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Synthesis of Thymidine Dimers Containing a New Internucleosidic Amide Linkage and their Incorporation into Oligodeoxyribonucleotides

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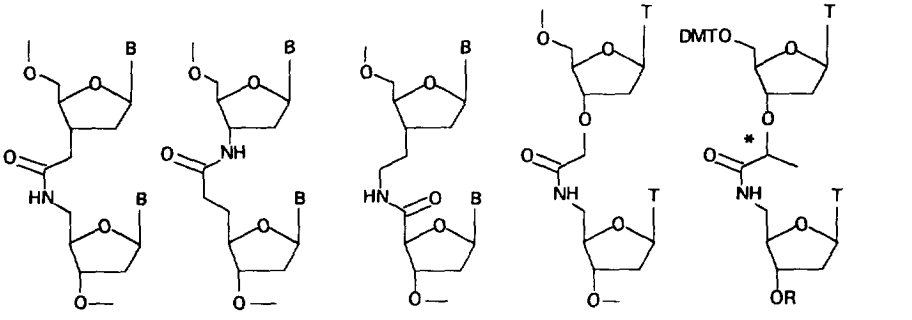
Abstract: The synthesis of thymidine dimers 5a,b containing a chiral carbon in the propionamide internucleosidic linkage is described. These new epimeric dimers were incorporated in oligodeoxyribonucleotides and the melting temperature (Tm) with complementary DNA and RNA as well as the resistence of the single strand against exonuclease were determined.

Oligonucleotides having unnatural internucleosidic linkages have emerged as potential antiviral and antitumoral agents in antisense and antigene strategies[1]. Chemical modifications in the sugar-phosphate backbone of oligonucleotide chains are basically addressed to the resolution of two main problems associated with the use, *in vivo*, of natural oligonucleotides: *i*) their low stability towards cellular nucleases and *ii*) poor cell penetration due to their polyanionic structure. Among the various internucleosidic linkages so far proposed, the amide function seems to show interesting properties. A non-ionic amide bridge (**1-3**) replacing the natural 3'-5' phosphodiester linkage, once incorporated in an oligomer, results in a net charge reduction, thus possibly enhancing its cellular uptake. In addition, such modification is known to be stable under physiological conditions and compatible with standard solid phase oligonucleotide synthesis.

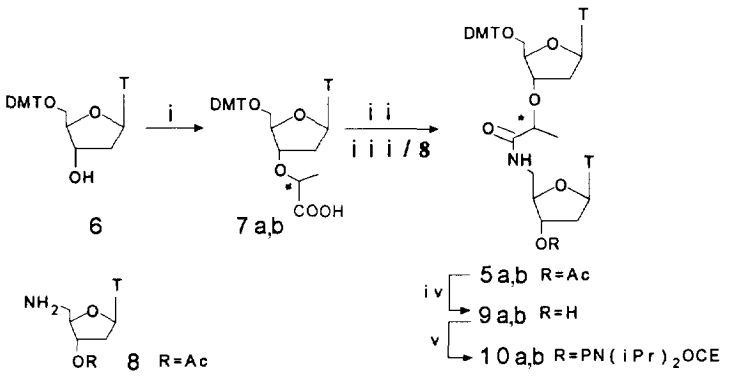


1 2 3 4 5a,b

Promising results, also in terms of hybridization properties, have been shown by a number of recently described[2] amide linkages (**1-3**) having a four atoms bridge between the C-3' carbon of the upper sugar and the C-4' carbon of the lower one. The introduction of such modification requires, in order to mantain the same atoms number present in natural phosphodiester backbone, deoxygenation at the 3' and the 5' positions of the nucleoside sugars, thus necessitating several synthetic steps. On the other hand, a five atoms bridge (**4-5**) containing an amide function results to be much more easily accessible, only few easy reactions being requested to introduce it starting from commercially available nucleosides. Though an acetamido group (**4**) has been known since long time[3] as an alternative to the phosphodiester in the oligonucleotide backbone and examination of models suggested that it would not hamper the hybridation capabilities of a resulting oligonucleotide, it seemed to us that this kind of amide linkages was not well explored, especially as far as its potentialities in an antisense approach are concerned.

