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Synthesis of 2′-*O*-[2-[(*N*,*N*-dialkylamino)oxy]ethyl]-modified oligonucleotides: hybridization affinity, resistance to nuclease, and protein binding characteristics

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Abstract—Synthesis of a series of 2′-*O*-[2-[(*N*,*N*-dialkylamino)oxy]ethyl]-modified 5-methyluridine nucleoside phosphoramidites and solid supports are described. Using these monomers, modified oligonucleotides containing phosphodiester linkages were synthesized in high yields. These modified oligonucleotides showed enhanced binding affinity to the complementary RNA (and not to DNA) and excellent nuclease stability with $t_{1/2}$>24 h. The human serum albumin binding properties of modified oligonucleotides have been evaluated to assess their transport and toxicity properties.

1. Introduction

Structural analogues of antisense oligonucleotides with modified heterocycles, sugars, and/or backbones have been synthesized in an attempt to improve binding affinity and nuclease resistance, to modulate binding to selected transport proteins, and to enhance cell permeation in vivo.[1] Some of the most successful analogues have resulted from modification of the sugar at the 2′-position.[2] The 2′-*O*-modified oligonucleotides used with the 'gapmer' technology[3] have emerged as the leading second generation candidates for clinical applications. Gapmer oligonucleotides have one or two 2′-*O*-modified regions and a 2′-deoxy phosphorothioate region; the 2′-deoxy region is required for RNase H digestion of mRNA. We recently reported synthesis and properties of 2′-*O*-(2-aminooxyethyl) modification (2′-*O*-AOE), the pseudoisostere of 2′-*O*-methoxyethyl (2′-*O*-MOE) modification.[4] Unfortunately, due to high chemical reactivity of 2′-*O*-AOE, several modified residues cannot be conveniently incorporated into an oligonucleotide. Moreover, this modification did not impart enhanced nuclease stability relative to the 2′-*O*-MOE modification, although duplexes formed with RNA had comparable affinity to 2′-*O*-MOE:RNA duplexes. The dimethyl derivative of the 2′-*O*-AOE modification, 2′-*O*-dimethylaminooxyethyl (2′-*O*-DMAOE), has been synthesized and showed considerable promise.[5] The stability of a 2′-*O*-DMAOE:RNA duplex was comparable to that of the 2′-*O*-MOE:RNA duplex of the same sequence. The 2′-*O*-DMAOE modification also imparted excellent resistance to nuclease digestion. Here we describe the design and synthesis of several additional analogues of the 2′-*O*-AOE in order to test two hypotheses. We postulated that increasing the steric bulk at the 2′-position would improve the nuclease stability of oligonucleotides. In addition, as these dialkyl modifications should be more lipophilic than either the 2′-*O*-AOE or the 2′-*O*-MOE modifications, the 2′-*O*-AOE analogues may offer more desirable protein binding and cellular permeation properties.

We envisaged that due to high reactivity of the aminooxy group to aldehydes and ketones,[5] it could be easily converted into dialkyl derivatives by reaction with aldehydes and ketones of interest followed by reduction of the resulting methyleneaminooxy derivatives. The modified nucleosides 2′-*O*-[2-[(*N*,*N*-diethylamino)oxy]ethyl] (2′-*O*-DEAOE), 2′-*O*-[2-[(*N*-isopropyl-*N*-methylamino)oxy]-ethyl] (2′-*O*-IPMAOE), and the cationic 2′-*O*-[2-[[*N*-methyl-*N*-[2-(amino)ethyl]amino]oxy]ethyl] (2′-*O*-AEMAOE) were synthesized using this strategy. Each novel nucleoside was incorporated into oligonucleotides (Fig. 1). The thermal melting temperatures (T_m's) of these modified oligonucleotide:RNA duplexes were determined and their stability to nuclease digestion and their albumin binding properties were evaluated.

Keywords: hybridization affinity; nuclease resistance; protein binding.
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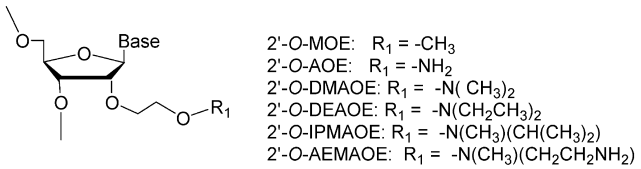
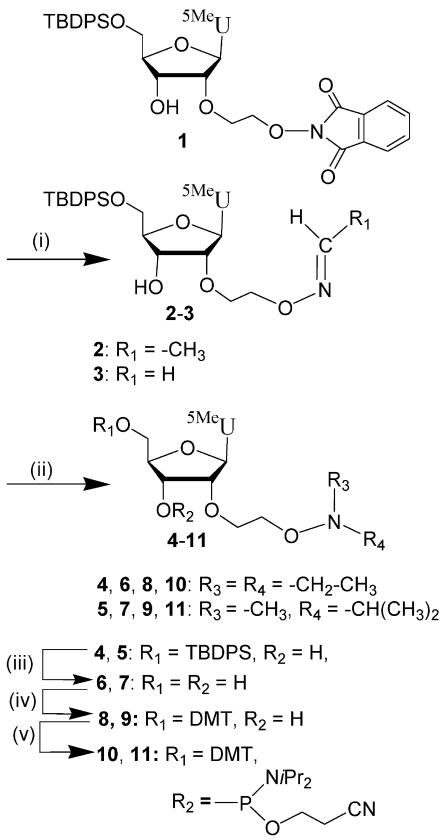


Figure 1. 2′-modifications.

2. Results and discussion

2.1. Synthesis of 2′-*O*-modified nucleoside phosphoramidites and solid supports

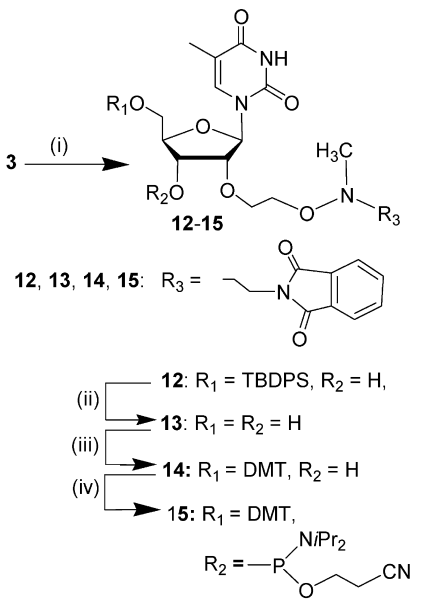
We recently described the synthesis of 2′-*O*-DMAOE (Fig. 1) nucleosides and phosphoramidites.[5] We used a similar synthetic strategy to synthesize all novel nucleosides described in this report. The 5′-*O*-(*tert*-butyldiphenylsilyl)-2′-*O*-[2-[(1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)oxy]-ethyl]-5-methyluridine **1** was a convenient intermediate for the synthesis of all phosphoramidites described in this report and was synthesized as reported.[5] The phthalimido group in compound **1** (Scheme 1) was deprotected with *N*-methylhydrazine (NMH) at 0°C to give the aminooxy derivative. The aminooxy derivative was converted into the ethylideneaminooxy derivative **2** (55% yield) and methyleneaminooxy derivative **3** (74% yield) using acetaldehyde in MeOH and formaldehyde in MeOH, respectively.



