Synthesis and Hybridization Properties of an Oligonucleotide Consisting of 1′,4′-Anhydro-2′,5′-dideoxy-2′-(thymin-1-yl)-D-altritol

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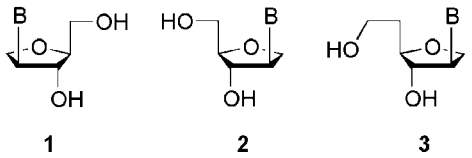
The novel oligonucleotide analogue **7**, consisting of 1′,4′-anhydro-2′,5′-dideoxy-2′-(thymin-1-yl)-D-altritol (**4**), residues was synthesized by the phosphoramidite approach on an automated DNA synthesizer. The phosphoramidite building block **6** was obtained by phosphitylation of the corresponding isonucleoside **5**. Oligoisonucleotide **7** contains an extended phosphodiester linkage with a higher flexibility. Oligoisonucleotide **7** was studied with respect to hybridization properties, enzymatic stability, and CD spectra. It exhibits a high stability towards snake-venom phosphodiesterase and an acceptable hybridization to complementary single-stranded DNA and RNA.

Introduction. – Much effort to enhance the biological activity of oligonucleotides as inhibitors of gene expression has focused on the improvement of their stability to nuclease digestion. This problem has been approached in several ways including alteration of the phosphate and sugar moieties in the oligonucleotides [1][2]. Most of the latter modifications contain a five-membered sugar ring closely resembling the natural deoxyribose [3–5]. Currently, one drug based on the antisense strategy has been approved by the Food and Drug Administration (FDA), and clinical evaluation of many other antisense phosphorothioate oligodeoxynucleotides and DNA·RNA oligomer chimeras is underway [6]. Oligonucleotides incorporating hexose nucleoside analogues were reported to possess significantly increased stability towards phosphodiesterases thereby retaining hybridization properties [7]. Recently, LNA (locked nucleic acid) was introduced, a modification where the backbone of the oligonucleotide consists of a conformationally restricted nucleotide with a 2′-*O*,4′-*C*-methylene bridge. Such nucleic acids have shown unprecedented helical thermal stability when hybridized to their complementary DNA and RNA [8].

We are interested in the synthesis and hybridization properties of oligonucleotides incorporating isonucleosides. Isonucleosides are a new class of nucleoside analogues in which the nucleobase is linked to a ribose position other than C(1′). We have reported the syntheses of 1′,4′-anhydro-2′-deoxy-2′-nucleobase-L-arabinitol **1** and 1′,4′-anhydro-2′-deoxy-2′-nucleobase-D-arabinitol **2** from D- or L-xylose (*Fig. 1*) [9][10]. Oligonucleotides bearing the isonucleosides **1** or **2** show increased stability towards snake-venom phosphodiesterase (SVPDE), but only oligonucleotides built up from the isonucleoside **2** retained their hybridization property [11]. To investigate whether extension of the phosphodiester linkage formed could improve the hybridization properties, we synthesized 1′,4′-anhydro-2′,5′-dideoxy-2′-nucleobase-D-altritol **3** in which an additional methylene group is introduced at the 5′-position of compound **2** [12]. We report here the synthesis of a homo-oligonucleotide consisting of 1′,4′-

anhydro-2′,5′-dideoxy-2′-(thymin-1-yl)-D-altritol (**4**) residues and the study on its duplex stability when it is hybridized with complementary single DNA and RNA strands.



1 **2** **3**

Fig. 1. *Isonucleosides* **1**–**3**

Results and Discussions. – The building block **5**, suitable for solid-phase oligonculeotide synthesis, was obtained starting from 1′,4′-anhydro-2′,5′-dideoxy-2′-(thymin-1-yl)-D-altritol (**4**), which was prepared earlier in our laboratory from D-glucose [12]. Compound **4** was protected with a dimethoxytrityl group ($(MeO)_2Tr$) to give **5** which was phosphitylated to the phosphoramidite **6** by standard procedures in good yield (*Fig. 2*). The oligonucleotide analogue **7**, fully based on **6**, was assembled in an automated DNA synthesizer on a 1-μmol scale. For synthetic convenience, the synthesis started with commercially available thymidine-loaded controlled pore glass (CPG). The synthesis followed the standard protocol, except for a prolonged coupling time of 200 s to ensure adequate coupling yields. The coupling efficiency was determined by measuring the release of $(MeO)_2Tr^+$: 66% of total incorporation yield was obtained. HPLC Analysis of the crude product showed that a uniform oligomer was formed (*Fig. 3*). The composition of oligomer **7** was confirmed by MALDI-TOF mass spectrometry.