In the present communication we report the synthesis of the two thymidine dimers **5a,b** having opposite chirality (R and S respectively) at the α-carbon of the five atoms propionamide internucleosidic bridge and their incorporation in DNA fragments. The melting temperatures of the duplexes formed with the complementary natural DNA and RNA strands have been determined. Furthermore we valued the resistance of the 5'-end modified oligomers towards exonuclease degradation.

The two diastereomers **5a,b**, could be easily separately synthesized using the commercially available pure enantiomers of 2-chloropropionic acid as starting reagent. The synthesis of dimers **5a,b** and their corresponding phosphoramidite derivatives **10a,b** is outlined in the Scheme. 5'-O-dimethoxytriphenylmethylthymidine **6** was reacted with (S) or (R)-2-chloropropionic acid and NaH in anhydrous DMF-dioxane. The resulting 3'-O-(2-propionate) derivative was obtained in 65% yield for R isomer **7a** and 55% yield for S isomer **7b**. ^{1}H NMR analysis of these compounds clearly indicated that this step is totally stereospecific, as already recognized by chromatographic investigation of the reaction mixtures. **7a** and **7b** were each then converted into the related 4-nitrophenyl esters by reaction with 4-nitrophenol in dioxane in the presence of the condensing agent N,N'-dicyclohexylcarbodiimide (DCCI), and, without isolation, coupled with 5'-amino-5'-deoxy-3'-O-acetylthymidine[4] **8** dissolved in pyridine/Et_3N, thus affording the 3'-O-acetylated dimers **5a** (73% yield) and **5b** (70% yield) respectively. Successive treatment of **5a,b** with conc. aq. NH_3/CH_3OH (3:1, v:v) allowed the removal of the acetyl protecting groups at the 3'-OH ends, thus obtaining the desired dimers **9a,b** (95% yields).



Scheme: i) NaH (5.0 eq), (R) or (S) 2-chloropropionic acid (25 eq), DMF/dioxane (1:1), 14 h, 65 °C; ii) 4-nitrophenol (1.2 eq), DCCI (1.9 eq), pyridine, 4 h, r.t.; iii) **8** (1.4 eq), pyridine/Et_3N (1:1), 15 h, r.t.; iv) NH_3 aq. (32%)/CH_3OH (3:1), 3 h, r.t.; v) $EtN(iPr)_2$, $P(Cl)N(iPr)_2OCH_2CH_2CN$ (1.8 eq), CH_2Cl_2, 1 h, r.t..

The dimers **9a,b** were converted into the corresponding 3'-phosphoramidite derivatives **10a,b** (70 and 75% yield respectively) following the standard 3'-phosphitylation procedures[5]. All the structures of the synthesized compounds were confirmed by spectroscopic data[6].

The automated solid phase syntheses[7] of the oligodeoxyribonucleotides were performed using commercially available 2-cyanoethyl(CE)phosphoramidite nucleosides having as protecting groups for the exocyclic amino functions more easily removable groups[8] than the classical benzoyl or isobutyroyl in order to use milder deprotection conditions in the final aqueous ammonia treatment (r.t., 2 h) that excluded even partial fission of the internucleosidic amide linkages. The coupling yields observed with the phosphoramidites **10a,b** were in the range 94-96%, as determined by DMT cation assay. The totally deprotected oligodeoxyribonucleotides were purified by HPLC on anion exchange column, detritylated and successively desalted by standard procedures[9]. The modified sequences synthesized (**11-14**) after purifications were subjected to PAGE (18% polyacrylamide) under non denaturing conditions and showed, in all cases, a single band. Analysis of the purified oligomers was performed by MALDI-TOF-MS; from the spectra of the four tetradecamers the peaks attributed to the molecular ions $(M\text{-}nH^+)^{n-}$ could be found[10]; peaks at higher m/z could be assigned to sodium and/or potassium complexes of the corresponding molecular ions.

