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Chiral and steric effects in the efficient binding of α -anomeric deoxyoligonucleoside *N*-alkylphosphoramidates to ssDNA and RNA

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ABSTRACT

We report hybridization properties of new phosphate-modified α -oligonucleoside analogs with non-ionic or cationic internucleotide linkages such as methoxyethylphosphoramidate (PNHME), phosphoromorpholidate (PMOR) and dimethylaminopropylphosphoramidate (PNHDMAP). First we evaluated the chirality effect of the phosphorus atom on the affinity of α - or β -dodecanucleoside phosphodiester containing one chirally enriched *N*-alkylphosphoramidate linkage located in the middle of the sequence d(TCTT-AA*CCCACA). As for P-substituted β -oligonucleotides, a difference in binding behavior between the two diastereoisomers (difference in ΔT_m) exists in the hybridization properties of α -analogs when DNA was the target but this effect was not detrimental to duplex stability. This effect was considerably reduced when RNA was the target. Secondly we studied the effect of steric hindrance around phosphorus on the affinity of fully modified β - and α -oligonucleoside *N*-alkylphosphoramidates for their DNA and RNA targets. This effect was very weak with α -analogs whereas it was more pronounced with β -oligos. PNHME-modified α -oligonucleosides formed more stable duplexes with DNA ($\Delta T_m +9.6^\circ\text{C}$) and RNA ($\Delta T_m +1.4^\circ\text{C}$) targets than the 'parent' phosphodiester. Finally, base pairing specificity of these α -oligonucleoside *N*-alkylphosphoramidates for their targets was found to be as high as for natural oligonucleoside phosphodiester.

INTRODUCTION

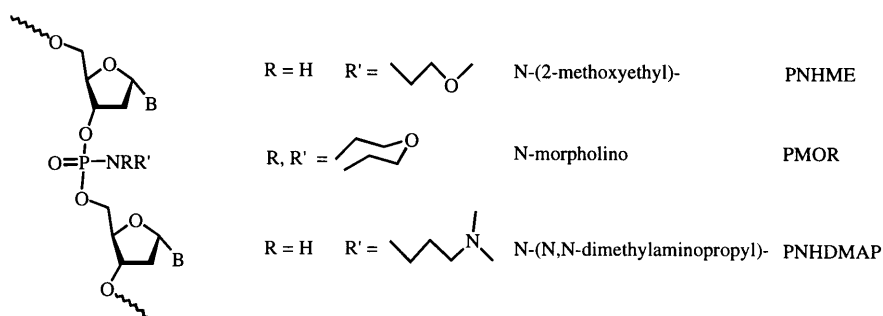
The high affinity and specificity of Watson–Crick hybridization has made oligonucleotides (ODNs) attractive agents for diagnostic and therapeutic applications via the antisense or antigene mode of action (1–5). Much effort has focused on the chemical modification of oligonucleotides (6–9) to improve their hybridization affinity, their biostability and their cellular uptake. Among these modifications, several phosphate-modified oligonucleotides, in particular ionic phosphorothioate (PS) (10) and non-ionic

methylphosphonate (MP) ODNs (11), have been investigated. These compounds are resistant to nucleases and have been used in various *in vitro* and *in vivo* systems as antisense agents (5,12). Phosphoramidate ODNs in which a non-bridged oxygen is substituted by primary or secondary amino groups have also been studied (13–22). The replacement of one non-bridging oxygen atom by any substituent induces the appearance of a new chiral center at the phosphorus. The acquired chirality of the new internucleoside linkages leads to a mixture of diastereoisomers, only a few of which possess the optimal combination of physical and chemical properties supporting the desired biological effects (23). It was recognized early that the stability of complexes formed between oligonucleotide analogs and complementary oligo- and polynucleotides depends on the stereochemistry of P-chiral internucleoside linkages. Most of the melting temperature (T_m) data were collected for complexes involving oligonucleotides with a single phosphoramidate internucleoside linkage (14), or oligomers with alternating phosphodiester/phosphoramidate linkages (16,20,21). Although oligonucleoside phosphoramidates have been studied, data on chirally pure diastereoisomers are limited (18,19). In addition to stereochemical effects, the steric hindrance of the P substituent also has a detrimental impact on the overall stability of the duplex (23).

Some years ago, our group developed nuclease-resistant α -anomeric oligonucleotides which form stable parallel-stranded duplexes with complementary natural β -DNA or β -RNA strands (24,25). Interestingly, we found that PS α -ODNs which combine two structural modifications, one located in the sugar moiety and the second in the internucleoside link, hybridize to their DNA and RNA targets more efficiently than do their PS β -homologs (26,27). The same behavior was observed with α - and β -dodecathymidylates containing non-ionic primary phosphoramidate (PNH₂) internucleoside linkages (28). Surprisingly, the non-ionic PNH₂ α -dT₁₂ hybridizes to its targets even better than does the parent β -oligonucleoside phosphodiester (PO). More recently, we reported that non-ionic MP α -ODNs form duplexes with DNA and RNA targets more stable than MP β -ODNs. In addition, MP α -ODNs bind to complementary DNA more tightly than do the corresponding natural PO β -oligomers (29).

These data obtained with PS, PNH₂ and MP α -ODNs prompted us to study new phosphate-modified α -oligonucleoside analogs with non-ionic *N*-alkylphosphoramidate linkages

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Scheme 1. *N*-alkylphosphoramidate internucleoside linkages used in this study.

which are chemically more stable than PNH_2 links and allow variation of the steric hindrance around phosphorus as a function of the alkyl groups attached to the nitrogen atom. Here we report hybridization properties of α -ODNs with non-ionic or cationic internucleotide linkages such as methoxyethylphosphoramidate (13,15–17), phosphoromorpholidate (13,18,19) and dimethylaminopropylphosphoramidate (20–22) (Scheme 1). In this paper we focus on the effect of the chirality of the internucleotide linkage and the effect of steric hindrance around the phosphorus on the hybridization properties of these new α -analogs with single-stranded DNA and RNA targets.

First, to evaluate the effect of chirality, we prepared α - or β -dodecanucleoside phosphodiester containing one chirally enriched phosphoramidate modification in the middle of the sequence β -3'-d(TCTTAA*CCCACA)-5' or α -5'-d(TCTTAA*CCCACA)-3' complementary to the splice acceptor site of HIV-1 *tat* RNA. The effect of chirality on the stability of the duplexes formed with complementary single-stranded (ss)DNA or RNA strands was measured by the variation in T_m (ΔT_m).

