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2′-Modified-2-thiothymidine Oligonucleotides

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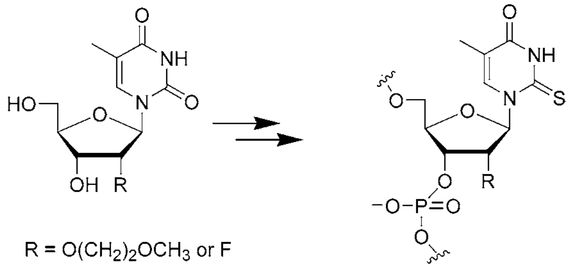
ABSTRACT



R = $O(CH_2)_2OCH_3$ or F

Oligonucleotides with novel modifications, 2′-*O*-[2-(methoxy)ethyl]-2-thiothymidine and 2′-deoxy-2′-fluoro-2-thiothymidine, have been synthesized. These modified oligonucleotides exhibited very high thermal stability when hybridized with complementary RNA. 2-*O*-(2-Methoxy)ethyl-2-thiothymidine modified oligonucleotide phosphodiesters showed enhanced resistance toward nucleases ($t_{1/2}$ > 24 h), but 2′-deoxy-2′-fluoro-2-thiothymidine modified oligonucleotide phosphodiesters showed limited stablity to nucleotytic degradation.

Oligonucleotides that form stable duplexes with RNA and that are resistant to digestion by nucleases are needed for antisense applications.[1] In addition, to be therapeutically useful, the synthesis of the modifications and of the oligonucleotides must be straightforward and economical. 2-Thiouridine residues are known to adopt a $C_{3'}$-*endo* sugar pucker[2] and oligonucleotides with this modification form thermodynamically stable duplexes with complementary RNA.[3] Furthermore, due to steric hindrance and the weaker H-bonding ability of sulfur relative to oxygen, 2-thiouridine destabilizes U-G wobble[2a] and U-2-amino-A base pairs[4] compared to uridine. As a result, a 2-thiouridine exhibits excellent hybridization specificity. Recently, Sekine et al. have reported the synthesis of 2′-*O*-methyl-2-thiouridine modified oligonucleotides[5] and have demonstrated better selectivity of 2′-*O*-methyl-2-thiouridine for adenine than guanine relative to unmodified uridine.

The 2′-*O*-[2-(methoxy)ethyl] (2′-*O*-MOE)-modified gapmer oligonucleotides have emerged as a promising choice for antisense applications.[1,6] 2′-*O*-MOE-modified nucleotides prefer to adopt the $C_{3'}$-*endo* sugar pucker favorable for RNA binding, due to an extended gauche effect and hydration involving the 2′-*O*-MOE side chain.[6c] Another 2′-modification that favors a $C_{3'}$-*endo* sugar pucker and A-type conformation when hybridized with RNA is 2′-deoxy-2′-fluoro (2′-F).[7] Though the 2′-deoxy-2′-fluoro ribo modification containing oligonucleotides favors A-form helices while binding to

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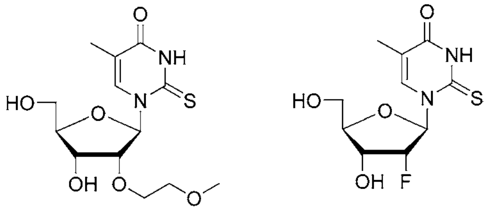
complementary nucleic acids, its resistance to nucleolytic degradation was very poor.[7a] On the other hand, the 2′-*O*-MOE side chain provided better resistance to nucleolytic degradation compared to the corresponding unmodified 2′-deoxy sugar-containing oligonucleotides.

We envisioned that oligonucleotides modified with 2-thiouridine and a 5-methyl substituent along with the use of either 2′-*O*-MOE (**1a**, Figure 1) or 2′-F (**1b**, Figure 1) sugar modifications would significantly improve the hybridization affinity toward RNA relative to an unmodified DNA oligonucleotide and the use of 2′-*O*-MOE should provide resistance to nucleolytic degradation.



