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SYNTHESIS AND HYBRIDIZATION STUDIES OF UREA AND CARBAMATE LINKED THYMIDINE DIMERS INCORPORATED INTO OLIGODEOXYNUCLEOSIDES

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Abstract: An efficient synthesis of urea, N-methyl urea and carbamate linked thymidine dimers has been accomplished. Incorporation of the dimers one or three times into a DNA oligonucleotide, via solid-phase synthesis, was performed. Thermal denaturation studies indicated that the urea-linked 12-mers selectively bind complementary DNA, whereas, the carbamate-linked oligomers bind poorly to both DNA and RNA.

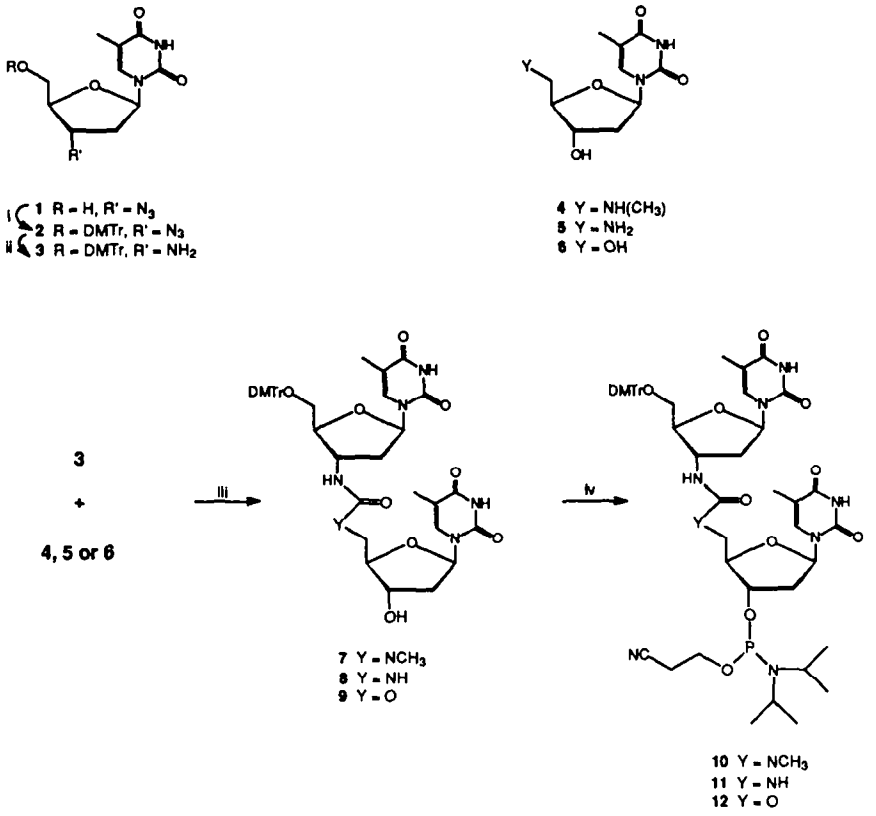
Inhibition of the expression of a targeted gene via selective binding of specific antisense oligonucleosides to a complementary region of a mRNA strand allows for the rational design of therapeutic agents[1]. The pharmacological viability of modified oligonucleosides is dependent on their ability to hybridize to complementary RNA, via Watson-Crick base pairing, at or above physiological temperature, be resistant to enzymatic degradation, have increased cellular permeability and exhibit stability to solid-support synthesis conditions. Many backbone modified oligonucleosides have been synthesized[1,2,3] and two of them, the phosphorothioates[4] and the methylphosphonates,[5] are undergoing clinical trials.[6]

Encouraged by the recent results from our laboratory showing excellent hybridization of amide (3'-CH_2CONR-5') backbone modified oligonucleosides to RNA,[3] we decided to investigate the effect of altering the 3' methylene to an amine group. These ureas have the advantage of being synthesized more easily. Other advantages include the stability of the urea moiety to physiological conditions and compatibility with automated oligonucleotide synthesis and purification conditions.

In this article we describe the relatively inexpensive and short synthesis of two novel urea linked dimers, **TmuT** (5'-N-methylurea) and **TuT** (urea), and one carbamate linked dimer, **TcT** (Scheme 1). These were then incorporated, one and three times, into regular DNA strands to form 12-mer deoxynucleoside sequences (Table 1, **B-G**).