Secondly, we studied the effect of steric hindrance around the phosphorus on the hybridization properties of fully modified phosphorus-substituted dodecamers β -3'- and α -5'-d(TCTTAA*CCCACA). Finally, base pairing specificity of these α -oligonucleoside *N*-alkylphosphoramidates with their complementary DNA and RNA targets was investigated.

MATERIALS AND METHODS

Except as noted, chemicals were reagent grade or better and used without further purification. Acetonitrile was distilled over calcium hydride, tetrahydrofuran was distilled over LiAlH_4 and methylene chloride was distilled over phosphorus pentoxide. 2-Methoxyethylamine (Aldrich) and *N,N*-dimethyl-3-aminopropylamine (Aldrich) were distilled over calcium hydride whereas morpholine (Aldrich) was used without purification. Silica gel TLC was carried out on Kieselgel 60 F₂₅₄ plates (Merck), and compounds were visualized by UV shadowing. Silica gel 60 (230–240 mesh; Merck) was used for flash column chromatography. FAB mass spectra were recorded on a JEOL DX300 spectrometer operating with a JMA-DA 5000 mass data system in positive or negative ion mode; MALDI-TOF mass spectra of oligonucleotides were obtained on a Linear Voyager DE instrument (PerSeptive Biosystems) equipped with a 337 nm UV laser. The ^{31}P NMR spectra were recorded with a Bruker AC 250 spectrometer, and chemical shifts were

measured relative to 85% H_3PO_4 as external reference. Reverse phase HPLC was performed on a Waters 600E system equipped with a Model 990 or 996 photodiode array detector and using an EC Nucleosil 5 μ C₁₈ column (150 \times 4.6 mm; Macherey-Nagel) for analytical purposes (flow rate 1.0 ml/min) and a Waters Delta Pak 15 μ C₁₈ 100 Å column (300 \times 7.8 mm) for preparative work (flow rate 2.0 ml/min). Elution was performed with linear gradients of acetonitrile in 50 mM triethylammonium acetate (TEAAC), pH 7.

P-chirally enriched α -dinucleoside phosphoramidates

O-[5'-*O*-(4,4'-dimethoxytrityl)-6-*N*-benzoyl- α -2'-deoxyadenosin-3'-yl]-*O*-[3'-*O*-(*tert*iobutyldimethylsilyl)-4-*N*-benzoyl- α -2'-deoxycytidin-5'-yl]-*N*-alkylphosphoramidates (**3–5**). The α -dinucleoside phosphoramidates **3–5** were prepared from 5'-*O*-dimethoxytrityl-*N*-6-benzoyl-2'-deoxy- α -adenosine-3'-*O*-(methyl *N,N*-diisopropylphosphoramidite) **1** synthesized as described previously (30) and 3'-*O*-*t*-butyldimethylsilyl-*N*-4-benzoyl-2'-deoxy- α -cytidine **2** obtained according to standard procedures (tritylation and detritylation) (31) with 77% overall yield from α -dC^{Bz} (30).

2. ^1H NMR (CDCl_3 , 250 MHz) δ 8.72 (br s, 1H, NH), 8.13 (d, 1H, H-6), 7.95–7.50 (m, 6H, H-5, H ar), 6.32 (dd, 1H, H-1', $J = 6.9, 1.4$), 4.43 (m, 2H, H-3', H-4'), 3.73 (m, 2H, H-5', H5''), 2.74 (m, H, H-2'), 2.51 (br s, 1H, OH-5'), 2.24 (m, 1H, H-2''), 0.82 [s, 9H, C(CH₃)₃], 0.04, 0.00 (2s, 6H, 2 \times CH₃). FAB mass spectrum (positive mode, glycerol/thioglycerol 50/50 v/v): 446 [M + H]⁺.

Compounds **1** (745 mg, 0.91 mmol) and **2** (290 mg, 0.65 mmol) were dried three times by evaporation from dry CH_3CN , then the residue was dissolved in dry CH_3CN (3.2 ml) and tetrazole (273 mg, 3.9 mmol) was added to the mixture. The reaction was complete after stirring for 30 min at room temperature. A solution of iodine (230 mg, 0.91 mmol) and amine (24.7 mmol) (methoxyethylamine, morpholine or dimethylaminopropylamine) in dry THF (8 ml) was added to the reaction mixture. After 5 min, the mixture was diluted with CH_2Cl_2 (20 ml), poured into 5% aqueous sodium bisulfite (150 ml) and extracted with CH_2Cl_2 (3 \times 50 ml). The organic layer was washed repeatedly with saturated NaCl, then dried over Na_2SO_4 and concentrated under reduced pressure. Efforts to separate the two diastereoisomers of compounds **3** and **4** have been unsuccessful. Compound **3** was purified (98% yield) as a diastereoisomeric mixture by silica gel chromatography using a 0–5% MeOH gradient in CH_2Cl_2 in the presence of 0.5% triethylamine.

3. ^{31}P NMR (CDCl_3 , 101 MHz) δ 9.18 and 9.10. FAB mass spectrum (negative mode, thioglycerol): 1221 [M – H][–].

Crude **4**. ^{31}P NMR (CDCl_3 , 101 MHz) δ 8.42 and 8.25. FAB mass spectrum (negative mode, thioglycerol): 1233 $[\text{M} - \text{H}]^-$.

The two diastereoisomers of **5** were separated by silica gel chromatography as the 'fast' **5F** (25% yield) and 'slow' **5S** (29% yield) eluted using 4% isocratic EtOH in CH_2Cl_2 in the presence of 0.1% Et_3N .

5F. ^{31}P NMR (CD_3CN , 101 MHz) δ 10.11.

5S. ^{31}P NMR (CD_3CN , 101 MHz) δ 10.12.

5F and **5S** FAB mass spectra (negative mode, NOBA) 1247 $[\text{M} - \text{H}]^-$.

