Hybridization properties of deoxyoligonucleotides containing anthraquinone pseudonucleosides

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ABSTRACT

Achiral pseudonucleosides bearing an anthraquinone moiety have been incorporated into deoxyoligonucleotides in internal, 3' and 5' positions. The ability of these modified deoxyoligonucleotides to hybridize to complementary RNA and DNA was investigated using T_m measurements. The anthraquinone was shown to enhance binding to a complementary RNA when linked to the 3' and 5' end. Pseudonucleoside substitutions on the 3' end of an oligonucleotide are also shown to confer serum stability to the oligonucleotide.

INTRODUCTION

The use of modified deoxyoligonucleotides for the regulation of gene expression is a current area of intense interest (1, 2). One approach is the incorporation of an intercalating agent into an oligonucleotide in order to enhance its affinity for a complementary sequence. This concept was pioneered by Letsinger using phenanthridinium as the intercalating agent (3) and has been extended by Helene using acridine (4, 5, 6, 7, 8, 9). One report has used anthraquinone as the intercalating moiety (10). We wish to report the use of anthraquinone conjugates on a pseudonucleoside scaffold, their incorporation into multiple positions within deoxyoligonucleotides, and their binding properties to complementary single stranded DNA and RNA.

The use of pseudonucleosides in which the deoxyribose is replaced by another diol moiety has been reported (11, 13). 1-Amino 2,3-dihydroxy propane has been used wherein the amino group is used as a derivatizable handle for reporter groups. The primary hydroxy serves as the 5' hydroxyl mimic of the ribose, and the secondary hydroxy serves as the 3' hydroxyl. The disadvantage of these derivatives is the chiral nature of the derivatized propane. This may not be an important issue for reporter groups, but with applications using the pseudonucleosides as scaffolds for intercalating agents, such chirality is undesirable. Chirality leads to diastereomers in the final product, each of which has potentially different binding properties. This differential diastereomer-binding problem has been recently demonstrated in modified oligonucleotides in the case of the chiral methyl phosphonate internucleotide linkages (14).

A solution to this problem in the pseudonucleoside case is to use of a non-prochiral amine. Diethanol amine and its homologs are simple, commercially available reagents which meet the

requirements. The amine nitrogen is used as the handle by which the binding agent, in this case the intercalator anthraquinone, is attached.

We have derivatized anthraquinone on the 2 position with diethanol amine, and appropriately derivatized this diol for incorporation into deoxyoligonucleotides (Scheme 1). A dimethoxytrityl (DMT) H-phosphonate derivative was prepared for incorporation into multiple sites of the oligonucleotide. A DMT derivative was also conjugated to Controlled Pore Glass (CPG) through an ester linkage (8, 15) for incorporation at the 3' end of an oligonucleotide. A variety of sequences was synthesized and tested for binding affinity to complementary RNA and DNA.

Scheme 1 - Synthesis of Pseudonucleosides

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MATERIALS AND METHODS

All oligonucleotide synthesis was done using H-phosphonate chemistry (8, 15) on a Biosearch 8750 synthesizer. T_m measurements were done on a Gilford Response II Spectrophotometer. The complementary RNA sequence was prepared from a DNA template using T7 polymerase (16). NMR spectra were recorded on a GE QE300 instrument.

Preparation of Anthraquinone Pseudonucleosides

Mono Dimethoxytrityl Ether of 2-(N,N-diethanolamino)-anthraquinone, 2: A mixture of 2-chloroanthraquinone (2.42g; 10mmole) and an excess (3.8ml; 40 mmole) of diethanolamine in DMSO (20ml) was heated to 150°C. After 24 hours, the reaction mixture was cooled to room temperature, then poured into water (70ml). The red precipitate was filtered off, washed thoroughly with water, and dried under vacuum. The crude product **1** was used without further purification.