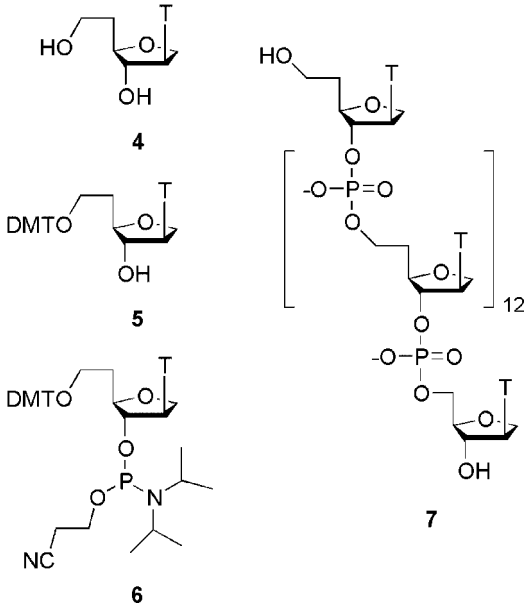


Fig. 2. *Oligonucleotide* **7** *with isonucleoside residues and the synthetic intermediates* **4**–**6**

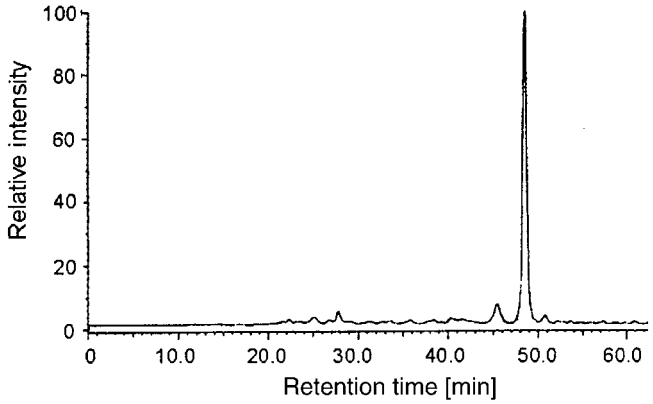


Fig. 3. *HPLC Profile* (detection at 260 nm) *of crude oligomer* **7**

The enzymatic stability of oligomer **7** was analyzed by monitoring the hyperchromic effect upon addition of SVPDE (3′-exonuclease) to a buffered solution of the oligomer. The result indicated that no significant change of UV absorbance occurred within 30 min, while regular dT_{14} gave a time-dependent increase of absorbance. This means that oligomer **7** consisting of isonucleoside residues is resistant to degradation by 3′-exonuclease.

The hybridization property of oligomer **7** with complementary dA_{14} was investigated in a thermal denaturation study. A stable duplex of oligomer **7** with dA_{14} was formed with a slightly reduced T_m value of 28.8°, *i.e.*, $\Delta T_m = -0.51°$ per modification, as compared to the T_m of 35.5° for the control duplex $dT_{14} \cdot dA_{14}$. A more stable duplex was formed when **7** was hybridized with the complementary RNA strand polyA (*Table*).

Table. *Melting Temperature of Duplexes Formed in 0.14*M *NaCl, 0.01*M Na_2HPO_4, *and 1.0 m*M *EDTA at pH 7.2*

	7·dA_{14}	**7**·polyA	$dT \cdot dA_{14}$
T_m [°]	28.8	31.5	35.5

As we described previously, the oligonucleotide consisting of isonucleoside **2** can form a duplex with its complementary single strand. The hyperchromicity value for this duplex was much less than for the control duplex $dT_{14} \cdot dA_{14}$, reflecting poor base stacking within the duplex [11]. Oligomer **7** has an extended phosphodiester linkage, and the backbone is more flexible compared to the oligomer consisting of isonucleoside **2**. It was expected that the perturbation of the base stacking in the oligomer consisting of **2** could be compensated by the higher backbone flexibility in **7**.

