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2′-*O*-{2-[*N*,*N*-(Dialkyl)aminooxy]ethyl}-Modified Antisense Oligonucleotides

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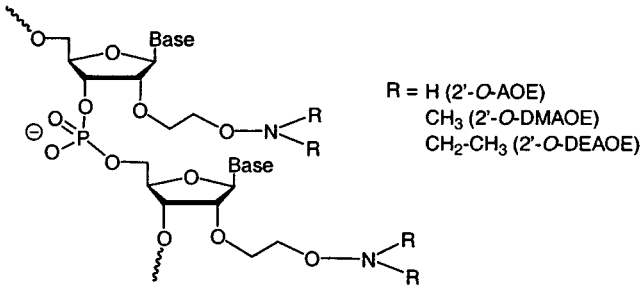
ABSTRACT



Oligonucleotides with two novel modifications, 2′-*O*-{2-[*N*,*N*-(dimethyl)aminooxy]ethyl} (2′-*O*-DMAOE) and 2′-*O*-{2-[*N*,*N*-(diethyl)aminooxy]ethyl} (2′-*O*-DEAOE), have been synthesized. These modifications exhibit high binding affinity to target RNA (and not to DNA) and enhance the nuclease stability of oligonucleotides considerably with $t_{1/2}$ > 24 h as a phosphodiester.

An ideal antisense oligonucleotide should have high binding affinity to the target RNA, high nuclease resistance, should bind selectively to transport proteins, and should be cell permeable in vivo.[1] 2′-*O*-Modified oligonucleotides[2,3] used with the "gapmer" technology[2,3] have emerged as leading second generation candidates for clinical applications. Among the 2′-modifications reported in the literature, the 2′-*O*-(2-methoxyethyl)[4] modification, abbreviated as 2′-*O*-MOE, offers a 2 °C increase in melting temperature (T_m) per modification as a diester (2′-*O*-MOE/PO) compared to the first generation 2′-oligodeoxyribonucleotide phosphorothioate (2′-H/PS) compounds. This modification as a phosphodiester linkage exhibits nuclease resistance (measured as the half-life of the full-length oligonucleotide, $t_{1/2}$) at approximately the same level as a 2′-deoxyphosphorothioate modification. To improve upon the 2′-*O*-MOE modification, we recently reported[5] the synthesis and properties of the 2′-*O*-(2-aminooxyethyl) modification (2′-*O*-AOE), the pseudoisostere of the 2′-*O*-MOE modification. Unfortunately, due to high reactivity of 2′-*O*-AOE, several modified residues cannot be conveniently incorporated into antisense oligonucleotides. However, this modification is extremely valuable as a conjugation site for various ligands.[6]

Here we report the synthesis of two dialkyl derivatives of 2′-*O*-AOE, 2′-*O*-{2-[*N*,*N*-(dimethyl)aminooxy]ethyl} (2′-*O*-DMAOE) and 2′-*O*-{2-[*N*,*N*-(diethyl)aminooxy]ethyl} (2′-*O*-DEAOE) modified oligonucleotides (Figure 1), their

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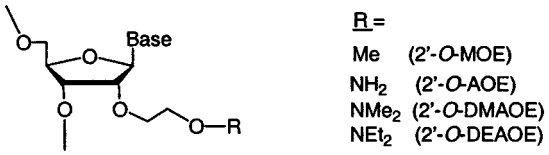
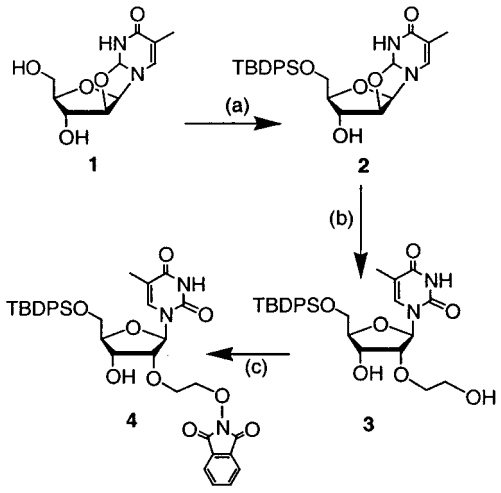


