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Alternating α,β-Oligothymidylates with Alternating (3′→3′)- and (5′→5′)-Internucleotidic Phosphodiester Linkages as Models for Antisense Oligodeoxyribonucleotides

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Summary: Novel oligothymidylate analogues, achiral at phosphorus, exhibiting enhanced resistance to the action of nucleases and satisfactory hybridizing properties toward complementary DNA and RNA sequences, are proposed as models for antisense oligonucleotides.

The strategy of using synthetic oligonucleotides complementary to mRNA or double-stranded DNA to impair gene expression in living cells has provided the impetus to design and develop oligonucleotide analogues for therapeutic purposes.[2-5] For instance, it has been reported that an oligodeoxyribonucleoside phosphorothioate (28-mer) complementary to the mRNA of the HIV-1 *rev* gene inhibited the cytopathic effect of the virus in chronically infected H9 cells.[6] It could be argued, however, that the anti-HIV activity of the phosphorothioate oligomer may not result from an equal contribution of each of the diastereoisomers stemming from inherent chirality at phosphorus. Consequently, the structure–activity relationship of oligonucleotide analogues could be better rationalized with oligonucleotides achiral at phosphorus. We wish to report the preparation of novel oligothymidylate analogues as models for antisense oligonucleotides. These oligonucleotides, achiral at phosphorus, were designed to form stable hybrids with native complementary DNA and RNA sequences and provide resistance against the nucleolytic activity of extracellular and intracellular phosphodiesterases.

The synthetic design was based on the assumption that oligonucleotides having alternating (3′→3′)- and (5′→5′)-internucleotidic phosphodiester links may not be recognized as rapidly by nucleases as natural oligomers carrying exclusively (3′→5′)-phosphodiester linkages and, therefore, may lead to significant hydrolysis retardation.[7,8] This strategy, however, would reduce by half the number of hybridizing nucleobases and, hence, severely restrict the application of similar oligonucleotides as antisense molecules. Theoretically, the hybridizing abilities of such oligonucleotide analogues could be optimized by the alternate substitution of a β-monodeoxyribonucleotide by an α-monodeoxyribonucleotide in the (3′→3′)- and (5′→5′)-internucleotidic linkage motif. This concept was tested by the first-time synthesis of the α,β-dT_{28} oligomers **1** and **2** via automated solid-phase phosphoramidite methodology.[9,10] The oligothymidylate analogues **3**, α-dT_{28},[11] S-dT_{28}[12,13] along with the natural β-dT_{28} and β-dA_{28} were also

(1) University of California–Berkeley.
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(7) Upon completion of this paper, it has been brought to our attention that oligonucleotides carrying terminal (3′→3′)- and (5′→5′)-internucleotidic phosphodiester linkages were stabilized against intracellular degradation.[8]
(8) Seliger, H.; Fröhlich, A.; Montenarch, M.; Ramalho Ortigao, J. F.; Rösch, H. *Nucleosides Nucleotides* **1991**, in press.
(9) (a) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859–1862. (b) McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245–248. (c) Adams, S. P.; Kavka, K. S.; Wykes, E. J.; Holder, S. B.; Galluppi, G. R. *J. Am. Chem. Soc.* **1983**, *105*, 661–663. (d) Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucl. Acids Res.* **1984**, *12*, 4539–4557.
(10) The deoxyribonucleoside phosphoramidites **4** and **5** required for the synthesis of **1**, **2**, and **3** were easily prepared from nucleosidic precursors according to standard procedures (see supplementary material). This work has been presented, in part, at the International Conference on Nucleic Acid Therapeutics held on January 13–17, 1991 in Clearwater Beach, FL.
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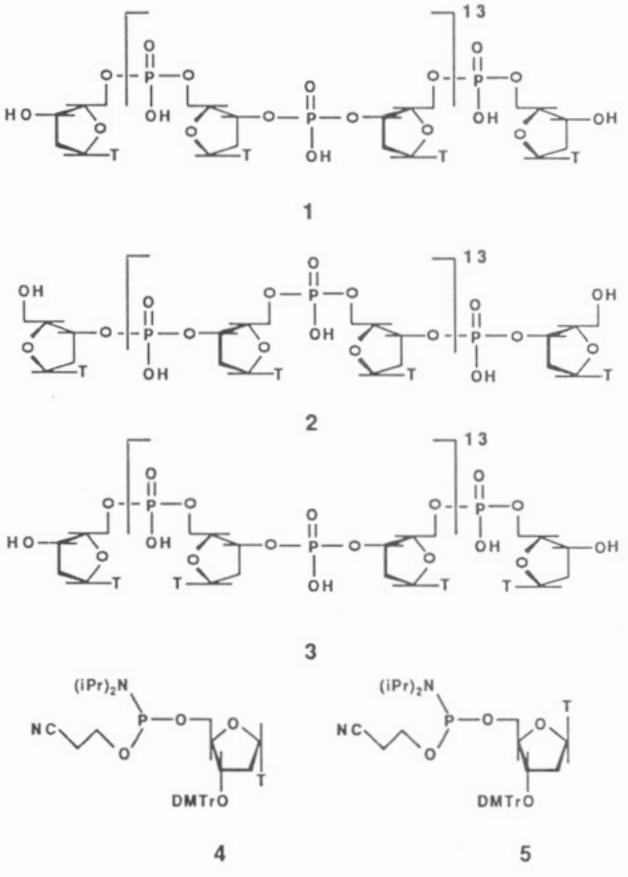
Table I. Thermal Denaturation Temperatures of Hybrids Formed with Oligothymidylate Analogues and Their Complementary Unmodified DNA and RNA Sequences