To study the global conformation of the duplex, the CD spectra of a duplex composed of oligomer **7** and the complementary dA_{14} and polyA were measured. The spectrum of the control duplex $dT_{14} \cdot dA_{14}$ showed a positive *Cotton* effect at 218 nm and a negative *Cotton* effect at 248 nm (*Fig. 4*). The shape of the spectrum of duplex **7**·dA_{14} was very similar to that of the control duplex. On the other hand, the CD spectrum of duplex **7**·polyA showed a very strong positive *Cotton* effect at 265 nm which

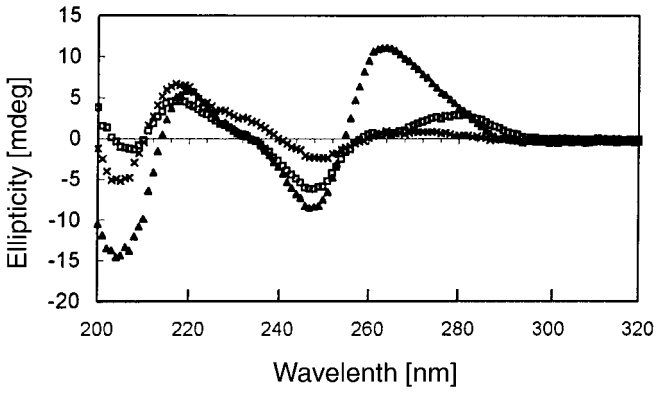
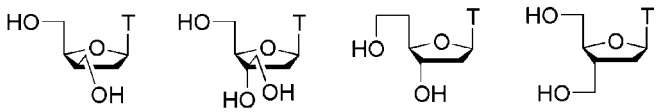


Fig. 4. *CD Spectra of duplexes of oligonucleotide* **7** *consisting of isonucleoside residues with its complementary sequences:* **7**·dA_{14} (×), **7**·*polyA* (▲), *and* dT_{14}·dA_{14} (□)

indicated that an A-like conformation was formed in solution. Oligomer **7** can form duplexes with both DNA and RNA complementary strands which indicates that the extended phosphodiester linkage in oligomer **7** renders the sugar moiety more flexible to fit the required A or B conformation of the duplex.

Wengel and co-workers have synthesized the thymidine analogs **8** and **9** in which a hydroxymethyl group was inserted at the 3′-position of the sugar moiety (*Fig. 5*). It was found that the homo-oligomer containing **8** or **9** increased the stability toward SVPDE and hybridized with a complementary strand with a slightly reduced T_m value [14][15]. *Kofoed et al.* exploited a comparative T_m between normal oligonucleotides and oligonucleotides incorporating analogs **10** or **11** for the formation of duplexes with a complementary hairpin sequence [16]. In this context, it is significant that oligomer **7** has desirable features such as resistance to cellular nucleases and the ability to form a stable duplex with the A-DNA conformation.



8 **9** **10** **11**

Fig. 5. *Some methylene-expanded nucleosides*

Experimental Part

General. The 1′,4′-anhydro-2′,5′-dideoxy-2′-(thymin-1-yl)-D-altritol (**4**) was synthesized by the protocol described earlier. dA_{14} was purchased from *Beijing AuGCT Biotechnology.* PolyA, diisopropylethylamine ($^iPr_2EtN$), and snake-venom phosphodiesterase (SVPDE) were purchased from *Sigma.* The 2-cyanoethyl diisopropylphosphoramidochloridite and thymidine-loaded controlled pore glass (CPG) were purchased from *Applied Biosystems.* All solvents were dried and distilled prior to use. Evaporations were carried out under reduced pressure and a bath temp. of $< 45°$. Column chromatography (CC): silica gel (200–300 mesh; *Qingdao Chemical Co.*). Optical rotations: *Perkin-Elmer 243B* polarimeter. UV Spectra: *Pharmacia LKB-Biochrom-4060* spectrophotometer. NMR Spectra: *Varian VXR-300* or *Bruker DPX-400* instrument; δ in ppm, J in Hz. MS and MALDI-TOF-MS: *ZAB-HS*, *KYKY-ZHP-5*, and *LSI-1700* (*Linear Scientific Inc.*) instruments; *m*/*z*.

