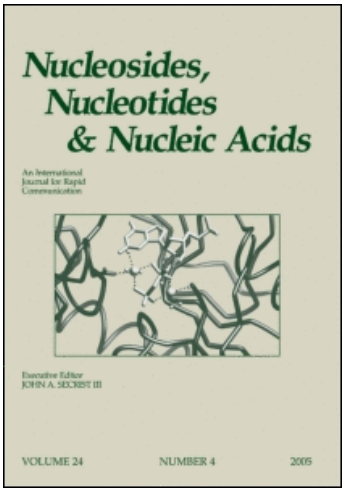

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Diastereomer Separation of Azobenzene-Tethered Oligodeoxyribonucleotides and Determination of Their Absolute Configurations by Enzymatic Digestion

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DIASTEREOMER SEPARATION OF AZOBENZENE-TETHERED OLIGODEOXYRIBONUCLEOTIDES AND DETERMINATION OF THEIR ABSOLUTE CONFIGURATIONS BY ENZYMATIC DIGESTION

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□ *Two diastereomers were produced by the introduction of azobenzene-tethering prochiral linker (2,2-bis(hydroxymethyl)propionic acid) in the modified ODN, which had been used for the photoregulation of DNA functions. We found that this modified ODN with sequence 5′-. . .pNpXpN. . .-3′ (p = phosphate; N = nucleoside; X = azobenzene residue) could be digested to pX (the phosphate at the 5′ side of X was left) by an over excess of Phosphodiesterase I. By comparing the retention time of pX from the separated diastereomer with that of authentic R- or S-pX on chiral HPLC, absolute configuration could be easily determined.*

Keywords Diastereomer separation; modified oligodeoxynucleotide; absolute configuration; enzymatic digestion; Phosphodiesterase

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Supporting Information Available. Supplemental Figure 1 (Molecular structures of the modified polysaccharide used for coating silica-gel in chiral columns), Supplemental Figure 2 (HPLC patterns of the products obtained from the digestion of β−**AXT9nt** by Phosphodiesterase I), Supplemental Figure 3 (HPLC patterns of pX obtained from the digestion of **AXG7nt** and **CXA9nt** by Phosphodiesterase I), Supplemental Figure 4 (HPLC patterns on CHIRALCEL AD-RH column of the diastereomers of 5′-TpX-3′ obtained from the digestion of $\mathbf{T_6XT}$ by Phosphodiesterase I), Supplemental Figure 5 (HPLC patterns of the products obtained from the digestion of α-$\mathbf{TXT_6}$ and $\mathbf{T_7X}$ by Phosphodiesterase I), and Supplemental Scheme (Synthetic route of pX) are available.

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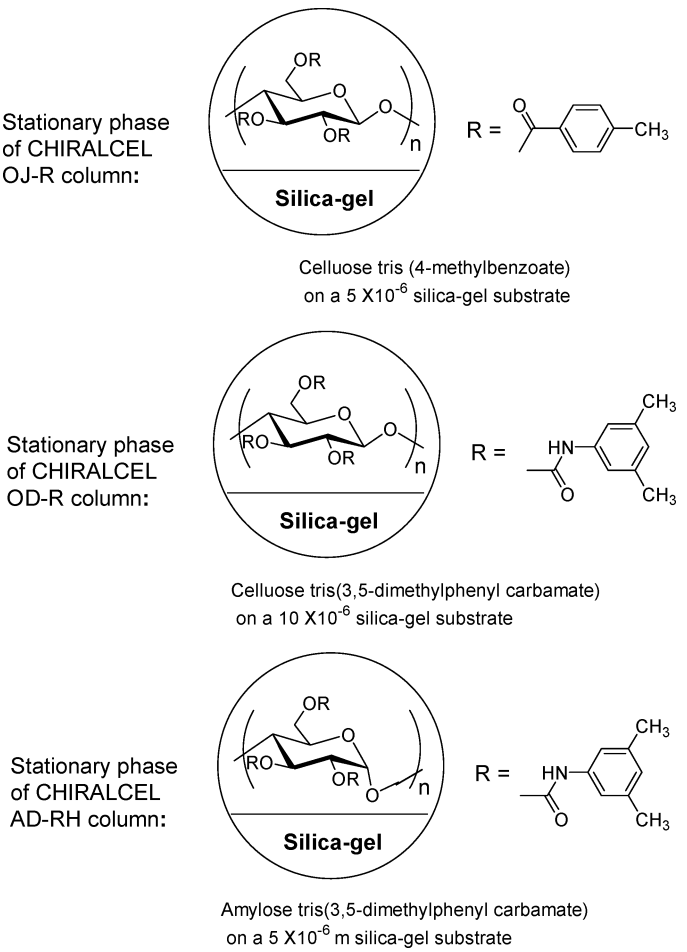
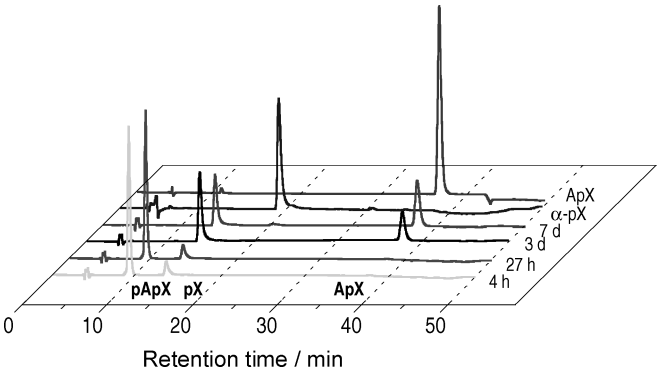
INTRODUCTION

