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2′-*O*-[2-(Guanidinium)ethyl]-Modified Oligonucleotides: Stabilizing Effect on Duplex and Triplex Structures†

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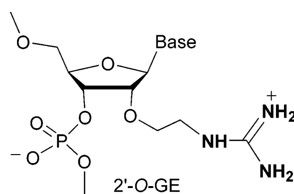
ABSTRACT



Oligonucleotides with a novel 2′-*O*-[2-(guanidinium)ethyl] (2′-*O*-GE) modification have been synthesized using a novel protecting group strategy for the guanidinium group. This modification enhances the binding affinity of oligonucleotides to RNA as well as duplex DNA (ΔT_m 3.2 °C per modification). The 2′-*O*-GE modified oligonucleotides exhibited exceptional resistance to nuclease degradation. The crystal structure of a palindromic duplex formed by a DNA oligonucleotide with a single 2′-*O*-GE modification was solved at 1.16 Å resolution.

Oligonucleotides have been modified to make them useful for therapeutic and diagnostic applications,[1,2] and 2′-*O*-modified oligonucleotides are candidates for clinical applications.[3] The 2′-*O*-aminopropyl (2′-*O*-AP, Figure 1) modification has extremely high nuclease resistance due to its cationic nature and good hybridization properties.[4] The pK_a of the primary amino group is around 9, and thus, the group is protonated under physiological conditions. Another interesting highly basic, cationic group is the guanidinium group (pK_a = 12.5). The guanidinium group contains three amines in a plane, remains protonated over a wide pH range, and can form up to five hydrogen bonds when present within the arginine side chain.[5]

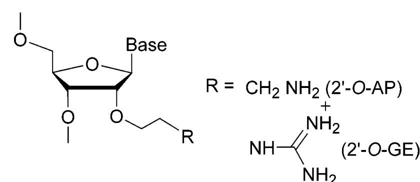


Figure 1. 2′-Modifications described in the text.

Unfortunately, conventional guanidinium protecting groups such as benzyloxycarbonyl (Cbz) or *tert*-butyloxycarbonyl

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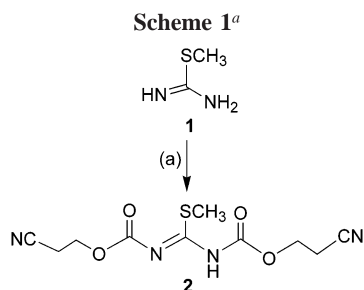
(Boc)[6] are not compatible with solid-phase DNA synthesis protocols. Recently, we reported use of a novel amino protecting group compatible with oligonucleotide synthesis, the *N*-(2-(cyanoethoxycarbonyl) group (CEOC).[7] Here, we report use of this protecting group in the synthesis of 2′-*O*-[2-(guanidinium)ethyl] (2′-*O*-GE) modified oligonucleotides. The guanylating reagent was prepared by treatment of CEOE-succinimide[7] with carbamimidothioc acid methyl ester **1** to yield protected [[(2-cyanoethoxy)carbonyl]amino(methylthio)methylene] carbamic acid 2-cyanoethyl ester **2** (Scheme 1).

Scheme 1[a]

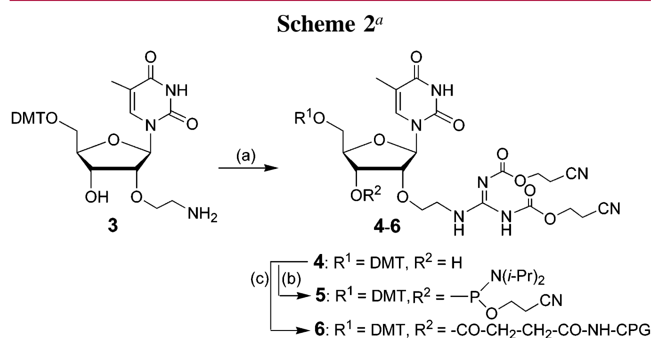


[a] Key: (a) CEOC-succinimide, CH_2Cl_2, $NaHCO_3$, rt.

Scheme 2 shows the synthesis of the CEOC-protected 2′-*O*-GE-5-methyluridine-3′-phosphoramidte **5** and solid support **6**. The 2′-*O*-[2-(amino)ethyl]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine **3** was synthesized using previously reported procedures.[7] Compound **3** was treated with guanylating reagent **2** and triethylamine in DMF at room temperature to yield **4** (66%) with a protected guanidinium functionality. Compound **4** was converted into 3′-amidite **5** using standard procdures.[7] Compound **4** was converted into the 3′-*O*-succinyl derivative and loaded onto amino alkyl controlled pore glass (CPG) according to previously published procedures[8] to yield solid support **6** (32.8 μmol/g).

