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Properties of Novel Oligonucleotide Analogues Containing an Acyclic Nucleoside and a Carbamate Linkage

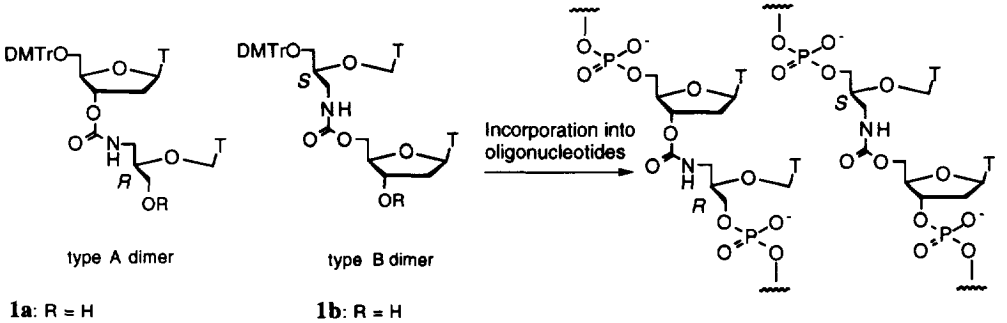
Satoshi Obika, Yoshihiro Takashima, Yasuhide Matsumoto, Atsuko Shimoyama, Yasuo Koishihara,† Yoshiyuki Ohsugi,† Takefumi Doi, and Takeshi Imanishi*

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan

†Fuji Gotemba Research Labs., Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412, Japan

Abstracts: Novel heterodimers containing an acyclic nucleoside and a carbamate linkage were incorporated into oligonucleotides, and the melting temperatures of the DNA-DNA and DNA-RNA duplexes as well as the nuclease resistance of the modified oligonucleotides were studied. Copyright © 1996 Elsevier Science Ltd

The ability of antisense oligonucleotides to interact with a complementary sequence on mRNA by Watson-Crick base pairing and inhibit the expression of the specific gene has been studied for their use as potential molecular biological tools and therapeutic agents. In fact, antisense natural oligonucleotides have been employed successfully for the inhibition of viral replication[1] and of cell proliferation[2] *in vitro* and/or *in vivo*. However, the low stability of natural oligonucleotides in biological media[3] has hitherto limited the development of antisense strategies. To overcome this problem, various modifications of internucleotide linkages were proposed.[4, 5] The other requirement for the modified oligonucleotides is that they must hybridize selectively to their target mRNAs. Sequence specific interactions could achieve highly effective results, but non-specific interactions could bring on unfavorable side effects. In some cases, several toxic side effects were detected when modified oligonucleotides were used *in vivo*. For instance, it was reported that the phosphorothioate oligonucleotides, which are most widely used as 'antisense oligonucleotides', had caused serious side effects which were not due to the antisense action.[6]



1a: R = H
2a: R = P(-OCH$_2$CH$_2$CN)[-N(*i*-Pr)$_2$]

1b: R = H
2b: R = P(-OCH$_2$CH$_2$CN)[-N(*i*-Pr)$_2$]

3a-7a, 13

3b-7b

In this paper, we wish to describe the incorporation of the thymidine heterodimers (type A, B) into oligonucleotides. Evaluation of the hybridization properties and the resistance to the snake venom phosphodiesterase (SVPDE) of our modified oligonucleotides are also reported.

The phosphoramidite building blocks **2a** and **2b** were prepared from the corresponding alcohols **1a** and **1b**[7] by the usual method.[8] The modified oligonucleotides **3-7** were synthesized using the standard phosphoramidite protocol on the DNA synthesizer (Gene Assembler® Plus, Pharmacia, 0.2 μmol scale, 5'-dimethoxytrityl on). The coupling efficiency was monitored by the absorbance of the dimethoxytrityl group after each detritylating step and no coupling loss was detected with the heterodimer (type A or type B). After cleavage from the support and deprotection with conc. ammonia at 70 ℃ for 1 h, removal of the 5'-*O*-dimethoxytrityl group and purification of the modified oligomers were performed on Oligo-Pak™ reverse-phase columns. The purity of these oligonucleotides was verified using analytical HPLC and the compositions were determined by ESI (electrospray ionization)-mass spectrometry. The sequences of modified oligonucleotides are shown in Table 1.[9]

Table 1. Sequences of Oligonucleotides

Oligonucleotides	Sequences (5'→ 3')
3a	d(GCGTTTT-**X**-GCT)
3b	d(GCGTTTT-**Y**-GCT)
4a	d(GCGTT-**X**-TTGCT)
4b	d(GCGTT-**Y**-TTGCT)
5a	d(GCG-**X**-TTTTGCT)
5b	d(GCG-**Y**-TTTTGCT)
6a	d(GCGTT-**X**-**X**-GCT)
6b	d(GCGTT-**Y**-**Y**-GCT)
7a	d(GCG-**X**-**X**-**X**-GCT)
7b	d(GCG-**Y**-**Y**-**Y**-GCT)
8 (complementary DNA)	d(AGCAAAAAACGC)
9 (complementary RNA)	r(AGCAAAAAACGC)
10 (native DNA)	d(GCGTTTTTTGCT)
11 (mismatch DNA)	d(AGCAATAAACGC)
12 (mismatch DNA)	d(AGCAAATAACGC)
13	d(GTTTTTTTTT-**X**-C)
14 (native DNA)	d(GTTTTTTTTTTTC)