O-[5'-*O*-(4,4'-dimethoxytrityl)-6-*N*-benzoyl- α -2'-deoxyadenosin-3'-yl]-*O*-(4-*N*-benzoyl- α -2'-deoxycytidin-5'-yl)-*N*-alkylphosphoramidates **6F** and **6S**, **7F** and **7S**, and **8F** and **8S**. Compounds **3**, **4**, **5F** and **5S** (1 mmol) were treated with tetrabutylammonium fluoride (2 mmol) in dry THF (25 ml) for 1 h. After evaporation of THF, the residue was dissolved in CH_2Cl_2 (150 ml) and washed with water (3×100 ml). The organic layer was dried over Na_2SO_4 , filtered and evaporated under reduced pressure. Purification of compounds **6**, **7**, **8F** and **8S** was performed as follows.

The two diastereoisomers of **6** were separated by silica gel chromatography as the 'fast' **6F** and 'slow' **6S** eluting isomers using 2% isocratic EtOH in CHCl_3 in the presence of 0.1% Et_3N ; six consecutive chromatographic steps were necessary to separate the two isomers.

6F. Yield 18%; HPLC R_T 20.58 min (91% diastereoisomeric purity) with a 10 min linear gradient of 72–90% CH_3CN in 50 mM TEAAC followed by 90% isocratic CH_3CN . ^{31}P NMR (CDCl_3 , 101 MHz) δ 8.97. FAB mass spectrum (negative mode, thioglycerol) 1106 $[\text{M} - \text{H}]^-$.

6S. Yield 27%; HPLC R_T 19.15 min (86% diastereoisomeric purity) with a 10 min linear gradient of 72–90% CH_3CN in 50 mM TEAAC followed by 90% isocratic CH_3CN . ^{31}P NMR (CDCl_3 , 101 MHz) δ 9.22. FAB mass spectrum (negative mode, thioglycerol) 1106 $[\text{M} - \text{H}]^-$.

The two diastereoisomers of **7** were separated by silica gel chromatography as the 'fast' **7F** and 'slow' **7S** eluting isomers using 1% isocratic EtOH in CHCl_3 in the presence of 0.15% Et_3N ; five consecutive chromatographic steps were necessary to separate the two isomers.

7F. Yield 35%; HPLC R_T 12.68 min (88.5% diastereoisomeric purity) with a 20 min linear gradient of 48–64% CH_3CN in 50 mM TEAAC. ^{31}P NMR (CD_3CN , 101 MHz) δ 8.38. FAB mass spectrum (negative mode, thioglycerol) 1118 $[\text{M} - \text{H}]^-$.

7S. Yield 25%; HPLC R_T 12.06 min (80% diastereoisomeric purity) with a 20 min linear gradient of 48–64% CH_3CN in 50 mM TEAAC. ^{31}P NMR (CD_3CN , 101 MHz) δ 8.21. FAB mass spectrum (negative mode, thioglycerol) 1118 $[\text{M} - \text{H}]^-$.

Diastereoisomers **8F** and **8S** were purified by silica gel chromatography using a 0–5% MeOH gradient in CH_2Cl_2 in the presence of 0.5% Et_3N .

8F. Yield 17%; HPLC R_T 16.28 min (93% diastereoisomeric purity) with a 30 min linear gradient of 48–64% CH_3CN in 50 mM TEAAC. ^{31}P NMR (CDCl_3 , 101 MHz) δ 9.45. FAB mass spectrum (positive mode, NOBA) 1134 $[\text{M} + \text{H}]^+$.

8S. Yield 21%; HPLC R_T 17.61 min (85% diastereoisomeric purity) with a 30 min linear gradient of 48–64% CH_3CN in 50 mM TEAAC. ^{31}P NMR (CDCl_3 , 101 MHz) δ 9.39. FAB mass spectrum (positive mode, NOBA) 1134 $[\text{M} + \text{H}]^+$.

Phosphoramidites of α -dinucleoside phosphoramidates **9F** and **9S**, **10F** and **10S** and **11F** and **11S**

A mixture of compound **6F** (0.5 mmol) and diisopropylammonium tetrazolide (0.25 mmol) was dried by co-evaporation three times with anhydrous pyridine and then acetonitrile. The dry residue was suspended in freshly distilled CH_2Cl_2 (10 ml) and *O*-(2-cyanoethyl)-*N,N*-bis(diisopropylamino)phosphine (0.6 mmol) was added under argon. After stirring at room temperature for 16 h, the reaction mixture was diluted with ethyl acetate (200 ml), the solution filtered and washed successively with saturated aqueous NaHCO_3 (250 ml) and saturated NaCl (250 ml). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography using a 60–98.5% CH_2Cl_2 gradient in cyclohexane in the presence of 1.5% Et_3N , then a 0–2% MeOH gradient in CH_2Cl_2 in the presence of 1.5% Et_3N . The phosphoramidite derivative **9F** was obtained in 43% yield as a white powder after lyophilization from benzene. Isomer **9S** was prepared similarly from **6S**.

9F. ^{31}P NMR (CD_3CN , 101 MHz) δ 150.11 and 149.65 (stereoisomeric phosphoramidite groups) and 10.31 and 10.26 (internucleoside phosphoramidate group) in a 1:1 ratio for the two sets of signals.

9S. Yield 64%, ^{31}P NMR (CD_3CN , 101 MHz) δ 150.21, 149.69 and 10.49, 10.40 (phosphoramidite/phosphoramidate, 1:1).

9F and **9S** FAB mass spectrum (positive mode, NOBA) 1308 $[\text{M} + \text{H}]^+$ and (negative mode, NOBA) 1306 $[\text{M} - \text{H}]^-$.

The other phosphoramidite dinucleoside blocks **10F**, **10S**, **11F** and **11S** were prepared in the same manner. Each exhibited signals for phosphoramidite and phosphoramidate in a 1:1 ratio.

10F. Yield 31%, ^{31}P NMR (CD_3CN , 101 MHz) δ 150.12, 149.77 and 8.50, 8.44.

10S. Yield 21%, ^{31}P NMR (CD_3CN , 101 MHz) δ 150.15, 149.78 and 8.42, 8.35.

11F. Yield 65%, ^{31}P NMR (CD_3CN , 101 MHz) δ 150.16, 149.69 and 10.39, 10.34.

11S. Yield 58%, ^{31}P NMR (CD_3CN , 101 MHz) δ 150.36, 149.75 and 10.50, 10.41.

