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Synthesis of Chimeric Oligonucleotides Containing Phosphodiester, Phosphorothioate, and Phosphoramidate Linkages

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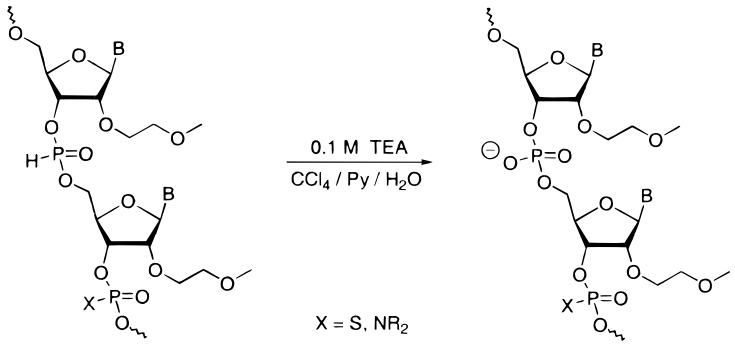
ABSTRACT



***H*-Phosphonate monomers of 2′-*O*-(2-methoxyethyl) ribonucleosides have been synthesized. Oxidation of oligonucleotide *H*-phosphonates has been optimized to allow the synthesis of oligonucleotides containing either 2′-deoxy or 2′-*O*-(2-methoxyethyl) ribonucleoside residues combined with three different phosphate modifications in the backbone, i.e., phosphodiester (PO), phosphorothioate (PS), and phosphoramidate (PN). Phosphodiester linkages were introduced by oxidation with a cocktail of 0.1 M Et_3N in CCl_4/Pyr/H_2O (5:9:1) without affecting phosphorothioate or phosphoramidate linkages. For the synthesis of phosphoramidate-modified oligonucleotides, N^4-acetyl deoxycytidine-3′-*H*-phosphonate monomers were used to avoid transamination during the oxidation step.**

A potent antisense drug should ideally have resistance to nucleases, high affinity for the target mRNA, and favorable pharmacokinetics and should cause inactivation of the target mRNA either by RNase H-mediated cleavage or by non-RNase H mechanisms.[1] One approach meeting these requirements involves the construction of "designer" oligonucleotides containing segments of different chemistries involving carbohydrate, backbone, and heterocyclic modifications.[2] Recently, a number of 2′-modifications at the pentofuranose sugar moiety have been reported that offer the dual advantages of high nuclease resistance and enhanced binding affinity.[3,4] Most second generation antisense compounds currently undergoing clinical trials are phosphorothioate (PS)

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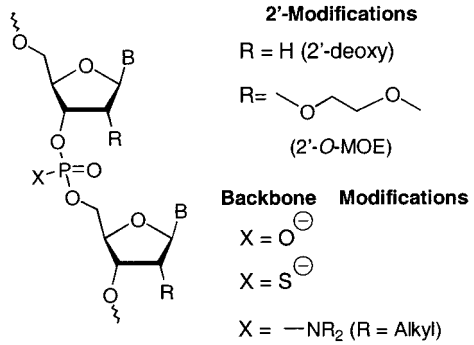
"gapmer" oligonucleotides that contain 2′-modified ribonucleotides on one or both ends. A 2′-deoxy (2′-H) segment in the center is required to maintain RNase H activity.[5] Among the 2′-modifications reported in the literature, the 2′-*O*-(2-methoxyethyl)[6] abbreviated as (2′-*O*-MOE) modification (Scheme 1) offers a 2 °C increase in melting

Scheme 1



temperature (T_m) per modification as a phosphodiester (2′-*O*-MOE/PO) compared to the first generation 2′-deoxyphosphorothioate (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. Since 2′-modifications such as 2′-*O*-MOE provide sufficient nuclease resistance even with a phosphodiester (PO) backbone, use of these modifications can reduce nonspecific protein binding properties characteristic of oligonucleotides with PS linkages. A third type of backbone linkage known from the literature is the cationic phosphoramidate (PN).[7] This linkage is expected to provide both nuclease resistance and improved cellular permeation, as do cationic tethers such as the 2′-*O*-aminopropyl.[8]

The ideal antisense drug molecule should have virtues derived from each of these backbone linkages (PO, PS, and PN) and the 2′-modifications. (Scheme 1). However, the achievement of synthesis of such a designer construct is limited by the present methods of oligonucleotide synthesis. Generally, antisense oligonucleotides are synthesized by the phosphoramidite methodology[9] which does not permit a straightforward preparation of analogues at phosphorus other than PS and PO. In contrast, *H*-phosphonate chemistry[10] offers a versatile route to synthesis of various phosphate analogues by oxidation of a single precursor under different conditions. Herein we report the synthesis of combined backbone linkage oligonucleotides having PO, PS, and PN linkages using the *H*-phosphonate methodology. In addition we have employed two variations in 2′-carbohydrate chemistries, namely, 2′-deoxy and 2′-*O*-methoxyethyl. (Scheme 1). We rationalized that the 2′-*O*-MOE/PO segment would provide high binding affinity, the 2′-H/PS region would allow RNase H activation and protein binding properties, and the PN linkages would impart nuclease resistance. This is the first literature report of a convenient, successful method for the synthesis of a "combined" backbone oligonucleotide.