	T_m (°C)		
oligomer	β-dA_{28} (PBS)	β-dA_{28} (1 M NaCl PBS)	Poly rA (1 M NaCl PBS)
β-dT_{28}	54	68	63
α,β-dT_{28} (1)	37	55	44
α,β-dT_{28} (2)	37	55	44
β-dT_{28} (3)		22	22
α-dT_{28}	52	70	77
S-dT_{28}	37	51	45

prepared to instigate meaningful control experiments. The relative electrophoretic mobility of each purified oligothymidylate on a 20% polyacrylamide-7 M urea gel is shown in Figure 1.



Equimolar amounts of oligothymidylate and β-dA_{28} or poly rA (MW > 100 000) were annealed in PBS[14] or 1 M NaCl PBS at 20 °C. The thermal denaturation profile of each complex was recorded[15] and the T_m values reported in Table I. The oligomer **1** or **2** hybridized to β-dA_{28} and poly rA, as the resulting complexes had a T_m of 55 and 44 °C, respectively, in 1 M NaCl PBS. These hybrids were considerably more stable than those formed with the β-dT_{28} oligomer **3**, which exhibited a T_m of only 22 °C under the same conditions (Table I).

The difference in the length of the (3′→3′)- and (5′→5′)-phosphodiester bridges relative to the natural (3′→5′)-phosphodiester bridge may account for the lower T_m values observed with the complexes formed with **1** or **2** relative to those formed with the native β-dT_{28} oligomer. Interestingly, the phosphorothioate oligomer S-dT_{28} generated complexes with β-dA_{28} and poly rA having stabilities similar to those formed with **1** or **2** in PBS or 1 M NaCl PBS.

The oligothymidylates α,β-dT_{28} **1** and **2** exhibited enhanced resistance to hydrolysis by snake venom (SVP) and

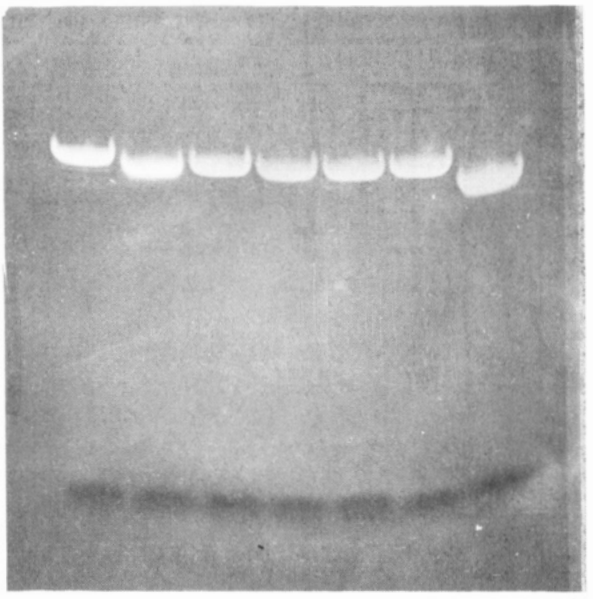


Figure 1. Relative electrophoretic mobility of oligothymidylates (28-mers) at pH 8.3 (1X TBE buffer) on a 20% polyacrylamide–7 M urea gel. From left to right: S-dT_{28}; α-dT_{28}; β-dT_{28} (**3**); α,β-dT_{28} (**2**); α,β-dT_{28} (**1**); β-dT_{28}; β-dA_{28}.

