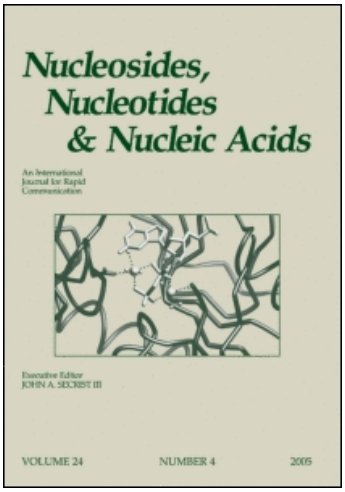

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Novel Chiral Phosphoramidite Monomers from (*R*)- and (*S*)-1,3-Butanediol: Synthesis of Modified Oligonucleotides and Binding Affinity Studies

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ABSTRACT

Two optically active phosphoramidite monomers for modified oligodeoxyribonucleotides were prepared. These monomers were then introduced into dodecanucleotides in the middle of the sequences. The modified dodecanucleotides were characterized by various analytical methods including MALDI-TOF mass spectrometry and Tm values were obtained to appraise the binding affinity, by measuring change in UV absorbance at 260 nm.

Key Words: Phosphoramidite; Oligonucleotide; 1,3-Butanediol; Chirality; Monomer.

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INTRODUCTION

Synthesis of modified oligodeoxyribonucleotides (ODNs) is an important subject in the field of antisense and antigene based drug discovery.[1] Recently, we have reported the design and synthesis of ODNs containing isoxazoline linkage in place of normal nucleotide phosphodiester links.[2,3] We have long been speculating that the chiral center at 3′-position would have a major role to play in defining the optical and binding properties of ODNs. In the field of modified ODNs, some research groups have been discussing the binding affinity with synthesized ODNs, which are mixtures of diastereomers with undefined absolute configuration.[4] Phosphothioate, one of the most famous backbone modifications of ODNs, has its own limitation of chirality control. That is, it mostly results in a diastereomeric mixture. These diastereomeric problems have been recently discussed by Asanuma et al. and Tanaka group.[5] They designed and synthesized optically pure phosphoramidite building blocks, and utilized them for diastereoselective ODN syntheses. In order to study the effect of chirality difference of *one chiral center* in backbone, we have chosen two functional monomers with different but known configurations; namely, optically pure *R*-(−) and *S*-(+)-1,3-butanediols. The number of atoms between two oxygens in *R*-(−)-1,3-butanediol and *S*-(+)-1,3-butanediol is three and this moiety is considered to be isosteric to nucleosides with no base units. 1,3-Butanediol has been selected so as to ensure that the effect is solely due to chiral center difference and not caused by any other interactions, generally associated with nucleobases or sugar moieties. This paper discusses our results on the synthesis of phosphoramidite monomers based on 1,3-butanediol and their subsequent introduction into the middle of dodecanucleotides. Also their binding affinities were estimated from Tm values.

RESULTS AND DISCUSSION

Primary hydroxyl groups of *R*-(−)-1,3-butanediol and *S*-(+)-1,3-butanediol were selectively protected with 4,4′-dimethoxytrityl (DMTr) while the secondary hydroxyl groups reacted with chloro-(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphine in the presence of DIPEA in THF to yield the enantiomeric monomers **R** and **S** in 52 and 85 percent yields, respectively (Sch. 1). The modified monomers (**R** and **S**) were introduced site-specifically by trityl-off synthesis of the oligonucleotide on the PerSeptive Biosystems Expedite 8909 (DNA synthesizer),[6] followed by the manual coupling of the desired modified phosphoramidite monomer. In a typical synthetic procedure, the modified monomer (0.02 mmol) was dissolved in 200 μL of 0.5 M (1*H*)-tetrazole in acetonitrile. This activated phosphoramidite solution was immediately injected into the synthesis cartridge containing CPG-bound ODN. Coupling times for the modified phosphoramidites were extended to 40 min. Then, the column was rinsed with acetonitrile (2 mL), repeating the coupling process once more to ensure maximum coupling efficiency. The synthesis cartridge was returned to the automated synthesizer for standard oxidation and capping steps and the remainders of the desired oligonucleotides were synthesized.