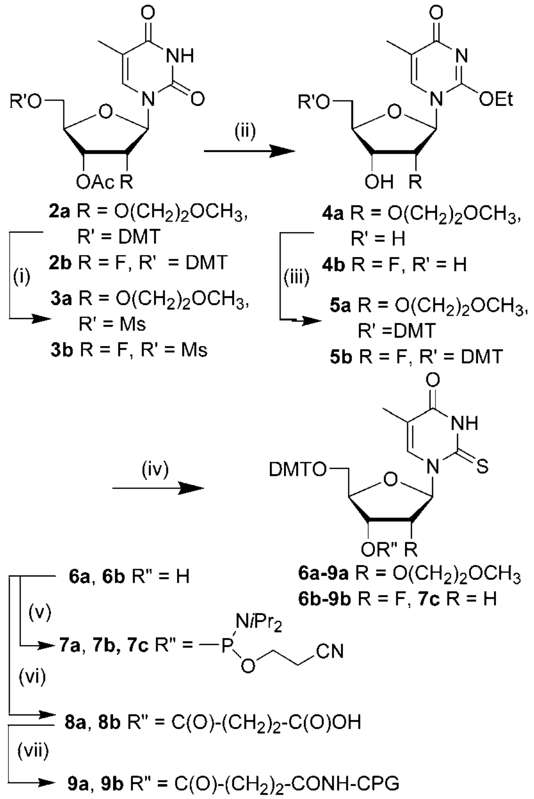
1a **1b**

Figure 1. 2′-*O*-MOE−2-thiothymidine (**1a**) and 2′-deoxy-2′-F-2-thiothymidine (**1b**).

Here we report the facile synthesis of 5′-*O*-DMT-2′-*O*-MOE-5-methyl-2-thiouridine-3′-phosphoramidite (**7a**), 5′-*O*-DMT-2′-deoxy-2′-fluoro-5-methyl-2-thiouridine-3′-phosphoramidite (**7b**), and the corresponding solid supports (**9a** and **9b**) (Scheme 1). Also reported are incorporation of these novel nucleoside modifications into oligonucleotides, thermal stability of the modified oligonucleotide duplexes with complementary DNA and RNA, and resistance to snake venom phosphodiesterase (SVPD) digestion relative to unmodified DNA.

Syntheses of phosphoramidites **7a** and **7b** are shown in Scheme 1. Compound **3a** was prepared from 5′-*O*-DMT-2′-*O*-MOE-thymidine according to standard procedures.[8] The methanesulfonate **3a** was refluxed in absolute ethanol in the presence of 2.5 molar equiv of anhydrous $NaHCO_3$ under argon atmosphere for 36 h to afford 2′-*O*-MOE-2-*O*-ethylthymidine (**4a**) in 75% yield.[9] Reaction of compound **4a** with DMT-Cl in anhydrous pyridine in the presence of DMAP afforded 5′-*O*-DMT-2′-*O*-MOE-2-*O*-ethylthymidine (**5a**) in 90% yield. Treatment of compound **5a** with H_2S and 1,1,3,3-tetramethylguanidine (TMG) in anhydrous pyridine gave 5′-*O*-DMT-2′-*O*-MOE−5-methyl-2-thiouridine (**6a**) in 85% yield.[10] Flash column chromatography with a 1:1 mixture of ethyl acetate and hexane was used to remove TMG-H_2S salt contamination from the product. Phosphitylation of compound **6a** gave the desired phosphoramidite **7a** in over 90% yield.[11]

Scheme 1[a]



[a] Reagents and conditions: (i) (a) HOAc/H_2O (4:1), rt, (b) MsCl/Py-dichloromethane (1:1), −20 °C; (ii) $NaHCO_3$ (2.5 mol equiv)/EtOH, reflux, 36 h; (iii) DMT-Cl, DMAP/Py, rt; (iv) for **6a**: H_2S, TMG (10 mol equiv)/Py, rt, 72 h; for **6b**: H_2S, Py, 60 °C, 72 h; (v) 2-cyanoethyltetraisopropylphosphorodiamidite, *N*,*N*-diisopropylammonium tetrazolide, CH_3CN, rt; (vi) succinic anhydride, 1,2-dichloroethane, DMAP, $(C_2H_5)_3N$, rt; (vii) 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU), 4-methylmorpholine, DMF, aminoalkyl controlled pore glass (CPG), rt.

