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Syntheses and binding studies of oligonucleotides containing *N*-hydroxycarbamate linkages: potential DNA cleaving antisense oligomers

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Abstract

An efficient synthesis of *N*-hydroxycarbamate containing thymidine dimer **9** was accomplished and it was incorporated into automatic DNA syntheses to make thymidine 16mers **T*-1**, **T*-2** and **T*-3**. Binding abilities of **T*-1**, **T*-2** and **T*-3** with poly (dA)-16mer were assayed by melting denaturation (T_m) studies of the corresponding duplexes. Iron binding ability of a thymidine oligomer with *N*-hydroxycarbamate linkages was studied by MALDI-MS. Nuclease stability assays showed that the modified oligonucleotides have enhanced resistance toward nuclease S1 (endonuclease) compared to natural oligonucleotides. © 2000 Elsevier Science Ltd. All rights reserved.

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The use of oligonucleotide analogs as rationally designed drugs continues to attract attention in medicinal chemistry. In principle, antisense oligonucleotides target, in a sequence specific manner, a particular gene, or mRNA to inhibit the expression of that gene.^{1–5} In recent years, chemical modifications of antisense oligonucleotides have resulted in improved solubility, stability towards nucleases, cellular uptake, and appropriate hybridization to target genes or mRNAs.^{6,7} In the antisense approach, oligonucleotides have been designed to bind to a given mRNA to inhibit the translation process via duplex formation and/or RNase H-induced degradation of the message. We proposed a novel backbone replacement for antisense oligonucleotides with *N*-hydroxycarbamates as linkages (Fig. 1).^{8,9} Hydroxamic acids can effectively chelate Fe(III), which can undergo Fenton chemistry to generate hydroxyl radicals.¹⁰ Therefore, in addition to potential RNase-H cleavage processes, antisense oligonucleosides with *N*-hydroxycarbamate linkages might cleave the target mRNA through radical reactions. Moreover, the Fe(III) binding ability of the antisense oligonucleoside would have the potential to assist in its cell permeability

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through iron uptake systems.¹¹ In order for the proposed Fenton chemistry to work, the oligonucleotides with *N*-hydroxycarbamate linkages are required to bind both iron and target DNA or mRNA strands with reasonable affinity. Herein, we report the syntheses of thymidine oligomers with *N*-hydroxycarbamate linkages and their binding ability toward poly(dA) and Fe(III).

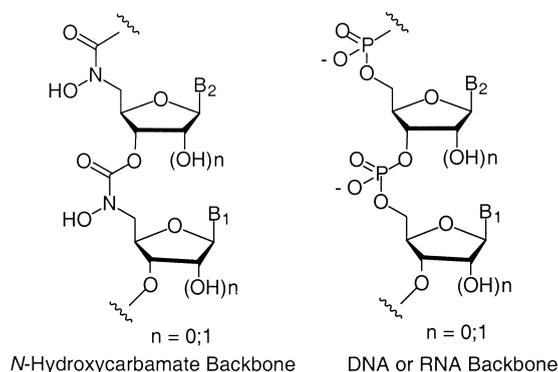
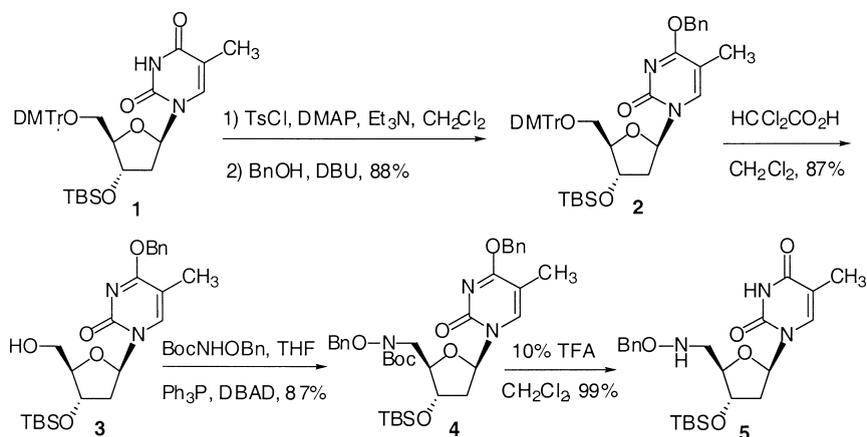