Oligonucleotides shown in Table 1 were synthesized using phosphoramidite **5** and solid support **6**, and the standard phosphoramidites and solid supports for incorporation of A, T, G, and C residues. Oxidation of the internucleosidic phosphite groups was carried out using 1-*S*-(+)-(10-camphorsulfonyl)oxaziridine[9] or *tert*-butylhydroperoxide/acetonitrile/water (10:87:3). The solid supports bearing the oligonucleotides were treated with 50% piperidine[10] in water and kept at room temperature for 24 h to remove the CEOC protecting groups from the guanidinium groups and internucleoside phosphates and simultaneously release the oligonucleotides from solid supports. The solid supports were removed by filtration and the filtrates were concentrated to dryness. The oligonucleotides were then heated with aqueous ammonia (28−30 wt %) at 55 °C for 6 h to complete deprotection of the exocyclic amino protecting groups. Removal of the 2-cyanoethoxy groups from modified oligonucleotides with 50% piperidine in water prior to aqueous ammonia treatment was required to prevent formation of triazine derivative. The oligonucleotides were purified by reversed-phase HPLC and characterized by ES-MS, HPLC, and capillary gel electrophoresis.

Scheme 2[a]



[a] Key: DMT = 4,4′-dimethoxytrityl; (a) **2**, anhydrous DMF, triethylamine, rt; (b) *N,N*-diisopropylammonium tetrazolide, 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite, CH_3CN, rt; (d) (i) succinic anhydride, pyridine, CH_2Cl_2, DMAP, rt, (ii) 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU), DMF, amino alkyl controlled pore glass (CPG), rt.

Table 1. Oligonucleotides Used for the Studies[a]

no.	sequences
7	5′ d(TCC AGG TGT CCG CAT C) 3′
8	5′ d(T*CC AGG T*GT* CCG CAT* C) 3′
9	5′ d(CTC GTA CTT TTC CGG TCC) 3′
10	5′ d(CTC GTA CT*T* T*T*C CGG TCC) 3′
11	5′ d(TTT TTC TCT CTC TCT) 3′
12	5′ d(T*T*T* T*T*C TCT CTC TCT) 3′
13	5′ d (T*TT* TT*C TCT CTC TCT) 3′
14	5′ d(TT*T TT*C TCT* CTC T*CT) 3′
15	5′ d(ttt ttC TCT CTC TCT) 3′
16	5′ d(GCG TAT* ACG C) 3′
17	5′ d(TTT TTT TTT TTT TTT TTT T*) 3′
18	5′ d(TTT TTT TTT TTT TTT TT*T T*) 3′
19	5′ d(TTT TTT TTT TTT TTT T*T*T* T*) 3′

[a] All oligonucleotides were phosphodiesters; T* = 2′-*O*-[2-(guanidino)ethyl]-5-methyluridine, t = 2′-*O*-[2-(amino)ethyl]-5-methyluridine.

Hybridization of the modified oligonucleotides **8** and **10** (Table 1) to complementary RNA and DNA was evaluated (Table 2). In oligonucleotide **8**, modifications were dispersed throughout the sequence, and we observed a duplex stabilization of 2 °C per modification as compared to the DNA analogue (**7**, Table 2). However, when the modifications were consecutive, as in sequence **10**, the duplex with RNA was

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Table 2. Effect of 2′-*O*-GE Modifications on Duplex Stability with Complementary RNA and DNA[a]

	vs complementary RNA			vs complementary DNA	
no.	T_m, °C	ΔT_m, mod °C	$-\Delta G°_{37}$, kcal mol^{-1}	T_m, °C	$-\Delta G°_{37}$, kcal mol^{-1}
7	62.4		−18.0	62.7	−17.9
8	70.4	2.0	−20.6	67.3	−20.0
9	62.1		−17.6	58.1	−16.2
10	61.4	−0.1	−17.5	58.0	−15.0

[a] T_m values were assessed in 100 mM Na^+, 10 mM phosphate buffer, 0.1 mM EDTA, pH 7, at 260 nm, and 4 μM oligonucleotides and 4 μM complementary length matched RNA. Standard deviation did not exceed ±0.5 °C.

slightly destabilized relative to the control oligonucleotide **9** (Table 2). These observations are in agreement with reported hybridization behavior of oligonucleotides bearing 2′-*O*-aminopropyl or homologous groups.[4] Hybridization with the complementary DNA led to duplexes less stable than those formed with the complementary RNA (Table 2).