With the development of phosphoramidite chemistry, various organic molecules have been incorporated into oligodeoxyribonucleotides (ODNs) to provide specific functions. For this purpose, functional molecules have been commonly introduced into either 2′-O-position of nucleoside or 5-position of uracil or cytosine to minimize the destabilization of duplex.[1–4] Besides such modifications, incorporation of a functional molecule to ODN via corresponding phosphoramidite monomer as a "cartridge" is another promising way of DNA modification.[5–8] In this case, a functional molecule is tethered on a diol derivative, which is thereafter converted to phosphoramidite monomer. Use of such a "cartridge" allows the introduction of functional molecules in ODN sequences at any chosen position on a DNA

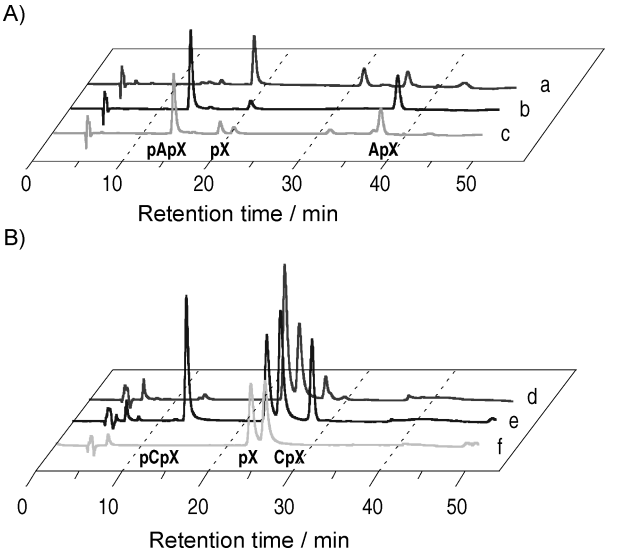


SUPPLEMENTAL FIGURE 1 Molecular structures of the modified polysaccharide used for coating silica-gel in chiral columns.

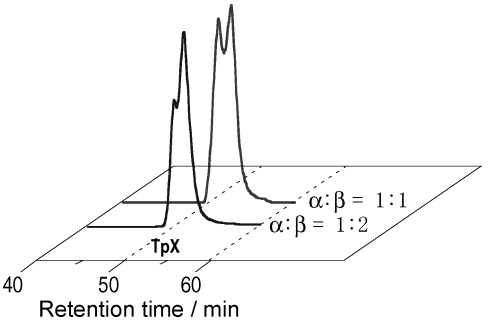


SUPPLEMENTAL FIGURE 2 HPLC patterns on a chiral CHIRALCEL OJ-R column of the products obtained from the digestion of β-**AXT9nt** by 0.8 U/mL of Phosphodiesterase I. A linear gradient of 15–18.5% acetonitrile/water was used in the first 30 minutes. The reaction time is shown in the figure. The standard sample of α-Xp and ApX are also shown here.

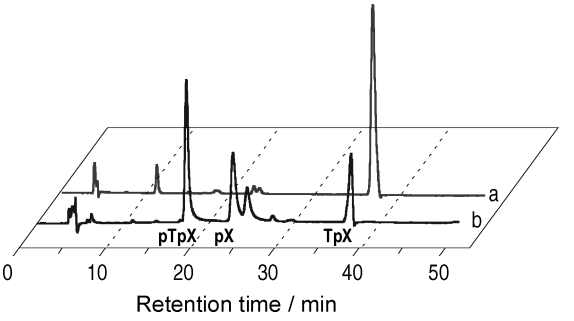
synthesizer. Although introduction of such a "cartridge" elongates the main chain of ODN and leads distortion of the duplex, destabilization is fairly offset by the stacking interaction of the intercalated functional molecule.



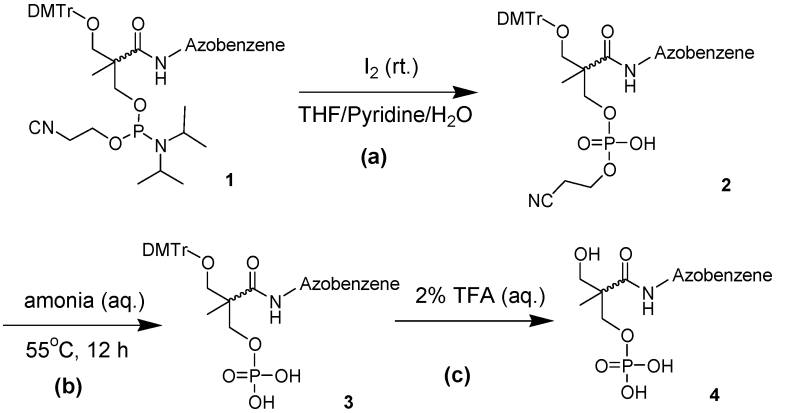
SUPPLEMENTAL FIGURE 3 HPLC patterns of pX on a CHIRALCEL OJ-R column obtained from the digestion of (A) **AXG7nt** (5′-CpGpApXpGpTpCp-3′) and (B) **CXA9nt** (5′-GpGpCpXpApCpCpTpC-3′) by 10 U/mL of Phosphodiesterase I for 8 hours. In the first 30 minutes, a linear gradient of 15–18.5% acetonitrile/water was used in (A); and a linear gradient of 14.5–20% acetonitrile/water was used in (B). (a) Digestion products of α-**AXG7nt**, (b) digestion products of β-**AXG7nt**, (c) mixture of both diastereomers (α:β = 1:3) after digestion; (d) mixture of digestion products from α-**CXA9nt** with synthesized racemate of pX, (e) mixture of digestion products from β-**CXA9nt** with synthesized racemate of pX, (f) racemate of pX (the retention time is 23.1 and 24.7 minutes, respectively).