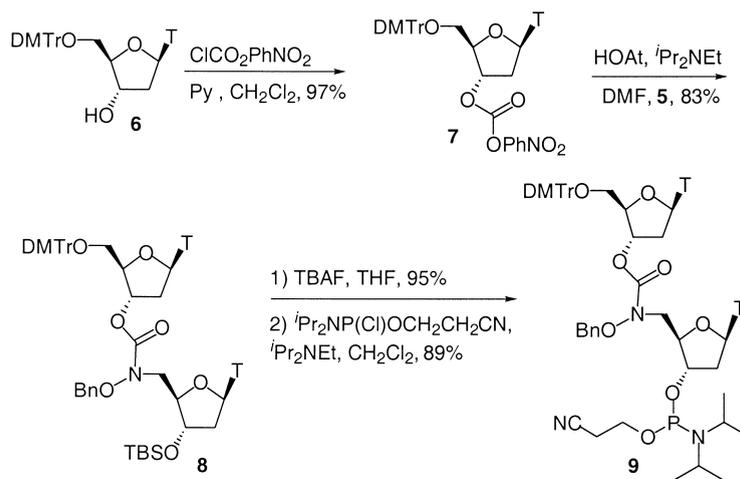
Figure 1.

In a previous report,⁸ we described the syntheses of the building blocks of oligonucleotides with *N*-hydroxycarbamate linkages. The conversion of the 5'-hydroxyl group of a nucleoside into the corresponding 5'-*N*-hydroxylamino substituted derivatives can be achieved by Mitsunobu reactions of *N*-(*tert*-butoxycarbonyl)-*O*-(benzyloxycarbonyl)hydroxylamine (BocNHOCbz) with nucleosides. Scheme 1 shows the application of this methodology to thymidine for the syntheses of thymidine oligomers with *N*-hydroxycarbamate linkages. In order for the Mitsunobu reaction to work, proper protecting groups were introduced to thymidine. Starting from 3'-*O*-*tert*-butyldimethylsilyl-5'-*O*-dimethoxytritylthymidine **1**, a benzyl group was introduced to the 4-*O*-position of the thymidine in two steps by base-aided condensation with tosyl chloride in dichloromethane, followed by the reaction of the resulting sulfonate with benzyl alcohol, to give **2** in 88% overall yield (Scheme 1). Removal of the dimethoxytrityl group in a dichloroacetic acid in dichloromethane



Scheme 1.

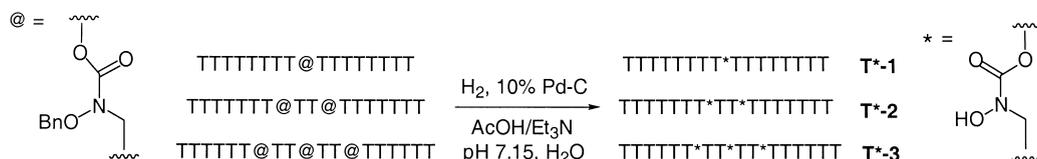
solution afforded an 87% yield of desired compound **3**. The Mitsunobu reaction of **3** with *N*-(*tert*-butoxycarbonyl)-*O*-(benzyl)hydroxylamine (BocNHOBn) gave desired hydroxyamino derivative **4** in good yield (Scheme 1). When **4** was exposed to 10% trifluoroacetic acid in dichloromethane, both the Boc and 4-*O*-Bn groups were removed to give **5** in 99% yield. Use of **5** in a coupling reaction provided the corresponding thymidine dimer with the desired *N*-hydroxycarbamate linkage as described below (Scheme 2).



Scheme 2.

The *p*-nitrophenyl (PNP) carbonate **7** was obtained in 97% yield by reaction between 5'-*O*-dimethoxytritylthymidine **6** and *p*-nitrophenyl chloroformate. Since direct reaction of **5** with **7** was sluggish, 1-hydroxy-7-azabenzotriazole (HOAt) was used to form a more active carbonate reaction intermediate for the coupling and generated dimer **8** in 83% yield. Compound **8** was then treated with *n*-tetrabutylammonium fluoride (TBAF) to remove the silyl group in 95% yield. The resulting 3'-hydroxy intermediate was reacted with chlorophosphoramidite to afford **9** in 89% yield (Scheme 2).