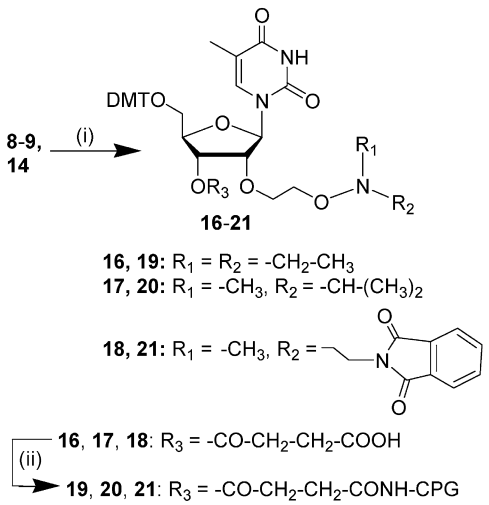
Scheme 1. (i) (a) CH_2Cl_2, *N*-methylhydrazine (b) for **2**: MeOH, CH_3CHO, rt; for **3**: MeOH, HCHO, rt; (ii) (a) 1 M PPTS in MeOH, $NaBH_3CN$, rt (b) for **4**: 1 M PPTS in MeOH, CH_3CHO, $NaBH_3CN$, rt, for **5**: 1 M PPTS in MeOH, CH_3COCH_3, $NaBH_3CN$, rt; (iii) triethylamine trihydrofluoride, THF, $(C_2H_5)_3N$, rt; (iv) 4,4′-dimethoxytrityl chloride, pyridine, DMAP, rt; (v) (2-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, *N*,*N*-diisopropylammonium tetrazolide, CH_3CN, rt.

Reduction of compound **2** in 1 M pyridinium *p*-toluenesulfonate (PPTS) in MeOH and $NaBH_3CN$ gave monoethylaminooxy derivative. This was followed by treatment with acetaldehyde under reductive conditions to give diethylaminooxy derivative **4** in 75% yield. Compound **3** was reduced to the monomethylaminooxy derivative under reductive conditions described for compound **2**; this was followed by treatment with acetone under reductive conditions to give compound **5** in 67% yield. Reduction of the compounds **2** and **3** proceeded smoothly at ambient temperature. Desilylation of compounds **4** and **5** with triethylamine trihydrofluoride and triethylamine in THF gave **6** (74% yield) and **7** (71% yield). These nucleosides were then selectively protected at 5′-position with 4,4′-dimethoxytrityl chloride (DMTCl) and DMAP in anhydrous pyridine to yield compounds **8** (86% yield) and **9** (92% yield). The compounds **8** and **9** were converted into 3′-phosphoramidites using 2-cyanoethyl tetraisopropylphosphorodiamidite and *N*,*N*-diisopropylammonium tetrazolide in acetonitrile at room temperature to yield **10** (70% yield) and **11** (85% yield), respectively.

The synthesis of phosphoramidite **15** is described in Scheme 2. Compound **3** was reduced in 1 M PPTS in MeOH with $NaBH_3CN$ to yield the methylaminooxy derivative. The methylaminooxy derivative was treated with 2-phthalimidoacetaldehyde under reductive conditions to yield **12** in 60% yield. The 2-phthalimidoacetaldehyde was generated from commercially available 2-phthalimidoacetaldehyde diethyl acetal by stirring in $CHCl_3$ with 50% trifluoroacetic acid for 24 h followed by usual work-up. The desilylation of compound **12** with triethylamine trihydrofluoride and triethylamine in THF gave **13** (52% yield). Selective 4,4′-dimethoxytritylation of compound **13** at 5′-position gave **14** (73% yield). Finally, phosphitylation of **14** at the 3′-positon yielded **15** (85% yield). The phthalimido group was used as the protecting group for the amino



Scheme 2. (i) (a) 1 M PPTS in MeOH, $NaBH_3CN$, rt; (b) 1 M PPTS in MeOH, 2-phthalimidoacetaldehyde, $NaBH_3CN$, rt; (ii) triethylamine trihydrofluoride, THF, $(C_2H_5)_3N$, rt; (iii) 4,4′-dimethoxytrityl chloride, pyridine, DMAP, rt; (iv) (2-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, *N*,*N*-diisopropylammonium tetrazolide, CH_3CN, rt.



Scheme 3. (i) Succinic anhydride, 1,2-dichloroethane, DMAP, $(C_2H_5)_3N$, 60°C; (ii) 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU), 4-methyl morpholine, DMF, aminoalkyl controlled pore glass (CPG), rt.

functionality during oligonucleotide[6] synthesis. The phthalimido group was deprotected, to provide amino functionality, during the deprotection of protecting groups at the exocyclic amino groups and cyanoethyl groups of the backbone phosphate of oligonucleotides by the treatment with aqueous ammonia containing 4% methylamine at 55°C for 6 h.

Compounds **8**, **9**, and **14** were also converted into the 3′-succinyl derivatives **16**, **17**, and **18** (Scheme 3) and loaded on to the aminoalkyl controlled pore glass (CPG) according to the literature procedure[7] to yield the functionalized solid supports **19**, **20**, and **21** (51–60 μmol g^{-1}).

2.2. Synthesis of 2′-modified oligonucleotides

The modified oligonucleotides (Table 1) were synthesized using phosphoramidites **10**, **11**, and **15** on a solid-phase DNA synthesizer. Standard phosphoramidites were used for incorporation of A, C, G, and T residues. Solid supports **19**, **20**, and **21** were used for the synthesis of oligonucleotides **34**, **36**, and **37**. A 0.1 M solution of phosphoramidite in anhydrous acetonitrile was used for the synthesis. Oxidation of the internucleoside phosphite to the phosphate was carried out using 1-*S*-(+)-(10-camphorsulfonyl)oxaziridine (CSO).[8] The overall coupling efficiency for oligonucleotides containing each of the modified phosphoramidites was more than 97%. The oligonucleotides were characterized by ES MS (Table 2) and purity was assessed by HPLC and capillary gel electrophoresis.

2.3. Evaluation of hybridization of the 2′-*O*-DMAOE, 2′-*O*-DEAOE, 2′-*O*-IPMAOE and 2′-*O*-AEMAOE modified oligonucleotides to complementary RNA and DNA by thermal denaturation studies

Table 1 shows the T_m values of the 2′-*O*-DMAOE, 2′-*O*-DEAOE, 2′-*O*-IPMAOE and 2′-*O*-AEMAOE modified oligonucleotides hybridized to complementary RNA. The T_m's of the modified oligonucleotides were compared to those formed with unmodified DNA oligonucleotides. Free energies of duplex formation ΔG^0_{37} (Table 1) were obtained from UV absorbance vs. temperature curves assuming a two-state model with a linear sloping baseline.[9] Substitution with the 2′-*O*-DMAOE, 2′-*O*-DEAOE and 2′-*O*-IPMAOE nucleosides led to an increase in the melting temperatures relative to unmodified oligonucleotides. A T_m enhancement of 0.8–1.1°C per substitution was observed when modified bases were not contiguous, as in the oligonucleotides **23**, **24**, and **25** (Table 1). In the oligonucleotides with ten adjacent substitutions, **27**, **28**, and **29**, the T_m enhancements were 1.4–1.5°C per substitution. The DNA phosphorothioates (PS) forms a duplex with complementary RNA destabilized by approximately −0.8°C per modification.[10] Therefore, stabilization due to the 2′-modifications described in this report corresponds to a net stabilization of more than 2°C per modification when compared to DNA PS, the first generation antisense drug. Although the 2′-*O*-DEAOE and 2′-*O*-IPMAOE modifications are bulkier than the 2′-*O*-DMAOE modification, there is no significant T_m differences. These data suggest that the RNA heteroduplex tolerates significant bulk at 2′-position without affecting binding affinity. Charge is not as well tolerated, as shown by melting of oligonucleotide **31**. This oligonucleotide has adjacent cationic 2′-*O*-AEMAOE modifications and there is only a moderate increase in T_m (0.5°C per modification). This observation is in agreement with the reported T_m for cationic 2′-*O*-(aminoalkyl)-modified oligonucleotides.[11]

In contrast to the increase in the T_m observed with RNA, the hybridization of oligonucleotides **27**, **28**, and **29** with complementary DNA led to duplexes less stable than those formed with unmodified DNA oligonucleotides (−0.9–−1.1°C per modification, Table 3). These data

Table 1. Effect of 2′-*O*-DMAOE, 2′-*O*-DEAOE, 2′-*O*-IPMAOE and 2′-*O*-AEMAOE modification on duplex stability against complementary RNA targets