SUPPLEMENTAL FIGURE 4 HPLC patterns on a chiral column CHIRALCEL AD-RH of the diastereomers of TpX obtained from the digestion of **T_6XT** by Phosphodiesterase I. The mixtures of the obtained two diastereomers with various ratios were injected for analysis. A constant mobile phase composition of 10.4% AN aqueous solution containing 20 mM NaH_2PO_4 (pH3.0) was used. It can be confirmed that *α*-diastereomer of **T_6XT** has *S*-configuration, whereas *β*-diastereomer has *R*-configuration.

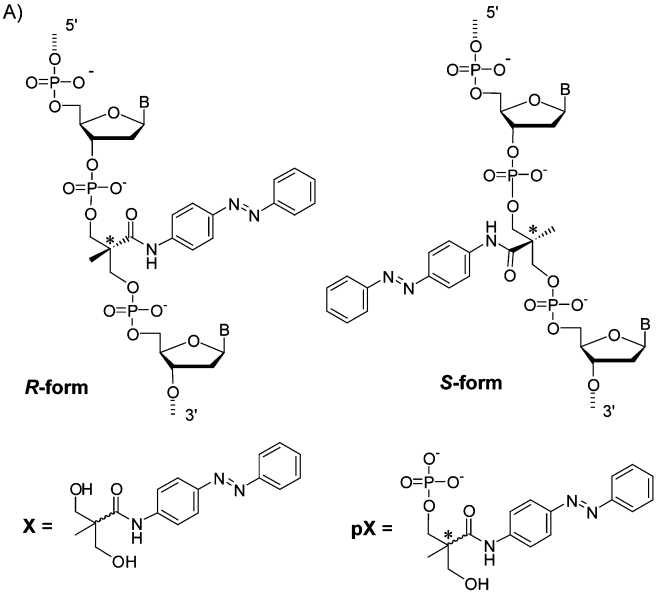


SUPPLEMENTAL FIGURE 5 HPLC patterns of the products on an CHIRALCEL OJ-R column obtained from the digestion of (a) *α*-**TXT_6** and (b) **T_7X** by 10 U/mL of Phosphodiesterase I overnight. A linear gradient of 14.5–20% acetonitrile/water was used in the first 30 minutes. No pX but only TpX was obtained from the digestion of **TXT_6**. Digestion of **T_7X** (mixture of the two diastereomers) showed again that *S*-configuration is more difficult to be digested by Phosphodiesterase I than *R*-configuration.



SUPPLEMENTAL SCHEME Synthetic route of pX (**4**). (a) The phosphoramidite monomer **1** was oxidized by 0.02 M I_2 in Tetrahydrofuran/Pyridine/H_2O (88:10:2) solution; and (b) 2-cyanoethyl was removed by 25% NH_4OH aqueous solution at 55°C for 12 hours; then (c) DMTr was removed by 2% Trifluroacetic acid aqueous solution.

So far, we have incorporated various azobenzenes into ODN for the photoregulation of DNA functions based on its trans-cis photoisomerization.[9–11] In our cases, an azobenzene was introduced to ODN on an appropriate diol linker through a corresponding phosphoramidite monomer (as a "cartridge"), and the formation and dissociation of DNA duplex and triplex, as well as the reactions of DNA and RNA polymerase, have been successfully photocontrolled.[12–17] In order to introduce 4-aminoazobenzene, we used prochiral 2,2-bis(hydroxymethyl)propionic acid as a linker. After the azobenzene-tethering linker was introduced into DNA, two diastereomers due to the chirality of C^2–carbon atom of the linker coexisted for every modified ODN (Scheme 1A). The two diastereomers,



B)

Name	Sequence
AXA9nt	GpGp**A**p**X**p**A**pCpTpTpC
AXC9nt	GpGp**A**p**X**p**C**pCpTpTpC
AXG9nt	GpGp**A**p**X**p**G**pCpTpTpC
AXT9nt	GpGp**A**p**X**p**T**pCpTpTpC
CXA9nt	GpGp**C**p**X**p**A**pCpTpTpC
CXC9nt	GpGp**C**p**X**p**C**pCpTpTpC
CXG9nt	GpGp**C**p**X**p**G**pCpTpTpC
CXT9nt	GpGp**C**p**X**p**T**pCpTpTpC
GXA9nt	GpGp**G**p**X**p**A**pCpTpTpC
GXC9nt	GpGp**G**p**X**p**C**pCpTpTpC
GXG9nt	GpGp**G**p**X**p**G**pCpTpTpC
GXT9nt	GpGp**G**p**X**p**T**pCpTpTpC
TXA9nt	GpGp**T**p**X**p**A**pCpTpTpC
TXC9nt	GpGp**T**p**X**p**C**pCpTpTpC
TXG9nt	GpGp**T**p**X**p**G**pCpTpTpC
TXG9nt	GpGp**T**p**X**p**G**pCpTpTpC

Name	Sequence
AXG7nt	CpGpAp**X**pGpTpC
AXA3nt	Ap**X**Ap
$\mathbf{A_mXA_n}$	$(Ap)_m\mathbf{X}(pA)_n$ (m + n = 7)
$\mathbf{T_mXT_n}$	$(Tp)_m\mathbf{X}(pT)_n$ (m + n = 7, or m + n =13)

SCHEME 1 Structures of the two diastereomers of modified ODNs (A) and sequences used in this study (B). All the sequences are shown from 5′ to 3′.

which could be separated by conventional reversed-phase HPLC with ODS column, showed very different photoregulation ability: basically, the isomer with shorter retention time was more efficient than the longer one.[12–14] For further understanding of this difference and the photoregulation mechanism, the absolute configuration of the diastereomers has to be determined. By the structural analysis of the duplex 5′-CpGpApXpGpTpC-3′/5′-GpApCpTpCpG-3′ (X = azobenzene residue) on the basis of 2D-NMR, the absolute configuration of 5′-CpGpApXpGpTpC-3′ was determined, disclosing that the more efficient one has R-configuration.[18] We also described that R-configuration of azobenzene-tethered ODN preferred to form right-hand helix as compared with S-configuration.[18] However, the absolute configurations of other modified ODNs with different sequence still remained unknown. It is highly time consuming and complicated to determine the configurations of all the modified ODNs by NMR.