In employing the *H*-phosphonate method for the synthesis of modified backbone linkages bearing oligonucleotides, we realized that oxidation is the most troublesome step of this approach. Since *H*-phosphonate linkages are highly susceptible to alkaline hydrolysis, incomplete oxidation results in strand cleavage during the subsequent deprotection with ammonia. Under aqueous conditions, partial cleavage of the backbone may also occur during the oxidation step. Consequently, the oxidation of *H*-phosphonates has been the subject of numerous studies in which various reagents that convert *H*-phosphonate diesters to PO,[11] PS,[12] and PN linkages[13] have been tested.

Taking the next step, preparation of oligonucleotides that contain different backbone modifications in the *same* oligonucleotide is more problematic and requires carefully controlled coupling and oxidation conditions. The oligonucleotide is synthesized and oxidized in blocks. The desired number of nucleotides are coupled, and the support-bound material is treated with the first oxidizer. Then, the next segment is assembled and treated with the second oxidizer. This block-wise synthesis and oxidation is continued until all segments of the oligonucleotide strand are completed.

In our experience, the modifications introduced by the first oxidation were often affected by the subsequent oxidative treatment. Hence, in the present study we determined appropriate oxidation conditions for the preparation of chimeric oligonucleotides containing PO, PS, and PN linkages and 2′-*O*-MOE sugar modifications.

5′-*O*-DMT-2′-*O*-methoxyethyl nucleoside-3′-*H*-phosphonates were synthesized by using the methods reported for 2′-deoxy monomers,[14] with minor modifications. Automated oligonucleotide synthesis was performed on a commercially available support as outlined in Scheme 2.

(5) Clinical trials with second generation antisense compounds are in progress, for example, at Isis Pharmaceuticals, Inc. and at Hybridon, Inc.

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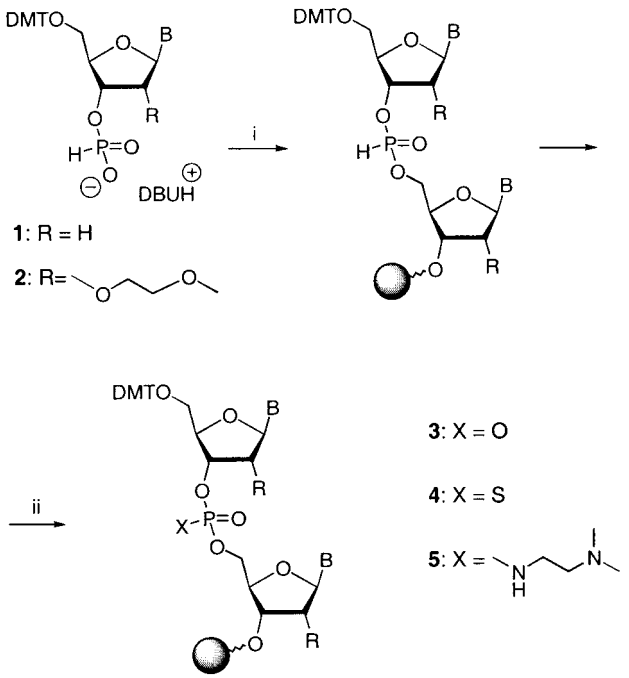
Scheme 2[a]



[a] Reaction conditions: (i) Nucleoside-CPG (1 equiv), **1** or **2** (10 equiv; 0.05 M in CH_3CN/Py, 1:1), pivaloyl chloride (40 equiv; 0.2 M in CH_3CN/Py, 1:1); (ii) For **3**: 0.1 M TEA in CCl_4/Py/H_2O (5:9:1). For **4**: S_8 (10% in CS_2/Py/TEA, 35:35:1). For **5**: DMAEA (5% in CCl_4).