To determine the modification effect, we designed poly T and poly A sequence. **Oligo 1** and **Oligo 2** were prepared by incorporating **R** and **S** into dT_{11}. **Oligo 3** and

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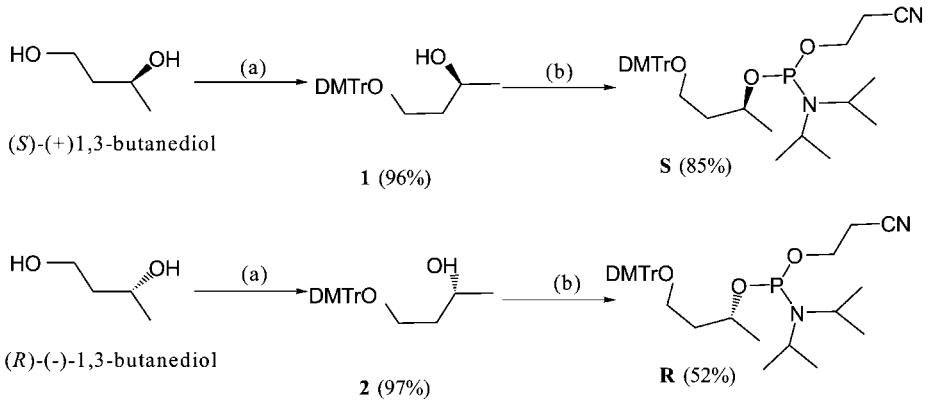

Scheme 1. *Reagents and conditions*: (a) DMTrCl (2eq.), DMAP, Py; (b) Chloro-(2-cyanoethyl)-*N,N*-diisopropylaminophosphine, DIPEA, THF.

Oligo 4 are dA_{11} containing **R** and **S** in the middle of sequence. For the unmodified counterparts, dT_{12} (**Oligo 5**) and dA_{12} (**Oligo 7**) were synthesized. dT_{11} (**Oligo 6**) and dA_{11} (**Oligo 8**) were prepared because they could have the same numbers of hydrogen bonds with each other as compared to modified duplexes. The sequences and Tm values of dodecanucleotides are given in Table 1. Figure 1 shows the typical curves of relative absorbance at 260 nm vs. temperature for Tm value determination.

Compared to unmodified duplex (dA_{12}/dT_{12}), **Oligo 1**, one *R*-(−)-1,3-butanediol incorporated into dT_{11}, decreased the duplex stability by −12°C (entry 1). Another type of duplex with *S*-(+)-1,3-butanediol similarly decreased (−12°C) duplex stability (entry 2). The duplexes between dT_{12} and modified dA_{11} (**Oligo 3** and **Oligo 4**)

Table 1. Sequence and Tm values of dodecanucleotides containing modified monomer.

Entry	Duplex	Sequence	Tm (°C)[a]	ΔTm (°C)
1	**Oligo 1**/**Oligo 7**[b]	5′d-T_6**Rp**T_5/5′d-A_{12}	26	−12
2	**Oligo 2**/**Oligo 7**[b]	5′d-T_6**Sp**T_5/5′d-A_{12}	26	−12
3	**Oligo 3**/**Oligo 5**[c]	5′d-A_5**Rp**A_6/5′d-T_{12}	28	−10
4	**Oligo 4**/**Oligo 5**[c]	5′d-A_5**Sp**A_6/5′d-T_{12}	27	−11
5	**Oligo 1**/**Oligo 3**[c]	5′d-T_6**Rp**T_5/5′d-A_5**Rp**A_6	15	−23
6	**Oligo 1**/**Oligo 4**[c]	5′d-T_6**Rp**T_5/5′d-A_5**Sp**A_6	14	−24
7	**Oligo 2**/**Oligo 3**[c]	5′d-T_6**Sp**T_5/5′d-A_5**Rp**A_6	15	−23
8	**Oligo 2**/**Oligo 4**[c]	5′d-T_6**Sp**T_5/5′d-A_5**Sp**A_6	15	−23
9	**Oligo 5**/**Oligo 7**[b]	5′d-T_{12}/5′d-A_{12}	38	0
10	**Oligo 6**/**Oligo 8**[c]	5′d-T_{11}/5′d-A_{11}	35	−3

[a]Melting temperatures were determined by measuring change in absorbance at 260 nm (cuvette, 1 cm path length) as a function of temperature in Tris-HCl buffer (10 mM, pH 7.2) containing 100 mM NaCl and 20 mM $MgCl_2$. Temperature was raised 1.0°C/min.
[b]Total strands concentration was 4.0 μM.
[c]Total strands concentration was 6.6 μM.