*1′,4′-Anhydro-2′,5′-dideoxy-6′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-(thymin-1-yl)-*D*-altritol* (**5**). Compound **4** (300 mg, 1.17 mmol) was dissolved in anh. pyridine (20 ml), then dimethoxytrityl chloride (477 mg, 1.40 mmol) was added under stirring with ice-water cooling. The soln. was stirred at r.t. for 24 h. After evaporation, the mixture was purified by CC (silica gel, AcOEt/$CHCl_3$/Et_3N 7:2:1: **5** (640 mg, 97.8%). ^{1}H-NMR ((D_6)DMSO): 1.75 (*s*, Me–C(5)); 1.95 (*m*, 2 H–C(5′)); 3.05 (*t*, *J* = 6.6, 2 H–C(6′)); 3.63 (*m*, H–C(4′)); 3.72 (*s*, 2 MeO); 3.74 (*d*, *J* = 7.5, 2 H–C(1′)); 3.91 (*t*, *J* = 8.9, H–C(3′)); 4.76 (*m*, H–C(2′)); 6.86–7.40 (*m*, 14 arom. H). FAB-MS: 559 ($[M+H]^+$).

*1′4′-Anhydro-2′,5′-didehydro-6′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-(thymin-1-yl)-*D*-altritol 3′-*O*-(2-Cyanoethyl) Diisopropylphosphoramidite* (**6**). To a soln. of **5** (477 mg, 0.877 mmol) in anh. THF (7.0 ml), ^{i}Pr_2EtN (0.5 ml, 2.85 mmol) was added under Ar. To this soln., 2-cyanoethyl diisopropyl phosphoramidochloridite (0.46 ml, 1.9 mmol) was added slowly under ice-water cooling. After stirring at 0° for 10 min and at r.t. for 30 min, the mixture was quenched by addition of MeOH (1 ml). After stirring for 10 min, AcOEt (20 ml) was added, the org. layer washed twice with 5% aq. $NaHCO_3$ soln. (5.0 ml) followed by H_2O (5 ml), dried (Na_2SO_4), and evaporated, and the oily residue purified by CC (silica gel, petroleum ether/AcOEt/CH_2Cl_2 2:1:1 (1% Et_3N)): **6** (649 mg, 96%). Colorless foam. ^{31}P-NMR ((D_6)DMSO): 150.7.

Solid-Phase Synthesis of Oligonucleotide **7**. Oligonucleotide synthesis was carried out on a 1-μM scale with a DNA synthesizer (model *391A*; *Applied Biosystems*) applying regular phosphoramidite chemistry (DMT off). Cleavage and deprotection of the oligonucleotide were performed in conc. aq. ammonia soln. at 50° for 24 h. The oligomer was purified by HPLC (*ZOBAX Oligo* semi-prep. column (9.4 mm × 250 mm), gradient elution with eluants *A* (MeCN/0.02M NaH_2PO_4 1:4) and *B* (1.0M NaCl in eluant *A*), flow rate 1.0 ml/min). The product-containing fraction was desalted by means of a *Sephadex-G-15* column. The pure oligonucleotide was lyophilized and stored at −20°. Total incorporation yield: 66%. MALDI-TOF-MS: 4368.88 (calc. 4365.06).

Enzymatic Stability of Oligonucleotide **7**. Oligonucleotide **7** (0.2 *OD*) in 1.0 ml of buffer soln. (0.1M, NaCl, 0.14 mM $MgCl_2$, 0.1M *Tris*·HCl, pH 8.6) was digested with 1.2 U of SVPDE at 37°. During digestion, the increase in absorbance at 260 nm was followed. The absorption *vs.* time curve of the digestion was plotted and the hyperchromicity evaluated.