Table II. Relative Stability of Oligothymidylate Analogues to the Nucleolytic Activity of S1 Nuclease, Snake Venom (SVP), and Calf Spleen (CSP) Phosphodiesterases

	estimated percentage of hydrolysis (%)		
oligomer	S1	SVP	CSP
β-dT_{28}	100 (0.25 h)	100 (0.25 h)	100 (0.25 h)
α,β-dT_{28} (1)	12 (8 h)	45 (0.5 h);[c] 73 (1 h)[c]	6 (4 h); 11 (24 h)
α,β-dT_{28} (2)	23 (2 h);[a] 43 (8 h)[a]	49 (0.5 h); 78 (1 h)	50 (24 h)[e]
β-dT_{28} (3)	31 (2 h); 78 (8 h)	19 (0.5 h);[d] 49 (1 h)[d]	5 (24 h)
α-dT_{28}	9 (8 h)	96 (0.5 h)	16 (4 h); 40 (24 h)
S-dT_{28}	100 (0.5 h)[b]	11 (1 h); 27 (2 h)	5 (24 h)

[a] Based on α,β-$d(T_{28},T_{27})$. [b] Incubation of S-dT_{28} in S1 buffer without added enzyme at 37 °C for ca. 4 h did not promote detectable desulfurization of the oligomer according to ^{31}P NMR spectroscopy. [c] Based on α,β-$d(T_{28}, T_{27}, T_{26})$. [d] Based on β-$d(T_{28}, T_{27}, T_{26})$. [e] The only degradation product (50%) is tentatively ascribed to α,β-dT_{27}.

(12) Like **1**, **2**, **3**, α-dT_{28}, β-dT_{28}, and β-dA_{28}, the preparation of S-dT_{28} was achieved according to the solid-phase phosphoramidite approach. However, the standard aqueous oxidation step of the synthetic cycle was replaced by a sulfurization reaction effected by a 0.05 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile.[13]

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(14) The composition of PBS (1× phosphate buffered saline pH 7.2; S. and S. Media, Inc.) is as follows: NaCl (8.5 g/L), NaH_2PO_4 (0.73 g/L), KH_2PO_4 (0.21 g/L).

(15) The thermal denaturation of DNA complexes and DNA/RNA hybrids in proper buffers was performed at a rate of 1 °C/min at 260 nm by a thermoelectrically controlled Perkin-Elmer Lambda 6 UV/vis spectrophotometer interfaced with an Epson Equity II computer.

calf spleen (CSP) phosphodiesterases (a 3′-exonuclease and a 5′-exonuclease, respectively) relative to β-dT_{28} and α-dT_{28} (Table II).[16] Particularly, CSP hydrolyzed the oligothymidylate 1 to the extent of 11% after 24 h at 37 °C. Under these conditions, α-dT_{28} was 40% hydrolyzed whereas β-dT_{28} was completely hydrolyzed within 15 min. β-dT_{28} was also completely digested by SVP within 15 min at 37 °C. However, the hydrolysis of α-dT_{28} by this phosphodiesterase was slower than the hydrolysis of β-dT_{28} but was at least 2 times faster than the hydrolysis of the oligothymidylate 2. The incubation of α,β-dT_{28} (1) with S1 nuclease (predominantly an endonuclease) for 8 h at 37 °C led to the partial hydrolysis of the oligothymidylate (12%, Table II). In contrast, β-dT_{28} and S-dT_{28} were completely hydrolyzed by S1 nuclease within 15 and 30 min, respectively, under identical conditions.

In conclusion, we have demonstrated that the α,β-oligothymidylates 1 and 2 were easily prepared and formed stable hybrids with complementary DNA and RNA sequences that had T_m values similar to those obtained with the phosphorothioate oligomer S-dT_{28}. We have also shown that 1 and 2 were resistant to endonucleolytic and exonucleolytic hydrolysis relative to β-dT_{28}, α-dT_{28}, and S-dT_{28}. To further assess the potential of alternating α,β-oligodeoxyribonucleotides with alternating (3′→3′)- and (5′→5′)-internucleotidic phosphodiester linkages as antisense molecules, the preparation of oligomers having the four different nucleobases is in progress in our laboratory. The ability of these oligonucleotide analogues to form a sequence-specific triple helix with large double-helical DNA in vitro or target particular mRNA in HIV-infected cells will be determined, and the data will be reported in due course.

