you get"*. Or perhaps we should take Yogi Berra's advice: *"You can observe a lot by watching"*. Bob communicated the paper to *PNAS*, and it still constitutes one of very few experimental studies of the mechanism of nuclease inhibition by an oligonucleotide modification. It demonstrates that both steric and electronic effects need to be taken into consideration.

13. STILBENE−DIAMIDE OLIGONUCLEOTIDE CONJUGATION

Bob was also a pioneer in the field of DNA conjugates. I will mention just one system here that we helped analyze in structural terms: stilbene-capped DNA hairpins, in particular stilbene dicarboxamides as shown schematically in the center of Figure 13. One key property of the stilbene cap is that it shifts the equilibrium toward the monomolecular species, unlike in the case of, say, a $d(T)_4$ loop. The examples at the bottom of Figure 13 demonstrate the high stability afforded by the stilbene cap for a short DNA hairpin with extensive noncanonical secondary structure. Higher stability is not the only interesting property because the singlet state of these stilbene−diamides also acts as an electron acceptor and such conjugates were therefore of interest in connection with electron-transfer studies through stacks of DNA base pairs. Jacky Barton viewed "DNA as a wire" at the time, and Bob, Fred Lewis, and Mike Wasilewski at Northwestern subsequently embarked on a multiyear investigation of stilbene-conjugated and other species, their photochemistry, DNA-guided redox processes, and their theoretical treatment.

14. CRYSTAL STRUCTURES OF STILBENE-CAPPED DNA HAIRPINS

In our studies of stilbene-capped hairpins, we also looked at crystal structures of stilbene−diether conjugates. Unlike the diamides, the singlet state here is an electron donor. In the first structure, we found that four hairpins interacted with each other under formation of a pinwheel-like arrangement, whereby stilbene moieties engaged in face-on stacking interactions with adjacent base pairs but interacted with other stilbenes in an edge-on manner.[16] Interestingly, in a second structure of a stilbene-capped hairpin, the stilbene moiety displayed both the face-on (Figure 14, yellow and cyan) and the edge-on (red) stacking interaction, whereby the *trans*-stilbene lost planarity in the latter case.

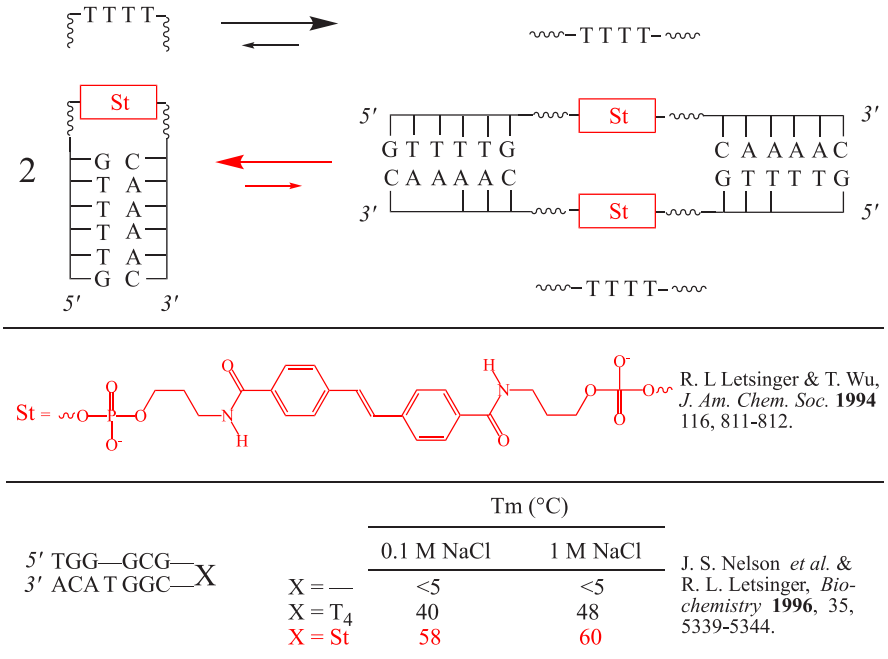

Figure 13. Stilbene−diamide conjugation: electron transfer and record thermal stability.

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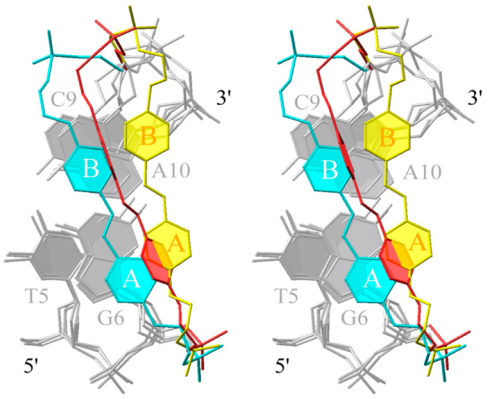

Figure 14. Stereo diagram depicting face-to-face and edge-to-face interactions between *trans*-stilbene and G:C base pairs in the crystal structure of stilbene–diether (Sd)-capped DNAs 5′-GTTTTG-Sd-CAAAAC-3′.[16,17] Adapted with permission from Egli, M.; Tereshko, V.; Murshudov, G. N.; Sanishvili, R.; Liu, X.; Lewis, F. D. Face-to-Face and Edge-to-Face $\pi-\pi$ Interactions in a Synthetic DNA with a Stilbenediether Linker. *J. Am. Chem. Soc.* **2003**, *125*, 10842–10849. Copyright 2003 American Chemical Society.

15. CONCLUSION

During those five years at Northwestern University, I got to appreciate Bob as a mentor, collaborator, and friend who taught me important lessons in DNA synthesis and the exploration of chemically modified and conjugated species. He was generous with his time and sharing his long years of insights in this field. Marv Caruthers mentioned in his obituary of Bob that he challenged students and was willing to take risks.[2] Let me tell you one small anecdote that truly illustrates the latter point. We were both invited to a nucleic acid summer school outside Copenhagen organized by Jesper Wengel in June of 1999. After all the hard work in the classroom, we visited the local amusement park, the Tivoli. The place features a lot of crazy rides, and as we were standing next to one of them, this one pulling a bunch of screaming teenagers high up in the air and then plunging them in a simulated free fall almost to the bottom (Figure 15, left), Bob went over to the booth and purchased a ticket. And there he was, with us standing there in disbelief and astonishment, being lifted up among screaming kids and clearly having a great time. Some risk-taking indeed: "Research is a way of taking calculated risks to bring about incalculable consequences" (Celia Green). Thank you, Bob, and thank you for the opportunity to pay tribute to this inspiring scientist at this symposium.

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Notes

The author declares no competing financial interest.

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Figure 15. Tivoli Gardens in Copenhagen, Denmark. https://www.tivoli.dk/en. Photo credit: "One Day in Copenhagen Itinerary" by Gina: https://www.onedayinacity.com/one-day-in-copenhagen/.

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