Deoxyoligonucleotides Bearing Neutral Analogues of Phosphodiester Linkages Recognize Duplex DNA via Triple-Helix Formation

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The sequence-specific recognition of duplex DNA using unmodified deoxyoligonucleotides (DONs) via triple-strand formation has been demonstrated[1] and offers a significant opportunity to develop a new class of pharmaceutical agents. Unmodified DONs are susceptible to nucleases and may prove to have difficulty in crossing cell membranes.[2] Neutral analogues of phosphodiesters have attracted attention as solutions to the nuclease and cellular permeation problems[3] and have been used to target messenger RNA in cell culture systems. Little has been reported on the ability of DONs containing neutral replacements of the phosphodiester to form a triple helix with duplex DNA under physiological salt conditions.

Methylphosphonates (MPs) have been reported to form triple helices with very short DONs[4] and longer sequences,[5] however, MPs are also reported not to form triple-stranded structures.[6] We report the synthesis and triple-helix formation of DONs containing the diastereomeric MP[7] and (methoxyethyl)phosphoramidate (MEA),[8] and the achiral formacetal[9] and the previously unreported 5′ thioformacetal. A footprint assay[10] was used which allows for the assessment of the modified linkages' effect on binding affinity and specificity.

Synthesis. The sequences tested along with the synthetic scheme are shown in Figure 1. Each analogue linkage was incorporated into a 5-methyl-N-benzoyl-2′-deoxycytidine–thymidine (5-MeC–T) dimer protected at the 5′ end with dimethoxytrityl (DMT) and derivatized at the 3′ end with a H-phosphonate salt.[11] The MP 1,[7] MEA 2,[8] and formacetal 3[9a] linkages were synthesized as previously reported. The thioformacetal 4 was synthesized in an analogous manner to that of the formacetal.[12] Phosphonylation

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t. R = CH₃
2. R = —NH—CH₂—CH₂OCH₃

3. X = O
4. X = S

5

6

Sequence: 5′ TpCpTpC^Me•TpC^Me•TpC^Me•TpC^Me•TpTpTpT

7 • phosphodiester
8 • formacetal
9 • thioformacetal
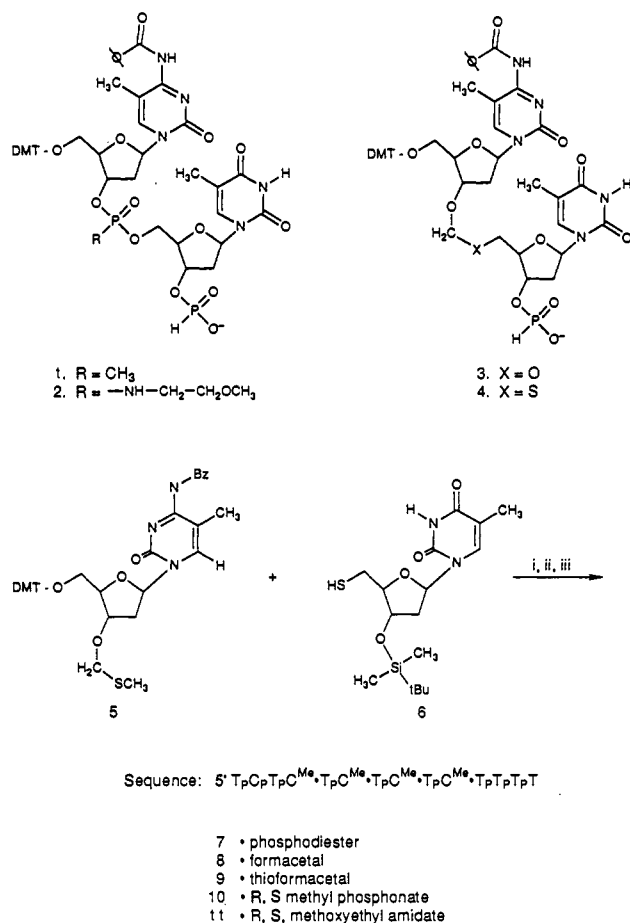10 • R, S methyl phosphonate
11 • R, S, methoxyethyl amidate

Figure 1. Dimer synthons, sequences, and synthetic scheme for thioformacetal: (i) Br₂, 4-Å molecular sieves, 2,6-diethylpyridine/benzene; (ii) Bu₄NF/THF; (iii) 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one/pyridine.

of all dimers and subsequent incorporation into DONs on a controlled pore glass (CPG) support was as previously reported.[11,15]

Characterization. The DONs bearing formacetal 8 and thioformacetal linkages 9 were characterized by end labeling and selective partial degradation of the modified linkage.[16] Limited formic acid treatment resulted in partial cleavage at the formacetal[9a] and thioformacetal linkages showing the presence of the four analogue linkages in each DON. The control DON 7, containing only phosphodiester linkages, showed no cleavage. Limited bromine treatment partially cleaved the thioformacetal linkages in 9,[17] with the formacetal 8 and phosphodiester 7 DONs showing no cleavage under the same conditions. The MEA 11 and MP 10 were also radiolabeled, and the modified linkages were mapped using piperidine as previously reported[7,8] (data not shown).