Oligo No.	Sequence 5′–3′	Modification	T_m (°C)	ΔT_m (°C)	ΔT_m/unit (°C)	$-\Delta G^0_{37}$ (kcal mol^{-1})
22	d(TCC AGG TGT CCG CAT C)	DNA parent	62.3			14.9
23	d(T*CC AGG T*GT* CCG CAT* C)	2′-*O*-DMAOE	66.8	4.5	1.1	17.8
24	d(T▲CC AGG T▲GT▲ CCG CAT▲ C)	2′-*O*-DEAOE	65.5	4.2	1.1	19.8
25	d(T$CC AGG T$GT$ CCG CAT$ C)	2′-*O*-IPMAOE	65.4	3.1	0.8	19.5
26	d(GCG TTT TTT TTT TGC G)	DNA parent	48.3			13.0
27	d(GCG T*T*T* T*T*T* T*T*T* T*GC G)	2′-*O*-DMAOE	62.9	14.6	1.5	17.8
28	d(GCG T▲T▲T▲ T▲T▲T▲ T▲T▲T▲ T▲GC G)	2′-*O*-DEAOE	63.3	15.0	1.5	17.0
29	d(GCG TTT$ TTT$ TTT$ T$GC G)	2′-*O*-IPMAOE	62.6	14.3	1.4	15.8
30	D(CTC GTA CTT TTC CGG TCC)	DNA parent	61.8			17.6
31	d(CTC GTA CT†T† T†T†C CGG TCC)	2′-*O*-AEMAOE	63.8	2.0	0.5	19.1

T*=2′-*O*-DMAOE-5-methyluridine, T▲=2′-*O*-DEAOE-5-methyluridine, T$=2′-*O*-IPMAOE-5-methyluridine, T†=2′-*O*-AEMAOE-5-methyluridine. Each T_m and ΔG^0_{37} was the average of at least three experiments with standard deviations not exceeding ±0.5°C for T_m and ±0.1 kcal mol^{-1} for $\Delta G°_{37}$ values.

Table 2. HPLC and mass spectral analysis of the 2′-*O*-DMAOE, 2′-*O*-DEAOE, 2′-*O*-IPMAOE and 2′-AEMAOE oligonucleotides used for T_m analysis

Oligo No.	Mass		HPLC t_R (min)[a]
	Calcd	Found	
23	5246.4	5245.6	23.6
24	5358.5	5358.9	25.5
25	5358.5	5358.3	22.9
27	5906.4	5905.6	26.2
28	6187.6	6188.1	32.4
29	6187.6	6188.1	37.7
31	5919.2	5919.8	23.8

[a] Water C-4, 3.9×300 mm, A=50 mM triethyl ammonium acetate, pH 7, B=acetonitrile, 5–60% B in 55 min, flow 1.5 mL min^{-1}, λ=260 nm.

Table 3. T_m data for the oligonucleotides containing 2′-*O*-DMAOE, 2′-*O*-DEAOE, 2′-*O*-IPMAOE modification hybridized to complementary DNA

Oligo No.	Modifications	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/unit
26	DNA parent	54.2		
27	2′-*O*-DMAOE	45.4	−8.8	−0.9
28	2′-*O*-DEAOE	42.9	−11.3	−1.1
29	2′-*O*-IPMAOE	43.5	−10.7	−1.1

Each T_m value was the average of at least three experiments with standard deviations not exceeding ±0.5°C.

demonstrated an RNA selective hybridization of 2′-modified oligonucleotides described in this report.

2.4. Resistance of 2′-modified oligonucleotides to degradation by snake venom phosphodiesterase

To evaluate the relative resistance of the 2′-modified oligonucleotides toward nucleases, the oligonucleotides **32**–**37** (Fig. 2) were digested with snake venom phosphodiesterase (SVPD).[12] The modifications were placed at the 3′-end of the oligonucleotide and all internucleoside linkages were phosphodiesters. The oligonucleotides were 5′-^{32}P labeled and the percentage of full-length oligonucleotide was estimated after separation by gel electrophoresis. Figure 2 shows the relative exonuclease stability of the modified oligonucleotides compared to the unmodified DNA. The half-lives of the disappearance of the full-length 2′-*O*-AOE analogues were more than 24 h. All are more resistant to degradation than 2′-*O*-AOE[4] and 2′-*O*-MOE modified oligonucleotide. This observation suggests that modifications with significant steric bulk impart enhanced nuclease stability to oligonucleotides. Both the 2′-*O*-AEMAOE and 2′-*O*-IPMAOE modified oligonucleotides were extremely stable to nuclease digestion. The amino group in the 2′-*O*-AEMAOE modification is protonated under physiological conditions (pK_a=9). The positive charge probably contributes to enhanced nuclease resistance of the 2′-*O*-AEMAOE modified oligonucleotide. The observed order of nuclease stability of cationic 2′-*O*-AEMAOE modified oligonucleotide is consistent with the reported data for other cationic modifications.[6,11]

2.5. Binding of oligonucleotides to human serum albumin

The distribution of oligonucleotide PS to peripheral tissues

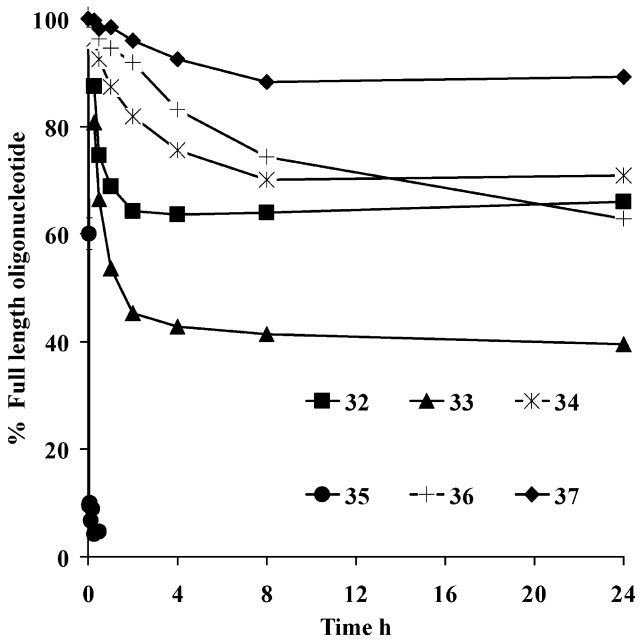


Figure 2. Time-dependent disappearance of oligonucleotides **32**–**37** in the presence of snake venom phosphodiesterase. The oligonucleotides were phosphodiester TTT TTT TTT TTT TTT T*T*T*T*T*, where T* is 2′-*O*-DMAOE-5-methyluridine for oligonucleotide **32**, 2′-*O*-MOE-5-methyluridine for **33**, 2′-*O*-DEAOE-5-methyluridine for **34**, T for **35**, 2′-*O*-IPMAOE for **36**, and 2′-*O*-AEMAOE for **37**. Each symbol represents the average of two separate measurements (n=2).

and their ultimate uptake into cells of target organs is due to their avid binding to plasma proteins.[13] Albumin and α_2-macroglobulin are the major protein species that bind to PS antisense oligonucleotides with relatively high capacity.[14] However, non-specific protein binding characteristics of PS oligonucleotides are responsible for some of the undesired hemodynamic effects.[13] Ideally, modifications to antisense oligonucleotides will either reduce the requirement for PS linkages or moderate the non-specific binding of PS oligonucleotides.