Here we report an enzyme-chromatographic method for determining the absolute configuration of these diastereomers. We found that a natural enzyme could digest the azobenzene-tethered ODN under certain conditions. After the modified ODN was digested to pX (phosphomonoester of X residue) by Phosphodiesterase I, the absolute configuration was analyzed by comparing its retention time with that of the configuration-known one (determined by NMR) on chiral HPLC. It is promising to be used as a facile and general method for determining the absolute configuration of modified ODNs attaching an intercalator.

MATERIALS AND METHODS

Materials

Azobenzene-tethered ODNs were synthesized using typical phosphoramidite chemistry as described in our previous report.[9] All the conventional phosphoramidite monomers, CPG columns, other regents for DNA synthesis, and Poly-Pak cartridges were purchased from Glen Research Co. (Sterling, VA, USA). The synthesized modified ODNs were purified by reversed-phase HPLC (ODS column: Merck LiChrospher 100 RP-18(e)) and characterized by negative mode of MALDI-TOFMS (Shimadzu, Japan). Sequences of synthesized modified ODNs were shown in Scheme 1B. Phosphodiesterase I (Snake Venom Phosphodiesterase) and Alkali Phosphatase (AP) were purchased from Boehringer Mannheim (Germany). Nuclease S1 (Aspergillus orzyae), Mung Bean nuclease, and Micrococcal Nuclease were purchased from Life Technologies (Carlsbad, CA), Promega (Tokyo), and Takara (Tokyo), respectively. Chiral column (5 μm, 150 × 4.6 mm i.d.) CHIRALCEL OJ-R, CHIRALCEL OD-R, and CHIRALCEL AD-RH, were purchased from Daicel Chemical Industries (Tokyo). In these columns,

polysaccharide involving various residues is attached to the silica-gel substrate containing 18C residues (see Supplemental Figure 1 for detailed structures).[19]

Monophosphate of 2,2-bis(hydroxymethyl)-propion-(*p*-aminoazobenzene) (pX, see Scheme 1A) was synthesized from the corresponding phosphoramidite monomer via a conventional method (Supplemental Scheme).[9] Racemic mixture of the synthesized pX was purified by reversed-phase HPLC (Merck LiChrospher 100 RP-18(e) column) and characterized by ESI-MS and ^{1}H NMR. ESI-MS: pX Obsd. 392.0 (Calcd. for [pX—H^+]: 392.1). ^{1}H NMR for pX [270 MHz, D_2O]: $\delta = 7.8$–7.4 (m, 9H, aromatic protons of azobenzene), 3.92 and 3.91 (s, 2H, -CH_2OP), 3.74 (s, 2H, -CH_2OH), 1.17 (s, 3H, -CH_3).

Separation of Diastereomers of Modified ODNs. Two diastereomers of the synthesized ODN involving X residue were separated by reversed phase HPLC (Merck LiChrospher 100 RP-18(e) column) with acetonitrile/water containing 50 mM ammonium formate (pH7.0) as mobile phase. In most cases, diastereomers were efficiently separated by the linear gradient 7.5–17.5% acetonitrile/water (40 minutes, 0.5 mL/min). For the modified ODNs whose diastereomers were difficult to be separated, a much slower gradient (0.1% increase of acetonitrile per minute) was used.[20]

Separation of Enantiomers of pX. Enantiomers of pX were separated on a chiral column CHIRALCEL OJ-R with acetonitrile/water containing 50 mM ammonium formate (pH7.0) as mobile phase. The following gradient program was used: first a linear gradient 15–18.5% acetonitrile/water in 30 minutes, then another linear gradient 18.5–50% acetonitrile/water in 10 minutes, at last washing with 50% acetonitrile/water for 10 minutes. In some cases, a linear gradient 14.5–20% acetonitrile/water in the first 30 minutes was used. The diastereomers of 5′-TpX-3′, which was very difficult to be separated with ODS column, was separated on chiral column CHIRALCEL AD-RH with a constant composition containing 14.5% acetonitrile/water containing 20 mM NaH_2PO_4 (pH3.0).

Digestion of Modified ODN. Modified ODNs were digested by Phosphodiesterase I at 37°C under the following conditions: 10 μM DNA, 100 mM NaCl, 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.4). Three concentrations (0.8 U/mL, 0.25 U/mL, and 10 U/mL) of phosphodiesterase I (one unit hydrolyzes 1.0 μmol *p*-nitrophenyl thymidine-5-phosphate per minutes at 25°C) were used. The samples were taken and analyzed by HPLC at various time intervals. In some cases, Alkali phosphatase (AP) was added to the above reaction solution for further digestion to remove the phosphate under the following conditions: 10 mM NaCl, 10 mM Na_2HPO_4 (pH7.0), at 37°C for 1.0 hour, 1.0 U/mL AP. 10 μL sample was directly injected for HPLC analysis, and the retention time was monitored at 350 nm, which is the λ_{max} of azobenzene.

RESULTS

Strategy for the Determination of Absolute Configuration of Modified ODNs

Our strategy for absolute configuration determination is to digest the azobenzene-tethered ODN whose absolute configuration is unknown to pX and compare it with the configuration-known one on chiral HPLC. Concretely, the approach includes three steps:

1. diastereomers of the modified ODN involving X residue are separated by reversed-phase HPLC with conventional ODS column;
2. each diastereomer is digested by an enzyme to optically pure pX (R-pX or S-pX);
3. the absolute configuration of the azobenzene-tethered ODN is assigned by comparing the retention time of the optically pure pX obtained in step (2) with the standard samples of R-pX and S-pX.