Protected 2'-deoxy- α -ribonucleoside-3'-*O*-*H*-phosphonates **12**, **13** and **14** (Scheme 3)

2'-Deoxy- α -ribonucleosides-3'-*O*-*H*-phosphonate (α -dT **12**, α -dC^{Bz} **13** and α -dA^{Bz} **14**) were prepared from base-protected 5'-*O*-dimethoxytrityl-2'-deoxy α -nucleosides (**30**) according to a published procedure (**32**).

5'-*O*-(4,4'-dimethoxytrityl)- α -thymidine-3'-*O*-*H*-phosphonate (triethylammonium salt) **12**. Yield 70%. ^{31}P NMR (CDCl_3 , 200 MHz) δ 4.46, $^1J_{\text{P-H}} = 626$ Hz, $^3J_{\text{P-H}} = 8.9$ Hz. FAB mass spectrum (negative mode, thioglycerol) 607 $[\text{M} - \text{H}]^-$.

5'-*O*-(4,4'-dimethoxytrityl)-4-*N*-benzoyl-2'-deoxy- α -cytidine-3'-*O*-*H*-phosphonate (triethylammonium salt) **13**. Yield 70%. ^{31}P NMR (CDCl_3 , 101 MHz) δ 3.62, $^1J_{\text{P-H}} = 626$ Hz, $^3J_{\text{P-H}} = 8.9$ Hz. FAB mass spectrum (negative mode, thioglycerol) 696 $[\text{M} - \text{H}]^-$.

5'-*O*-(4,4'-dimethoxytrityl)-6-*N*-benzoyl-2'-deoxy- α -adenosine-3'-*O*-*H*-phosphonate (triethylammonium salt) **14**. Yield 76%.

^{31}P NMR (CDCl_3 , 101 MHz) δ 3.69, $^1J_{\text{P-H}} = 623$ Hz, $^3J_{\text{P-H}} = 8.7$ Hz. FAB mass spectrum (negative mode, thioglycerol) 720 [M – H] $^-$.

Oligonucleotides synthesis

β - and α -oligonucleoside phosphodiester **15**, **15a**, **15b**, **15c**, **16**, **16a**, **16b**, **16c**, **17** and **18**. Protected deoxy-2'- β -ribonucleoside-3'-*O*-(*N,N*-diisopropyl)cyanoethylphosphoramidites were purchased from PerSeptive Biosystems GmbH and deoxy-2'- α -ribonucleoside-3'-*O*-(*N,N*-diisopropyl)cyanoethylphosphoramidites (α -dT, α -dC^{Bz} and α -dA^{Bz}) were prepared according to published procedures (30). The RNA target oligomer r(AGA-AUUGGGUGU) **15** was a gift from ISIS Pharmaceuticals (Carlsbad, CA). The DNA target oligomer d(AGAATT-GGGTGT) **16** and the complementary phosphodiester β -d(ACACCCAATTCT) **17** and α -d(TCTTAACCCACA) **18** were prepared on the 1 μmol scale with an Applied Biosystems Model 381A DNA synthesizer. The protected oligomers were cleaved from the support and deprotected upon treatment with concentrated ammonium hydroxide for 5 h at 55°C. The oligomers were purified by preparative reverse phase HPLC using a linear gradient of 5–50% acetonitrile in 50 mM TEAAC.

β - and α -oligonucleoside phosphodiester containing a single chirally enriched phosphoramidate linkage **19F**, **19S**, **20F**, **20S**, **21F**, **21S**, **22F**, **22S**, **23F**, **23S**, **24F** and **24S**. Oligonucleotides were prepared on the 1 μmol scale following standard procedures of phosphoramidite chemistry except that the coupling step of the chirally enriched dinucleotide was repeated three times with extended (3 min) coupling times. The oligomers were purified by preparative reverse phase HPLC using a linear gradient of 7.5–25% acetonitrile in 50 mM TEAAC.

MS [negative mode MALDI-TOF, matrix: 2,4,6-trihydroxyacetophenone (THAP) and ammonium citrate]: **19F** m/z calculated 3605.5, found 3603.2; **19S** m/z calculated 3605.5, found 3606.4; **20F** m/z calculated 3632.5, found 3631.8; **20S** m/z calculated 3632.5, found 3632.1; **21F** m/z calculated 3617.5, found 3618.5; **21S** m/z calculated 3617.5, found 3618.6; **22F** m/z calculated 3605.5, found 3603.1; **22S** m/z calculated 3605.5, found 3606.4; **23F** m/z calculated 3632.5, found 3629.4; **23S** m/z calculated 3632.5, found 3631.3; **24F** m/z calculated 3617.5, found 3617.7; **24S** m/z calculated 3617.5, found 3616.7.

β - and α -oligonucleoside phosphodiester containing a single chirally enriched methylphosphonate linkage **25f** and **25s** and **26f** and **26s**. Oligonucleotides were prepared on the 1 μmol scale using the appropriate protected nucleoside phosphoramidite and methylphosphonamidite synthons. The oligomers **25** and **26** were deprotected and cleaved from the support by sequential treatment with ammonium hydroxide and ethylenediamine (33). The β -oligomer **25** was then purified by reverse phase HPLC using an EC Nucleosil 5 μ C₁₈ EC column (150 \times 4.6 mm; Macherey-Nagel) and a linear gradient of 11–13% acetonitrile in 50 mM TEAAC, pH 7. The diastereoisomers of the β -oligomer **25** were resolved into two peaks and each diastereoisomer was collected separately as **25f** for the 'fast' eluting isomer on reverse phase C₁₈ (HPLC R_T 33.07 min,

100% diastereoisomeric purity) and **25s** for the 'slow' eluting isomer on reverse phase C₁₈ (HPLC R_T 34.76 min, 100% diastereoisomeric purity). The diastereoisomers of the α -oligomer **26** were separated into two peaks by reverse phase HPLC using a Lichrospher 5 μ C₁₈ EC column (150 \times 4.6 mm; Phase Sep) and a linear gradient of 7–9.5% acetonitrile in 50 mM ammonium acetate, pH 5.6.

The two diastereoisomers were collected separately as: **26f**, HPLC R_T 28.77 min (100% diastereoisomeric purity); **26s**, HPLC R_T 32.32 min (90% diastereoisomeric purity). MS (negative mode MALDI-TOF, matrix: THAP and ammonium citrate) m/z calculated 3546.4, **25f** found 3545.7, **25s** found 3545.3, **26f** found 3547.6, **26s** found 3544.0.