Compound **3b** was prepared in quantitative yield from compound **2b** according to standard procedures.[8] Treatment of compound **3b** with anhydrous $NaHCO_3$ in ethanol under reflux gave a mixture of products with close R_f values, not observed when **4a** was synthesized from compound **3a**. This may be due to side reactions such as 2,2′-anhydro formation in the presence of $NaHCO_3$ and subsequent ring opening at elevated temperature in ethanol.[12] The mixture of products was treated with DMT-Cl in pyridine in the presence of a catalytic amount of DMAP to obtain compound **5b** in 33% over-all isolated yield from compound **3b**. Compound **5b** was heated with saturated H_2S in anhydrous pyridine at 60 °C for 72 h to afford compound **6b** in 53% yield. TMG was excluded to avoid the possible formation of the 2,2′-anhydro

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derivative of **6b**. At room temperature the thiolation reaction of compound **5b** with H_2S in anhydrous pyridine was very slow. Phosphitylation of compound **6b** gave compound **7b** in 60% yield. Compounds **6a** and **6b** were converted into 3-*O*-succinyl derivatives, **8a** and **8b**, and loaded on to amino alkyl controlled pore glass (CPG, Scheme 1) to obtain supports **9a** (loading capacity: 56.96 μmol/g) and **9b** (loading capacity: 58.31 μmol/g).[11]

Commercially available phosphoramidites were used for oligonucleotide synthesis unless otherwise specified. Phosphoramidite **7a** was incorporated into oligonucleotides **10**, **17** (Table 1), and **23** (Figure 2a).[8] Oxidation of the internucleotide phosphite to the phosphate was carried out with *tert*-butylhydroperoxide/acetonitrile/water (10:87:3) with a 10 min oxidation wait period.[13] The oligonucleotides were characterized by ES-MS, capillary gel electrophoresis, and HPLC. Oligonucleotides containing 2′-deoxy-2-thiothymidine (**12** and **18**, Table 1, and **27**, Figure 2b) were synthesized according to the same protocols with use of commercial phosphoramidite **7c** obtained from Glen Research Inc.

Table 1. Effect of the 2′-*O*-MOE−2-thiothymidine (X), 2′-Deoxy-2′-fluoro-2-thiothymidine (Y), 2′-Deoxy-2-thiothymidine (Z), 2′-*O*-MOE-thymidine (*t*), and 2′-Deoxy-2′-flourouridine (*u*) Modifications on Stability of Duplexes with Complementary RNA

seq no.	sequence 5′−3′	T_m,[8] °C	ΔT_m,[16] (°C)/unit
10	d(XCCAGGXGXCCGCAXC)	74.1	+2.9
11	d(YCCAGGYGYCCGCAYC)	77.3	+3.7
12	d(ZCCAGGZGZCCGCAZC)	65.8	+0.9
13	d(*t*CCAGG*t*G*t*CCGCA*t*C)	66.0	+0.9
14	d(TCCAGGTGTCCGCATC)	62.4	
15	d(*u*CCAGG*u*G*u*CCGCA*u*C)[17]	64.8	+0.6
16	d(UCCAGGUGUCCGCAUC)[17]	60.0	−0.6
17	d(GCGXXXXXXXXXXGCG)	82.9	+3.4
18	d(GCGZZZZZZZZZZGCG)	65.4	+1.7
19	d(GCG*tttttttttt*GCG)	60.5	+1.2
20	d(GCGTTTTTTTTTTGCG)	48.5	
21	d(CTCGTACYYYYCCGGTCC)	76.9	+3.8
22	d(CTCGTACTTTTCCGGTCC)	61.8	

The synthesis of oligonucleotides **11**, **21** (Table 1), and **26** (Figure 2b) was first attempted with phosphoramidite **7b** and standard phosphoramidites of A, G, and C. However, deprotection with aqueous ammonia gave a complex mixture of products as evident from ES-MS analysis. Therefore, 2-cyanoethyl phosphoramidites and solid supports with N^6-phenoxyacetyl-A, N^2-(4-isopropyl-phenoxyacetyl)-G, and N^4-acetyl-C (Glen Research Inc.) were used for the synthesis. The coupling and oxidation protocols were the same as those used for the synthesis of oligonucleotide **10**. The solid-support bound oligonucleotides were treated with 1 M piperidine in anhydrous acetonitrile, to remove the cyanoethyl protection from the phosphate,[14] and subsequently with freshly prepared 0.05 M K_2CO_3 in methanol to complete the deprotection.[15] HPLC purification, detritylation, and desalting gave **11**, **21** (5−10% isolated yield), and **26** (30% isolated yield).

