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Synthesis of Oligodeoxyribonucleotides containing Dimers with Carbamate Moieties as Replacement of the Natural Phosphodiester Linkage

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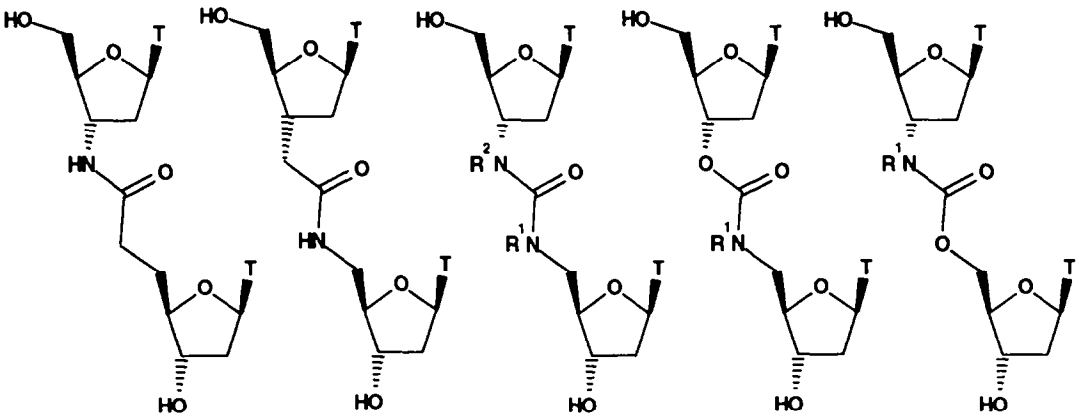
Abstract: The synthesis of thymidine dimers containing both possible regioisomeric carbamate moieties in place of the natural phosphodiester backbone is described. These dimers were incorporated in oligodeoxyribonucleotides and melting temperatures (T_m) of the DNA/RNA and DNA/DNA duplexes are reported. In addition the nuclease resistance of these single strands were determined.

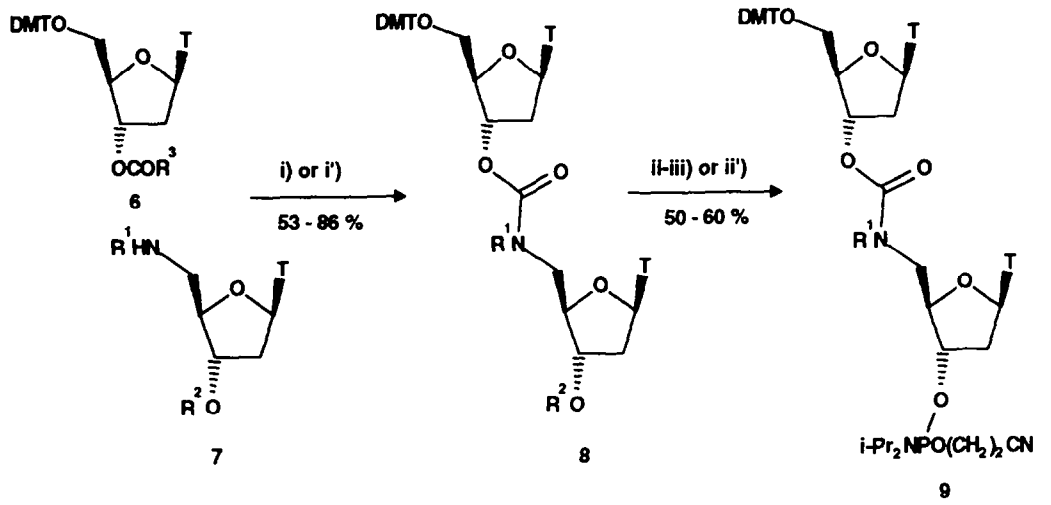
We recently reported the synthesis and incorporation of dinucleotides containing amide linkages[1] **1** and **2** as well as urea linkages[2] **3** in oligodeoxyribonucleotides. These modifications were designed in order to improve the nuclease stability and the capability of cell penetration of the corresponding oligonucleotides. In addition, a good affinity for an RNA target was expected. These are, amongst others, three important prerequisits for the application of modified oligonucleotides in the antisense approach for the regulation of gene expression to treat pathogenic diseases.[3]