Figure 1.

affinity toward target RNA, and nuclease stability. These two modifications were selected for the following reasons: First, we envisaged that these modifications may retain the "gauche effect" [7] within the side chain maintaining the $C_{3'}$-endo sugar pucker shown by the 2′-*O*-MOE modification.[8] This should maintain the stable hybridization to the RNA target observed with the 2′-*O*-MOE modification. Second, these dialkyl modifications would be more lipophilic than the 2′-*O*-AOE and 2′-*O*-MOE modifications, a property that affects the protein binding and cellular permeation capabilities of antisense oligonucleotides.

For initial evaluations, we synthesized 2′-*O*-DMAOE and 2′-*O*-DEAOE modified 5-methyluridine phosphoramidites (**13** and **14)** and incorporated these phosphoramidites into oligonucleotides using a DNA synthesizer. The phosphoramidites **13** and **14** were synthesized according to Schemes 1−3. The 5′-hydroxy group of 2,2′-anhydro-5-methyluridine **1** was silylated with *tert*-butyldiphenylsilyl chloride (TBDPSCl) in pyridine (Py) to yield **2** in 75% yield (Scheme 1).[9] Product **2** was then subjected to ring opening[10] with a

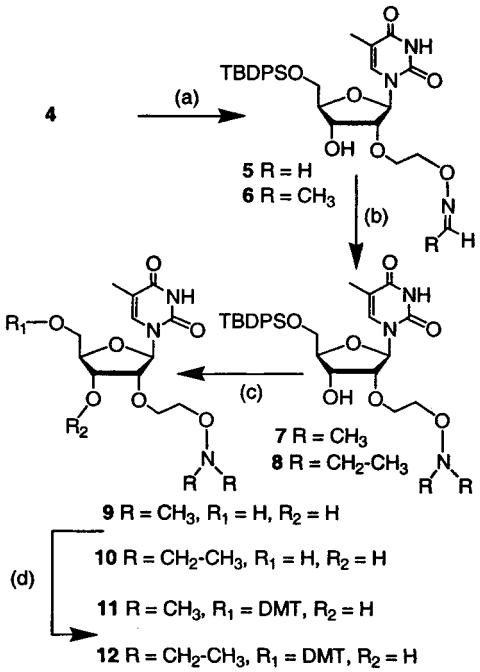
Scheme 1[a]



[a] (a) TBDPSCl, Py, rt; (b) BH_3·THF, ethylene glycol, 150 °C; (c) Ph_3P, *N*-hydroxyphthalimide, DEAD.

borate ester generated from ethylene glycol and borane in THF to provide 2′-*O*-(2-hydroxyethyl) nucleoside **3** in 50% yield. This simple procedure gave the intermediate for the construction of the 2′-*O*-DMAOE and 2′-*O*-DEAOE pyrimidine nucleosides. Compound **3**, treated under Mitsunobu[11] conditions with Ph_3P, *N*-hydroxyphthalimide, and diethylazodicarboxylate (DEAD) in THF, gave the phthalimido derivative **4** in 86% yield. Deprotection of the phthalimido group with *N*-methylhydrazine (NMH) at 0 °C gave the aminooxy derivative that was used without purification for the next step (Scheme 2). Treatment of the aminooxy

Scheme 2[a]