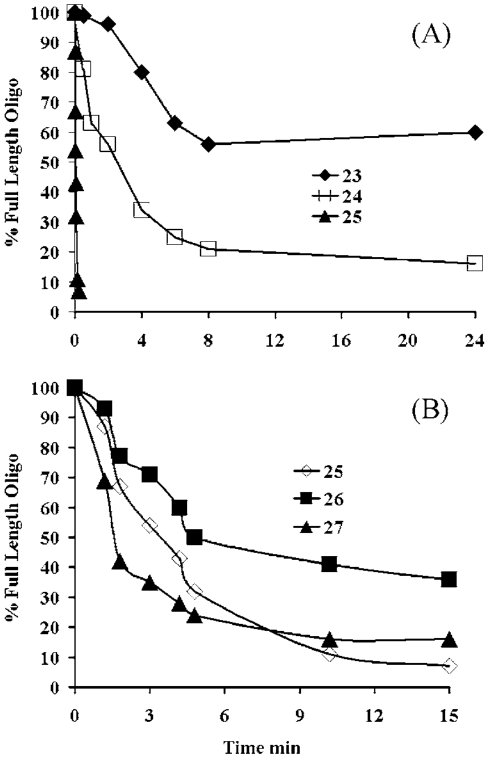

Figure 2. The disappearance of oligonucleotides **23**−**27** in the presence of SVPD as a function of time: **23**, 5′-$T_{15}X_4$-3′ (X = 2′-*O*-MOE-2-thiothymidine); **24**, 5′-$T_{15}t_4$-3′ (*t* = 2′-*O*-MOE-thymidine); **25**, T_{20}; **26**, 5′-$T_{15}Y_4$-3′ (Y = 2′-deoxy-2′-fluoro-2-thiothymidine); **27**, 5′-$T_{15}Z_4$-3′ (Z = 2′-deoxy-2-thiothymidine); each point is an average of two experiments.[8]

Thermal denaturation studies of duplexes formed between oligonucleotides **10**−**22** and complementary RNA are summarized in Table 1. Incorporation of 2′-*O*-MOE-2-thiothymidine (**10** and **17**, Table 1) provided a melting temperature (T_m) enhancement of 2.9−3.4 and 2.0−2.2 °C per modification compared to 2′-deoxy oligonucleotides (**14** and **20**) and 2′-*O*-MOE oligonucleotides (**13** and **19**), respectively. The T_m enhancement observed with oligonucleotides containing singly modified 2′-deoxy-2-thiothymidine (**12** and **18**) or 2′-*O*-MOE-thymidine (**13** and **19**) hybridized to complementary RNA was between 0.9 and 1.7 °C per modification when compared to the 2′-deoxyoligonucleotides (**14** and **20**).

Earlier studies showed that both 2′-deoxy-2-thiothymidine[2] and 2′-*O*-MOE-thymidine[6c] favor the $C_{3'}$-*endo* sugar conformation in the oligonucleotides and thus contribute to the stability of duplexes formed with complementary RNA. Replacement of thymidine with 2-thiothymidine, due to the

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intrinsic $C_{3'}$-*endo* conformational preference of the later, does not allow binding of human RNase H to RNA-DNA hybrid containing 2-thiothymidine in the DNA part.[18] Thus the advantage of using 2-thiothymidine (as 2′-deoxy) is lost in antisense applications due to its inability to recruit RNase H. This makes the combination of a 2′-modification and the 2-thiothymidine preferred. When these two modifications were combined in one nucleoside residue, as in 2′-*O*-MOE-2-thiothymidine, the T_m enhancement for the duplex formed with complementary RNA was additive.

