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SYNTHESIS OF DI-, TRI-, AND TETRAMERIC BUILDING BLOCKS WITH NOVEL CARBAMATE INTERNUCLEOSIDE LINKAGES AND THEIR INCORPORATION INTO OLIGONUCLEOTIDES

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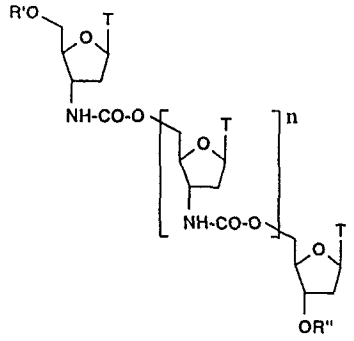
Summary: *Synthesis of di-, T*T, tri-, T*T*T, and tetrameric, T*T*T*T, building blocks with carbamate internucleoside linkage, *=3'-NH-CO-O-5', was achieved by the reaction of mono-, di-, or trimeric nucleoside 5'-O-p-nitrophenyl carbonate intermediates with 3'-amino-3'-deoxythymidine. These building blocks were suitably protected at 5'-position and converted into phosphoramidites, or attached onto CPG, and then used for the "chimeric oligonucleotide" synthesis. The novel oligonucleotides derived therefrom have been studied for their binding properties to complementary nucleic acids and for their nuclease sensitivity. Oligonucleotides containing one, two, or three carbamate linkages at 3'-end, were found to have increased nuclease resistance and did not effect the duplex stability significantly.*

Modulation of gene expression by antisense oligonucleotides requires the development of modified oligonucleotides which have increased nuclease resistance, bind to complementary nucleic acid targets effectively, and also are taken up by cells[1,2]. In earlier studies we have reported[3] the advantages of "chimeric oligonucleotides" - oligonucleotides which contain segments of phosphodiester or phosphorothioate linkages flanked by segments of methyl phosphonate, methyl phosphonothioate or phosphoramidate linkages. In the present study we have established synthetic protocols to introduce a carbamate linkage, 3'-NH-CO-O-5', which is non-ionic, achiral, and provide resistance to enzymatic cleavage, into chimeric oligonucleotides.

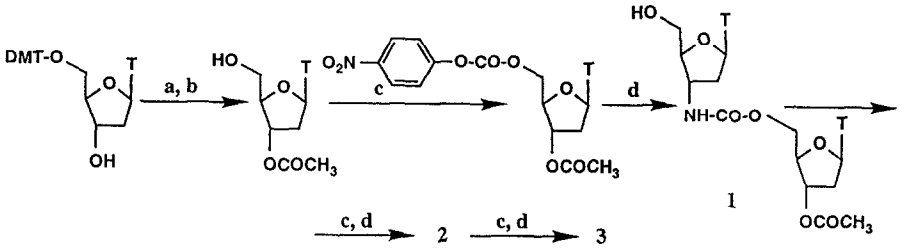
There are reports in the literature[4] describing the synthesis and characterization of oligonucleotides with a 3'-O-CO-NH-5' carbamate linkage, or a carbamate linkage with morpholine subunit incorporated in[5]. The present work has focused on the synthesis of di-, **1**, tri-, **2**, and tetrameric, **3**, building blocks containing carbamate internucleoside linkage, 3'-NH-CO-O-5', and their incorporation into oligonucleotides. The synthesis of building blocks **1**, **2**, and **3** was achieved by the reaction of mono-, di-, or trimeric nucleoside 5'-O-*p*-nitrophenyl carbonate intermediates with 3'-amino-3'-deoxythymidine in 99.4%, 92.7%, and 93.6%, respectively, (*Scheme 1*). The reaction occured regiospecifically at the 3'-amine, and no undesired product of attack on the activated carbonate by the 5'-hydroxyl was detected. Preparation of the active