The absolute configurations of the two diastereomers of **AXG7nt** (see Scheme 1B), a 7-nucleotide-long azobenzene-tethered ODN with the sequence of 5′-CpGpApXpGpTpC-3′, have been determined previously.[18] Thus, standard R-pX and S-pX could be obtained by digesting these configuration-known modified ODNs to pX.

From above strategy, we can see that two essential problems have to be solved. One is the digestion of azobenzene-tethered ODN to pX. The other is R-pX and S-pX should be separated on HPLC, that is, enough difference in retention time has to be obtained for distinguishing the two enantiomers of pX.

Separation of the Diastereomers of Modified ODNs

Sequences of the azobenzene-tethered ODNs used in this study are shown in Scheme 1B. Each of the modified ODNs involving 2,2-bis(hydroxymethyl)-propion-(*p*-aminoazobenzene) is a mixture of two diastereomers. Except for 5′-X$(pA)_7$_3′, 5′-X$(pT)_7$_3′, and 5′-$(Tp)_7$X-3′, in which the azobenzene residue was introduced at the terminus of ODN, the diastereomers of all the other sequences listed in Scheme 1B were successfully separated by the conventional reversed phase HPLC equipped with an ODS column. The HPLC pattern of **AXG7nt** (5′-CpGpApXpGpTpC-3′) is shown in Figure 1. The four peaks were assigned as previously described.[9] The diastereomer with shorter retention time is designated as α, the longer one as β. The two major peaks (α-trans and β-trans) present the two diastereomers of modified ODN in which azobenzene takes trans-form; and the two minor ones are the corresponding cis-form (α-cis and

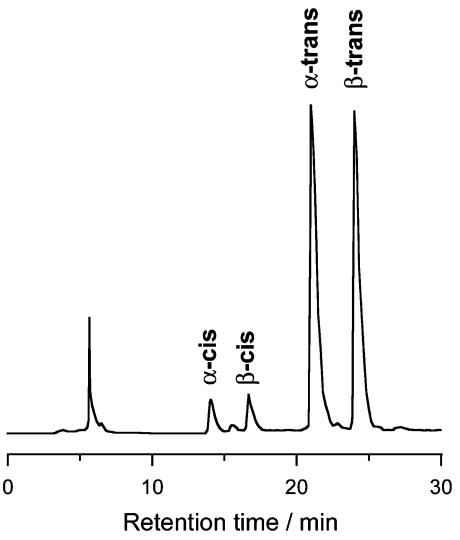


FIGURE 1 Separation of the diastereomers of azobenzene-tethered ODN **AXG7nt** (5′-CpGpA pXpGpTpC-3′) on conventional reversed-phase HPLC. A linear gradient of 12.5–16% acetonitrile/water in 40 minutes was used. The fraction with shorter retention time is designated as α diastereomer whereas that with longer retention time as β one. The retention time of α-cis, β-cis, α-trans, and β-trans are 14.4, 17.2, 21.6, and 24.6 minutes, respectively.

β-cis). The difference in retention time between α and β diastereomers (ΔRt) changes greatly with the bases adjacent to azobenzene moiety (Table 1). In principle, ΔRt is much larger (easier to be separated) when purine is adjacent to X in comparison with the case of pyrimidine. For example, ΔRt for trans-**GXG9nt** is 4.5 minutes, while it is only 0.4 mimute for trans-**CXT9nt**. More interestingly, ΔRt for the trans-form changes regularly in the order of G>A>T$\geq$C (the base at 5′-side of X) when the base at 3′ side of X is fixed (Table 1). The diastereomers of modified DNA could also be completely separated even when azobenzene was introduced into an ODN as long as 26 mer or as short as 2 mer.[20]

Separation of the Enantiomers of pX

The synthesized racemic mixture of pX was used as the sample for enantiomer separation (see Supplemental Scheme for details of synthesis). As expected, the conventional ODS column could not separate the enantiomers. For the three chiral columns (CHIRALCEL OJ-R, OD-R, and AD-RH) we tried, only CHIRALCEL OJ-R column, in which the cellulose tris(4-methylbenzoate) is coated on a 5 μm silica-gel substrate, could separate the two enantiomers of pX completely. When a slow linear gradient, 15% to 18.5% acetonitrile in water (50 mM ammonium formate, pH7.0) in 30 minutes, was used, two sharp peaks of the enantiomers appeared at 19.3 and 21.0 minutes, respectively (Figure 2). The difference in retention time

TABLE 1 Retention time of the diastereomers of azobenzene-tethered ODNs on a conventional ODS column[a]

Modified ODN[b)]	Retention time of *cis*-form/min			Retention time of trans-form/min		
	α-form	β-form	ΔRt (Rt_β—Rt_α)	α-form	β-form	ΔRt (Rt_β—Rt_α)
GXG9nt	14.5	17.3	2.9	17.9	22.4	4.5
GXA9nt	16.9	18.5	1.6	21.2	23.9	2.7
GXC9nt	16.3	18.4	2.1	21.3	24.4	3.1
GXT9nt	17.7	19.4	1.7	22.2	24.7	2.5
AXG9nt	16.7	18.5	1.8	20.5	23.4	2.8
AXA9nt	18.4	20.0	1.6	22.9	25.2	2.3
AXC9nt	18.0	19.3	1.3	22.9	25.0	2.1
AXT9nt	19.9	21.3	1.4	24.5	26.6	2.1
TXG9nt	18.9	19.9	1.0	23.3	25.1	1.8
TXA9nt	20.5	21.2	0.7	25.5	26.8	1.3
TXT9nt	21.3	22.0	0.7	27.0	27.7	0.7
TXC9nt	20.1	20.7	0.6	25.7	26.6	0.9
CXG9nt	16.1	17.1	1.0	20.3	21.9	1.6
CXA9nt	16.9	17.8	0.9	22.0	22.9	0.9
CXC9nt	16.9	17.6	0.7	22.4	23.2	0.8
CXT9nt	17.8	18.6	0.8	23.4	23.8	0.4