The sequence-specific recognition of duplex DNA by pyrimidine oligonucleotides involves the formation of triple helical structures stabilized by Hoogsteen hydrogen bonds between the base on the DNA target and the pyrimidine third strand.[11] Affinity of 2′-*O*-GE-modified oligonucleotides for a double-stranded DNA target was evaluated (Table 3, Figure 2). An increase in T_m of 4.1 °C per modification was observed for oligonucleotide **12**, with sequential modifications, as compared to control **11**. The observed enhancement in T_m was 0.6 °C per modification higher than observed with the 2′-*O*-aminoethyl-modified oligonucleotide **15** (Table 3).[12] When the modifications were dispersed as in oligonucleotides **13** and **14**, the T_m enhancement was increased relative to control **11** but was considerably less than that of oligonucleotide **12** with consecutive modifications. This is in contrast to the positional effect on duplex stability where the oligonucleotide with consecutive modifications formed a less stable duplex with complementary RNA than that with dispersed modifications. Like the previously observed enhanced affinity of oligonucleotides with 2′-amino ethyl groups for duplex DNA[12] (Table 3), the stable triplexes formed by 2′-*O*-GE oligonucleotides are presumably due to interaction of positively charged amino group and the negatively charged phosphate backbone.

Table 3. Effect of 2′-*O*-GE Modifications on Triplex Stability (Indicated Oligonucleotide Was Annealed with Target DNA Duplex)

no.	T^a triplex (°C)	ΔT triplex (°C)	ΔT triplex/mod (°C)	T_m duplex (°C)
11	21.7			60.9
12	42.3	20.6	4.1	61.4
13	29.3	7.6	2.5	61.0
14	32.4	10.7	2.7	60.6
15	39.2	17.5	3.5	60.7

[a] T = temperature of third strand dissociation. The DNA duplex was 5′d(GCTAAAAAGAGAGAGAGATCG)3′•5′d(CGATCTC TCTCTCTTTT-TAGC)3′. T values were assessed in 180 mM KCl, 20 mM Na^+, 10 mM phosphate (pH 7.0), and 0.1 mM ethylenediaminetetraacetate, and 3 μM each strand concentration. Standard deviation did not exceed ± 0.5 °C. Thermal denaturation results in two UV transitions. The first transition (T triplex) corresponds to the dissociation of the numbered strand and the second to melting of the DNA duplex (T_m duplex).

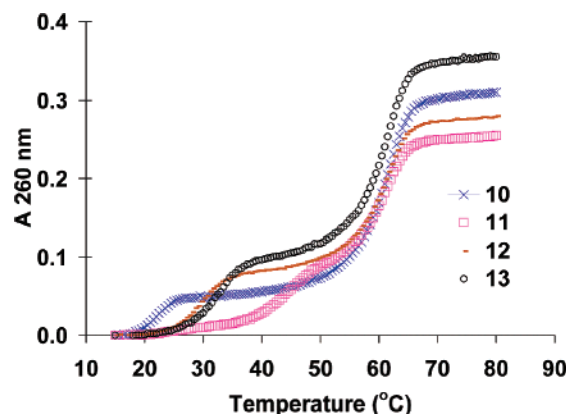

Figure 2. Effect of 2′-*O*-GE modification on affinity of oligonucleotide to duplex DNA.

The crystal structure of palindromic 2′-*O*-GE-modified oligonucleotide **16** was determined at 1.16 Å resolution and refined to an *R* factor of 13.2% (*R*-free = 17.4%). The decamer duplex adopted a standard A-type geometry and all sugars, including the ribose moieties of 2′-*O*-GE modified residues, exhibited $C_{3'-endo}$ pucker. Torsion angles O2′−CA′−CB′−NC′ (atoms of 2′-*O*-substituents are denoted CA′, CB′, NC′, CD′, NE′, and NF) for both T*6 and T*16 displayed synclinal conformations (−63° and −55°, respectively), consistent with a gauche effect between O2′ and NC′ (Figure 3). However, the CA′−CB′−NC′−CD′ torsion angles differed considerably for T*6 and T*16 (87° and −90°, respectively). As a result, the guanidinium moiety of T*6 was near the phosphate group of the 3′-adjacent residue A7 (the distances between terminal 2′-*O*-GE nitrogens and bridging and nonbridging phosphate oxygens were between 3.41 Å, to O3′, and 4.90 Å, to O5′). For T*16, these distances are considerably longer (4.88 Å to O5′, and 5.30 Å to O1P). Notably, only one of the terminal 2′-*O*-GE nitrogens of T*16 was directed toward the phosphate group of A17, while the other forms a hydrogen bond to O4′ of the same residue (3.58 Å).