X: type A dimer unit, Y: type B dimer unit

To study the hybridizing properties of the modified oligonucleotides **3-7** with their complementary DNA **8** or RNA **9**, the melting temperatures (Tm) were determined by UV measurements.[10] The results are summarized in Table 2. Oligonucleotides **3-5** with one heterodimer unit showed 7-12 ℃ depressions in Tm with DNA or RNA as compared with the native oligonucleotide **10**. From the results of the molecular modeling experiment, the type B dimer unit was expected to be more suitable for the hybridization with the complementary nucleotides,[11] but there were no significant differences in Tm between the oligonucleotides containing type A or type B dimer unit. Two modifications (**6**) led to depression in Tm of 12 ℃ per heterodimer, and the effect of three consecutive modifications (**7**) on the thermal stability was substantial so that the complete thermal hypochromicities was not observed.[12]

Table 2. Tm (°C) of Oligonucleotides **3-7** and **10** with Complementary DNA, RNA[a)]

Oligo-nucleotides	Complementary DNA **8**	Complementary RNA **9**
10 (native DNA)	45	44
3a	37	32
3b	36	35
4a	38	
4b	38	
5a	37	
5b	37	
6a	21	
6b	22	
7a	<0	
7b	*ca.* 5	

a) Conditions: 0.1M NaCl, 0.01M sodium phosphate (pH 7.2), conc.=4μM for each strand.

As shown in Table 3, study of the hybridization ability of the modified oligonucleotides **4a** and **4b** with the mismatch DNA gave interesting results. The duplexes between **4a** and mismatch DNA **11** or **12** were significantly destabilized by a T-T mismatch (ΔTm 11-12 °C), while the ΔTm by a T-T mismatch to the native oligonucleotide **10** was 9 °C. It is noted from that the modified oligonucleotides have a stricter base-pair recognition property than the native oligonucleotides.

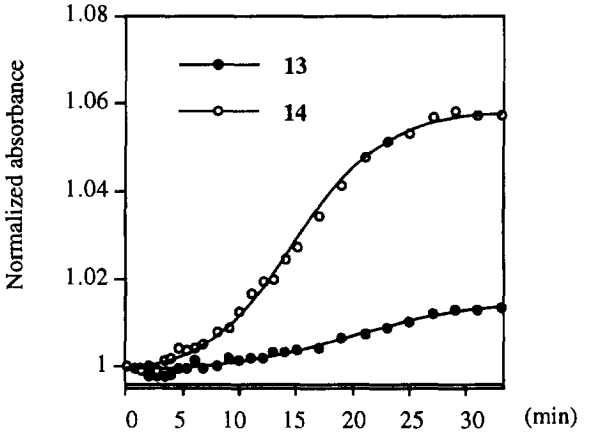
Table 3. Tm (°C) of Oligonucleotides **4** and **10** with Mismatch DNA[a)]

Oligonucleotides	Complementary DNA **8**	Mismatch DNA **11**	**12**
10 (native DNA)	45	36	36
4a	38	26	26
4b	38	27	26

a) Conditions: see Table 2

The 3'-exonucleases were thought to be a major factor leading to degradation of the antisense oligonucleotides *in vivo*.[4, 13] The stability of the modified oligonucleotide **13** to SVPDE, which is one of the 3'-exonucleases, was investigated. The unmodified **14** or 3'-end modified oligonucleotide **13** were digested with SVPDE at 37 °C.[14] During enzymatic digestion, the change in absorbance at 260 nm was monitored as shown in Figure 1. The result shows that the absorbance of the unmodified oligonucleotide **14** increased by the SVPDE digestion during the incubation. On the other hand, the change in the absorbance of the modified oligonucleotide **13** was negligible. This indicates that the modified oligonucleotide **13** was significantly stabilized compared to the unmodified oligonucleotide **14**.

Figure 1. Time Course of SVPDE Digestion of Oligonucleotides **13** and **14**



Conditions: oligonucleotide; 0.7 OD, enzyme; 0.006 U snake venome phosphodiesterase, buffer; 0.1 M Tris-HCl (pH 8.6); 0.1 M NaCl; 14 mM $MgCl_2$, temp.; 37 °C

In conclusion, we have prepared the modified oligonucleotides including our heterodimers. The modified oligonucleotides containing one heterodimer unit formed the duplexes with the complementary strands and had stricter base-pair recognition property than the native oligonucleotides. The excellent enzymatic stability of the modified oligomer was also found.

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10. Melting temperatures were determined by adding equimolar amounts of complementary or mismatch strands in the buffer. The mixture was heated to 95 ℃ and slowly cooled to 25 ℃ over 10 h, then to 0 ℃ over another 1 h. The UV absorption (260 nm) of the mixture at gradually increasing temperature (from 0 ℃ to 95 ℃, 0.2 ℃ per min) was measured on a UV-2100PC (Shimadzu) equipped with a thermocontroller SPR-8.
11. Molecular modeling experiments revealed that, in order to form B-form duplex between our dimer units and natural nucleotides, type A dimer unit requires *syn* orientation of the carbamate linkage, while type B requires *anti* orientation. The oligonucleotide containing type B dimer was expected to be more suitable for hybridization with complementary nucleotides, because *anti* carbamate linkage is thought to be more stable than *syn* carbamate linkage. See, Balaram, H.; Prasad, B. V. V.; Balaram, P. *J. Am. Chem. Soc.*, **1983**, *105*, 4065; Hoos, R.; Naughton, A. B.; Thiel, W.; Vasella, A.; Weber, W.; Rupitz, K.; Withers, S. G. *Helv. Chim. Acta.*, **1993**, *76*, 2666; Nguyen, M. T.; De Wael, K.; Zeegers-Huyskens, T. *J. Phys. Chem.*, **1995**, *99*, 9739.
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