[a]HPLC conditions: Merck LiChrospher 100 RP-18(e) column; linear gradient of 7.5–17.5% acetonitrile/water (50 mM ammonium formate (pH7.0)) in 40 minutes; 0.5 mL/min.
[b] See Scheme 1 for the sequences.

between R-pX and S-pX is as large as 1.7 minutes. Thus, R-pX and S-pX can be distinctly distinguished and separated by chiral HPLC analysis, which is sufficient to be used for the determination of the absolute configurations of azobenzene-tethered ODNs for our purpose.

Digestion of Modified ODNs to pX by Phosphodiesterase I

For configuration determination, azobenzene-tethered ODNs have to be digested to pX with the phosphate on a certain side of diol being left. At first, we tried several nucleases such as nuclease S1, nuclease P1, and Mung bean nuclease that decompose single-stranded ODN specifically. They are known to digest ODN to give 5′-nucleotides (5′-pN-3′) or short

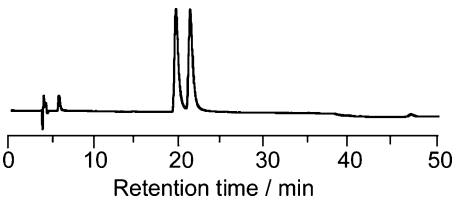


FIGURE 2 Separation of the two enantiomers from racemic mixture of pX on a chiral column CHIRALCEL OJ-R. A linear gradient of 15–18.5% acetonitrile/water in 30 minutes was used.

oligonucleotides (such as 5′-pNpN-3′). But digestion of our modified ODNs gave only 5′-pNpXpN-3′ or 5′-pNpX-3′ (data not shown). We also tried Micrococcal nuclease, which digests DNA to produce 3′-nucleotides (5′-Np-3′), but 5′-XpNp-3′ (not Xp) was obtained in this case (data not shown). With these enzymes, we could not obtain either pX or Xp. But fortunately, we found Phosphodiesterase I, an exonuclease that usually removes 5'-nucleotides successively from free 3′-hydroxyl terminus under normal conditions, could digest them to pX under specific conditions.[21,22]

Digestion of α diastereomer of AXT9nt. The modified ODN **AXT9nt** (5′-GpGpApXpTpCpCpTpC-3′) was first used as the substrate for analyzing the digestion process by Phosphodiesterase I. Aliquots of the products were taken after a certain time and analyzed by Chiral-HPLC. As shown in Figure 3A, α diastereomer of **AXT9nt** (Rt = 3.7 minutes) was completely digested to 5′-GpGpApX-3′ (Rt = 8.3 minutes) within 5 minutes in the presence of 0.8 U/mL of Phosphodiesterase I. Note that the intrinsic activity of Phosphodiesterase I is to remove 5'-nucleotide one by one from the free 3′-hydroxyl terminus. As the reaction proceeded, 5′-GpGpApX-3′ decreased and 5′-pApX-3′ (Rt = 12.3 minutes) increased gradually. After 4 hours of reaction, about 82% of 5′-GpGpApX-3′ was digested to 5′-pApX-3′. Further 23 hours of digestion gave pX (Rt = 18.5 minutes) as well as 5′-ApX-3′

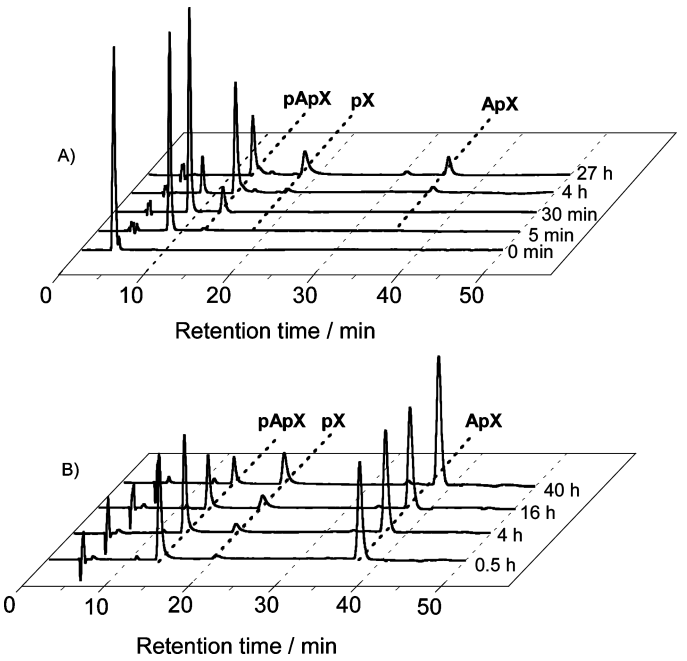


FIGURE 3 HPLC patterns of the products obtained from the digestion of (A) α and (B) β diastereomers of **AXT9nt** by Phosphodiesterase I on a chiral column CHIRALCEL OJ-R. For the digestion of α diastereomer, 0.8 U/mL of the enzyme was added, whereas 10 U/mL was added for β diastereomer. A linear gradient of 15–18.5% acetonitrile/water in the first 30 minutes was used.