[a] (a) For **5**, 1. *N*-methylhydrazine, CH_2Cl_2, −10 to 0 °C; 2. MeOH, HCHO; for **6**, 1. *N*-methylhydrazine, CH_2Cl_2, −10 to 0 °C; 2. MeOH, CH_3CHO; (b) for **7**, 1. 1 M PPTS in MeOH, $NaCNBH_3$; 2. HCHO, 1 M PPTS in MeOH, $NaCNBH_3$; for **8**, 1. 1 M PPTS in MeOH, $NaCNBH_3$; 2. CH_3CHO, 1 M PPTS in MeOH, $NaCNBH_3$; (c) TEA·3HF, TEA, THF; (d) DMTCl, Py, DMAP, rt.

compound with formaldehyde or acetaldehyde in methanol at room temperature gave the formaldoximino derivative **5** (78% yield) or the acetaldoximino derivative **6** (55.5% yield). Reduction of **5** gave the monomethylaminooxy derivative which was then treated with formaldehyde under reductive conditions to give the dimethylaminooxy derivative **7** in 80% yield.

Reduction of compound **6** gave the monoethylaminooxy derivative which was treated with acetaldehyde to give the diethylaminooxy derivative **8** in 75% isolated yield. Compounds **7** and **8** were then desilylated with triethylamine trihydrogenfluoride (TEA·3HF) and triethylamine (TEA) in THF to give **9** (92% yield) and **10** (74% yield), respectively.

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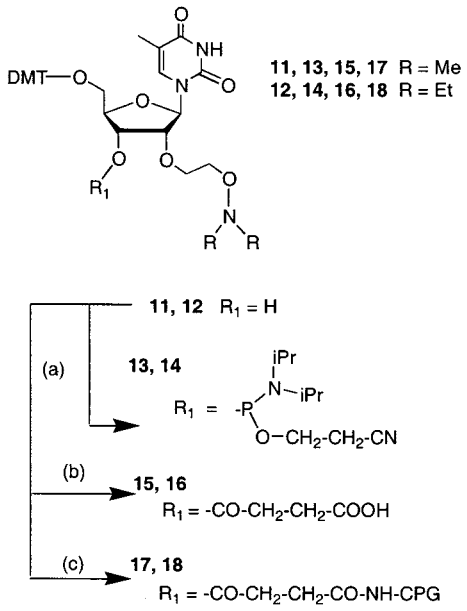
Table 1. Characterization of Oligonucleotides Containing 2′-*O*-DMAOE-5-methyluridine (T*) and 2′-*O*-DEAOE-5-methyluridine ($T^{§}$) Modifications

			ES MS		
no.	ISIS no.	sequence	calcd	found	HPLC t_R, min
19	17963	5′GCG T*T*T* T*T*T* T*T*T* T*GC G 3′	5906.4	5905.6	26.2
20	17961	5′T*CC AGG T*GT* CCG CAT* C 3′	5246.4	5245.6	23.6
21	20444	5′TTT TTT TTT TTT TTT T*T*T* T* 3′	6131.1	6130.0	25.4
22	32390	5′GCG $T^{§}T^{§}$ $T^{§}$ $T^{§}$ $T^{§}T^{§}$ $T^{§}$ $T^{§}$ $T^{§}$ $T^{§}$GC G 3′	6187.6	6188.1	32.4
23	32391	5′ $T^{§}$CC AGG $T^{§}$G $T^{§}$ CCG CAT§ C 3′	5194.4	5193.6	25.5
24	32388	5′TTT TTT TTT TTT TTT $T^{§}T^{§}T^{§}$ $T^{§}$ 3′	6243.2	6242.9	29.9

[a] Waters, C-4, 3.9 × 300 mm, A = 50 mM triethylammonium acetate, B = acetonitrile, 5 to 60% B in 55 min, flow 1.5 mL min^{-1}, λ = 260 nm.

Compounds **9** and **10** were converted into 5′-*O*-dimethoxytrityl derivatives **11** and **12** (80 and 87% yields, respectively) and then phosphytilated to yield compounds **13** (75% yield) and **14** (70% yield).[12] Compounds **11** and **12** were also converted into 3′-*O*- succinyl derivatives (**15** and **16)** and loaded on to amino alkyl controlled pore glass (CPG, Scheme 3) according to the standard synthetic procedure[13] to yield functionalized solid supports **17** (55 μmol/g loading) and **18** (57 μmol/g loading), respectively (Scheme 3).