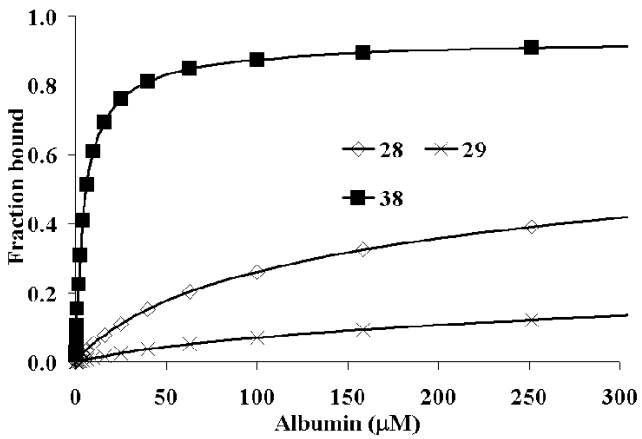


Figure 3. Fraction of oligonucleotides **28**–**29** and **38** bound to albumin ploted as a function of albumin concentration. Each symbol represents the average of three separate measurements (n=3). Oligonucleotides **26** and **27** did not show binding and could not fit into the no-linear regression equation. **38**: 5′ d(GCG T_{10} GC G) 3′, back bone phosphorothioates; **39**: 5′ d(GCG T^*_{10} GC G) 3′, T* is 2′-*O*-MOE-5-methyluridine, back bone phosphodiesters.

Table 4. Effect of 2′-modification on human serum albumin binding constant

Oligo[a] No.	Chemistry		K_d (μM)
	Back bone	T*	
26	P=O	Thymidine	No binding
27	P=O	2′-*O*-DMAOE-5-methyluridine	No binding
28	P=O	2′-*O*-DEAOE-5-methyluridine	153
29	P=O	2′-*O*-IPMAOE-5-methyluridine	No binding
38	P=S	Thymidine	30
39	P=O	2′-*O*-MOE-5-methyluridine	No binding

[a] 5′ d(GCG T^*_{10} GCG) 3′, P=O phosphodiesters, P=S phosphorothioates.

In order to evaluate the protein binding characteristics of 2′-modified oligonucleotides with phosphodiester (P=O) backbone described in this report, binding of the oligonucleotides **26**–**29** and oligonucleotides **38**–**39** (Fig. 3) to human serum albumin was measured by ultrafiltration techniques.[13] The equilibrium constant, K_d, (Table 4) was determined from the data of fraction of oligonucleotide bound to protein concentration. The constants were determined from non-linear regression analysis of a fraction of oligonucleotides bound as a function of free albumin monomer concentration. An oligonucleotide with a lower K_d value binds more tightly to the protein than one with higher value of K_d. An oligonucleotide with K_d value more than 300 μM is not considered to bind protein. The 2′-*O*-DEAOE-modified oligonucleotide with a phosphodiester backbone exhibited moderate protein binding (K_d=153 μM) while the 2′-deoxy, 2′-*O*-MOE,[15] 2′-*O*-DMAOE and 2′-*O*-IPMAOE/P=O showed no binding (Table 4). The PS oligonucleotide had a K_d=30 μM in these experiments. The moderate protein binding properties of 2′-*O*-DEAOE modified oligonucleotides indicates that, 2′-*O*-DEAOE modified P=O oligonucleotides are expected to bind less tightly to protein compared to oligonucleotide PS in vivo. Therefore, antisense drugs with 2′-*O*-DEAOE modified oligonucleotide P=O are expected to have fewer side effects than oligonucleotide PS.

In conclusion, we have designed and synthesized 2′-*O*-DEAOE, 2′-*O*-IPMAOE and the cationic 2′-*O*-AEMAOE modified oligonucleotides for antisense applications. Efficient synthetic strategy for the synthesis of pyrimidine nucleosides with these modifications and their phosphoramidites and solid supports were developed. The methods described herein provide general procedures for functionalization of nucleosides at the 2′-position. The use of the 2′-modified nucleoside building blocks in oligonucleotide synthesis resulted in high coupling efficiency. These modified oligonucleotides showed RNA-selective, high binding affinity, and excellent nuclease stability. These oligonucleotides showed reduced binding affinity to human serum albumin relative to phosphorothioate oligonucleotides. These favorable antisense properties make them valid candidates for further in vivo evaluation for antisense drug development. We have selected 2′-*O*-DEAOE modification for further in vivo antisense evaluation and such efforts are in progress in our laboratory based on the target optimal binding, enhanced nuclease resistance and protein binding compared to 2′-*O*-MOE.

3. Experimental

3.1. General procedures

Anhydrous MeCN (water content <0.001%), standard phosphoramidites and ancillary reagents for oligonucleotide synthesis were purchased from PE Biosystems (Foster City, CA). All other reagents and anhydrous solvents were purchased from Aldrich and used without further purification. Thin-layer chromatography was performed on precoated plates (silica gel 60 F254 from EM Science) and visualized with UV light and spraying with a solution of *p*-anisaldehyde (6 mL), H_2SO_4 (8.3 mL), CH_3COOH (2.5 mL) in C_2H_5OH (227 mL) followed by charring. ^1H NMR spectra were referenced using internal standard $(CH_3)_4Si$ and ^{31}P NMR spectra using external standard 85% H_3PO_4. Mass spectra were recorded by Mass Consortium, San Diego, CA and the College of Chemistry, University of California, Berkeley, CA.

3.1.1. 5′-*O*-(*tert*-Butyldiphenylsilyl)-2′-*O*-[2-(ethylideneaminooxy)ethyl]-5-methyluridine (2). Compound **1** (10 g, 14.6 mmol) was dissolved in CH_2Cl_2 (146 mL) and methylhydrazine (1.03 mL, 14.6 mmol) was added dropwise at −10°C. The reaction mixture was stirred at −10–0°C for 1 h. The white precipitate that formed was filtered and washed thoroughly with CH_2Cl_2 (ice-cold). The organic phase was evaporated to dryness. The residue obtained was dissolved in MeOH (210 mL), and acetaldehyde (0.89 mL, 16 mmol) was added and stirred at room temperature for 12 h. The solvent was removed under reduced pressure and the residue was purified by flash silica gel column chromatography and eluted with ethyl acetate/hexane (6:4) to yield compound **2** (4.64 g, 55%): R_f=0.42, (ethyl acetate/hexane, 7:3); ^1H NMR (400 MHz, DMSO-d_6) δ 1.02 (s, 9H), 1.44 (s, 3H), 1.69 (dd, 3H, *J*=5.6 Hz), 3.66 (m, 1H), 3.76 (m, 2H), 3.94 (m, 2H), 4.05 (s, 2H), 4.15 (m, 1H), 4.22 (m, 1H), 5.18 (d, 1H, *J*=6 Hz), 5.9 (dd, 1H, *J*=4.4 Hz), 7.36 (m, 1H), 7.40 (m, 7H), 7.63 (m, 5H), 11.38 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.8, 15.1, 19.4, 27.0, 63.0, 68.6, 70.3, 72.0, 72.1, 82.7, 84.3, 87.0, 111.1, 127.9, 130.0, 132.3, 133.0, 135.0, 135.1, 135.4, 147.9, 150.5, 164.1; HRMS (FAB) calcd for $C_{30}H_{39}N_3O_7SiNa^+$ 604.2455, found 604.2471.

3.1.2. 5′-*O*-(*tert*-Butyldiphenylsilyl)-2′-*O*-[2-(methyleneaminooxy)ethyl]-5-methyluridine (3). From compound **1**, compound **3** was synthesized as described in the literature.[5]