(Rt = 35.7 minutes).[23] Assignment of pX was confirmed by the comparison of its retention time with that of the synthesized pX. Since pX could not be obtained from 5′-ApX-3′,[24] we inferred that pX was derived from 5′-pApX-3′, indicating that the 5′-terminus phosphate group is essential.

Digestion of β diastereomer of AXT9nt. The other diastereomer (β) of **AXT9nt** could also be digested to pX, although it was much more difficult than α diastereomer. For example, under the same conditions (0.8 U/mL Phosphodiesterase I, 27 hours), pX could not be obtained. As the reaction time became longer, 5′-pApX-3′ was also digested gradually to 5′-ApX-3′. After digestion of 7 days, only a trivial amount of pX was obtained (supplemental Figure 2). Thus, the β-pApX preferred to be digested to β-ApX rather than to pX. In order to digest β-**AXT9nt** more efficiently, concentration of Phosphodiesterase I was increased to 10 U/mL. As a result, a clear peak at around 20 minutes assignable to pX appeared after 4 hours of digestion, and increased gradually as shown in Figure 3B. Although most of 5′-pApX-3′ was digested to 5′-ApX-3′ after 40 hours, sufficient amount of pX was obtained (Figure 3B).

As described above, β diastereomer of azobenzene-tethered ODN was also digested to pX via 5′-pApX-3′. But it seems much more difficult to be recognized by Phosphodiesterase I. It is interesting that the chirality of X residue influences this enzyme digestion quite differently.

For both diastereomers, this enzymatic digestion could not be applied to the modified ODN in which X residue is attached at the second position from 5′-terminus (e.g., $\mathbf{TXT_6}$ and $\mathbf{AXA_6}$). In these cases, only 5′-NpX-3′ from which pX could not be derived was detected (data not shown). But as long as the X residue is located far from 5′-terminus, optically pure pX could be obtained by the digestion with Phosphodiesterase I.

Determination of the Absolute Configuration by Comparison of the Obtained pX with the Standard One on Chiral HPLC

In our previous study, the absolute configurations of the two diastereomers of 5′-CpGpApXpGpTpC-3′ (**AXG7nt**) were determined by NMR structural analysis.[18] The isomer with shorter retention time on a conventional ODS column (α diastereomer) is determined as R-configuration, and the other isomer (β diastereomer) as S-configuration with respect to the central carbon of the linker. Here, standard samples of optically pure pX (R-pX and S-pX) were prepared by digesting **AXG7nt** with Phosphodiesterase I (see supplemental Figure 3A). According to HPLC analysis using CHIRALCEL OJ-R column, retention time of R-pX from R-form of **AXG7nt** (α diastereomer) and S-pX from S-form of **AXG7nt** (β diastereomer) was determined as 19.3 and 20.9 minutes, respectively (Table 2). We can conclude that R-pX eluted faster than S-pX when CHIRALCEL OJ-R column was used as the stationary phase. Thus, absolute configuration

TABLE 2 Retention time on a chiral CHIRALCEL OJ-R column of pX obtained from the digestion of diastereomers of modified ODNs with various sequences by Phosphodiesterase I

Modified ODN	Retention time/min		ΔRt/min
	α Diastereomer	β Diastereomer</tsh>	Rt_α—Rt_β
AXG7nt[a]	19.3 (*R*)	20.9 (*S*)	1.6
AXA9nt[a]	19.4 (*R*)	20.9 (*S*)	1.5
AXC9nt[a]	18.9 (*R*)	21.0 (*S*)	2.1
AXG9nt[a]	19.2 (*R*)	20.9 (S)	1.7
AXT9nt[a]	19.3 (*R*)	20.9 (*S*)	1.6
GXA9nt[a]	19.7 (*R*)	21.1 (*S*)	1.4
GXC9nt[a]	19.6 (*R*)	21.3 (*S*)	1.7
GXG9nt[a]	19.6 (*R*)	20.6 (*S*)	1.0
GXT9nt[a]	19.6 (*R*)	21.4 (*S*)	1.8
CXA9nt[b]	23.2 (*R*)	24.6 (*S*)	1.4
CXC9nt[b]	22.9 (*R*)	24.5 (*S*)	1.6
CXG9nt[b]	22.9 (*R*)	24.6 (*S*)	1.7
CXT9nt[b]	23.1 (*R*)	24.6 (*S*)	1.5
TXA9nt[b]	23.2 (*R*)	24.6 (*S*)	1.4
TXC9nt[b]	22.8 (*R*)	24.4 (*S*)	1.6
TXG9nt[b]	23.1 (*R*)	24.5 (*S*)	1.4
TXT9nt[b]	22.8 (*R*)	24.7 (*S*)	1.9
$\mathbf{T_2XT_5}$[b]	23.1 (*R*)	24.9 (*S*)	1.8
$\mathbf{T_3XT_4}$[b]	22.3 (*R*)	24.1 (*S*)	1.8
$\mathbf{T_4XT_3}$[b]	22.4 (*R*)	24.0 (*S*)	1.6
$\mathbf{T_5XT_2}$[b]	22.4 (*R*)	24.7 (*S*)	2.3
$\mathbf{T_6XT}$[b]	**25.0 (*S*)**	**23.1 (*R*)**	**−1.9**[c]
$\mathbf{A_2XA_5}$[b]	23.1 (*R*)	24.7 (*S*)	1.6
$\mathbf{A_3XA_4}$[b]	22.4 (*R*)	24.1 (*S*)	1.7
$\mathbf{A_6XA}$[b]	24.4 (*R*)	26.0 (*S*)	1.6

[a]The gradient program: 15–18.5% acetonitrile/water in 30 minutes; then 18.5–50% acetonitrile/water in 10 minutes; at last washing with 50% acetonitrile/water for 10 minutes.