Triple-Helix Binding. The affinity and specificity analysis was performed using the DNA footprinting technique that has been extensively exploited to elucidate the sequence specificity of DNA protein interactions. This technique has also been used to map

(12) 5^9a was activated with bromine in the presence of 4-Å molecular sieves and 2,6-diethylpyridine in benzene, and 6[13] was then added. Desilylation with TBAF in THF[14] followed by phosphonylation[11] yielded 4.
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(16) Data shown in supplementary material.
(17) Bromine has been used as the deprotection agent of the methylthioformacetal protecting group in organic synthesis and would be expected to cleave the thioformacetal linkages: Greene, T. W. *Prot. Groups Org. Synth.* **1981**, 297.
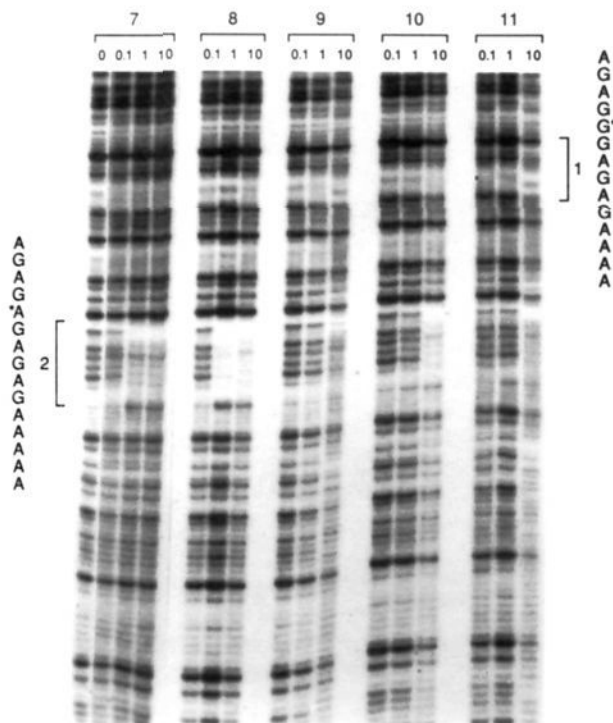
Figure 2. Footprint analysis. Conditions for footprint: Concentrations of DONs shown were in micromoles/liter, and the radiolabeled target duplex (370-bp restriction fragment derived from a PUC vector containing a synthetic DNA insert) was approximately 1 nM in 20 mM MOPS, pH 6.8, 140 mM KCl, 10 mM NaCl, 1 mM MgCl$_2$, 1 mM spermine hydrochloride. Triple-stranded hybridizations (50 μL) were done for 1 h at 37 °C followed by limited DNase I digestion (2 units for 1 min), followed by EDTA quenching, PAGE, and autoradiography.

triple-helix formation.[1b,18] The target sequence is shown in Figure 2 along with the autoradiogram resulting from the footprint experiment. The target sequence has been repeated a second time, an adenosine to guanosine base change having been introduced in the polypurine tract, creating a single triplet mismatch for the test DONs. This allows for the assessment of the ability of a given DON to discriminate a perfect match from one mismatch. The region of the mismatch target is shown by a bar labeled 1 on the autoradiogram, and the perfect match is labeled 2.

The control phosphodiester DON **7** shows protection of the target from DNase I when present at 1 μM, and binding to the single mismatched target occurs only at 100 μM. The formacetal DON **8** shows a similar specific footprint. The MP DON **10** requires a >10-fold-higher concentration to give similar protection as compared to the two previous DONs. The MEA **11** and thioformacetal **9** DONs show reduced binding relative to **10** with partial protection from DNase being observed at 10 μM.

The formacetal linkage is competent for sequence-specific triple-helix binding when placed in a 5-MeC–T context. The shorter 3'-oxygen to 5'-oxygen distance in the formacetal linkage relative to a phosphodiester is perhaps not a liability, given the shorter ribose to ribose distances in A-form helix versus B-form.[19] The reduced binding of the thioformacetal is surprising.[20] The

MP **10** required a >10-fold-higher concentration for binding relative to the diester and formacetal DONs.[21] The MEA **11** is clearly inferior to MP in this context. This result parallels the two linkages' ability to form duplex structures with single-stranded DNA and RNA.[8b,9c] These results show the promise of neutral achiral formacetal DON analogues as agents capable of sequence-specific triple-helix formation.

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(20) Model building suggests that the 3'-carbon to 5'-carbon distance in the thioformacetal is comparable to that of a phosphodiester, yet this linkage confers reduced triplex binding properties on the DON relative to a phosphodiester and formacetal. Modelling also suggests a close contact between the 5'-oxygen of the formacetal linkage and the 6-hydrogen of the thymine ring. This contact could be disturbed by the substitution at the 5'-position with the larger, less polar sulfur (modelling performed using Biograf software by Molecular Simulations).

(21) **10** and **11** are mixtures of 16 diastereomers. One of the isomers in **10** could be the active agent, and consequently, on a molar basis, this isomer would then have an affinity comparable to that of a phosphodiester.

Mechanistic Studies on DNA Photolyase. 3. The Trapping of the One-Bond-Cleaved Intermediate from a Photodimer Radical Cation Model System

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DNA photolyase[1] is the enzyme involved in the cleavage of pyrimidine photodimers in UV-damaged DNA (eq 1). Although several model systems for this reaction have been reported, their mechanisms remain poorly understood.[2] The quinone-sensitized
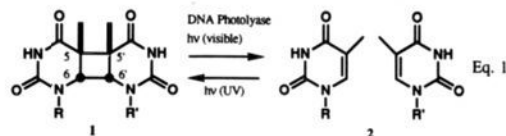
cleavage, for example, has been proposed[2] to proceed by electron transfer from the photodimer to the photoexcited quinone followed by sequential cleavage of the C6,C6' and the C5,C5' bonds of the photodimer and reduction of the uracil radical cation by the semiquinone radical (Scheme I). The only experimental evidence in support of this proposal is the observation of CIDNP in the product.[3] With the view of developing mechanistic probes for the enzymatic reaction, we have synthesized a photodimer substituted with a radical trap designed to block the quinone-sensitized photodimer fragmentation after the first CC bond cleavage.

The most frequently used trap for enzyme-generated radicals involves the rapid ring opening of the methylcyclopropyl radical.[4]

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