The crude compound **1** was dissolved in pyridine (20ml) and triethyl amine (1.7ml), cooled to 0°C, followed by addition of 4-dimethylaminopyridine (DMAP; 0.2g) and 4,4'-dimethoxytrityl chloride (DMT-Cl; 4.0g; 12mmol). The reaction mixture was warmed to room temperature. After 4 hours of reaction, more DMT-Cl (1g) was added to the reaction mixture and reacted one more hour, then concentrated to dryness. The residue was then partitioned between methylene chloride and saturated sodium bicarbonate solution. The organic solution was separated and dried, purified by flash column chromatography on silica gel, eluted with 1% Et₃N/1% CH₃OH/CH₂Cl₂ to afford the product mono-DMT-protected 2-N,N-diethanolaminoanthraquinone, **2**, in an amount of 0.7g (13% overall yield) as a purple oil; 3.1g of unreacted starting material was recovered. NMR(CDCl₃): 8.29 (dd, 1H), 8.25 (dd, 1H), 8.08 (d, 1H), 7.75 (m, 2H), 7.42 (d, 1H), 7.38 (d, 2H), 7.20–7.30 (m, 7H), 6.92 (d, 1H), 6.24 (d, 4H), 3.88 (q, 2H), 3.68–3.80 (m + s, 10H), 3.48 (t, 2H).

Mono Dimethoxytrityl Ether of 2-(N,N-diethanolamino)-anthraquinone was prepared by same method as compound **2**. NMR (CDCl₃): 8.30 (d, 1H), 8.25 (d, 1H), 8.10 (d, 1H), 7.75 (m, 2H), 7.15–7.55 (m, 11H), 6.24 (d, 4H), 3.80 (s, 6H), 3.70 (t, 2H), 3.42 (q, 4H), 3.08 (m, 2H), 1.03–1.80 (m, 16H).

Conjugation to a CPG Support, 3: A mixture of the mono dimethoxytrityl ether of 2-(N,N-diethanolamino) anthraquinone (0.5g; 0.81mmol), DMAP (0.1g) and succinic anhydride (0.325g; 3.26mmol) in pyridine (10ml) was stirred at room temperature for 4 hours, and more succinic anhydride (0.1g) was added to

the reaction. After 2 more hours of reaction, the residue was dissolved in methylene chloride, washed with 1M triethylammonium bicarbonate (TEAB) aqueous solution. The organic solution was isolated, dried over Na₂SO₄, concentrated, then purified by flash column chromatography and eluted with 1% Et₃N/2% CH₃OH/CH₂Cl₂, 1% Et₃N/5% CH₃OH/CH₂Cl₂, to afford the succinylated product. The succinylated form of the subject compound (0.39g) was isolated as a red solid (yield 59%). NMR (CDCl₃): 8.28 (dd, 1H), 8.25 (dd, 1H), 8.12 (d, 1H), 7.68–7.80 (m, 2H), 7.48 (d, 1H), 7.36 (d, 2H), 7.20–7.30 (m, 7H), 6.96 (dd, 1H), 6.75 (d, 4H), 4.28 (t, 2H), 3.80 (t, 2H), 3.65–3.75 (m, 8H), 3.40 (t, 2H), 2.85 (q, 6H), 2.48–2.62 (m, 4H), 1.20 (t, 9H).

Succinylated dihexanolamine derivative was prepared as described on diethanolamino compound. NMR (CDCl₃): 8.27 (dd, 1H), 8.23 (dd, 1H), 8.15 (d, 1H), 7.66–7.78 (m, 4H), 7.38–7.48 (m, 3H), 7.18–7.38 (m, 7H), 6.88 (d, 1H), 6.82 (d, 2H), 4.07 (t, 2H), 3.77 (s, 6H), 3.35–3.50 (m, 4H), 3.06 (t, 2H), 2.87 (q, 6H), 2.50–2.70 (2d, 4H), 1.30–1.75 (m, 16H), 1.18 (t, 9H).

132 mg (150μmoles) of the succinylated compound **2**, 40 mg (300μmoles) of diisopropylcarbodiimide, a catalytic amount of DMAP (10 mg) and aminopropyl (70μmoles/g) CPG in DMF/pyridine (4/1; 4ml) were shaken at 20°C overnight and then acetylated with acetic anhydride/pyridine (1/1). After 4 hours acetylation at room temperature and quenching by slow addition of methanol, the CPG was filtered off and washed thoroughly with methylene chloride, methanol, and ether, and dried under vacuum overnight. The loading is 15μmole/g. The resulting CPG derivative with anthraquinone-coupled pseudonucleoside is then used to provide the pseudonucleoside at the 3' terminus of an oligonucleotide.