Scheme 3[a]



[a] (a) *N*,*N*-Diisopropylamine tetrazolide, (2-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphophoramidite, CH_3CN, rt; (b) succinic anhydride, TEA, $ClCH_2-CH_2Cl$, DMAP, rt; (c) TBTU, DMF, 4-methylmorpholine, CPG, rt.

Oligonucleotides listed in Table 1 were synthesized using these building blocks.[14] Oxidation of the internucleotide phosphite to phosphate was carried out using CSO [1-*S*-(+)-(10-camphorsulfonyl)oxaziridine].[15]

(12) All compounds were characterized by ^1H, ^{13}C, and, where appropriate, by ^{31}P NMR and mass spectroscopy.

Hybridization properties of the modified oligonucleotides **19**, **20**, **22**, and **23** against complementary RNA and DNA were studied (Tables 2 and 3). When RNA (Table 2) was used as a target, oligonucleotides **19** and **22**, which contained 10 consecutive modifications, demonstrated a duplex stabilization of 1.5 °C per modification as compared to the unmodified DNA analogue and 2.3 °C per modification as compared to the phosphorothioate, respectively.[16] Similarly, oligonucleotides **20** and **23**, which contained 4 dispersed modifications, stabilized the duplex with RNA by 1.1 and

Table 2. T_m Data for the Oligonucleotides Containing 2′-*O*-DMAOE and 2′-*O*-DEAOE Modifications against Complementary RNA

oligo no.	no. of substituents	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/ modification
parent DNA1		48.3		
19	10	62.9	14.6	1.46
22	10	63.3	15.0	1.50
parent DNA2		62.3		
20	4	66.83	4.5	1.12
23	4	65.5	4.2	1.05

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(14) 0.1 M solution of amidites **13** and **14** in anhydrous acetonitrile was used for the synthesis of the modified oligonucleotides. For incorporation of **13** and **14**, phosphoramidite solutions were delivered in two portions, each followed by a 5 min coupling wait time. All other steps in the protocol supplied by Millipore were used without modification except for the oxidation step.[15] The coupling efficiencies were more than 97%. After completion of the synthesis, CPG was suspended in aqueous ammonium hydroxide (30 wt %) and kept at room temperature for 2 h. The CPG was filtered and the filtrate was heated at 55 °C for 6 h to complete the removal of all protecting groups. Crude oligonucleotides were purified by high performance liquid chromatography (HPLC, Waters, C-4, 7.8 × 300 mm, A = 50 mM triethylammonium acetate, pH = 7, B = acetonitrile, 5 to 60% B in 55 min, flow 2.5 mL min^{-1}, λ = 260 nm). Detritylation with aqueous 80% acetic acid followed by desalting gave 2′-modified oligonucleotides. Oligonucleotides were analyzed by HPLC, CGE, and mass spectroscopy. The isolated yields for modified oligonucleotides were 30−40%. These yields are similar to those obtained during unmodified DNA oligomer synthesis.

(15) See: Manoharan, M.; Lu, Y.; Casper, M. D.; Just, G. *Org. Lett.* **2000**, *2*, 243−246 and references cited.

(16) The 2′-deoxyphosphorothioates cause −0.8 °C/modification in RNA hybridization compared to DNA. See ref 17.