3.1.3. 5′-*O*-(*tert*-Butyldiphenylsilyl)-2′-*O*-[2-[(*N*,*N*-diethylamino)oxy]ethyl]-5-methyluridine (4). Compound **2** (4.5 g, 7.74 mmol) was dissolved in 1 M pyridinium *p*-toluenesulfonate (PPTS) in MeOH (77.4 mL). To this, $NaBH_3CN$ (0.97 g, 15.5 mmol) was added at 10°C and the mixture was stirred for 10 min. The reaction mixture was allowed to come to room temperature and stirring continued for an additional 4 h. The reaction was monitored by TLC (ethyl acetate/hexane, 70:30). The solvent was removed under reduced pressure and the oily residue obtained was diluted with ethyl acetate (100 mL) and washed with water (75 mL), aqueous $NaHCO_3$ (5 wt%, 75 mL), and brine (75 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was dissolved

in 1 M PPTS in MeOH (77.4 mL) and acetaldehyde (0.48 mL, 8.52 mmol) was added and stirred at ambient temperature for 10 min. To this $NaBH_3CN$ (0.97 g, 15.50 mmol) was added at 10°C and stirred for 10 min. The mixture was allowed to come to room temperature and stirring continued for an additional 4 h. The solvent was removed in vacuo and the residue obtained was dissolved in ethyl acetate (100 mL), washed with water (75 mL), 5% $NaHCO_3$ (75 mL), and brine (75 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was purified by flash silica gel column chromatography and eluted with CH_2Cl_2/MeOH/NEt_3, 94:5:1 to afford **4** (3.55 g, 75.1%) as a white foam: R_f=0.41 (ethyl acetate/hexane, 7:3); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.95 (t, 6H, J=7.2 Hz), 1.03 (s, 9H), 1.43 (s, 3H), 2.58 (q, 4H, J=7.2 Hz), 3.59 (m, 1H), 3.73 (m, 3H), 3.81 (m, 1H), 3.88 (m, 1H), 3.96 (m, 2H), 4.23 (m, 1H), 5.21 (d, 1H, J=5.6 Hz), 5.95 (d, 1H, J=6.4 Hz), 7.43 (m, 7H), 7.76 (m, 4H), 11.39 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.8, 19.4, 27.0, 52.3, 63.3, 68.8, 70.3, 72.3, 82.6, 84.5, 86.8, 111.0, 127.9, 130.0, 132.3, 133.0, 135.1, 135.4, 141.3, 150.5, 164.0; HRMS (FAB) calcd for $C_{32}H_{45}N_3O_7SiCs^+$ 744.2081, found 744.2067.

3.1.4. 5′-*O*-(*tert*-Butyldiphenylsilyl)-2′-*O*-[2-[(*N*-isopropyl-*N*-methylamino)oxy]ethyl]-5-methyluridine (5). Compound **3** (12.12 g, 21.38 mmol) was dissolved in 1 M PPTS in MeOH (213.8 mL). To this, $NaBH_3CN$ (2.69 g, 42.75 mmol) was added at 10°C and the mixture was stirred for 10 min. The reaction mixture was allowed to come to room temperature and stirring continued for an additional 2 h. Solvent was removed under reduced pressure. The residue obtained was dissolved in ethyl acetate (200 mL), washed with water (150 mL), aqueous $NaHCO_3$ (5 wt%, 150 mL), and brine (150 mL). The organic layer was evaporated to dryness. The residue was dissolved in 1 M PPTS in MeOH (213.8 mL). To this, acetone (1.88 mL, 25.66 mmol) was added and the reaction mixture was stirred at room temperature for 15 min. $NaBH_3CN$ (2.69 g, 42.75 mmol) was added at 10°C and the mixture was stirred for 10 min. The reaction mixture was warmed up to room temperature and stirring continued for 4 h. The solvent was removed under reduced pressure and the residue obtained was dissolved in ethyl acetate (300 mL), washed with water (200 mL), aqueous $NaHCO_3$ (5 wt%, 200 mL), and brine (200 mL). The organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The residue obtained was purified by flash silica gel column chromatography and eluted with ethyl acetate/hexane (70:30) to afford **5** (8.73 g, 66.8%): R_f=0.3 (ethyl acetate/hexane, 70:30); ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.89 (d, 3H, J=2.2 Hz), 0.92 (d, 3H, J=1.96 Hz), 1.42 (s, 3H), 2.38 (s, 3H), 2.64 (m, 1H), 3.56–3.71 (m, 4H), 3.75–3.8 (m, 2H), 3.87–4.02 (m, 2H), 4.2–4.27 (m, 1H), 5.22 (d, 1H, J=5.42 Hz), 5.94 (d, 1H, J=5.94 Hz), 7.37–7.48 (m, 6H), 7.51–7.7 (m, 4H), 11.39 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$, 3′-*O*-acetyl derivative) δ 11.9, 19.1, 19.4, 20.8, 27.0, 41.8, 58.3, 63.3, 69.6, 70.4, 71.5, 80.3, 82.3, 86.2, 111.5, 127.7, 128.0, 130.1, 130.2, 132.0, 132.8, 134.9, 135.2, 135.5, 150.5, 163.7, 170.2; MS (ES) m/z 610.3 $(M-H)^-$; HRMS (FAB) calcd for $C_{32}H_{45}N_3O_7SiCs^+$ 744.2041, found 744.2017.

3.1.5. 2′-*O*-[2-[(*N*,*N*-Diethylamino)oxy]ethyl]-5-methyluridine (6). A mixture of triethlyamine trihydrofluoride (4.39 mL, 26.81 mmol) and triethylamine (1.87 mL, 13.41 mmol) in THF (53.6 mL) was added to compound **4** (3.28 g, 5.36 mmol). The reaction mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the residue obtained was purified by flash silica gel column chromatography and eluted with CH_2Cl_2/MeOH/NEt_3 (89:10:1) to afford **6** (1.49 g, 74.3%): R_f=0.38 (10% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.97 (t, 6H, J=7.2 Hz), 1.75 (s, 3H), 2.58 (q, 4H, J=7.2 Hz), 3.55 (m, 4H), 3.66 (m, 2H), 3.83 (br s, 1H), 3.95 (t, 1H, J=5.6 Hz), 4.11 (q, 1H, J=4.8, 5.6 Hz), 5.05 (d, 1H, J=5.6 Hz), 5.87 (d, 1H, J=6 Hz), 7.75 (s, 1H), 11.31 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.8, 12.3, 52.2, 61.3, 68.9, 70.2, 72.3, 81.5, 85.1, 90.3, 110.6, 137.8, 150.6, 164.4; HRMS (FAB) calcd for $C_{16}H_{28}N_3O_7^+$ 374.1927, found 374.1919.

3.1.6. 2′-*O*-[2-[(*N*-Isopropyl-*N*-methylamino)oxy]ethyl]-5-methyluridine (7). Compound **7** was obtained from compound **5** (4.62 g, 7.55 mmol) and triethylamine trihydrofluoride (6.15 mL, 37.76 mmol) and triethylamine (2.63 mL, 18.88 mmol) in anhydrous THF (50.53 mL) according to the procedure used for the synthesis of compound **6** from **4** with the minor variations that follow. After work up, the solvent was removed under reduced pressure and the residue obtained was dissolved in ethyl acetate (50 mL) and filtered. The filtrate was added dropwise to hexane (1000 mL). The precipitate formed was decanted, dissolved in CH_2Cl_2, and evaporated to dryness to yield **7** (1.89 g, 71.31%): R_f=0.36 (10% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.91 (s, 3H), 0.94 (s, 3H), 1.76 (s, 3H), 2.39 (s, 3H), 2.66 (m, 1H), 3.51–3.66 (m, 6H), 3.84 (d, 1H, J=3 Hz), 3.94 (t, 1H, J=5.28 Hz), 4.08–4.15 (m, 1H), 5.07 (d, 1H, J=5.48 Hz), 5.87 (d, 1H, J=5.52 Hz), 7.76 (s, 1H), 11.27 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.4, 18.7, 18.9, 41.6, 58.3, 61.6, 69.1, 70.7, 71.3, 81.6, 85.4, 91.0, 110.7, 138.1, 150.6, 164.3; HRMS (FAB) calcd for $C_{16}H_{28}O_7N_3^+$ 374.1927, found 374.1933.