β - and α -oligonucleoside *N*-alkylphosphoramidates **27–30**. These were synthesized via hydrogen phosphonate chemistry (34). Amidative oxidation of *H*-phosphonate diesters was performed manually using a 10% solution of either 2-methoxyethylamine or morpholine in an anhydrous CCl₄/pyridine solution (50:50 v/v). The ODNs were removed from the solid support and deprotected with concentrated NH₄OH at 55°C for 5 h. Purification of ODNs was performed by preparative reverse phase HPLC using a 50 min linear gradient of 8–40% acetonitrile in 50 mM TEAAC. They were characterized by MALDI-TOF mass spectrometry. MS (negative mode MALDI-TOF, THAP and ammonium citrate): **27** m/z calculated 4176.4, found 4174.8; **28** m/z calculated 4308.5, found 4307.2; **29** m/z calculated 4176.4, found 4175.6; **30** m/z calculated 4308.5, found 4306.2.

β - and α -oligonucleoside methylphosphonates. These were synthesized according to published procedures (29,33) on the 1 μmol scale using either β -methylphosphonamidite or α -methylphosphonamidite synthons. Their purification and characterization were described in a previous publication (29).

Melting experiments

The concentration of each separated oligonucleotide was determined spectrophotometrically at 260 nm and at 80°C assuming that the molar extinction coefficient of the α - and β -oligomers is the sum of the molar extinction coefficients of the constitutive deoxynucleotides. Optical measurements were performed on a UVIKON 931 spectrophotometer (Kontron). The temperature of the cell was controlled by a Huber PD415 temperature programmer connected to a refrigerated ethyleneglycol/water bath (Huber Ministat). Cuvettes were 1 cm path length quartz cells. The cell compartment was continuously flushed with dry nitrogen when the temperature was below room temperature. Prior to the experiments, the oligonucleotides were mixed together each at a final concentration of 3 μM in 100 mM sodium chloride, 10 mM sodium cacodylate (pH 7) in the presence of 0.3% dioxane and allowed to incubate at 90°C for 30 min. During the hybridization (melting) experiments the cooling (heating) rate was 0.5°C/min. Digitized absorbance and temperature values were stored in a computer for subsequent plotting and analysis. T_m values were determined from the maxima of the first derivative plots of absorbance versus temperature.

Table 1. Oligonucleotides synthesized

No.	Oligonucleotide	Sequence	Backbone
15	RNA target	5'-AGA AUU GGG UGU-3'	PO
15a	RNA target (CC mismatch)	5'-AGA AUU CGG UGU-3'	PO
15b	RNA target (CU mismatch)	5'-AGA AUU UGG UGU-3'	PO
15c	RNA target (CA mismatch)	5'-AGA AUU AGG UGU-3'	PO
16	DNA target	5'-AGA ATT GGG TGT-3'	PO
16a	DNA target (CC mismatch)	5'-AGA ATT CGG TGT-3'	PO
16b	DNA target (CT mismatch)	5'-AGA ATT TGG TGT-3'	PO
16c	DNA target (CA mismatch)	5'-AGA ATT AGG TGT-3'	PO
17	β -oligo DNA	5'-ACA CCC AAT TCT-3'	PO
18	α -oligo DNA	5'-TCT TAA CCC ACA-3'	PO
19F and 19S	β -oligo with 1 modification	5'-ACA CCC *AAT TCT-3'	PO/PNHME
20F and 20S	β -oligo with 1 modification	5'-ACA CCC *AAT TCT-3'	PO/PNHDMAP
21F and 21S	β -oligo with 1 modification	5'-ACA CCC *AAT TCT-3'	PO/PMOR
22F and 22S	α -oligo with 1 modification	5'-TCT TAA* CCC ACA-3'	PO/PNHME
23F and 23S	α -oligo with 1 modification	5'-TCT TAA* CCC ACA-3'	PO/PNHDMAP
24F and 24S	α -oligo with 1 modification	5'-TCT TAA* CCC ACA-3'	PO/PMOR
25f and 25s	β -oligo with 1 modification	5'-ACA CCC *AAT TCT-3'	PO/MP
26f and 26s	α -oligo with 1 modification	5'-TCT TAA* CCC ACA-3'	PO/MP
27	β -oligo fully modified	5'-ACA CCC AAT TCT-3'	PNHME
28	β -oligo fully modified	5'-ACA CCC AAT TCT-3'	PMOR
29	α -oligo fully modified	5'-TCT TAA CCC ACA-3'	PNHME
30	α -oligo fully modified	5'-TCT TAA CCC ACA-3'	PMOR

*Chirally enriched phosphoramidate or methylphosphonate linkage.

F, S for 'fast' and 'slow' isomers according to their chromatographic mobility on a silica gel column of the P-modified dinucleotide incorporated in the ODN (see Materials and Methods).

f, s for 'fast' and 'slow' isomers according to their chromatographic mobility on a reverse phase HPLC C₁₈ column.

RESULTS AND DISCUSSION

Preparation of oligonucleotides containing a single chirally enriched phosphoramidate linkage

To examine the effect of chirality on binding interactions, α - and β -oligodeoxynucleotides containing a single chirally enriched phosphoramidate linkage in the middle of the sequence (Table 1) were prepared on a solid support using the appropriately protected nucleoside phosphoramidite synthons. These ODNs were complementary to the splice acceptor site AGAA-UUGGUGU of the mRNA coding for HIV-1 *tat* protein, a target **15** already used in our previous studies (26,29). The central phosphoramidate linkage was introduced through a 3'-phosphoramidite dinucleoside building block containing a chirally enriched phosphoramidate internucleoside linkage. The fully protected α -dinucleoside phosphoramidates **3-5** were prepared according to a procedure described in the literature for the synthesis of β -analogs (21) (Scheme 2). After removal of the TBDMS group, separation from the mixture of each diastereoisomer was achieved by chromatography on silica gel. The absolute configuration of the phosphoramidate linkage in the dinucleotide was not assigned. The two diastereoisomers of each dinucleotide were identified as 'fast' (or F) and

'slow' (or S) isomers according to their chromatographic mobility on a silica gel column. Subsequent phosphitylation afforded the phosphoramidite synthons **9F**, **9S**, **10F**, **10S**, **11F** and **11S** employed for the synthesis of oligonucleotides **22F** and **22S**, **23F** and **23S**, and **24F** and **24S**, respectively. Similarly, the β -oligonucleotides **19F**, **19S**, **20F**, **20S**, **21F** and **21S** were synthesized.