1 R' = H; R" = $COCH_3$; n = 0
2 R' = H; R" = $COCH_3$; n = 1
3 R' = H; R" = $COCH_3$; n = 2
4 R' = DMT; R" = H; T*T; n = 0
5 R' = DMT; R" = H; T*T*T; n = 1
6 R' = DMT; R" = H; T*T*T*T; n = 2
7 R' = DMT; R" = $P(OCH_2CH_2CN)[N(i\text{-}Pr)_2]$; n = 0

carbonates was achieved by treatment of mono-, di, or trimeric nucleoside with bis(*p*-nitrophenyl)carbonate in 89.7%, 83.7%, and 83.0% yield[4a,b]. Thus obtained

Scheme 1



a. $(CH_3CO)_2O$/Pyridine; b. 80% CH_3COOH; c. bis(*p*-nitrophenyl)carbonate/Pyridine; d. 3'-amino-3'-deoxythymidine/Pyridine

1, **2**, and **3** were further protected at the 5'-position with dimethoxytrityl, DMT, followed by hydrolysis of the 3'-acetyl in ammonia to give T*T, **4**, T*T*T, **5**, and T*T*T*T, **6**, *=3'-NH-CO-O-5'. The building blocks **4**, **5**, and **6** were attached to long-chain alkyl amidopropanoic acid modified controlled pore glass support[6], CPG, via an ester bond formation. Loading values for CPG-T*T, CPG-T*T*T, and CPG-T*T*T*T were found to be 37.4, 27.5, and 2.2 μmole/g CPG, respectively. To improve the loading of **5** and **6**, they were first succinylated at the 3'-OH position and then attached to CPG via an amide bond formation[6,7]. While the loading value for **5** remained unchanged, the same for **6** was significantly increased to 14.6 μmole/g CPG. Dimeric building block **4** was also converted[8] into phosphoramidite **7**, precipitated in hexane at -78°C to give white powder and directly used for the oligonucleotide synthesis with 96% coupling efficiency. Oligonucleotides, Table 1, were synthesized using β-cyanoethyl phosphoramidite approach, on 1 μmole scale using a standard coupling cycle. The tritylated oligonucleotides containing T*T, T*T*T, or T*T*T*T building blocks were purified by reverse phase HPLC at pre- and post-DMT removal stages and

Table 1. Hybridization Data of Oligonucleotides[a]

Oligonucleotide	Sequence (5'——►3')	Length	Tm^b, (°C)	$Tm^{b,c}$, (°C)
Seq.# 1	CTCGCACCCATCTCTCTCCT*T	21	63.3	63.8
Seq.# 2	CTCTCGCACCCATCTCTCTCCT*T*T	24	67.5	67.7
Seq.# 3	CTCTCGCACCCATCTCTCTCCT*T*T*T	25	67.4	67.7
Seq.# 4	CTCGCACCCATCTCTCTCCT*TT*TT*T	25	65.3	66.5
Seq.# 5	T*TCTCGCACCCT*TT*TATCTCTCTCCT*T	27	58.6	65.5
Seq.# 6	T*TGT*TCT*TCT*TGT*TGT*TCT*TCT*T*T*T	25	16.9	56.2
Seq.# 7	CTCTCGCACCCATCTCTCTCCT*T*T*T	25	59.8	60.1

[a]Oligonucleotides Seq.#1-7 were hybridized with complementary DNA; Seq.#1-6, phosphodiester, (PO); Seq.#7, phosphorothioate, (PS); *=3'-NH-CO-O-5'. [b]Absorbance vs temperature profiles were measured at 0.2 A_{260} units of each strand in 1 ml of buffer, (100 mM Na^+, 10 mM phosphate, pH 7.5). [c]Same sequences as above, without carbamate linkages: Seq.#1-6, PO analogs; and Seq.#7, PS analog.

exhibited a single band on polyacrylamide gel-electrophoresis. Furthermore, HPLC analysis following enzymatic degradation[9] of the oligonucleotides indicated the expected ratios of nucleosides and the incorporated building blocks.