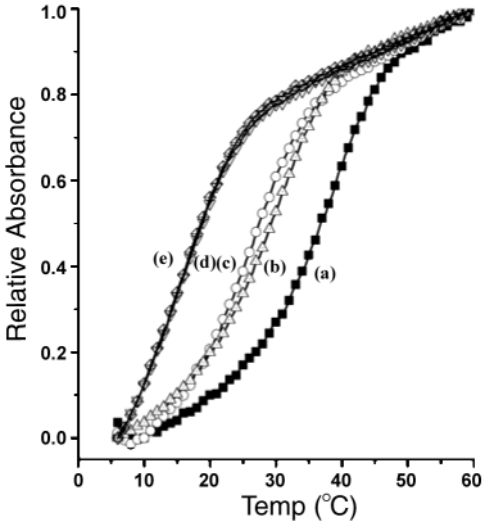

Figure 1. Selected Tm value curves of synthetic ODNs. (a) **Oligo 5**/**Oligo 7** (entry 9), (b) **Oligo 3**/**Oligo 5** (entry 3), (c) **Oligo 1**/**Oligo 7** (entry 1), (d) **Oligo 1**/**Oligo 3** (entry 5), (e) **Oligo 2**/**Oligo 3** (entry 7).

decreased the duplex stability by of $-10°C$ (entry 3) and $-11°C$ (entry 4). On the other hand, the duplexes between the modified dT_{11} (**Oligo 1**, **Oligo 2**) and modified dA_{11} (**Oligo 3**, **Oligo 4**) showed drastic difference in ΔTm (entry 5–8). These negative ΔTm values were caused by the substitution of nucleoside with 1,3-butanediol that reduced the contribution of the base hydrogen bonding of the oligonucleotides and increased the flexibility more than sugar moiety. Thus we could not observe the significant difference in their Tm values (Table 1). The CD spectroscopy has been a useful method for distinguishing the structures of ODNs. The CD pattern of double helical B-form DNA from 5′d-A_{12} and 5′d-T_{12} (entry 9) had characteristic negative maximum at 250 nm and positive maximum at 280 nm. Based on the CD spectra (Fig. 2 and Fig. 3), we could assume that all the synthetic ODNs (entry 1–8) also formed similar double helical structures.

In summary, we report the synthesis of non-nucleoside monomers based on 1,3-butanediol and their specific incorporation into ODNs. In principle, there should be some difference between ODNs of **R** and **S** epimers. However the change in configuration of one chiral center does not bring about any meaningful change in properties of ODNs in this case. This may be attributable to the real insignificance of one chirality difference in duplex formation of the nature of minimal base-free nucleoside isostere, 1,3-butanediol.

EXPERIMENTAL

All solvents were carefully dried and distilled prior to use. ^{1}H NMR and ^{13}C NMR spectra were obtained on FT-300MHz Bruker Apect 3000 spectrometer.

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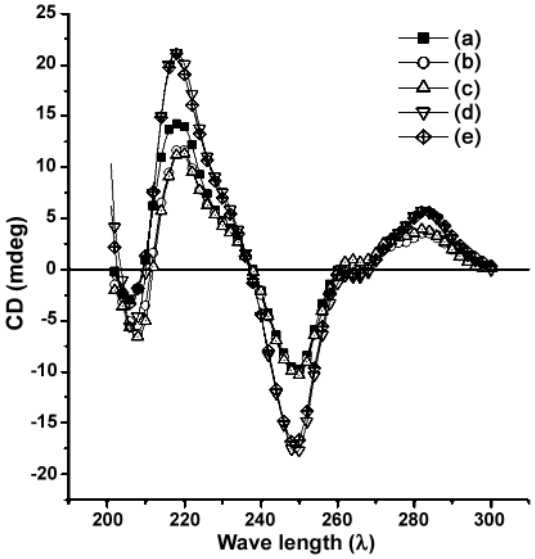

Figure 2. CD spectra of synthetic ODNs. The sample conditions are summarized in Table 1; (a) entry 9, (b) entry 1, (c) entry 2, (d) entry 3, (e) entry 4.