1 **2** **3** **4** **5**

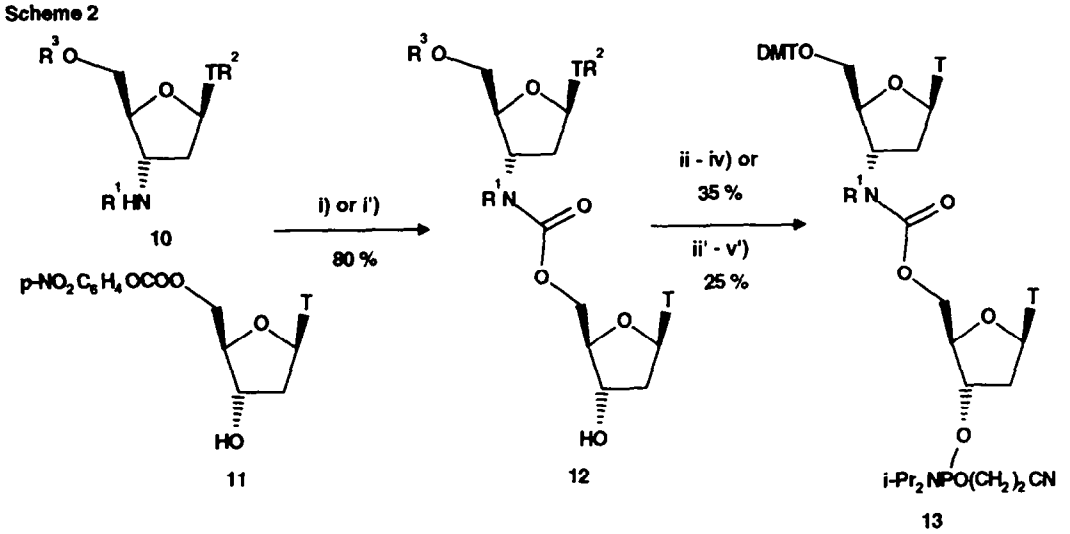
The present study deals with the synthesis and incorporation in oligodeoxyribonucleotides of dimers **4** and **5** which contain the carbamate moiety as an internucleoside linkage. The flexibility of the carbamate moiety with respect to the barrier of rotation around the N-C(O) bond ranges between the one for amide and the one for urea linkages. The incorporation of carbamate **4** in oligonucleotides was already reported, but the results on the duplex formation between these modified oligonucleotides and their complements are contradictory.[4]

Scheme 1



For R^1=alkyl, R^2=diphenyl-t-butylsilyl, R^3=OC_6H_4-p-NO_2 i) pyridine, 80°, 16 h; ii) Bu_4NF, THF, AcOH, RT, 16 h; iii) $((i\text{-}Pr)_2N)_2POCH_2CH_2CN$, $(i\text{-}Pr)_2NH_2^+$ tetrazole$^-$, CH_2Cl_2, RT, 16 h. For R^1, R^2=H, R^3=imidazole i') pyridine, RT, 16 h; ii') as iii).

Scheme 2



For R^1, R^2=H, R^3=Trityl i) pyridine, 80°, 16 h; ii) 80% AcOH, RT, 48 h; iii) DMTCl, pyridine, RT, 16 h; iv) $((i\text{-}Pr)_2N)_2POCH_2CH_2CN$, $(i\text{-}Pr)_2NH_2^+$ tetrazole$^-$, CH_2Cl_2, RT, 5 h. For R^1=Me, R^2=BOM, R^3=thexyl-dimethylsilyl i') DMF, DMAP, 80°, 2 h; ii') Bu_4NF, THF, AcOH, RT, 19 h; iii') H_2, Pd/C (10%), MeOH, RT, 16 h; iv'-v') as iii-iv).

The synthesis of the phosphoramidite building blocks (**TcT**) are outlined in **Scheme 1** and **2**.[5] The 5'-O-DMT-protected derivatives **6** (**Scheme 1**), obtained by reaction of the free 3'-alcohol with either p-nitrophenyl chloroformate or carbonyldiimidazole, were coupled with the 5'-amino[2] derivatives **7** in pyridine at 80°. In case of the alkyl substituted carbamates **8** subsequent desilylation with fluoride ion on the 3'-position had to occur in a buffered medium, adding acetic acid, in order to prevent cleavage of the carbamate moiety. The final phosphoramidite formation was carried out under standard conditions.[6]

3'-Amino-5'-trityl derivative **10**[2] (**Scheme 2**) was coupled with the p-nitrophenylcarbonate **11**, prepared from thymidine; the trityl group in **12** (R^1, R^2=H, R^3=Trityl) was replaced by the DMT group and phosphoramidite **13** was obtained as described earlier.[5] The dimer **12** (R^1=Me, R^2=BOM on N(3) of thymine, R^3=thexyldimethylsilyl) was desilylated followed by catalytic hydrogenolysis of the BOM group. 5'-Protection and phosphitylation of the 3'-OH occurred under standard conditions. Yields given in **Schemes 1** and **2** are overall yields for steps indicated. It was observed by ^{1}H-NMR, that the dimeric carbamates are mixtures of two rotamers of varying composition.