UV Melting Experiments. UV Melting experiments were recorded with a *Pharmacia-LKB-Biochrom-4060* spectrophotometer. Oligomers were dissolved in a buffer soln. containing 0.14M NaCl, 0.01M Na_2HPO_4, 1.0 mM EDTA (pH 7.2). The soln. containing oligonucleotide **7** at a concentration of 2 μM was mixed with an equimolar amount of dA_{14} or equimolar per nucleotide amount of polyA. Samples were incubated at 80° for 5 min, slowly cooled to 4°, and kept at this temp. overnight. These samples were used for the thermal denaturation studies. Thermally induced transitions of every mixture were monitored at 260 nm. Sample temp. was increased at 0.2°/min between 15 and 75°.

CD Spectra. CD Spectra were measured at 20° with a *J715 CD* spectrophotometer (*JAC*) in thermostatically controlled 1-cm cuvettes. The oligomers were dissolved and analyzed in buffer containing 10 mM Na_2HPO_4, 0.14M NaCl, and 1.0 mM EDTA (pH 7.2), and at a oligonucleotide concentration of 4 μM or equimolar per nucleotide amount.

The project was supported by the *National Natural Science Foundation of China* and the *Ministry of Science and Technology of China* (G1998051103).

REFERENCES

[1] C. A. Stein, J. Tonkinson, L. Yakubov, *Pharmacol. Ther*. **1991**, *52*, 365.
[2] W. S. Marshall, M. H. Caruthers, *Science (Washington, D.C.)* **1993**, *259*, 1564.
[3] F. Debart, B. Rayner, G. Degols, J.-L. Imbach, *Nucleic Acids Res.* **1992**, *20*, 1193.
[4] B. S. Sproat, B. Beijer, A. Iribarren, *Nucleic Acids Res.* **1990**, *18*, 41.
[5] J. Sagi, J. Szemzo, J. Szecsi, L. Otvos, *Nucleic Acids Res.* **1990**, *18*, 2133.
[6] J. Nemunaitis, J. T. Holmlund, M. Kraynak, D. Richards, J. Bruce, N. Ognoskie, T. J. Kwoh, R. Geary, A. Dorr, D. von Hoff, S. G. Eckhardt, *J. Clin. Oncol*. **1999**, *17*, 3586.
[7] K. Augustyns, F. Vandendriesshe, A. van Aerschot, R. Busson, C. Urbanke, P. Herdewijn, *Nucleic Acids Res.* **1992**, *20*, 4711.
[8] J. Wengel, A. Koshkin, K. S. Singh, P. Nielson, M. Meldgaard, V. K. Rajwanshi, R. Kumar, J. Skour, C. B. Nielson, J. P. Jacobsen, N. Jacobsen, C. E. Olsen, *Nucleosides Nucleotides* **1999**, *18*, 1365.
[9] H. W. Yu, L. R. Zhang, L. T. Ma, L. H. Zhang, *Bioorg. Med. Chem*. **1996**, *4*, 609.
[10] H. W. Yu, H. Y. Zhang, Z. J. Yang, J. M. Min, L. T. Ma, L. H. Zhang, *Pure Appl. Chem*. **1998**, *70*, 435.

[11] Z. J. Yang, H. Y. Zhang, J. M. Min, L. T. Ma, L. H. Zhang, *Helv. Chim. Acta* **1999**, *82*, 2037.
[12] X. B. Tian, J. M. Min, L. H. Zhang, *Tetrahedron: Asymmetry* **2000**, *11*, 1877.
[13] G. M. Hashem, L. Pham, M. R. Vaughan, D. M. Gray, *Biochemistry* **1998**, *37*, 61.
[14] M. L. Svendsen, J. Wengel, O. Dahl, R. P. Kirpekar, *Tetrahedron* **1993**, *49*, 11341.
[15] P. N. Jorgensen, M. L. Svendson, C. Scheuer-Larsen, J. Wengel, *Tetrahedron* **1995**, *51*, 2155.
[16] T. Kofoed, P. B. Rasmussen, P. Valentin-Hansen, E. B. Pedersen, *Acta Chem. Scand*. **1997**, *51*, 318.

Received December 3, 2001