The main monomeric component for the formation of all three dimers was 5'-O-dimethoxytrityl-3'-amino-3'-deoxythymidine[7] **3**. This component was synthesized from 3'-azido-3'-deoxythymidine (AZT) by first dimethoxytritylating the 5'-hydroxyl group and then reducing the 3'-azide to the amine by catalytic hydrogenation in 92% and 95% yield, respectively (Scheme 1). Triethylamine was present in the hydrogenation solvent to avoid loss of the dimethoxytrityl protecting group. The second monomeric component in the synthesis of the urea dimers **7** and **8** and the carbamate dimer **9** were 5'-N-methylamino-5'-deoxythymidine[3] **4**, 5'-amino-5'-deoxythymidine[8] **5** and thymidine **6**, respectively. Dimer formation was performed via the reaction of **3** with 1/3 equivalent of triphosgene in the presence of triethylamine at 0°C, followed, after 10 min., by the addition of a solution of the second monomeric component (**4**, **5** or **6**) in either methylene chloride / triethylamine or N,N-

Scheme 1. Synthesis of Functionalized Dimers



Experimental conditions: (i) 3 eq DMTrCl, 3 eq NEt_3, pyridine, 1/2 h, 92%; (ii) 0.5 eq Pt(IV)O, ethanol/NEt_3 (99:1, v/v), 45 lbs H_2, 2 h, 95%; (iii) a) 1/3 eq triphosgene, 2 eq NEt_3, CH_2Cl_2, 0°C, 10 min, b) (**4**), CH_2Cl_2, NEt_3, 0°C to rt, 1 h, 78% (**7**)or (**5**), DMF, NEt_3, 0°C to rt, 1 h, 70% (**8**)or (**6**), DMF, NEt_3, 0°C to rt, 1 h, 60% (**9**); (iv) 2.2 eq 2-cyanoethyl *N,N*-diisopropylchloro-phosphoramidite, 5 eq NEt_3, CH_2Cl_2, 1 h, 87% (**10**), 75% (**11**), 93% (**12**).

dimethylformamide/triethylamine. Unoptimized yields ranging from 60% to 78% were obtained for compounds **7**, **8** and **9**. Confirmation of the structures of these dimers was obtained by ^1H, ^{13}C and COSY NMR and FAB HRMS of the M+NEt_3·H^+ ion[10]. Only one rotamer was observed in all three dimers and the presence of a strong NOE effect between the 3'-NH proton and the 5'-N-CH_3 protons of compound **7** indicate that the urea linkage is in a trans conformation, as depicted in Scheme 1. We can also deduce that the proton of the 3'-NH is oriented towards the back and somewhat closer to the 4'-H of the same sugar ring because of an observed NOE effect between the 3'-NH and the 4'-H as well as a slightly weaker NOE effect between the 3'-NH and the 2'-H. Finally, phosphitylation of the 3'-position resulted in dimeric units (**10**, **11** and **12**)[11] properly functionalized for incorporation into normal DNA sequences.[12]

The solid phase synthesis of the modified DNA oligomers listed in Table 1 was performed in an average of 95% or greater overall coupling yield using the standard protocol for a 1.3 μmole synthesis scale in a Pharmacia LKB Gene Assembler Plus, with a dimer solution concentration of 0.15M in acetonitrile. The 12-

mers were cleaved off the solid support and deprotected with concentrated ammonia at 50°C for 16 hours and then subjected to purification on an oligonucleotide purification cartridge from Applied Biosystems. A test of the stability of the urea and carbamate linkages to deprotection and cleavage was performed by subjecting the dimers to the conditions used and no decomposition or cleavages of the linkages was detected. Polyacrylamide gel electrophoresis of oligomers **A** to **G** as well as the DNA and RNA complement exhibited a single band for each sequence.

Thermal denaturation studies of oligonucleotides **B** to **G** were carried out in a 1.0M NaCl, 10mM phosphate buffer at pH 6.95 (DNA) and pH 7.01 (RNA). The absorbance vs. temperature profiles in the DNA/DNA hybridization studies were all of a well-defined sigmoid shape. Tm values obtained for both the **TmuT** and **TuT** sequences (**B, C, D** and **E**) were comparable and substantially higher than the Tm values for the **TcT** sequences (**F** and **G**). This indicates stronger binding to DNA of the urea as compared to the carbamate oligomers, although the binding of the **TmuT** and **TuT** sequences with three dimer insertions (**C** and **E**) were slightly lower than that of the standard DNA sequence **A** (Table 1). Surprisingly, and in contrast to the excellent RNA binding ability of amide oligomers, we observed very weak binding of our urea oligomers to RNA. Hypochromicity was observed in all cases but in some cases, such as the binding of sequences **E** and **G** to RNA, it was very low. Of the three linkages the methylurea, **TmuT** sequence **C**, showed the strongest hybridization characteristics, highest hypochromicity and Tm. The carbamate sequence **G** exhibited poor binding to DNA and RNA, just like the known inverse carbamate (3'-O-C(O)-NH-5').[13]