Phosphorylation of Mono-DMT-protected Anthraquinone Pseudonucleosides, 4: To a 0°C cold methylene chloride solution (8ml) of the mono dimethoxytrityl ether of 2-(N,N-diethanolamino)anthraquinone, **2** (0.24g; 0.39mmol) were added pyridine (0.1ml) and 2-chloro-4H-1,2,3-benzodioxaphosphorin-4-one (1.17ml of 1M methylene chloride solution; 1.17mmol). After 0.5 hours of reaction at 0°C, the reaction mixture was washed with 1M TEAB aqueous solution. The organic solution was separated, dried over Na₂SO₄, and purified by flash column chromatography, eluted with 1.5% Et₃N/5% CH₃OH/CH₂Cl₂. Fractions of the product were combined, washed with 1M TEAB aqueous solution, dried over Na₂SO₄, and concentrated, affording phosphorylated DMT-protected anthraquinone

Table 1.

No.	Oligomer	ΔT_m °C ^a		T_m °C ^a	
		RNA ^b	DNA ^c	RNA ^b	DNA ^c
1	5'-CCC-TCT-CTT-TTT-CCP ^d	+4.0	+4.5	64.0	56.5
2	5'-CCC-TCT-PCT-TTT-TCC	-0.5	+3.0	59.5	55.0
3	5'-CCC-TCT-CPT-TTT-TCC	-0.5	+2.5	59.5	54.5
4	5'-PCC-CTC-TCT-TTT-TCC	+4.0	+5.0	64.0	57.0
5	5'-CCC-TCT-PCT-TTT-TCC-P	+4.0	+7.5	64.0	59.5
6	5'-PCC-CTC-TCT-TTT-TCC-P	+8.0	+9.0	68.0	61.0
7	5'-PCC-CTC-TPC-TTT-TTC-CP	+7.5	+11.0	67.5	63.0
Control	5'-CCC-TCT-CTT-TTT-CC	-	-	60.0	52.0

^a T_m values $\pm 0.2^\circ\text{C}$

^b ~1.6μM DNA/RNA (1/1) in 150mM NaCl/50mM Tris, pH 7.5

^c ~5μM DNA/DNA (1/1) in 150mM NaCl/10mM Na₂HPO₄, pH 7.5

^d P = 2-(N,N-Diethanolamino)anthraquinone pseudonucleoside

pseudonucleotide, 4. NMR (CDCl₃): 8.28 (dd, 1H), 8.25 (dd, 1H), 8.12 (d, 1H), 7.89 (s, 1/2H), 7.65–7.80 (m, 2H), 7.48 (d, 1H), 7.38 (d, 2H), 7.15–7.30 (m, 7H), 7.0 (d, 1H), 6.75 (d, 4H), 5.85 (s, 1/2H), 4.08 (q, 2H), 3.75–3.80 (m, 4H), 3.72 (s, 6H), 3.38 (t, 2H), 3.0 (q, 6H), 1.30 (t, 9H).

Phosphorylated DMT-protected dihexanolamino anthraquinone pseudonucleotide was prepared as compound 4. NMR (CDCl₃): 8.30 (dd, 1H), 8.28 (dd, 1H), 8.15 (d, 1H), 7.90 (s, 1/2H), 7.65–7.80 (m, 2H), 7.15–7.50 (m, 10H), 6.75–6.93 (m, 5H), 5.85 (s, 1/2H), 3.85–3.95 (q, 2H), 3.80 (s, 6H), 3.30–3.50 (m, 4H), 2.95–3.20 (m, 8H), 1.15–1.80 (m, 25H).

RESULTS AND DISCUSSION

The sequences synthesized are shown in Table 1 along with the T_m of each on complementary DNA and RNA. It is interesting to note that the anthraquinone is stabilizing in all positions when the complement is DNA. This is not the case with RNA as the target when the anthraquinone is incorporated into the middle of the oligonucleotide. In these cases the effect is slightly destabilizing. In the case of RNA hybridization the most stable oligonucleotide is one bearing the anthraquinone pseudonucleoside on both the 3' and 5' ends. The combination of these two substitutions has a roughly additive effect on the delta T_m .

One question resulting from the above study is the effect of linker arm length on the T_m stabilization values observed with the anthraquinone pseudonucleoside. Selected sequences were resynthesized with di-hexanol amine as the pseudonucleoside in

a manner analogous to the use of diethanol amine in Scheme 1. The sequences and their resulting T_m properties with complementary RNA are shown in Table 2. These results are roughly similar to those observed with the diethanol pseudonucleoside (Table 1). The largest difference is the combination of a 5' and 3' pseudonucleoside has less of an additive effect on T_m as compared to the diethanol amine series. The conclusion is that the length of the linker arm is not very pronounced, but the diethanol amine series appeared to be superior.