Hybridization studies indicated that incorporation of 1-3 consecutive carbamate linkages, 3'-NH-CO-O-5', contiguously at the 3'-end of oligonucleotides, Seq.#1, 2, 3, and 7, had little or no effect on the stability of the

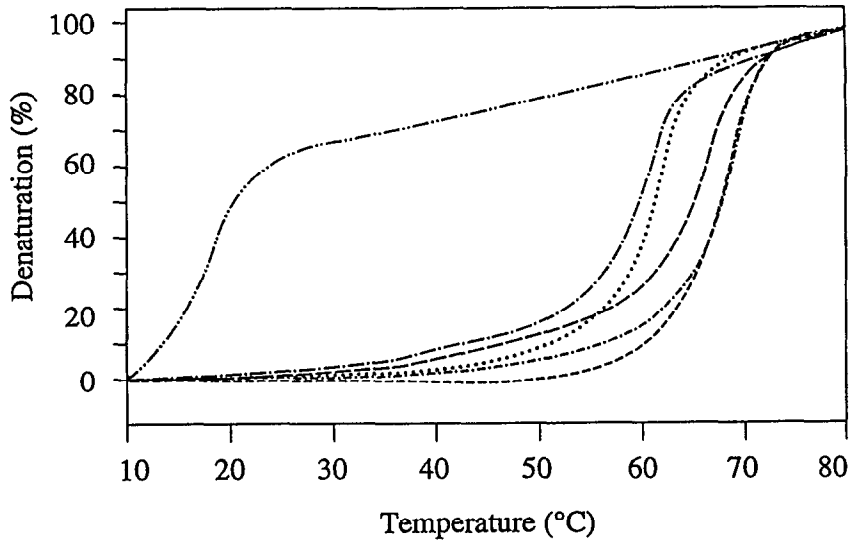


Figure 1. Melting temperature curves of oligonucleotides Seq.#2, (·–·–·); Seq.#3, (– – –); Seq.#4, (——); Seq.#5, (—·—·); Seq.#6, (··—··); and Seq.#7, (·····). Oligonucleotides Seq.#2, 3, 4, 5, 6, and 7 were mixed with complementary DNA at equimolar concentration, (0.2 A_{260} units each), in 1 ml of buffer, (100 mM Na+, 10 mM phosphate, pH 7.5). The duplex was heated to 80°C, then cooled down to room temperature. The duplex was then heated from 10°C to 80°C at a rate of 1°C/min and A_{260} was recorded against temperature.

duplexes formed between oligonucleotides and their complementary nucleic acids, (Table 1, Figure 1). In contrast, the introduction of three carbamate linkages from 3'-end alternating with phosphodiester linkages has modest inhibitory effect on duplex formation, Seq.#4, ΔTm = -1.2°C compared to the parent DNA:DNA duplex, (Table 1). Furthermore, the introduction of two or more carbamate linkages at more interior sites causes a significant reduction in binding, or no binding effect, Seq.#5, ΔTm = -6.9°C and Seq.#6, ΔTm = -39.3°C.

The resistance of carbamate containing oligonucleotides to a 3'-exonuclease was determined[10]. Half life, ($T_{1/2}$), of oligonucleotides Seq.#1, 2, 3, 4, 5, 6, and 7 was found to be: 3.70; 5.85; 6.0; 2.15; 4.05; 3.33; and >30 minutes, respectively, (Table 2; unmodified all phosphodiester oligonucleotide, 27-mer, $T_{1/2}$ = 1.25 minutes). Incorporation of one carbamate linkage at the 3'-end, Seq.#1, or a combination of one carbamate linkage at each end with two in the middle, Seq.#5, did not increase the stability of the oligonucleotides. The introduction of three or more carbamate linkages from 3'-end alternating with

Table 2. Resistance To Degradation by 3'-Exonuclease[10]

Oligonucleotide	Sequence (5'——→3')	Length	$T_{1/2}$ of Digestion, (min.)
Seq.# 1	CTCGCACCCATCTCTCTCCT*T	21	3.70
Seq.# 2	CTCTCGCACCCATCTCTCTCCT*T*T	24	5.85
Seq.# 3	CTCTCGCACCCATCTCTCTCCT*T*T*T	25	6.00
Seq.# 4	CTCGCACCCATCTCTCTCCT*TT*TT*T	25	2.15
Seq.# 5	T*TCTCGCACCCT*TT*TATCTCTCTCCT*T	27	4.05
Seq.# 6	T*TGT*TCT*TCT*TGT*TGT*TCT*TCT*T*T*T	25	3.33
Seq.# 7	CTCTCGCACCCATCTCTCTCCT*T*T*T	25	>30