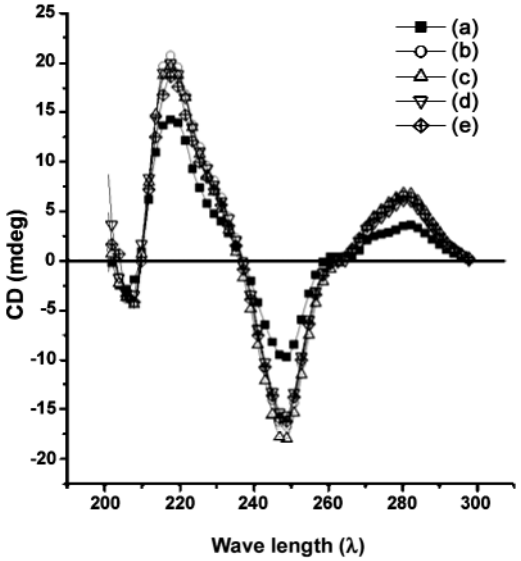

Figure 3. CD spectra of synthetic ODNs. The sample conditions are summarized in Table 1; (a) entry 9, (b) entry 5, (c) entry 6, (d) entry 7, (e) entry 8.

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Mass spectra (FAB) were performed by using Jeol JMS-AX505WA in Korea Basic Science Center, Daejeon, Korea. The IR spectra were obtained on Bruker FT-IR PS55+ and optical rotation was determined by RUDOLPH AUTOPOL III instrument. Tm values were detected by using Shimadzu UV-2501PC UV-VIS spectrophotometer. And the temperature was controlled by Polyscience 9110 programmable Digital temperature controller.

All reactions were performed in oven-dried glassware under a positive pressure of argon. Analytical TLC was performed on pre-coated silica gel plates and visualized with UV light and/or by spraying with *p*-anisaldehyde or phosphomolybdic acid solutions followed by heating with hotplate. Flash column chromatography was performed with silica gel.

S-(+)-1-*O*-(4,4′-Dimethoxytrityl)-1,3-butanediol (1)

A solution of *S*-(+)-1,3-butanediol (96 mg, 1.065 mmol) in pyridine (3 mL) was cooled in an ice-water bath and 4,4′-dimethoxytrityl chloride (430 mg, 1.27 mmol) was added. The resulting reaction mixture was allowed to warm to room temperature and then stirred for 6 hours. 5% $NaHCO_3$ aqueous solution (10 mL) was added and the reaction mixture was extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and evaporated off in vacuo. The residue was purified by silica gel column chromatography (Ethyl acetate:Hexane = 1:3) to give **2** as an oil (401 mg, 1.02 mmol, 96%): $R_f = 0.3$ (Ethyl acetate:Hexane = 1:2); IR (NaCl) ν (cm^{-1}) 3462, 3059, 3034, 2959, 2927, 2848, 2835, 1607, 1508, 1250; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.49 (br, 1H), 7.46 (br, 1H), 7.36–7.18 (m, 7H), 6.86 (t, 2H, $J = 2.6$ Hz), 6.84 (t, 2H, $J = 2.6$ Hz), 3.93 (br, 1H), 3.73 (s, 6H), 3.50 (br, 1H), 3.28–3.14 (m, 2H), 1.73 (m, 2H), 1.11 (d, 3H, $J = 6.2$ Hz); ^{13}C-NMR (75.5 MHz, acetone-d_6) δ 158.1, 145.3, 136.1, 136.0, 129.5, 127.6, 127.2, 126.1, 112.5, 85.4, 64.2, 60.6, 54.2, 39.0, 23.1; MS-FAB (m/z): $[M]^+$ calcd for $C_{25}H_{28}O_4$, 392; found 392.; $[\alpha]^{21}{}_D = +17.6$ (c = 1.0, $CHCl_3$).