Evaluation of the chirality effect on hybridization properties of α -oligonucleoside *N*-alkylphosphoramidates

For each phosphoramidate modification, two α - (F and S) and two β -oligonucleotides (F and S) were hybridized to their complementary RNA **15** and DNA **16** strands. These interactions were investigated by thermal denaturation and renaturation analyses using change in absorbance at 260 nm versus temperature. No significant differences were observed between thermal association and dissociation curves. Melting temperatures (T_m) were determined at the inflexion point of the sigmoid curves (Table 2). The thermal stability of duplexes having a single phosphoramidate linkage (F or S) was compared to that of the corresponding all-phosphodiester duplexes under identical conditions. The difference in T_m (ΔT_m) reported in Figure 1 accounts for the effect due to the stereoselective introduction

Table 2. Thermal stability (T_m and ΔT_m) of a duplex formed between α - or β -oligonucleotides containing one chirally enriched *N*-alkylphosphoramidate or methylphosphonate linkage and their DNA or RNA targets

Oligonucleotide		DNA target 16		RNA target 15	
		T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
β -PO	17	45.4		44.5	
β -PNHME	19F	44.4	-1.0	42.0	-2.5
β -PNHME	19S	43.4	-2.0	40.7	-3.8
β -PNHDMAP	20F	44.2	-1.2	42.6	-1.9
β -PNHDMAP	20S	43.6	-1.8	41.3	-3.2
β -PMOR	21F	43.4	-2.0	41.6	-2.9
β -PMOR	21S	42.9	-2.5	40.5	-4.0
β -MP	25f	44.7	-0.7	43.5	-1.0
β -MP	25s	43.7	-1.7	41.6	-2.9
α -PO	18	43.4		41.5	
α -PNHME	22F	42.9	-0.5	41.4	-0.1
α -PNHME	22S	44.9	+1.5	42.2	+0.7
α -PNHDMAP	23F	42.8	-0.6	41.3	-0.3
α -PNHDMAP	23S	46.0	+2.6	41.2	-0.2
α -PMOR	24F	42.4	-1.0	40.6	-0.9
α -PMOR	24S	43.4	0	41.0	-0.5
α -MP	26f	45.3	+1.9	41.8	+0.3
α -MP	26s	43.4	0	41.1	-0.4

Experiments were carried out at 3 μ M concentration for each strand in a buffer containing 10 mM sodium cacodylate, pH 7, 100 mM sodium chloride in the presence of 0.3% dioxane.

of one phosphoramidate linkage in the various duplexes. Additionally, data obtained with α - and β -oligonucleotides **26f** and **26s** and **25f** and **25s** bearing a single chirally enriched methylphosphonate linkage are reported.

With DNA target **16** (Fig. 1), regarding β -oligonucleotides, whatever the backbone modification the two diastereoisomers (designated **F** and **S**) formed duplexes less stable than the reference PO β -ODN **17** ($-2.5^\circ\text{C} < \Delta T_m < -0.7^\circ\text{C}$). As expected, one isomer bound to DNA target **16** less tightly than the other. With α -ODNs, a difference between the two isomers was also observed (from 1.0 to 3.2°C) and it was even more pronounced than with β -ODNs (from 0.5 to 1.0°C). It is noteworthy that with modified α -ODNs, one isomer had no effect or a stabilizing effect on the duplex ($0^\circ\text{C} < \Delta T_m < 2.6^\circ\text{C}$) whereas the other isomer had a slightly destabilizing effect ($-1.0^\circ\text{C} < \Delta T_m < 0^\circ\text{C}$).

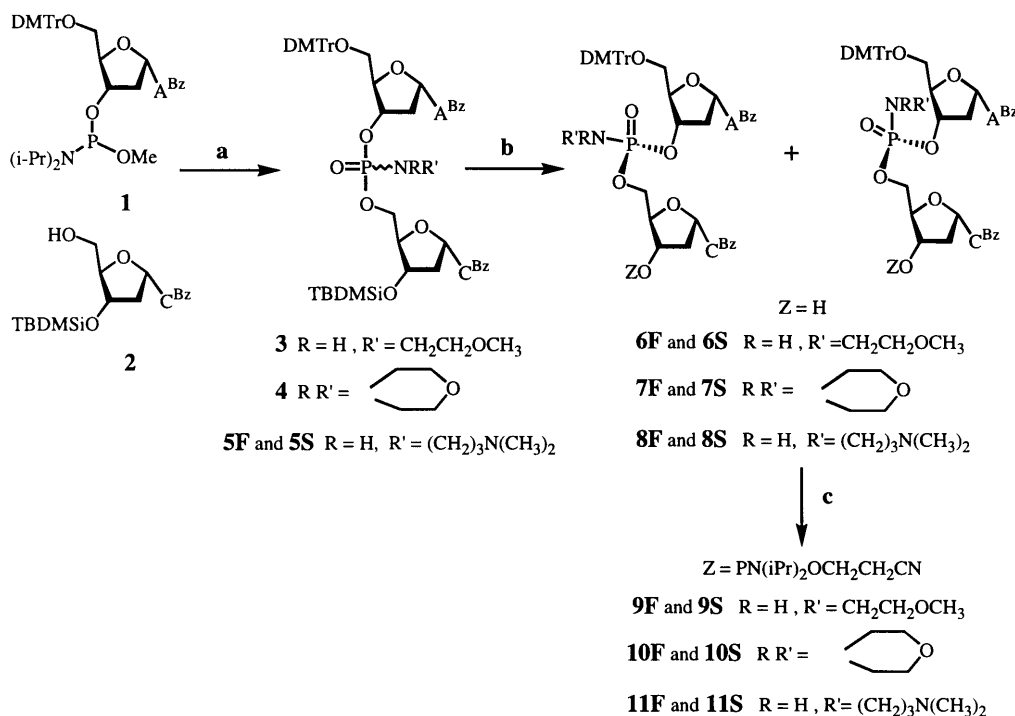
With RNA target **15** (Fig. 1), the backbone modification introduced in β -oligonucleotides destabilized duplexes more dramatically than with DNA target **16** ($-4.0^\circ\text{C} < \Delta T_m < -1.0^\circ\text{C}$). In contrast, the modifications of the internucleoside linkage in α -ODNs induced a weaker effect whatever the P substituent ($-0.9^\circ\text{C} < \Delta T_m < 0.7^\circ\text{C}$) and the stereoisomeric conformation (from 0.1 to 0.8°C) of the modified linkage. This suggests that the chirality effect in duplexes formed between phosphate-modified α -ODNs and the RNA target is reduced to such an extent that

a stereoselective synthesis of these ODN analogs would not be required for efficient binding to the target.