Oligonucleotide corresponding to Seq.#5 containing all phosphodiester linkages, $T_{1/2}$ = 1.25 minutes.

phosphodiester linkages, Seq.#4 and 6, did not significantly increase the stability of oligonucleotides either. However, the addition of two or three consecutive carbamate linkages at the 3'-ends of the oligonucleotides, Seq.#2 and 3, gave increased protection against exonuclease. The kinetic study showed most of Seq.#2 and 3 remained intact even after 15 minutes, while the unmodified analog of Seq.#3 was completely degraded, (Figure 2). The pattern of degradation observed on the gel, indicates that 3'-exonuclease did not hydrolyze the carbamates, but rather jumped over these linkages plus two more phosphodiester linkages, and then proceeded to hydrolyze the rest of the phosphodiester linkages. Similar degradation profile was observed for oligonucleotide phosphorothioate Seq.#7, ~90% of which remained intact for 24 hours. Whereas the unmodified all phosphorothioate analog of Seq.#7 was ~50% degraded.

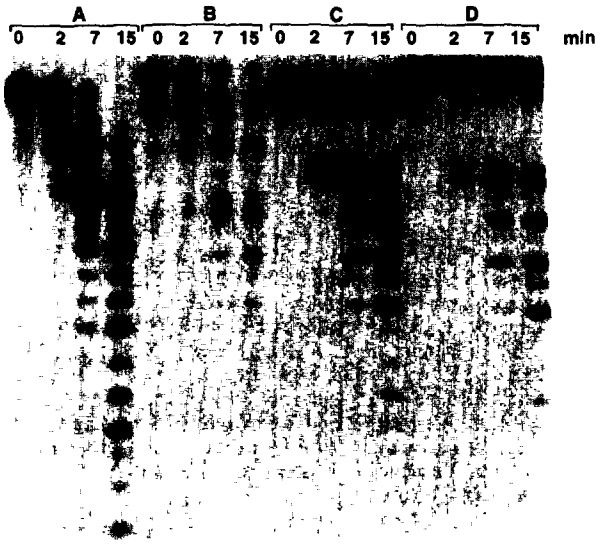


Figure 2. Polyacrylamide gel electrophoresis of enzymatically digested oligonucleotides containing carbamate linkages. Oligonucleotide corresponding to Seq.#3 containing all phosphodiester linkages, (A); oligonucleotides Seq.#5, (B); Seq.#2, (C); and Seq.#3, (D); were labeled[11] with [γ-^{32}P]ATP. Nuclease resistance was studied by incubating 100 ng of each oligonucleotide (10^6 cpm) with snake venom phosphodiesterase, (5 ng, 5 U/mg), at 37°C in 30 µl of buffer, (10 mM Tris-HCl, 100 mM Na^+, 10 mM Mg^{2+}, pH 8.5). At the indicated time, an aliquot, (4 µl), was removed and analyzed by electrophoresis on 20% polyacrylamide gel containing 8 M urea.

The synthesis of T*T, T*T*T, and T*T*T*T building blocks with the carbamate linkage, which is non-ionic, achiral, and resistant to enzymatic cleavage, has been accomplished. These were incorporated into novel chimeric oligonucleotides, thus replacing the negatively charged phosphodiester linkages of natural oligonucleotides. Some of the oligonucleotides, containing T*T, T*T*T, or T*T*T*T, exhibited increased stability toward 3'-exonuclease, and were found to hybridize to their complementary nucleic acids as effectively as the unmodified parent DNAs. These oligonucleotides are presently being studied for their gene regulation activity.

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10. 0.2 A_{260} units of oligonucleotide was lyophilized, suspended in 0.5 ml of buffer, (10 mM Tris, 10 mM Mg^{2+}, pH 8.5), and mixed with 5 μl, ($1.5x10^{-3}$ units), of snake venom phosphodiesterase. The mixture was incubated at 37°C and A_{260} was recorded for 30 minutes.
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