Table I. Hybridization Data

Oligomers	Sequences d (5' → 3')	Tm (°C)[a]	
		DNA[b]	RNA[c]
A	GCGTTTTTTGCT	59.1	55.0
B	GCGTTTmuTTTGCT	57.0	50.0
C	GCGTmuTTmuTTmuTGCT	41.1	28.2
D	GCGTTTuTTTGCT	54.8	46.4
E	GCGTuTTuTTuTGCT	42.9	21.1
F	GCGTTTcTTTGCT	51.1	42.7
G	GCGTcTTcTTcTGCT	30.8	18.9

a) experimental error is ± 0.5 °C
b) Complementary DNA sequence d(3'-CGCAAAAAACGA-5')
c) Complementary RNA sequence (3'-CGCAAAAAACGA-5')[14]

In conclusion, the synthesis of urea-linked dimers differing from previously reported amide linked dimers in only one position (the 3'-position of the backbone linkage) has been accomplished. The sequences resulting from the replacement of 3 phosphate linkages of a 12-mer oligonucleotide with either urea (ΔTm[15]=16.2 or 17.0°C) or amide (ΔTm=8.5 or 11.4°C)[4] linkages show somewhat lower hybridization to complementary DNA than normal DNA. But it is the results of the binding to complementary RNA that is of particular interest in that the modified amide oligomers anneal to RNA and the urea and carbamate oligomers do not. This leads us to believe that the amide-linked oligomers can adopt the A-form helix, preferred by RNA, and the urea-linkage imparts some other helical form on the oligomers, which is incompatible with the A-conformation. Modeling studies are currently underway to shed light on this theory.

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10. a) Compound **7**: (**I** represents the 3'-substituted thymidine moiety and **II** represents the 5'-substituted moiety) ^{1}H-NMR (500 MHz, $CDCl_3$ + 1 microdrop C_5D_5N): δ 10.23 (br, s, 2, NH **I** & **II**), 7.58 (s, 1, C6H **I**), 7.39-7.15 (m, 9, Ar of DMTr), 7.15 (s, 1, C6H **II**), 6.79 (d, 4, O-C-CH of Ar of DMTr, $J_{O\text{-}C\text{-}CH=CH}$ = 7.8 Hz), 6.33 (t, 1, C_1'**H I**, $J_{1',2'}$ = 5.6 Hz), 5.99 (s,1, C_1'**H II**), 5.86 (d,1, 3'-NH of urea, $J_{3',NH}$ = 1.5 Hz), 4.60 (d, 1, C_3'**H I**, $J_{3',NH}$ = 1.5 Hz), 4.31 (d, 1, C_3'**H II**, $J_{2',3'}$ = 2.9 Hz), 4.04 (s, 1, C_4'**H I**), 3.93 (d, 1, C_4'**H II**, $J_{4',5'}$ = 1.0 Hz), 3.74 (s, 6, OCH_3 from DMTr), 3.40 (m, 4, C_5'H_2 **I** and **II**), 2.91 (s, 3, 5'-N-CH_3 of urea), 2.37 (m, 4, C_2'H_2 **I** and **II**), 1.84 (s, 3, C5-CH_3 **II**), 1.36 (s, 3, C5-CH_3 **I**); HRMS (FAB-nitrobenzyl alcohol): *m/z* calcd. for $C_{49}H_{64}N_7O_{11}$ [M+NEt$_3$·H$^+$], 926.4663; found, 926.4664. b) Compound **8**: HRMS (FAB-nitrobenzyl alcohol): *m/z* calcd. for $C_{48}H_{62}N_7O_{11}$ [M+NEt$_3$·H$^+$], 912.4510; found, 912.4507.
11. a) Compound **10**: ^{31}P-NMR ($CDCl_3$ + 1 microdrop C_5D_5N): 149.34 and 149.28 ppm. b) Compound **11**: ^{31}P-NMR ($CDCl_3$ + 1 microdrop C_5D_5N): 149.62 and 149.08 ppm. c) Compound **12**: ^{31}P-NMR ($CDCl_3$ + 1 microdrop C_5D_5N): 149.56 and 149.24 ppm; HRMS (FAB-nitrobenzyl alcohol): *m/z* calcd. for $C_{57}H_{78}N_8O_{13}P$ [M+NEt$_3$·H$^+$], 1113.5427; found, 1113.5426.
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15. ΔTm refers to the temperature difference between the modified and natural strands.

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