3.1.7. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*,*N*-diethylamino)oxy]ethyl]-5-methyluridine (8). Compound **6** (1.25 g, 3.35 mmol) was dried over P_2O_5 in vacuo overnight. It was then co-evaporated with pyridine (10 mL) under reduced pressure and the residue was dissolved in anhydrous pyridine (9.32 mL). To this, 4,4′-dimethoxytrityl chloride (1.45 g, 3.99 mmol) and DMAP (0.041 g, 0.34 mmol) was added and the reaction mixture was stirred at ambient temperature for 12 h under argon atmosphere. Pyridine was evaporated under reduced pressure and the residue obtained was purified by flash silica gel column chromatography and eluted with CH_2Cl_2/MeOH/NEt_3 (89:10:1) to afford **8** (1.96 g, 86.64%): R_f=0.47 (10% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.98, (t, 6H, J=7.12 Hz), 1.39 (s, 3H), 2.6 (q, 4H, J=7.22 Hz), 3.22 (m, 2H), 3.73 (br s, 10H), 3.98 (br s, 1H), 4.07 (m, 1H), 4.23 (m, 1H), 5.18 (d, 1H, J=5.76 Hz), 5.9 (d, 1H, J=5.38 Hz), 6.9 (d, 4H, J=8.42 Hz), 7.24–7.41 (m, 9H), 7.49 (s, 1H), 11.39 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.7, 11.9, 52.3, 55.1, 62.2, 69.0, 70.4, 72.2, 82.8, 83.5, 86.7, 87.5, 110.8, 113.2, 127.0, 127.9, 128.1, 130.0, 135.3,

144.3, 150.5, 158.6, 164.2; HRMS (FAB) calcd for $C_{37}H_{46}N_3O_9^+$ 676.3234, found 676.3214.

3.1.8. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*-isopropyl-*N*-methylamino)oxy]ethyl]-5-methyluridine (9). Compound **9** (2.33 g, 92% yield) was prepared from compound **7** (1.39 g, 3.93 mmol) in anhydrous pyridine (6.8 mL), DMTCl (1.44 g, 4.2 mmol) and DMAP (0.072 g, 0.59 mmol) according to the procedure used for the synthesis of compound **8** from compound **6**: R_f=0.42 (ethyl acetate/hexane, 80:20); ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.91 (s, 3H), 0.94 (s, 3H), 1.37 (s, 3H), 2.4 (s, 3H), 2.66 (m, 1H), 3.2 (m, 2H), 3.69 (s, 4H), 3.72 (6H), 3.96–4.06 (m, 2H), 4.21 (m, 1H), 5.18 (d, 1H, *J*=5.8 Hz), 5.88 (d, 1H, J=5.3 Hz), 6.89 (d, 4H, *J*=8.62 Hz), 7.2–7.4 (m, 9H), 7.47 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.8, 18.9, 19.1, 41.7, 55.2, 58.3, 62.3, 69.2, 70.8, 71.3, 83.0, 83.6, 86.9, 87.8, 110.9, 113.3, 127.1, 128.0, 128.2, 130.2, 135.4, 144.4, 150.6, 158.7, 164.4; HRMS (FAB) calcd for $C_{37}H_{45}O_9N_3Na^+$ 698.3222, found 698.3221.

3.1.9. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*,*N*-diethylamino)oxy]ethyl]-5-methyluridine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (10). Compound **8** (1.02 g, 1.51 mmol)) was co-evaporated with toluene (10 mL) in vacuo. The residue was mixed with *N*,*N*-diisopropylamine tetrazolide (0.13 g, 0.76 mmol) and dried over P_2O_5 under reduced pressure overnight. To this, anhydrous acetonitrile (7.6 mL) was added, followed by 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisoporpylphosphorodiamidite (1.92 mL, 6.04 mmol). The mixture was stirred at room temperature under argon atmosphere until all of the starting material disappeared (TLC, CH_2Cl_2/MeOH/Pyridine, 94:5:1). The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (100 mL), washed with aqueous $NaHCO_3$ (5 wt%, 75 mL), and brine (75 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The oily residue obtained was dissolved in CH_2Cl_2 (5 mL) and added dropwise into vigorously stirring hexane (20 mL). The white solid formed was collected by decanting the hexane and dried under reduced pressure to afford **10** as a white foam (0.92 g, 70%): R_f=0.58 (CH_2Cl_2/MeOH/pyridine, 94:5:1); ^{31}P NMR (80 MHz, $CDCl_3$) δ 150.84, 150.54; HRMS (FAB) calcd for $C_{46}H_{62}N_5O_{10}PNa^+$ 898.4132, found 898.4113.

3.1.10. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*-isopropyl-*N*-methylamino)oxy]ethyl]-5-methyluridine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (11). Compound **11** (1.1 g, 85%) was prepared from compound **9** (1 g, 1.48 mmol), *N*,*N*-(diisopropyl)amine tetrazolide (0.25 g, 1.48 mmol), anhydrous acetonitrile and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.88 mL, 5.92 mmol) according to the procedure used for the synthesis of **10** from **8**, except that the residue obtained after work up was purified by flash silica gel column chromatography and eluted with ethyl acetate/hexane/pyridine (69:30:1): R_f=0.62 (ethyl acetate/hexane/pyridine, 69:30:1); ^{31}P NMR (80 MHz, $CDCl_3$) δ 150.86, 150.61; MS (FAB) *m*/*z* 898 $(M+Na)^+$; HRMS (FAB) calcd for $C_{46}H_{62}N_5O_{10}PNa^+$ 898.4162, found 898.4123.

3.1.11. 5′-*O*-(*tert*-Butyldiphenylsilyl)-2′-*O*-[2-[[*N*-methyl-*N*-(2-phthalimidoethyl)amino]oxy]ethyl]-5-methyluridine (12). Compound **3** (2.3 g, 4.17 mmol) was dissolved in 1 M PPTS in MeOH (41.7 mL) and $NaBH_3CN$ (0.52 g, 8.35) was added at 10°C and the mixture was stirred for 15 min. The reaction mixture was allowed to come to room temperature and stirring continued for an additional 4 h. The reaction was monitored by TLC (5% MeOH in CH_2Cl_2). The solvent was removed under reduced pressure and the residue obtained was diluted with ethyl acetate (50 mL) and washed with water (30 mL), aqueous $NaHCO_3$ (5 wt%, 30 mL), and brine (30 mL). The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness to yield a foam. This was then dissolved in 1 M PPTS in MeOH (34 mL). α-phthalimidoacetaldehyde (0.72 g, 3.78 mmol) was added and the reaction mixture was stirred at ambient temperature for 10 min. To this $NaBH_3CN$ (0.43 g, 0.89 mmol) was added at 10°C and stirred for 15 min. The reaction mixture was allowed to come to room temperature and stirring continued for an additional 4 h. The solvent was removed under reduced pressure and the oily residue obtained was diluted with ethyl acetate (50 mL) and washed with water (40 mL), aqueous $NaHCO_3$ (5 wt%, 40 mL), and brine (25 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was purified by flash silica gel column chromatography and eluted with ethyl acetate/hexane (60:40) to afford **12** (1.54 g, 60%): R_f=0.68 (ethyl acetate); ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.04 (s, 9H), 1.41 (s, 3H), 2.46 (s, 3H), 2.79 (t, 2H, *J*=6.34 Hz), 3.69–4.08 (m, 10H), 4.27 (m, 1H), 5.22 (d, 1H, *J*=5.7 Hz), 5.95 (d, 1H, *J*=5.86 Hz), 7.39–7.7 (m, 11H), 7.84 (s, 4H), 11.38 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.8, 19.4, 27.0, 35.6, 45.8, 58.2, 63.2, 68.9, 70.1, 71.1, 82.8, 84.4, 86.9, 111.1, 123.1, 126.3, 127.9, 128.0, 128.4, 130.1, 131.9, 132.1, 132.1, 133.9, 135.2, 135.5, 147.2, 150.2, 163.7, 168.5; HRMS (MALDI) calcd for $C_{39}H_{46}O_9N_4SiNa^+$ 765.2932, found 765.2922.