Dimer **9** was then incorporated into thymidine 16mers using phosphoramidite chemistry on solid support according to a standard 1 μmole synthesis cycle.¹² Oligonucleotides with *O*-benzyl-*N*-hydroxycarbamate (@) internucleoside linkages (up to three) were deprotected and removed from the solid support by treatment with concentrated aqueous ammonia solution at rt for 1 h. Benzyl groups were then removed by hydrogenolysis in acetic acid and triethylamine buffer solution (pH 7.15) to give **T*-1**, **T*-2** and **T*-3** (Scheme 3).



Scheme 3.

The binding of thymidine oligomers with *N*-hydroxycarbamate linkages to Fe(III) was studied by the synthesis of a model heptathymidylate analog using the same methodology mentioned above. When the heptamer T*TT*TT*TT was treated with iron(III)-acetylacetonate, Fe(acac)₃, MALDI-MS showed distinct Fe complex formation, indicating effective binding ability of *N*-hydroxycarbamate linkages toward Fe(III).

The stability of duplexes of the thymidine 16mer analogs with poly (dA) was investigated by thermal denaturation experiments (T_m).¹³ Incorporation of one, two and three modifications in the middle of the sequence led to a progressive decrease in the melting temperature of the duplex compared to the natural DNA duplex. The degree of the destabilization depended on the steric factors of the linkages. While a more pronounced destabilization was observed with the *O*-benzyl-*N*-hydroxycarbamate linked compounds ($\Delta T_m/\text{mod} \sim 3.7^\circ\text{C}$), the affinity of the deprotected *N*-hydroxycarbamate linked oligonucleotides for poly (dA) was only slightly decreased ($\Delta T_m/\text{mod} \sim 1.4^\circ\text{C}$). It was also observed that T*-3 did not further destabilize the duplex compared to T*-2 (Table 1).

Table 1

Thermal stability (T_m)^a of duplexes formed between oligothymidylate analogs and poly (dA) targets and half-lives of oligothymidylate analogs towards nuclease S1

Compounds	versus poly (dA)		Nuclease S1 t _{1/2} ^(b)
	T _m (°C)	$\Delta T_m/\text{mod}$ (°C)	
d(T) ₁₆	38.3		25.3 min
TTTTTTTT@TTTTTTTT	34.9	- 3.4	
TTTTTTT@TT@TTTTTTT	30.4	- 3.95	
TTTTTT@TT@TT@TTTTTT	27.1	- 3.7	
TTTTTTTT*TTTTTTTT T*-1	36.1	- 2.2	29.1 min
TTTTTTTT*TT*TTTTTTTT T*-2	34.2	- 2.05	32.7 min
TTTTTT*TT*TT*TTTTTT T*-3	34.1	- 1.4	44.4 min

^(a)Melting temperature (T_m) measured at 10 μM oligomer concentration in 10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA buffer solution (pH 7.0). ^(b)50 mM sodium acetate buffer (pH 4.5) containing 300 mM sodium chloride and 100 mM zinc acetate, 37 °C.

Nuclease resistance of *N*-hydroxycarbamate linked oligonucleotides towards nuclease S1 (endonuclease) was investigated in comparison with the resistance of unmodified d(T)₁₆.¹⁴ Due to the modified linkage, a stabilizing effect was observed. The half-life of oligonucleotides increased when more *N*-hydroxycarbamate linkages were incorporated into the sequence (Table 1).

N-Hydroxycarbamate linked oligonucleotides represent a new class of backbone modified DNA analogs. Thymidine 16mer analogs with alternating phosphodiester and *N*-hydroxycarbamate linkages were synthesized with one to three modifications and their biophysical properties were studied. Replacement of negatively charged phosphodiester linkages by *N*-hydroxycarbamate linkages only slightly weaken duplex stability of the oligonucleotide analogs when hybridized with poly (dA). The resistance of *N*-hydroxycarbamate linked oligonucleotides towards endonuclease is improved. Binding studies indicated a modified oligonucleotide with three *N*-hydroxycarbamate linkages does bind with Fe(III) and also binds to the target poly (dA). Work is currently in progress to study the ability of T*-3 to perform Fenton chemistry and cleave the DNA sense strand sequence-specifically.

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