The loss of stability observed for duplexes between RNA and oligonucleotides carrying consecutive 2′-*O*-GE modifi-

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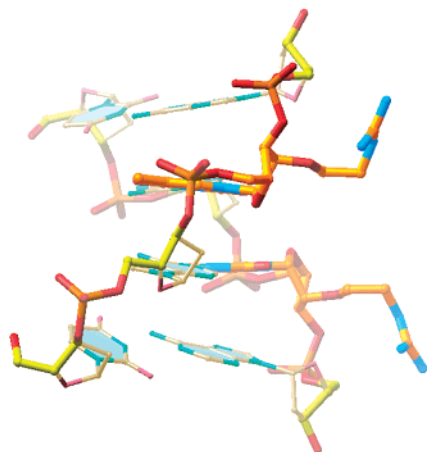

Figure 3. Stereoimage depicting the central $[d(TA)T^*d(A)]_2$ portion of the A-form duplex adopted by **16**. The 2′-*O*-GE substituents of residues T*6 (foreground) and T*16 (background) protrude into the minor groove. Atoms are colored yellow, red, cyan, and orange for carbon, oxygen, nitrogen, and phosphorus, respectively, and T* carbons are highlighted in gold.

cations relative to oligonucleotides with dispersed 2′-*O*-GE modified residues may be due to repulsions between positively charged guanidinium moieties in the minor groove. Although substituents and phosphate groups from 3′-adjacent residues in the crystal structure of **16** are relatively closely spaced, the resulting partial neutralization appears to be insufficient to prevent repulsive interactions between neighboring 2′-*O*-GE substituents.

The stability of 2′-*O*-GE oligonucleotides against nucleases was evaluated. The oligonucleotide phosphodiesters **17**−**19** with 2′-*O*-GE modified residues at the 3′-ends were synthesized and digested with snake venom phosphodiesterase (SVPD, Figure 3).[13] Oligonucleotide **19** with four modified residues exhibited the highest exonuclease stability and oligonucleotide **17** with one residue was least resistant to exonuclease digestion (Figure 4).

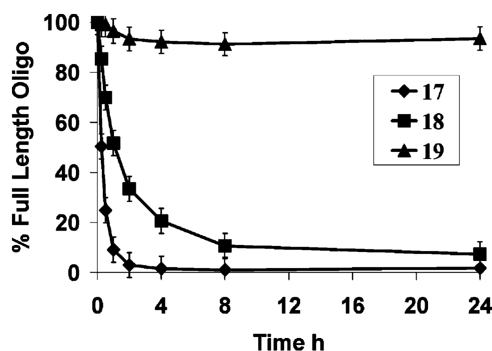

Figure 4. Disappearance of oligonucleotides **17**−**19** in the presence of SVPD as a function of time; 5′-^{32}P labeled oligonucleotides were digested with SVPD (5 × 10−3 U mL^{-1}) in 50 mM Tris-HCl buffer at pH 8.5, containing 72 mM NaCl and 14 mM $MgCl_2$ at 37 °C.

In conclusion, we have synthesized novel 2′-*O*-GE modified oligonucleotides that showed high affinity to RNA and double-stranded DNA and exceptional exonuclease stability. A novel guanylating reagent and a CEOC protecting group compatible with solid-phase oligonucleotide synthesis were used. When dispersed in the sequence, the 2′-*O*-GE modified oligonucleotides enhanced the T_m with a complementary RNA by 2 °C per modification compared to an unmodified oligonucleotide. This cationic modification also significantly enhanced the affinity of the oligonucleotides for triplex formation (ΔT_m 3.2 °C per modification). These properties make the 2′-*O*-GE modification useful for antisense and antigene strategy-based therapeutics and also as tools in molecular biology. The crystal structure of 2′-*O*-GE modified DNA duplex gives insight into the stabilization observed with 2′-*O*-GE modification.[14]

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