PS-linkages were introduced by treatment with S_8; this treatment did not affect PO or PN linkages introduced earlier in the synthesis. Conversion of *H*-phosphonate to PN-linkages was performed with *N*,*N*-dimethylaminoethylamine (DMAEA) in CCl_4 with no adverse effects on PO and PS linkages. However, N^4-acetyl deoxycytidine-3′-*H*-phosphonate (dC^{Ac})[14] rather than commercial dC^{Bz} *H*-phosphonate was used for oligonucleotide synthesis to prevent transamination with the primary amine. Partially deprotected exocyclic amino functions were reacylated in a subsequent capping step using a mixture of Ac_2O (10% in THF/lutidine, 8:1) and 1-methylimidazole (10% in THF). To determine how transamination and deprotection of exocyclic amino functions affected the synthesis quality, we prepared a 16-mer oligonucleotide 5′-T^{PN}CCAGG T^{PN}GT^{PN}CCG CAT^{PN}C with a PO backbone except where indicated. Oligonucleotides were prepared either with the standard method using dC^{Bz} monomers or using dC^{Ac} together with the capping step performed after PN oxidation. ES-MS and CGE analysis of the crude products clearly demonstrated that the former method produced a number of impurities, mainly due to transamination, while the latter procedure resulted in a product of significantly higher purity and homogeneity.

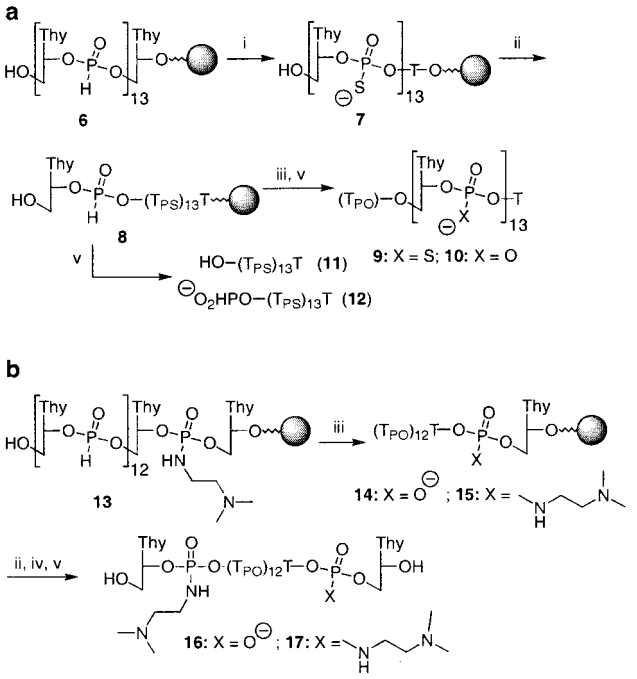
In a model experiment, oligothymidylate, **8**, was prepared, and various oxidation procedures (methods A to D) were tested with respect to their efficiency and compatibility with PS chemistry (Scheme 3 and Table 1). To evaluate the oxidation efficiency, the presence of (n − 1) fragments, **11** and **12**, resulting from hydrolysis of unreacted **8** on the deprotection step was followed by mass spectrometry. Compatibility with PS chemistry was considered acceptable when the extent of desulfurization *i.e.*, conversion of **9** to products containing two or more PO linkages, did not exceed 5%.

Table 1. Efficiency and Compatibility of Various Oxidation Reagents for PO-oxidation of *H*-Phosphonate Linkages

			compatibility	
method	oxidation reagent	yield %	with PS	with PN
A	I_2 in Py/H_2O (98:2)	97	no	no
B	10% *t*-BuOOH in CH_3CN/H_2O (20:1)	39	no	na[b]
C[a]	0.5 M CSO in CH_3CN	52	no	na[b]
D	CCl_4/Py/H_2O (5:9:1)	25	yes	yes
E	0.1 M Et_3N in CCl_4/Py/H_2O (5:9:1)	100	yes	yes

[a] CSO: 1-*S*-(+)-(10-camphorsulfonyl)oxaziridine. See ref 11c. [b] Not attempted, because incompatible with PS.

Scheme 3[a]



[a] Conditions: (i) S_8 (10% in CS_2/Py/TEA, 35:35:1; 3 h; (ii) 1. 3′-*H*-phosphonate/PivCl; 2. dichloroacetic acid/CH_2Cl_2; (iii) Table 1, methods A−E; (iv) DMAEA (5% in CCl_4; 2 h); (v) conc. aq NH_3, 55 °C, 6 h.