Supplementary Material Available: General procedures regarding the preparation of the deoxyribonucleoside phosphoramidites and their precursors required for the solid-phase synthesis of the oligothymidylates mentioned in this paper along with a general procedure for the derivatization of long chain alkylamine controlled pore glass (LCAA-CPG) with nucleoside analogues (3 pages). Ordering information is given on any current masthead page.

(16) The conditions for the enzymatic digestion of the oligothymidylates were the following: (i) To 0.6 $O.D_{260}$ of oligothymidylate was added 60 μL of 1 M Tris·HCl (pH 9), water to a total volume of 597 μL, and 3 μL of snake venom phosphodiesterase (*Crotallus durissus*, 9 × 10^{-3} U); (ii) To 0.6 $O.D_{260}$ of oligothymidylate was added 60 μL of 1 M ammonium acetate (pH 6.5), water to a total volume of 599 μL, and calf spleen phosphodiesterase (4 × 10^{-3} U, 1 μL); (iii) To 0.3 $O.D_{260}$ of oligothymidylate was added 30 μL of 10X S1 buffer (0.33 M sodium acetate pH 4.5, 0.5 M sodium chloride, 0.3 mM zinc sulfate), water to a total volume of 298 μL, and S1 nuclease (20 U, 2 μL). These digestion reaction were incubated at 37 °C. Aliquots (100 μL) of the digests were withdrawn at various time points, added to concentrated ammonium hydroxide (500 μL), and subsequently evaporated to dryness under reduced pressure. The hydrolyzates were dissolved in water (75 μL) and directly analyzed by an Applied Biosystems Model 270A Capillary Electrophoresis instrument equipped with Micro-Gel_{100} capillaries operating at 300 V/cm in 75 mM Tris–phosphate buffer (pH 7.6) containing 10% methanol. Unless otherwise indicated (Table II), the estimated percentage of oligonucleotide hydrolysis is defined as the integrated area under the peaks corresponding to fragments smaller than the full length oligomer.

Mechanism of Conjugate Additions of Dialkylcuprates to Bromonaphthoquinones

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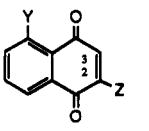
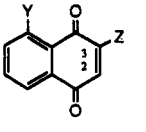
Summary: The correlation of bromonaphthoquinone redox potentials with reactivity toward dialkylcuprates reveals that the conjugate addition of Gilman reagents to bromojuglones proceeds via a single-electron-transfer mechanism.

Lithium dialkylcuprates (R_2CuLi·LiI) are known to react with α,β-unsaturated carbonyl compounds to give the corresponding β-alkylated enolate.[1] However, despite efforts since the early sixties, the detailed mechanism of this conjugate addition remains uncertain. Copper(III) is often suggested as a plausible intermediate,[2,3] but there remains the question of how the copper(III) intermediate is formed. Two accepted working hypotheses are: (1) direct nucleophilic addition of the organocopper species to the β-position and (2) stepwise addition consisting of an initial single-electron transfer (SET) from the cuprate to the carbonyl compound followed by combination of the carbonyl radical anion with the oxidized cuprate complex.[2]

In this paper we show that the regiochemistry of conjugate addition to bromonaphthoquinones 1 and 2 allows unambiguous distinction between the direct addition and SET pathways, and that the SET mechanism is in fact operative in these reactions.





1	Y = OH	Z = Br	2	Y = OH	Z = Br
3a	Y = OH	Z = t-Bu	4a	Y = OH	Z = t-Bu
3b	Y = OH	Z = Me	4b	Y = OH	Z = Me
6	Y = OAc	Z = Br	5	Y = H	Z = Br
9	Y = O-	Z = Br	7	Y = OAc	Z = Br
			8	Y = O-	Z = Br

According to FMO theory direct nucleophilic conjugate addition to bromojuglones 1 and 2 should occur at the unsubstituted carbon, which has the largest LUMO coefficient.[4,5] In fact, regiospecific nucleophilic additions

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