The oligonucleotides with the incorporated modifications **4** and **5** were synthesized on a solid phase support.[7] The thermal denaturation of the RNA/DNA and DNA/DNA hydrids was performed under standard conditions and the data are summarized in the **Table**.[8]

Table: Hybridization data

	Oligomer sequence (5' →3')[a]	T_m(°C) w.t.[b]	ΔT_m (°C)/modification **4a** (R^1=H)	**4b** (R^1=Me)	**4c** (R^1=Et)	**4d** (R^1=i-Pr)	**5a** (R^1=H)	**5b** (R^1=Me)
A	CTCGTACC**TcT**TCCGGTCC	63.3	-3.4	-3.2	-2.5	-1.1	n.m.[c]	-5.6
B	CTCGTAC**TcTTcT**CCGGTCC	61.6	-3.2	-2.5	n.m.[c]	-2.3	-5.0	-4.8
C	GCG**TcTTcTTcTTcTTcT**GCG	50.2	-4.8	-4.4	n.m.[c]	-3.9	n.c.[d]	n.c.[d]
D	TTT**TcT**CTCTCTCTCT	51.6	-2.9	-2.0	n.m.[c]	-1.2	-3.8	-3.9
E	TTT**TcT**CTCTCTCTCT	42.6	-1.8	-1.1	n.m.[c]	n.m.[c]	n.m.[c]	-4.8

a) **A - D** measured against RNA complement, **E** measured against DNA complement b) melting temperature of the wild type (unmodified) oligo c) not measured d) non-cooperative (no duplex formation > 20°C)

A slight improvement (ΔT_m/mod. = -3.2°C for **A**,**B** and **D**, **Table**) of the thermal stability of duplexes formed with carbamate modified oligos with their RNA complement was observed compared to the urea analogs, where an average ΔT_m/modification of -3.8°C was found.[2] Using the DNA complement instead a significant increase of the melting temperature was detected (**E**). Additional alkyl substituents on the 5'-nitrogen atom, as in **4b - 4d**, did not have a negative effect on the T_m's. This is also in agreement with our previous results on analogous amide and urea modifications. The thermal stability of oligos with the isomeric carbamates **5** proved to be worse and e.g. no duplex formation above 20°C could be observed with 5 modifications in a row with the sequence **C**.

As the **Table** reveals and in agreement with our results on the isomeric amides **1** and **2**,[1,2] introduction of a rigid moiety in the vicinity of the 3'-carbon center of the upper sugar, as in **1** and **5**, has a negative effect on the thermal stability of duplexes formed with the RNA complement. On the other hand, rigidity as well as additional substituents on the heteroatom attached to the 5'-carbon center of the lower sugar, as in **2** and **4**, have little to no effect.

The enzymatic stability in 10% fetal calf serum at 37°C using the sequence CGACTATGCAATTcTC was increased by a factor of up to 4 with the carbamates as modifications compared to the unmodified oligomer.[9]

In conclusion, our results on the carbamate modified backbone oligonucleotides represent the link between our results on the amide **1** and **2** and the urea modified analogs **3**. It becomes obvious from these results that restriction of rotation in the backbone has to occur preferentially right in the middle between the two sugar moieties in order to decrease the steric interactions. In addition, adjusting the distance between the sugars and the rigidity of the backbone turns out to be another important criterion in order to gain the desired thermal stability of the duplexes. However, it can be concluded that carbamates as well as ureas seem to be unsuitable for their use in the antisense approach due to their interference on binding affinities with complementary RNA.

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6. Sinha, N.D., Biernat, J., McManus, J., Koster, H. *Nucl. Acid Res.* **1984**, *12*, 4539.
7. Each oligonucleotide was prepared on an ABI 390 DNA synthesizer using standard phosphoramidite chemistry according to M. J. Gait, Oligonucleotides synthesis: A Pratical approach, IRL Press, Oxford 1984, but with prolonged times (10 min.). DMT oligonucleotides were purified by reverse phase HPLC. The oligodeoxynucleotides were controlled by capillary gel electrophoresis and their molecular weight was checked by mass spectrometry (MALDI-TOF: Pieles, U., Zürcher, W., Schär, M., Moser, H. *Nucl. Acids Res.*, **1993**, *21*, 3191.
8. The thermal denaturation of DNA/RNA hybrides was performed at 260 nm using a UV-spectrophotometer. Absorbance *vs.* temperature profiles were measured at 4 μM of each strand in 10 mM phosphate pH 7.0 (Na salts), 100 mM total [Na^+] (supplemented as NaCl), 0.1 mM EDTA. Tm's were obtained from fits of absorbance *vs.* temperature curves to a two state model with linear slope baselines (Freier, S. M., Albergo, D. D.,Turner, D. H. *Biopolymers*, **1982**, *22*, 1107). All values are the average of at least three experiments.
9. For experimental details see: Hoke, G.D., Draper, K., Freier, S.M., Gonzales, C., Driver, V.B., Zounes, M.C., Ecker, D.J. *Nucl. Acid Res.* **1991**, *19*, 3373.

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