When a standard procedure for PO-oxidation (method A) was used, full length product was observed. However, **9** suffered from a severe desulfurization to give **10** as a main product (Figure 1a). Methods B and C were also incompatible with the PS backbone. In contrast, when CCl_4 was used

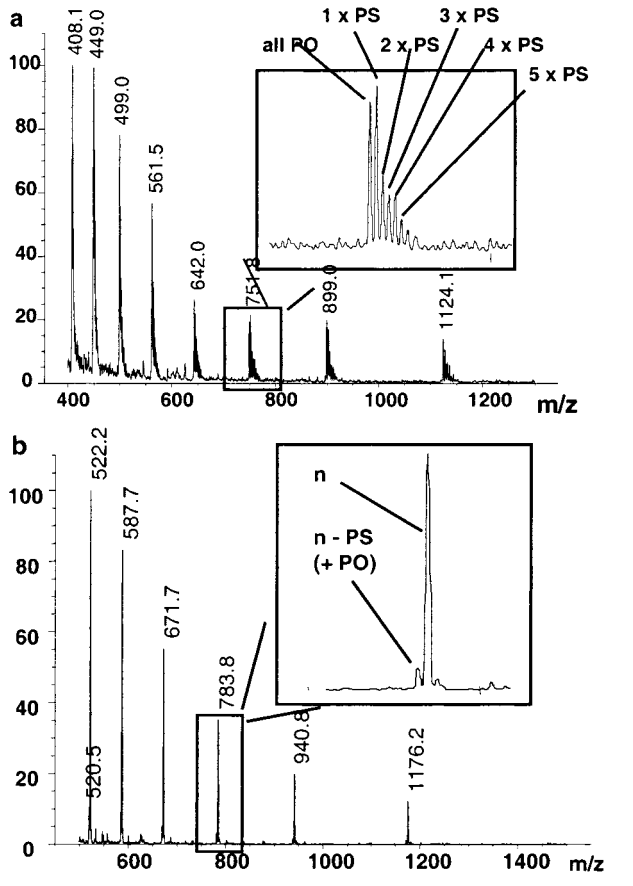


Figure 1. ES-MS spectrum of $T_{PO}(T_{PS})_{13}T$ synthesized by (a) method A and (b) method E. MW_{calc}: 4709.8. MW_{found}: 4708.6.

(method D) no desulfurization occurred, although the oxidation was sluggish. Addition of Et_3N (method E) dramatically improved the oxidation kinetics to give **9** in 90 min without affecting the PS-linkages in the backbone (Figure 1b). Similarly, when treated by method A, **13** gave primarily **14**, as judged by subsequent conversion to **16** rather than **17**. Conversely, by method D, **13** was oxidized to **15**, which was smoothly transformed to **17**.

These findings were used in preparation of a 20-mer oligonucleotide with an antisense sequence targeting murine c-*raf* message that contained 2′-*O*-MOE ribonucleoside residues at the 3′-terminus and three different phosphate modifications (Figure 2). First, DMAE−phosphoramidate linkages were introduced, and partially deprotected exocyclic amino functions were reacylated with Ac_2O. The chain elongation was resumed, until the 2′-*O*-MOE segment was completed, and the *H*-phosphonate diesters were oxidized by method E. Next, the 2′-deoxy center part was assembled to give, upon sulfurization, the PS segment of the backbone. Finally, the last segment was synthesized and oxidized using method E to complete the 5′-terminal PO fragment. Upon deprotection with ammonia, no evidence of backbone hydrolysis due to incomplete oxidation of *H*-phosphonate linkages or conversion of PS or PN to PO linkages was found by ES MS (Figure 2).

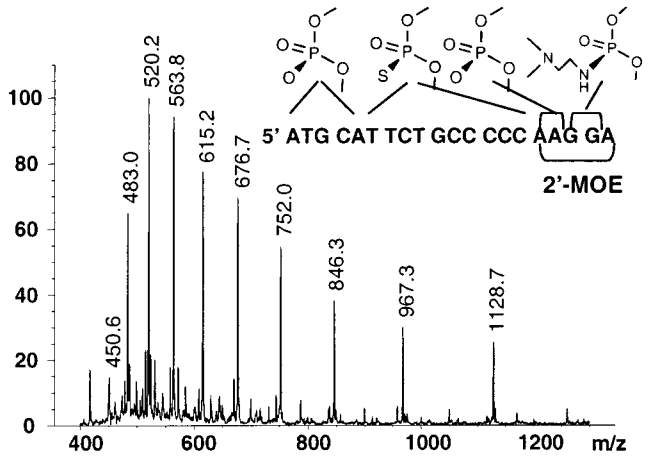


Figure 2. ES-MS spectrum of a 20-mer oligonucleotide with PO, PS, and PN phosphate modifications in the backbone. MW_{calc}: 6775.3. MW_{found}: 6777.8.

In conclusion, *H*-phosphonate chemistry offers a versatile method for synthesis of antisense oligonucleotides containing various phosphorus linkage modifications in their backbone. Using optimized oxidation conditions along with dC^{Ac} *H*-phosphonate monomer, we were able to synthesize chimeric oligonucleotides with phosphodiester, phosphorothioate, and phosphoramidate linkages and 2′-deoxy and 2′-*O*-MOE carbohydrate residues. Biological and biophysical evaluation of these "combined" backbone oligomers is in progress.

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