^1H NMR analyses of the model tetramers TT*TT, having the modification in the middle of the sequence, showed that, within the accurracy limit of this technique, no epimerization occurred at the α–carbon of the propionamide linkage during the oligomerization and deprotection procedures.

The synthesized sequences **11-14** and the related melting temperatures (Tm) of the amide modified oligonucleotides are reported in the Table. Melting curves[11] of the oligonucleotides **11-14** showed in each case a characteristic single sigmoid transition. These results suggest that the introduction of a five atom bridge as amide internucleosidic linkage doesn't dramatically affect the oligonucleotide hybridization properties. Interestingly, a higher affinity was shown towards RNA complementary fragments than to the corresponding DNA strands. It is to be noted that no sensible difference was also observed in the binding capabilities of the modified oligomers in relation to the chirality of the α-carbon of the propionamide linkage.

Table: Melting Temperatures (Tm)[a]

		(b)		(c)	
	Modified Oligomers (5'-3')	Tm (°C)	ΔTm (°C)	Tm (°C)	ΔTm (°C)
11	TACATGT*(R)TCATGTT	51.0	-1.6	41.8	0
12	TACATGT*(S)TCATGTT	51.2	-1.8	41.7	-0.1
13	T*(R)TCACACATTCTTT	63.0	-0.10	n.m.	n.m.
14	T*(S)TCACACATTCTTT	62.6	0.8	n.m.	n.m.

T*(R)T stands for **5a**; **T*(S)T** stands for **5b**.

a) experimental error is ± 0.2°; b) measured against DNA complement;

c) measured against RNA complement; n.m.= not measured.

The oligonucleotides **13** and **14** were tested for their resistance against a 5'-exonuclease. Incubation of **13** and **14** with calf spleen phosphodiesterase II[12] gave interesting results; while compoud **14** resulted to be totally unaffected even after 72 h, **13** was cleaved at the same rate as the corresponding unmodified sequence. HPLC analysis of the digested products indicated that in the latter case the enzyme showed an unexpected endonuclease activity, cutting the oligomer in correspondance of the second phosphodiester linkage starting from the 5'-terminus[13].

In conclusion, in this paper a convenient and easy synthesis of the new thymidine dimers containing a propionamide internucleosidic linkage **5a,b** is presented. These modified dinucleosides could be efficiently incorporated by automated procedures into oligonucleotide sequences, which displayed similar affinity for the complementary single stranded RNA fragments as the corresponding natural analogues and increased stability towards exonuclease degradation. Further experiments, based on biologically relevant modified sequences, are currently underway.