Effect of steric hindrance of the P substituent on hybridization properties

To study the steric hindrance effect, fully modified β - and α -oligonucleoside *N*-alkylphosphoramidates (PNHME or PMOR) **27** and **28** and **29** and **30** with the same sequence as mentioned above were synthesized. These analogs were prepared in a non-stereocontrolled way, via *H*-phosphonate chemistry using the appropriately protected nucleoside *H*-phosphonate synthons (Scheme 3) and following a standard procedure (34). The coupling yields averaged 96–97% per cycle. The *H*-phosphonate diester linkages were then oxidized by CCl_4 in the presence of 2-methoxyethylamine or morpholine. After deprotection, oligonucleoside *N*-alkylphosphoramidates were purified by reverse phase HPLC. The ODN *N*-alkylphosphoramidates obtained as diastereoisomeric mixtures were hybridized to their complementary DNA and RNA strands. The T_m values presented in Figure 2 (versus DNA target and RNA target) were compared to that obtained with PO β - and α -oligonucleosides **17** and **18**, and to β - and α -dodecanucleoside methylphosphonates previously studied (29).

First, in agreement with previous studies (13), the introduction of non-ionic internucleotide linkages into the β -ODNs greatly decreased the thermal stability of the hybrids formed with



Scheme 2. Synthesis of 3'-phosphoramidite α -dinucleoside *N*-alkylphosphoramidates. (a) Tetrazole, CH_3CN , 30 min, then I_2 , 2-methoxyethylamine or morpholine or dimethylaminopropylamine, THF, 5 min. (b) TBAF 1 M in THF. (c) $\text{P}[\text{O}(\text{CH}_2)_2\text{CN}][(\text{i-Pr})_2\text{N}]_2$, diisopropylammonium tetrazolidine, CH_2Cl_2 , 20 h.

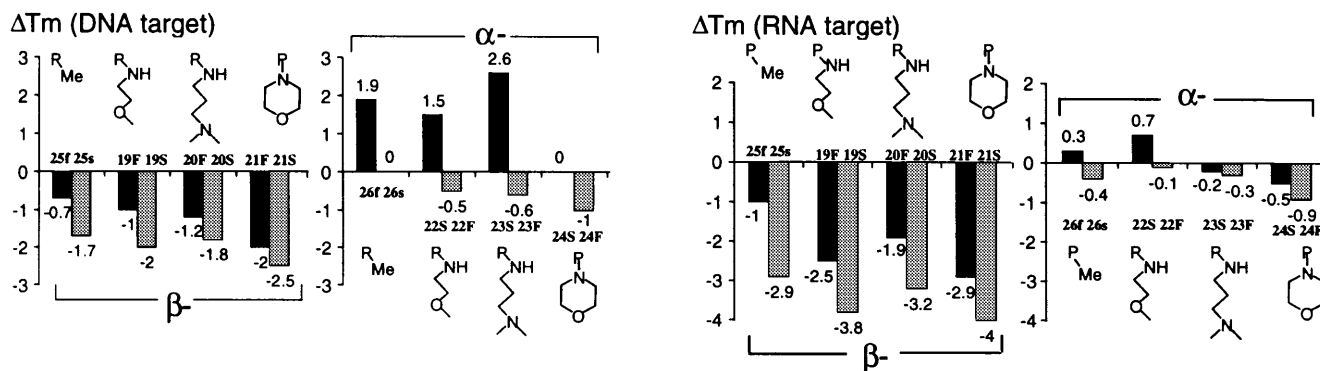
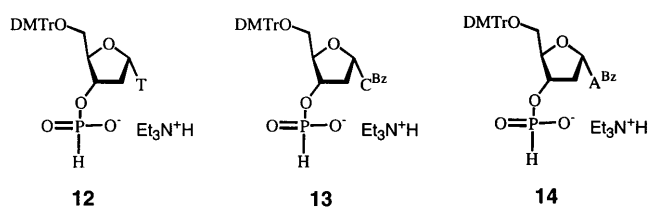


Figure 1. Variation in T_m induced by the stereoselective introduction of a single phosphoramidate or methylphosphonate linkage in duplexes formed between α - or β -oligonucleotides and their DNA target **16** or RNA target **15**. Position of the phosphoramidate or methylphosphonate linkage is indicated in Table 1.



Scheme 3. Protected 2'-deoxy- α -ribonucleoside-3'-*O*-*H*-phosphonates used for the synthesis of fully modified oligonucleoside phosphoramidates.

either complementary DNA or RNA strands according to the bulkiness of the phosphorus substituent. When PO linkages were replaced by PMOR linkages in β -ODNs, the T_m value of the duplex formed with DNA decreased by 32.4°C , whereas the ΔT_m between PO and PNHME was 15.9°C . This destabilization induced by the pendant group was more pronounced regarding hybrids formed between β -ODNs and their RNA target (MP ΔT_m 18.3°C , PNHME ΔT_m 29.6°C , PMOR ΔT_m 39.6°C). In contrast, the phosphorus substituent affects to a very low extent the hybridization of α -ODNs with DNA as well as with RNA targets. Surprisingly, the bulky morpholino group in the

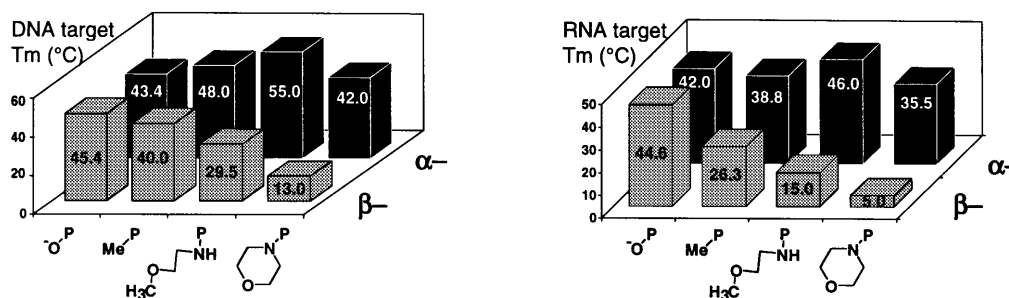


Figure 2. Thermal stability of fully phosphate-modified β - and α -ODNs with their DNA target **16** or RNA target **15**. Experimental conditions as indicated in Table 2.