3.1.12. 2′-*O*-[2-[[*N*-Methyl-*N*-(2-phthalimidoethyl)amino]oxy]ethyl]-5-methyluridine (13). Compound **13** (0.42 g, 52% yield) was prepared from compound **12** (1.2 g, 1.62 mmol), triethylamine trihydrofluoride (2.64 mL, 16.2 mmol) and triethylamine (1.13 mL, 8.1 mol) and THF (16 mL) according to the procedure used for the synthesis of compound **6** from compound **4**: R_f=0.34, 10% MeOH in CH_2Cl_2; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.70 (s, 3H), 2.46 (s, 3H), 2.78 (t, 2H, *J*=6.35 Hz), 3.54–3.74 (m, 8H), 3.80 (d, 1H, *J*=3.52 Hz), 3.97 (t, 1H, *J*=5.26 Hz), 4.10 (q, 1H, *J*=4.98 Hz), 5.05 (d, 1H, *J*=5.58 Hz), 5.12 (t, 1H, *J*=5.14 Hz), 5.86 (d, 1H, *J*=5.64 Hz), 7.75 (s, 1H), 7.84 (s, 4H), 11.29 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.4, 35.6, 45.6, 58.1, 61.6, 69.1, 70.0, 71.0, 81.4, 85.2, 90.7, 110.7, 123.2, 132.0, 133.9, 138.1, 150.5, 164.2, 168.5; HRMS (FAB) calcd for $C_{23}H_{28}O_9N_4Na^+$ 527.1927, found 527.1917.

3.1.13. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[[*N*-methyl-*N*-(2-phthalimido)ethylamino]oxy]ethyl]-5-methyluridine (14). Compound **14** (0.47 g, 73% yield) was prepared from compound **13** (0.4 g, 0.79 mmol), DMAP (0.019 g, 0.16 mmol), anhydrous pyridine (1.9 mL), and DMTCl (0.29 g, 0.87 mmol) according to the procedure described

for synthesis of compound **8** from compound **6**: R_f=0.35, (5% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.36 (s, 3H), 2.48 (s, 3H), 2.79 (t, 2H, J=6.34 Hz), 3.21 (m, 2H), 3.73 (br s, 12H), 3.97 (m, 1H), 4.07 (m 1H), 4.22 (m, 1H), 5.16 (d, 1H, J=6.12 Hz), 5.87 (d, 1H, J=4.94 Hz), 6.89 (d, 3H, J=4 Hz), 7.34–7.43 (m, 9H), 7.48 (s, 1H), 7.83 (s, 4H), 11.36 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.6, 35.4, 45.5, 54.8, 57.9, 62.0, 68.9, 69.9, 82.6, 83.2, 86.5, 87.3, 110.5, 113.0, 122.6, 125.8, 127.7, 128.0, 129.8, 131.8, 133.6, 135.3, 135.8, 144.2, 150.4, 158.3, 164.3, 168.2; HRMS (FAB) calcd for $C_{44}H_{46}O_{11}N_4Na^+$ 829.3061, found 829.3066.

3.1.14. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[[*N*-methyl-*N*-(2-phthalimidoethyl)amino]oxy]ethyl]-5-methyl-uridine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl)phosphoramidite (15). Compound **15** (0.28 g, 85%) was prepared from compound **14** (0.26 g, 0.32 mmol), *N*,*N*-diisopropylamine tetrazolide (0.06 g, 0.32 mmol), anhydrous acetonitrile (1.6 mL), and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-phosphorodiamidite (0.41 mL, 1.28 mmol) according to the procedure used for the synthesis of compound **10** from **8**, except that the residue obtained after work up was purified by flash silica gel column chromatography and eluted with ethyl acetate containing 0.5% of pyridine: R_f=0.28, ethyl acetate/hexane (60:40); ^{31}P NMR (80 MHz, $CDCl_3$) δ 150.82, 150.61; MS (FAB) m/z 1029 $[M+Na]^+$, HRMS (FAB) calcd for $C_{53}H_{63}O_{12}N_6PNa^+$ 1029.4132, found 1029.4112.

3.1.15. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*,*N*-diethyl-amino)oxy]ethyl]-3′-*O*-succinyl-5-methyluridine (16). Compound **8** (0.2 g, 0.3 mmol) was mixed with succinic anhydride (0.05 g, 0.45 mmol) and DMAP (0.02 g, 0.15 mmol) and dried over P_2O_5 in vacuo overnight. This was then dissolved in anhydrous CH_2Cl_2 (0.8 mL) and anhydrous triethylamine (0.08 mL, 0.6 mmol) was added and the reaction mixture was stirred at r.t. for 8 h under inert atmosphere. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with aqueous citric acid (ice cold, 15 mL, 10 wt%) and water (2×15 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford **16** (0.23 g, 100%) as a foam: R_f=0.44 (10% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.96 (t, 6H, J=7.22 Hz), 1.44 (s, 3H), 2.55–2.7 (m, 8H), 3.26 (m, 2H), 3.42–3.7 (m, 4H), 3.74 (s, 6H), 4.14 (br s, 1H), 4.37 (m, 1H), 5.31 (br s, 1H), 5.90 (d, 1H, J=6.36 Hz), 6.91 (d, 4H, J=8.3 Hz), 7.23–7.41 (m, 9H), 7.50 (s, 1H), 11.47 (s, 1H), 12.26 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.6, 28.9, 52.3, 55.1, 62.2, 69.6, 70.8, 72.5, 80.4, 81.1, 86.9, 87.1, 111.2, 113.2, 127.1, 128.0, 130.0, 135.0, 135.5, 144.0, 150.5, 158.7, 164.5, 171.5, 175.6; HRMS (FAB) calcd for $C_{41}H_{50}N_3O_{12}^+$ 776.3395, found 776.3380.

3.1.16. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*-isopropyl-*N*-methylamino)oxy]ethyl]-3′-*O*-succinyl-5-methyluridine (17). Compound **17** (0.31 g, 98% yield) was prepared from compound **9** (0.28 g, 0.41 mmol), succinic anhydride (0.134 g, 1.34 mmol), and DMAP (0.071 g, 0.58 mmol) according to the procedure used for the synthesis of compound **16** from compound **8**: R_f=0.38 (10% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.10 (s, 3H), 1.13 (s, 3H), 1.39 (s, 3H) 2.61 (s, 3H), 2.68 (m, 4H), 2.93 (m, 1H), 3.41 (dd, 1H, J=2.12, 8.9 Hz), 3.54–4.02 (m, 5H), 3.82 (s, 6H), 5.37 (m, 1H), 6.07 (d, 1H, J=4.84 Hz), 6.86 (d, 4H, J=8.74 Hz), 7.27–7.43 (m, 9H), 7.61 (s, 1H), 8.56 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.8, 18.8, 29.2, 41.7, 55.3, 58.4, 62.4, 69.7, 71.0, 71.4, 80.6, 81.4, 87.0, 87.2, 111.4, 113.4, 127.3, 128.2, 130.2, 135.1, 135.6, 144.2, 150.8, 158.8, 164.6, 171.7, 175.5; MS (FAB) m/z 776 $(M+H)^+$; HRMS (FAB) calcd for $C_{41}H_{50}N_3O_{12}^+$ 776.8423, found 776.8412.

3.1.17. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[[*N*-methyl-*N*-(2-phthalimidoethyl)amino]oxy]ethyl]-3′-*O*-succinyl-5-methyluridine (18). Compound **18** (0.162 g, 90% yield) was prepared from compound **14** (0.16 g, 0.2 mmol), DMAP (0.013 g, 0.10 mmol), and succinic anhydride (0.03 g, 0.3 mmol) according to the procedure used for the synthesis of compound **16** from compound **8**: R_f=0.43 (10% MeOH in CH_2Cl_2); ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.4 (s, 3H), 2.42 (s, 3H), 2.56 (m, 4H, overlapping with DMSO peak), 2.75 (t, 2H, J=6.29 Hz), 3.24 (m, 2H, overlapping with H_2O peak), 3.53–3.8 (m, 6H), 3.72 (s, 6H), 4.13 (br s, 1H), 4.37 (t, 1H, J=5.86 Hz), 5.29 (t, 1H, J=4.4 Hz), 5.87 (d, 1H, J=6.36 Hz), 6.89 (d, 4H, J=8.72 Hz), 7.21–7.39 (m, 9H), 7.49 (s, 1H), 7.82 (s, 4H), 11.42 (s, 1H), 12.24 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.7, 28.9, 29.4, 35.7, 45.7, 55.3, 58.0, 62.3, 69.7, 71.0, 71.2, 80.5, 81.2, 87.1, 111.2, 113.3, 123.3, 127.2, 128.0, 128.2, 130.1, 132.1, 133.9, 135.1, 135.2, 135.4, 144.1, 150.2, 158.8, 163.7, 168.5, 171.4, 173.9; MS (FAB) m/z 929 $[M+Na]^+$, HRMS (FAB) calcd for $C_{48}H_{50}N_4O_{14}Na^+$ 929.9253, found 929.9242.