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4. 3'-O-acetylthymidine was converted into the 5'-O-tosylderivative (TsCl/pyridine, 97%) which in turn, by reaction with sodium azide (in DMF, 100°C, 1h), yielded the corresponding 5'-azidoderivative (95%). Reduction of the azido group (H_2/Pd-C in CH_3OH) afforded **8** (90%).
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6. Selected ^1H NMR data [270 MHz, δ (ppm), J (Hz)]; **7a** (CD_3OD): 7.70 (1H, s, H-6); 7.45-6.80 (13H, m's, DMT protons); 6.35 (1H, dd, J=6.0 and 5.7, H-1'); 4.50 (1H, m, H-3'); 4.30 (1H, m, H-4'); 3.99 (1H, q, J=6.5, OCR<u>H</u>-CH_3); 3.81 (6H, s, 2OCH_3 of DMT); 3.43 (2H, m, H_2-5'); 2.52 and 2.28 (1H each, m's, H_2-2'); 1.37 (3H, d, J=6.5, OCRHC<u>H</u>$_3$); 1.31 (3H, s, CH_3-C-5). **7b** (CD_3OD) significative difference with at 4.13 (1H, m, H-4'); 3.89 (1H, q, J=6.5, OCR<u>H</u>CH_3); 1.39 (3H, s, CH_3-C-5); 1.31 (3H, d, J=6.5, OCRHC<u>H</u>$_3$). **9a** ($CDCl_3$): 7.55 and 7.03 (1H each, s's, 2H-6); 7.45-6.80 (13H, m's, DMT protons); 6.52 and 5.82 (1H each, dd's, 2H-1'); 4.23 (2H, m's, 2H-3'); 4.13 (1H, m, H-4'); 3.87 (2H, complex signal, H-4' and OCR<u>H</u>CH_3); 3.82 (6H, s, 2OCH_3 of DMT); 3.48 (2H, m. H_2-5'); 2.66-2.18 (4H, m's, 2H_2-2'); 1.90 and 1.50 (3H each, s's, 2CH_3C-5); 1.36 (3H, d, J=6.5, OCRHC<u>H</u>$_3$). **9b** ($CDCl_3$), significative difference at 6.39 and 5.81 (1H each, dd's, 2H-1'); 1.27 (3H, d, J=6.5, OCRHC<u>H</u>$_3$). **10a** ($CDCl_3$) 7.58 and 7.15 (1H each, s's, 2H-6); 7.42-6.78 (13H, m's, DMT protons); 6.52 (1H, m ,H-1'); 5.82-5.70 (1H, m's, H-1'); 3.85 (2H, m, OC<u>H</u>$_2$$CH_2$CN); 3.80 (6H, s, 2$OCH_3$ of DMT); 3.75 (2H, m, OCH_2C<u>H</u>$_2$CN); 1.91 and 1.51 (3H each, d's, 2CH_3-C-5); 1.37 (3H, d, J=6.4, OCRHC<u>H</u>$_3$). ^{31}P ($CDCl_3$): 149.01 and 150.02. **10b** ($CDCl_3$) significative difference at 6.42 and 5.80 (1H each, m's, 2H-1'); 1.31 (3H, d, J=6.6, OCRHC<u>H</u>$_3$). ^{31}P ($CDCl_3$): 149.32 and 149.90.
7. The syntheses of DNA and RNA fragments were performed on CPG support (30 μmol/g) using a Cyclone Plus automated synthesizer (Millipore) following the standard (CE) phosphoramidite method.

8. *t*-butylphenoxyacetyl (tBPA) base-protecting group in "Expedite" amidites (Millipore).
9. HPLC purification and analysis were carried out on a Partisil 10 SAX column (Whatman, 25 cm, 4.6 mm i.d.) using linear gradients of KH_2PO_4 (20% CH_3CN, pH 7.0) from 1 to 350 mM in 30 min, flow 0.7 ml/min; retention time **11**: 19.9 min, **12**: 20.2 min, **13**: 19.7 min, **14**: 19.8 min.
10. MALDI-TOF MS spectra were obtained using 2–cyano-4-hydroxycinnammic acid as matrix. Experimentally determined masses (m/z) were: 4221.3 for **11** and 4221.0 for **12** (calculated mass for the molecular ions: 4220.8); 4202.0 for **13** and 4201.8 for **14** (calculated mass for the molecular ions: 4201.7).
11. Melting curves were recorded on Cary 1E Spectrophotometer equipped with a temperature programmer. Oligonucleotides **11-14** were mixed with complementary unmodified DNA and RNA sequences at equimolar concentration (4 μM each) in Na_2HPO_4 (1 mM) pH 7.5. The absorbance at 260 nm was recorded against temperature (from 22° to 75 °C, rate 0.5 °C/min).
12. 0.5 A_{260} units of oligonucleotides were dissolved in 0.5 ml of sodium succinate (0.1 M, pH 7) and 0.25 units of calf spleen phosphodiesterase II were added. The mixture was incubated at 37 °C and analyzed by HPLC at fixed time intervals in a 72 hours period.
13. This unexpected enzymatic behaviour couldn't be explained as the result of an endonucleases contamination; in fact when cyclic oligodeoxyribonucleotides[14] were incubated under the same experimental conditions with the same enzyme stock, they were completely unaffected even after prolonged treatment.
14. De Napoli L; Galeone A.; Mayol L.; Messere A.; Montesarchio D.; Piccialli G.; *BioMed. Chem.* **1995**, in press.

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