Table 3. Influence of a single mismatch on the T_m of duplexes with *N*-alkylphosphoramidates α -oligonucleosides

ODN	T_m (°C)	DNA targets				RNA targets			
		16 CG	16a CC	16b CT	16c CA	15 CG	15a CC	15b CU	15c CA
β -PO 17	45.4	25.9	28.2	25.2	44.6	24.8	23.3	28.7	
α -PNHME 29	55.0	36.2	35.5	36.5	46.0	25.0	22.5	26.3	
α -PNMOR 30	42.0	23.0	22.5	22.5	35.5	15.0	14.0	15.9	

Experiments were carried out at 3 μ M concentration for each strand in a buffer containing 10 mM sodium cacodylate, pH 7, 100 mM sodium chloride in the presence of 0.3% dioxane.

α -series slightly destabilized the hybrid with the DNA target compared to the parent PO β -ODN–DNA duplex (ΔT_m -3.4°C). When RNA was the target, the destabilization was more pronounced (ΔT_m -9.1°C).

Secondly, the results indicate that whatever the backbone modification, the α -oligonucleotides formed much more stable duplexes with the DNA target than did their β -homologs. This difference in stability of the hybrids was more pronounced with ODN *N*-alkylphosphoramidates (25.5°C for PNHME, 29.0°C for PMOR) than with MP oligos (8.0°C for MP). The same effect was observed when RNA was the target (31.0°C for PNHME, 30.5°C for PMOR, 12.5°C for MP) (Fig. 2).

Finally, MP α -ODN and PNHME α -ODN formed more stable duplexes with the DNA target than did the natural PO β -ODN (ΔT_m $+2.6^\circ\text{C}$ and $+9.6^\circ\text{C}$, respectively). Among all the oligonucleotides studied, the α -oligonucleoside methoxyethyl-phosphoramidate has the best affinity for its DNA (ΔT_m $+9.6^\circ\text{C}$) and RNA (ΔT_m $+1.4^\circ\text{C}$) targets.

Specificity of hybridization of α -oligonucleoside *N*-alkyl-phosphoramidates

To examine whether the high affinity of the PNHME α -ODN for complementary strands was detrimental to the base pairing specificity, we measured the influence of a single mismatch in the DNA **16** or RNA **15** targets on the stability of the hybrids (Table 3). Introduction of one CC, CT or CA mismatch instead of the regular match CG in the hybrids formed with PNHME α -ODN **29** and DNA targets **16a**, **16b** and **16c** decreased the melting temperature by 18.8, 19.5 and 18.5°C, respectively. These values compare well with those obtained when the same mismatches were introduced into the corresponding duplexes formed with unmodified PO β -oligomer **17** (19.5, 17.2 and

20.2°C, respectively). When PMOR α -ODN **30** was hybridized to DNA targets **16a**, **16b** and **16c**, an average decrease of 19.3°C was found, similar to that (18.9°C) observed with the PNHME α -ODN and β -PO ODN. The specificity of hybridization of α -oligonucleoside *N*-alkylphosphoramidates was also studied with RNA targets and similar results were obtained. Destabilization of the duplex relative to the perfectly matched duplex was not dependent upon the bulkiness of the P-substituent. These data indicate that the Watson–Crick base pairing specificity of α -ODN phosphoramidates is as high as that of natural oligonucleoside phosphodiester despite their relatively high affinity.

Influence of salt concentration on the thermal stability of hybrids

Experiments at 0.1 M NaCl or in the absence of NaCl showed that the T_m values for the hybrids formed between fully modified PNHME α -ODN **29** and DNA **16** or RNA **15** targets were not modified by changes in the ionic strength of the medium (Table 4). This behavior reflects the absence of charge repulsion between the non-ionic oligonucleotide and the negatively charged phosphodiester backbone of the target strand as observed for neutral backbone β -analogs (13,35). In the absence of NaCl, PNHME α -ODN **29** formed much more stable duplexes with DNA **16** (ΔT_m 26.7°C) and RNA **15** (ΔT_m 13.8°C) than PO β -ODN **17**.

In conclusion, we have demonstrated that inversion of the anomeric configuration (from β to α) of the sugar moieties in fully modified oligonucleoside *N*-alkylphosphoramidates increases their affinity for ssDNA and ssRNA targets. An enhancement of affinity for DNA and RNA induced by the combination of two structural modifications was previously observed with non-ionic oligonucleoside PNH₂ (28) and MP

Table 4. Salt concentration dependence of the T_m (°C) of the fully modified phosphoramidate oligomer **29** with RNA **15** and DNA **16** targets compared with the natural duplex formed with β -PO **17**

ODN	T_m (°C)	T_m (°C)			
		DNA target 16		RNA target 15	
		0.1 M NaCl	No NaCl	0.1 M NaCl	No NaCl
β -PO 17		45.4	28.3	44.6	31.2
α -PNHME 29		55.0	55.0	46.0	45.0
ΔT_m (°C)		+9.6	+26.7	+1.4	+13.8

Experiments were carried out at 3 μ M concentration for each strand in a buffer containing 10 mM sodium cacodylate, pH 7, in the presence of 0.3% dioxane at the indicated NaCl concentrations.

ODNs (**29**) and, to a lesser extent, with ionic PS ODNs (**27**), and this finding seems to be a general phenomenon for backbone-modified oligodeoxynucleotides in the α -anomeric configuration. The high binding of 2-methoxyethylphosphoramidate ODNs to DNA is promising for triplex formation and we are currently studying the affinity of these backbone-modified α -oligonucleotides for dsDNA. Furthermore, α -oligonucleoside *N*-alkylphosphoramidates offer several advantages over phosphodiester ODNs and phosphorothioate analogs that include increased affinity, high specificity and ability to hybridize under low salt conditions. These properties make them attractive for antisense purposes as well as for use as diagnostics probes.

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SUPPLEMENTARY MATERIAL

See Supplementary Material available in NAR Online.

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