R-(–)-1-*O*-(4,4′-Dimethoxytrityl)-1,3-butanediol (2)

4,4′-Dimethoxytrityl chloride (460 mg, 1.27 mmol) was added to a solution of *R*-(−)-1,3-butanediol (103 mg, 1.14 mmol) in pyridine (3 mL) which was cooled in an ice-water bath. The reaction mixture was allowed to warm to room temperature and then stirred for 6 h. 5% $NaHCO_3$ aqueous solution (10 mL) and ethyl acetate was added. The organic layer was separated, dried over $MgSO_4$, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (Ethyl acetate:Hexane = 1:3) provided the product as an oil (437 mg, 1.11 mmol, 97%): $R_f = 0.3$ (Ethyl acetate:Hexane = 1:2); IR (NaCl) ν (cm^{-1}) 3462, 3059, 3034, 2960, 2929, 2835, 1607, 1508, 1250; ^{1}H NMR (300MHz, acetone-d_6) δ 7.47 (t, 1H, $J = 1.7$ Hz), 7.45 (br, 1H), 7.35–7.20 (m, 7H), 6.87 (t, 2H, $J = 2.6$ Hz), 6.84 (t, 2H, J = 2.6 Hz), 3.92 (br, 1H), 3.73 (s, 6H), 3.47(d, 1H, $J = 3.7$ Hz), 3.25–3.14 (m, 2H), 1.71 (m, 2H), 1.09 (d, 3H, $J = 6.2$ Hz); ^{13}C-NMR (75.5 MHz, acetone-d_6) δ 158.1, 145.2, 136.1, 136.0, 129.5, 127.6, 127.2, 126.1, 112.5, 85.4, 64.2, 60.5, 54.1, 38.9,

23.0; MS-FAB (m/z): $[M]^+$ calcd for $C_{25}H_{28}O_4$, 392; found 392.; $[\alpha]^{21}{}_D = -9.9$ ($c = 1.0$, $CHCl_3$).

Compound S

DIPEA (140 μL, 0.804 mmol) was added to a solution of *S*-(−)-1-*O*-(4,4′-dimethoxytrityl)-1,3-butanediol (**1**, 158 mg, 0.402 mmol) in THF (3 mL). The reaction mixture was stirred at room temperature for 30 min, and then charged with chloro-(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphine (177 μL, 0.80 mmol). After 30 min stirring, a white precipitant was filtered off and the solvent was evaporated off in vacuo. The oily reaction mixture was extracted with ethyl acetate and 5% $NaHCO_3$ aqueous solution. The organic layer was dried over $MgSO_4$ and evaporated off in vacuo. The residue was purified by silica gel column chromatography (Ethyl acetate:Hexane = 1:5) to give **S** as a colorless oil (203 mg, 0.34 mmol, 85%): $R_f = 0.45$ (Ethyl acetate:Hexane = 1:5); ^{1}H-NMR (300 MHz, acetone-d_6) δ 7.47–7.43 (2H, m), 7.34–7.25 (5H, m), 7.22–7.16 (1H, m), 6.89–6.80 (4H, m), 4.15 (1H, m), 3.74 (3H, s), 3.73 (3H, s), 3.63–3.51 (3H, m), 3.20–3.16 (2H, m), 2.68 (1H, t, $J = 6.0$ Hz), 2.55 (1H, t, $J = 6.0$ Hz), 1.94–1.73 (3H, m), 1.21–1.11 (12H, m), 1.07 (1.5H, s), 1.05 (1.5H, s); ^{13}C-NMR (75.5 MHz, acetone-d_6) δ 158.1, 145.2, 136.0, 129.6, 129.5, 127.7, 127.6, 127.2, 126.1, 117.7, 117.6, 112.5, 85.4, 68.0, 67.7, 67.4, 67.2, 60.0, 59.8, 59.2, 58.1, 57.8, 57.5, 54.2, 42.4, 42.2, 38.3, 23.7, 23.6, 23.6, 23.5, 23.4, 21.6, 19.5, 19.4; ^{31}P-NMR (121 MHz, acetone-d_6) **δ** 149.0, 148.3; MS-FAB (m/z): $[M + Na]^+$ calcd for $C_{34}H_{45}N_2O_5P_1Na_1$, 615; found 615.