1.9 °C, compared to phosphodiester and phosphorothioate analogues, respectively.[17] Comparison of the T_m values of the oligonucleotides modified with 2′-*O*-DMAOE (**19** and **20**) with those modified with 2′-*O*-DEAOE (**22** and **23**) demonstrates no significant difference between the two substituents. This suggests that the addition of the steric bulk in the 2′-*O*-DEAOE modified oligonucleotides does not result in any substantial destabilization of hybridization with RNA. Furthermore, even with the increased bulk, the novel modifications demonstrate a palpable increase in binding affinity compared to the 2′-*O*-MOE modification.[4,17]

In contrast, hybridization of **19** and **22** with complementary DNA led to duplexes less stable than those formed with unmodified DNA oligonucleotides (−0.9 and −1.1 °C per modification, respectively, as shown in Table 3). These results demonstrate a high, RNA-selective hybridization of the 2′-*O*-DMAOE and 2′-*O*-DEAOE oligonucleotides.

Table 3. T_m Data for the Oligonucleotides Containing 2′-*O*-DMAOE and 2′-*O*-DEAOE Modifications against Complementary DNA

oligo no.	no. of substituents	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/ modification
parent DNA1		54.2		
19	10	45.4	−8.8	−0.88
22	10	42.9	−11.3	−1.13

To evaluate the stability of 2′-*O*-DMAOE and 2′-*O*-DEAOE oligonucleotides against nucleases, phosphodiester oligonucleotides **21** and **24** were synthesized and digested with snake venom phosphodiesterase (SVPD).[18] The modifications were placed at the 3′-terminal ends. Figure 2 shows the relative nuclease stability of these modified oligonucleotides compared to unmodified DNA oligonucleotide of the same sequence. Half-lives of modified oligonucleotides were more than 24 h. The ethyl analogue has a longer lifetime than the methyl analogue. The nuclease stability of 2′-*O*-DMAOE and 2′-*O*-DEAOE capped oligonucleotides was higher than that of the 2′-*O*-MOE oligonucleotide. Furthermore, we compared the relative lipophilicity of these two modifications. As expected, 2′-*O*- DEAOE is more lipophilic than 2′-*O*-DMAOE as assessed by reverse phase HPLC retention times (Table 1).

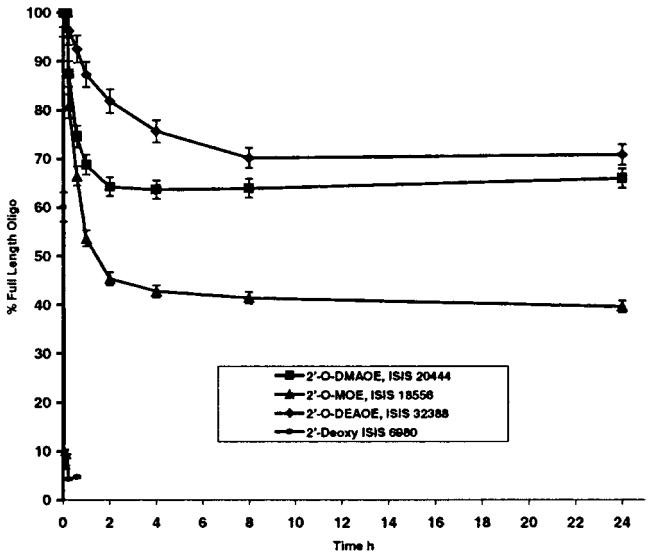

Figure 2. SVPD assay demonstrating relative nuclease resistance of 2′-*O*-DMAOE and 2′-*O*-DEAOE capped oligonucleotides TTT TTT TTT TTT TTT T*T*T*T* T*.

In conclusion, we have synthesized two novel types of 2′-modified oligonucleotides that showed high binding affinity to complementary RNA and high nuclease stability. High binding affinity to RNA, favorable lipophilicity, and high nuclease resistance makes these modifications ideal candidates for further evaluation for antisense drug development. Evaluation of other antisense properties such as in vivo pharmacology is in progress.

Acknowledgment. We are grateful to Dr. P. Dan Cook for his impetus and support in the design of the modifications described in this paper. We thank Drs. Bruce Ross and Bal Bhat for helpful discussions.

Supporting Information Available: Analytical data for compounds **11**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL006555G

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