3.1.18. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*,*N*-diethyl-amino)oxy]ethyl]-5-methyluridine-3′-*O*-succinyl-CPG (19). Compound **16** (0.21 g, 0.27 mmol) was dried over P_2O_5 in vacuo overnight. Anhydrous DMF (0.66 mL) was added, followed by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU, 0.09 g, 0.27 mmol) and *N*-methylmorpholine (0.06 mL, 0.54 mmol) and vortexed under argon atmosphere to yield a clear solution. An additional anhydrous DMF (2.1 mL) and CPG (1.17 g, 115.2 μmol g^{-1}, particle size 120/200, mean pore diameter 520 Å) was added and shaken for 18 h at room temperature. The solid support was filtered, washed with DMF, acetonitrile and diethyl ether dried in vacuo overnight and capped with a mixture of Ac_2O/pyridine/*N*-methylimidazole/THF (10:10:10:70) for 3 h at room temperature. The solid support was filtered and washed with acetonitrile and diethyl ether. The solid support (**19**) was dried in vacuo and final loading (57±0.5 μmol g^{-1}) was determined by the standard DMT assay.

3.1.19. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[(*N*-isopropyl-*N*-methylamino)oxy]ethyl]-5-methyluridine-3′-succinyl CPG (20). Compound **20** (51 μmol g^{-1}) was synthesized from **17** by a procedure similar to that used for the synthesis of compound **19** from compound **16**.

3.1.20. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-[[*N*-methyl-*N*-(2-phthalimidoethyl)amino]oxy] ethyl]-5-methyluridine-3′-*O*-succinyl CPG (21). Compound **18** (0.15 g, 0.17 mmol) and DMAP (0.021 g, 0.17 mmol) was dissolved in anhydrous acetonitrile. The reaction mixture was

protected from moisture and 2,2′-dithiobis(5-nitropyridine) (0.07 g, 0.19 mmol) was added and stirred for 5 min at room temperature. To this triphenyl phosphine (0.05 g, 0.17 mmol) in anhydrous acetonitrile (1.12 mL) was added. The solution was stirred at ambient temperature for 10 min. Activated CPG (1.12 g, 115.2 mmol g^{-1}, particle size 120/200, mean pore diameter 520 Å) was added and allowed to shake for 2 h. An aliquot was withdrawn and loading capacity was determined (61.52 mmol g^{-1}). The solid support was filtered and washed with CH_3CN, CH_2Cl_2 and Et_2O and dried in vacuo overnight and capped with a mixture of Ac_2O/pyridine/*N*-methylimidazole/THF (10:10:10:70) for 3 h at room temperature. Finally, the solid support was filtered and washed with CH_3CN, CH_2Cl_2 and Et_2O. The loading of **21** (60±0.5 μmol g^{-1}) was determined by standard DMT assay.

3.2. Oligonucleotide synthesis

A 0.1 M solution of the amidites **10**, **11** and **15** in anhydrous acetonitrile was used for the synthesis of modified oligonucleotides. Standard phosphoramidates were used for incorporation of A, G, C and T residues. The oligonucleotides were synthesized on solid support on a solid-phase DNA synthesizer. The solid supports **19**, **20** and **21** were used when necessary. For incorporation of **10**, **11**, and **15**, phosphoramidite solutions were delivered in two portions, each followed by a 5 min coupling wait time. All other steps in the protocol supplied by a manufacturer were used without modification. Oxidation of the internucleotide phosphite to the phosphate was carried out using CSO ([1-*S*-(+)-(10-camphorsulfonyl)oxaziridine], 0.5 M in acetonitrile) and 4 min oxidation wait time. The coupling efficiencies were more than 97%. After completion of the synthesis, the solid support-bound oligonucleotides **23**–**25** (2 μmol, Table 1) and **27**–**29** (2 μmol, Table 1) was suspended in aqueous ammonium hydroxide (2 mL, 30 wt%) and kept at room temperature for 2 h. The solid support was filtered and the filtrate was heated at 55°C for 6 h to complete the removal of all protecting groups. The solid support bound oligonucleotide **31** (2 μmol) was suspended in aqueous ammonium hydroxide (1.8 mL, 30 wt%) and kept at room temperature for 2 h. To this aqueous methylamine (0.2 mL, 40 wt%) was added to facilitate the deprotection of phthalimodo group and filtered. The filtrate was heated at 55°C for 6 h. Crude oligonucleotides were purified by HPLC (C-4 column, Waters, 7.8×300 mm, A=100 mM ammonium acetate, pH 6.5–7, B=acetonitrile, 5–60% of B in 55 min, flow 2.5 mL min^{-1}, λ 260 nm). Oligonucleotides were detritylated with aqueous 80% acetic acid at room temperature for 1 h. Purification and desalting by HPLC gave 2′-modified oligonucleotides in 30–40% isolated yield. The oligonucleotides were characterized by ES MS and purity was assessed by HPLC and capillary gel electrophoresis.

3.3. T_m analysis

The thermal stability of the duplexes formed by oligonucleotides with the 2′-modified residues and complementary RNA and DNA was studied by measuring the UV absorbance versus temperature curves as described previously.[16] Absorbance versus temperature curves were measured at 260 nm using a Gilford Response II spectrophotometer. The buffer contained 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7. The 500 mL of buffer for T_m analysis was prepared in a standard flask by adding 0.2669 g of $NaH_2PO_4.H_2O$, 0.8218 g $Na_2HPO_4.7H_2O$, 2.4482 g NaCl, 100 μL 0.5 M EDTA solution. The pH was adjusted to 7 and the volume was adjusted to 500 mL. Oligonucleotide and their complements were combined at 4 μM of each strand, heated for 5 min at 90°C, and cooled slowly to allow the formation of duplexes. Oligonucleotide concentrations were calculated from the oligonucleotide absorption at 260 nm at 85°C using extinction coefficients estimated according to the method of Puglisi and Tinoco.[17] Oligonucleotide solutions were heated at a rate of 0.7°C min^{-1} in 1 cm path length cells and then cooled to confirm reversibility and lack of evaporation. T_m values were obtained from the absorbance versus temperature curves. Standard deviations did not exceed ±0.5°C. Each T_m reported was an average of three experiments. The ΔT_m per modification was calculated by subtracting the T_m value of a duplex containing modified oligonucleotide from the T_m value of a duplex containing unmodified DNA (parent duplex), and dividing by the number of modified residues in the sequence.

3.4. Nuclease stability assay

The nuclease stability of the 2′-modified oligonucleotides was evaluated using SVPD assay as described previously.[12] Oligonucleotides were 5′ end-labeled using [γ^{-32}P]ATP and T4 polynucleotide kinase. After the labeling reaction, the T4 polynucleotide kinase was heat inactivated at 95°C for 3 min and oligonucleotides were used without further purification. The SVPD assay was performed at 37°C using 1 μM oligonucleotide in a buffer of 50 mM Tris–HCl, pH 8.5, 72 mM NaCl and 14 mM $MgCl_2$. The SVPD (USB, Cleveland, OH) enzyme concentration was 5×10^{-3} U mL^{-1}. The enzyme was shown to maintain its activity under these condition for 24 h. Aliquots of the reaction mixture were removed at the indicated times, quenched by addition to an equal volume of 80% formamide gel loading buffer containing tracking dyes, heated for 2 min at 95°C and then stored until analysis by denaturing polyacrylamide electrophoresis. Quantitation was performed on a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

3.5. Binding of oligonucleotides to human serum albumin

The equilibrium constants, K_d, of the oligonucleotides albumin binding were determined by following the procedure reported in the literature.[13,14b]

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