Compound R

In a solution of *R*-(−)-1-*O*-(4,4′-dimethoxytrityl)-1,3-butanediol (**2**, 138 mg, 0.315 mmol) in THF (2 mL), DIPEA (140 μL, 0.804 mmol) was added. The reaction mixture was stirred for 30 min and then chloro-(2-cyanoethyl)-*N*,*N*-diisopropylamino phosphine (157 μL, 0.70 mmol) was added. After 30 min stirring, a white precipitant was filtered off and the solvent was evaporated off in vacuo. The oily reaction mixture was extracted with ethyl acetate and 5% $NaHCO_3$ aqueous solution. The organic layer was dried over $MgSO_4$ and evaporated off in vacuo. The residue was purified by silica gel column chromatography (Ethyl acetate:Hexane = 1:5) to give **R** as a colorless oil (108 mg, 0.182 mmol, 52%): $R_f = 0.45$ (Ethyl acetate:Hexane = 1:5) ^{1}H NMR (300 MHz, acetone-d_6) δ 7.47–7.43 (2H, m), 7.34–7.25 (5H, m), 7.22–7.16 (1H, m), 6.89–6.80 (4H, m), 4.15 (1H, m), 3.76 (3H, s), 3.75 (3H, s), 3.63–3.51 (3H, m), 3.20–3.16 (2H, m), 2.68 (1H, t, $J = 6.0$ Hz), 2.55 (1H, t, $J = 6.0$ Hz), 1.94–1.73 (3H, m), 1.19–1.10 (12H, m), 1.05 (1.5H, s), 1.03 (1.5H, s); ^{13}C-NMR (75.5 MHz, acetone-d_6) δ 158.1, 145.2, 136.0, 129.5, 129.5, 127.6, 127.5, 127.2, 126.1, 117.6, 112.4, 85.3, 67.9, 67.7, 67.4, 67.2, 66.7, 59.9, 59.8, 58.0, 57.8, 57.5, 54.1, 42.4, 42.2, 38.3, 38.2, 24.8, 23.7, 23.6, 23.5, 23.4, 23.3, 21.6, 19.4, 19.3; ^{31}P-NMR (121 MHz, acetone-d_6) δ 149.0, 148.3; MS-FAB (m/z): $[M + Na]^+$ calcd for $C_{34}H_{45}N_2O_5P_1Na_1$, 615; found 615.

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Synthesis of ODNs

The synthesized oligonucleotides were cleaved from the solid support by treatment with 1.0 mL 30% aqueous ammonium hydroxide for 10 h at 55°C. The crude products from the automated oligonucleotide synthesis were lyophilized and diluted with 1 mL of distilled water. The oligonucleotides were purified by HPLC (Merck LichoCART C18 column, 10 × 250 mm, 10 μm, 100 Å pore size). The HPLC mobile phase was isocratically held for 10 min of 5% acetonitrile/0.1 M triethylammonium acetate (TEAA) pH 7.0 at a flow rate of 3 mL/min. The gradient was then linearly increased from 5% acetonitrile/0.1 M TEAA to 50% acetonitrile/0.1 M TEAA for 10 min at the same flow rate. The fractions containing the purified oligomer were pooled and lyophilized. Aqueous 80% acetic acid was added to the oligomer. After 30 min at ambient temperature, the acid was removed under reduced pressure. The residue was diluted with water (1 mL), and the solution was purified by HPLC at the same condition as in the previous one. The oligonucleotides were analyzed by HPLC (Agilent Eclipse XDB-C18 column, 4.6 × 150 mm, 80 Å pore size) with almost same eluent system but with different flow rate (1 mL/min).

Sequence of Synthetic ODNs and Maldi-Tof Mass Spectrometric Data

Oligo 1: (5′d-$T_6\mathbf{R_p}T_5$) calcd 3425.3; found 3433.3. **Oligo 2**: (5′d-$T_6\mathbf{S_p}T_5$) calcd 3425.3; found 3462.3. **Oligo 3**: (5′d-$A_5\mathbf{R_p}A_6$) calcd 3528.7; found 3534.5. **Oligo 4**: (5′d- $A_5\mathbf{S_p}A_6$) calcd 3528.7; found 3538.6. **Oligo 5**: (5′d-T_{12}) calcd 3577.4; found 3594.0. **Oligo 6**: (5′d-T_{11}) calcd 3274.2; found 3277.6. **Oligo 7**: (5′d-A_{12}) calcd 3690.2; found 3701.0. **Oligo 8**: (5′d-A_{11}) calcd 3377.6; found 3384.1.

Maldi-Tof: PE Biosystems Voyager System 4095; Accelerating voltage: 25000 V; Matrix: 3-Hydroxypicolinic acid + ammonium citrate; Polarity: Positive.

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