Another question is the effect of the anthraquinone pseudobases on the specificity of hybridization with RNA. Two experiments were performed addressing this issue. The first was an assessment of the effect of a 5' and 3' anthraquinone on the recognition of a mismatch in the middle of the oligonucleotide–RNA duplex, (Table 3). The conclusion is that the two anthraquinones discriminated a mismatch to approximately the same degree as an unmodified control. This preservation of specificity is a requirement for any modified oligonucleotide's use in an *in vivo* setting. The other experiment was the effect of a 3' anthraquinone on the discrimination of a mismatch at the 3' end. This context places the mismatched base pair next to the anthraquinone. The data are again shown in Table 3. The anthraquinone pseudobase actually enhances specificity in this particular case. This can be rationalized by the possible inability of the anthraquinone to stack efficiently on a mispaired base pair. This effect is provocative in that it suggests the potential to design oligonucleotide analogs which are more discriminating of mismatches in target RNA sequences than parent oligonucleotides.

Deoxynucleotide bearing the anthraquinone pseudobases at their 3' or 5' ends are interesting candidates for use in the *in vivo* regulation of gene expression by binding to specific messenger RNAs. A requirement for such an application is a reasonable stability of the oligonucleotide in blood. The stability in sera of a modified oligonucleotide bearing an anthraquinone pseudobase was compared to the control oligonucleotide without the anthraquinone (sequence 5' TTTTCTCCAT-anthra) (17). The conditions of the experiment used RPMI media with 10% fetal calf serum at 37°C. The control oligonucleotide was completely degraded in one hour while the anthraquinone oligonucleotide had a half-life of 5 hours. This substantial stabilization is consistent with other experiments in which the 3' end of an oligonucleotide has been blocked in some manner for the purpose of slowing or precluding 3' exonuclease attack (18, 19, 20).

Anthraquinone pseudonucleosides have been shown to increase the stability of oligonucleotide hybrids with complementary RNA when incorporated at the 3' and 5' end of an oligonucleotide. This stabilization does not result in a loss of specificity; in fact, experiments suggest that in the 3' end modification, specificity is enhanced. Additionally, 3' end modifications stabilize the oligonucleotide to nucleases found in sera. All these results suggest that oligonucleotides bearing anthraquinone pseudonucleotides are candidates for *in vivo* antisense RNA applications.

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Table 2.

Oligomer	T_m , °C ^a	ΔT_m , °C ^a
1* 5'-CCC-TCT-CTT-TTT-CCP*	64.0	4.0
2* 5'-CCC-TCT-P*CT-TTT-TCC	57.5	-2.5
4* 5'-P*CC-CTC-TCT-TTT-TCC	63.5	3.5
5* 5'-CCC-TCT-P*CT-TTT-TCC-P*	58.5	-1.5
6* 5'-P*CC-CTC-TCT-TTT-TCC-P*	65.0	5.0
Control 5'-CCC-TCT-CTT-TTT-CC	60.0	—

Salt condition and concentrations: same as Table 1

^a T_m values $\pm 0.2^\circ\text{C}$

P* = 2-(N,N-Dihexanolamino)anthraquinone

Table 3.

	T_m , °C ^a	ΔT_m , °C
Effect of Mismatch on Controls:		
complement 14mer 5'-CCC-TCT-CTT-TTT-CC	60.5	—
complement 15mer 5'-T-CCC-TCT-CTT-TTT-CC	61.0	+0.5
mismatch 14mer 5'-CCC-TCT-TTT-TTT-CC	52.5	-8.0
Effect of Mismatch with Anthraquinone:		
complement 5'-P-CCC-TCT-CTT-TTT-CCP	68.0	—
mismatch 5'-P-CCC-TCT-TTT-TTT-CCP	60.5	-7.5
Effect of 3'-Terminal Mismatch on Control:		
complement 5'-CCC-TCT-CTT-TTT-CC	60.0	—
mismatch 5'-CCC-TCT-CTT-TTT-CT	57.5	-2.5
Effect of 3'-Terminal Mismatch with Anthraquinone:		
complement 5'-CCC-TCT-CTT-TTT-CC-P	64	—
mismatch 5'-CCC-TCT-CTT-TTT-CT-P	59.5	-4.5

P = 2-(N,N-Diethanolamino)anthraquinone pseudonucleoside

^a T_m values $\pm 0.2^\circ\text{C}$

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