The oligonucleotide with 2′-deoxy-2′-fluorouridine (**15**), which lacks a 5-methyl group relative to 2′-fluoro-thymidine, stabilized the duplex by 0.6 °C per modification compared to DNA control **14**.[17] The loss in thermal stability due to the lack of a methyl group was 0.6 °C per modification (**16**),[17] and therefore, one would expect an increase of about 1.2 °C per modification for an oligonucleotide modified with 2′-deoxy-2′-fluorothymidine. The oligonucleotides **11** and **21** containing the 2′-deoxy-2′-fluoro-2-thiothymidine increased thermal stability of duplex with complementary RNA up to 3.8 °C per modification (Table 1) compared to the unmodified controls (**14** and **22**). These results indicate the synergistic stereoelectronic effect of 2-thio and 2′-*O*-MOE or 2′-F modifications on enhancement of thermal stability against complementary RNA. This is in contrast to the 2′-*O*-methyl-2-thiouridine modification where no additive effect on thermal stability was observed.[5]

Unlike the 2′-*O*-MOE-thymidine oligonucleotide that destabilizes a duplex with complementary DNA (**19**, Table 2) compared to the control oligonucleotide **20**, both 2′-*O*-MOE-2-thiothymidine (**17**, Table 2) and 2′-deoxy-2-thiothymidine (**18**, Table 2) oligonucleotides stabilize the duplexes with complementary DNA. However, when compared to the duplex with RNA (**17** and **18**, $\Delta T_m = 3.4$ and 1.7 °C per modification, respectively, Table 1), the stabilization of the duplex with DNA (**17** and **18**, $\Delta T_m = 1.9$ and 0.8 °C per modification, respectively, Table 2) was less. These data suggest an RNA selective preorganization and hybridization of the 2′-*O*-MOE-2-thiothymidine and 2′-deoxy-2-thiothymidine modified oligonucleotides.

Table 2. Effect of the 2′-*O*-MOE-2-thiothymidine (X), 2′-Deoxy-2-thiothymidine (Z), and 2′-*O*-MOE-thymidine (*t*) Modifications on Stability of Duplexes with Complementary DNA

seq no.	modification	T_m,[8] °C	ΔT_m, °C/modification
17	X	73.50	+1.9
18	Z	62.00	+0.8
19	*t*	42.40	−1.2
20	parent DNA	54.20	

The susceptibility of oligonucleotides containing novel modifications to nuclease digestion was evaluated by using a standard assay with SVPD.[19] The enzymatic hydrolysis of phosphodiester oligonucleotides **23**−**27**, capped at the 3′-terminus with four modified nucleoside residues, was evaluated. The plot of the time-dependent disappearance of the novel oligonucleotides and their 2′-deoxy and 2′-*O*-MOE analogues is shown in Figure 2. The half-life of the oligonucleotide with 2′-*O*-MOE-2-thiothymidine modification (**23**) was over 24 h and showed better resistance to SVPD than the 2′-*O*-MOE-thymidine modified oligonucleotide (**24**, Figure 2A). However, the 2′-deoxy-2′-fluoro-2-thiothymidine (**26**) and the 2′-deoxy-2-thiothymidine (**27**) modified oligonucleotides were degraded as rapidly as the 2′-deoxy-oligonucleotide (half-lives between 1.5 and 5 min, Figure 2B). Thus the replacement of thymine with 2-thiothymine did not improve the nuclease resistance of oligonucleotides. On the other hand, when the 2′-*O*-MOE-2-thiothymidine (Figure 2A) and the 2′-deoxy-2-thiothymidine modification (Figure 2B) are compared, it is clear that a combination of 2′-*O*-MOE ribose sugar and 2-thio-5-methyluracil in a modified pyrimidine nucleoside provides enhanced nuclease resistance compared to the corresponding singly modified nucleoside when incorporated into oligonucleotides..

In conclusion, facile syntheses of 2′-*O*-MOE-2-thiothymidine and 2′-F-2-thiothymidine and their incorporation into oligonucleotides were demonstrated. The additive effect of the 2′-*O*-MOE or 2′-F and the 2-thiothymine modifications on thermal stability suggests that there is a synergistic stereoelectronic effect of sugar and base modifications in preorganizing the modified oligonucleotides to form an A-type helix. High binding affinity, nuclease stability, and expected favorable pharmacokinetic properties (due to sulfur and 2′-*O*-MOE modifications) warrant further evaluation of 2′-*O*-MOE-2-thiothymidine modified oligonucleotides for antisense applications. The remarkably high binding affinity of 2′-F-2-thiothymidine makes it a good candidate for hybridization-dependent nontherapeutic applications as well.

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Supporting Information Available: Experimental procedures and spectroscopic data for compounds **2a**−**8a**, **2b**−**8b**, **10**, **11**, **17**, **21**, **23**, **26**, and **27**; procedures used for T_m and nuclease studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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