[b]The gradient program: 14.5–20% acetonitrile/water in 30 minutes; then 20–50% acetonitrile/water in 10 minutes; at last washing with 50% acetonitrile/water for 20 minutes.

[c]Only in the case of **T_6XT**, the pX obtained from α-diastereomer has longer retention than that from β-diastereomer.

of all the diastereomers listed in Table 1 could be assigned by analyzing the obtained pX from each diastereomer on CHIRALCEL OJ-R column (see Supplemental Figure 3B). If the pX has a shorter retention time, the original ODN before digestion should be R-form; if the retention time is longer, it should be S-form. We found that all the α diastereomers listed in Table 1 (with shorter retention time on ODS column) were assigned as R-form and β diastereomers (with longer retention time) as S-form (Table 2).

Using the above approach, we also determined the absolute configuration of a series of modified ODNs such as $\mathbf{A_mXA_n}$ and $\mathbf{T_mXT_n}$ (Scheme 1), in which position of X residue is varied. Interestingly, α diastereomer of **T_6XT** (with shorter retention time on ODS column) was assigned as S-form and β-**T_6XT** as R-form as shown by the HPLC chart in Figure 4. This also can

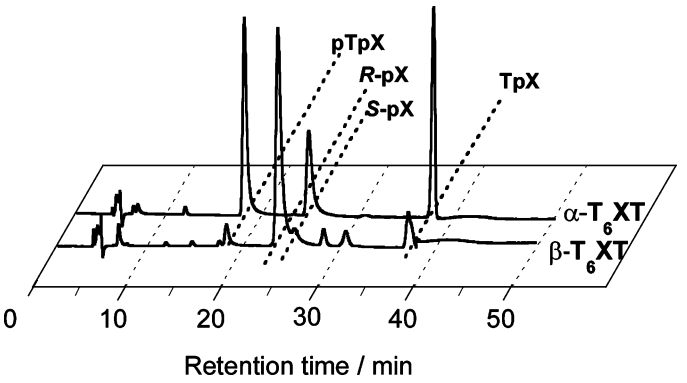


FIGURE 4 HPLC patterns of the products on a chiral CHIRALCEL OJ-R column obtained from the digestion of α-**T$_6$XT** and β-**T$_6$XT** by 10 U/mL of Phosphodiesterase I. A linear gradient of 14.5–20% acetonitrile/water in the first 30 minutes was used.

be confirmed by the fact that α-**T$_6$XT** is much more difficult to be digested by Phosphodiesterase I than β-**T$_6$XT** (compare the peak heights of pX and pTpX of β-**T$_6$XT** with those of α-**T$_6$XT** in Figure 4). Thus, importance of this method is evidenced: not all α diastereomers have the same configuration. All other α diastereomers we used (even **A$_6$XA**) were assigned as R-form (Table 2).

Determination of the Absolute Configuration of TXT$_6$, TXT$_{12}$, and AXA$_6$ That Cannot Be Digested to pX

The absolute configurations of modified ODNs were determined successfully by analyzing the Phosphodiesterase I digestion product on chiral HPLC with CHIRALCEL OJ-R column as long as the X residue was located far from 5′-terminus. In the case of **TXT$_6$** and **TXT$_{12}$**, for example, in which X residue is located next to the 5′-terminus, only 5′-TpX-3′ (not pX) could be obtained after the digestion because it could not be further digested to pX. But even for such sequences, we could determine their absolute configurations. The configuration-known two diastereomers of 5′-TpX-3′ were obtained from R-**T$_3$XT$_4$** and S-**T$_3$XT$_4$**, respectively (data not shown). As shown in Figure 5, these two diastereomers of 5′-TpX-3′ were distinguished by another chiral column CHIRALCEL AD-RH with a constant mobile phase composition of 10.4% AN aqueous solution in NaH_2PO_4 (pH3.0).[25] As the peaks of 5′-TpX-3′ were wide and the difference in retention time between the two diastereomers was small, we mixed the obtained two diastereomers with various ratios for clear distinction. It can be seen that R-TpX (from R-**T$_3$XT$_4$**) has a retention time (52.6 minutes) longer than that of S-TpX (51.2 minutes). As 5′-TpX-3′ obtained from α-**TXT$_6$** also has a longer retention time as compared with that from β-**TXT$_6$**, we can conclude that α-**TXT$_6$** has R-configuration, whereas the β-one has S- configuration (Figure 5).

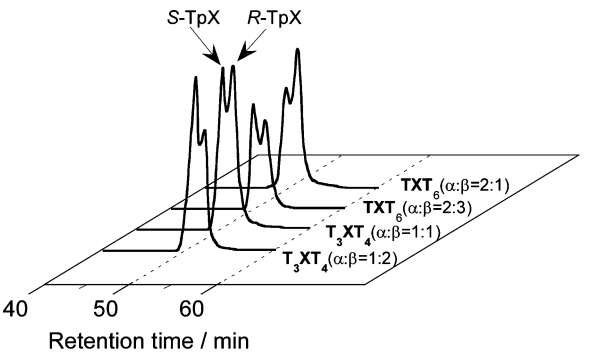


FIGURE 5 HPLC patterns on a chiral column CHIRALCEL AD-RH of the diastereomers of 5′-TpX-3′ obtained from the digestion of **TXT$_6$** and **T$_3$XT$_4$** by Phosphodiesterase I. The mixtures of the obtained two diastereomers with various ratios were injected for analysis. A peak of shorter retention time corresponds to S-TpX while that of longer one is R-TpX. A constant mobile phase composition of 10.4% AN aqueous solution containing 20 mM NaH_2PO_4 (pH3.0) was used.

Similarly, α-**TXT$_{12}$** and β-**TXT$_{12}$** were also assigned as R and S, respectively (data not shown). By analyzing 5′-ApX-3′, the digestion product of **A$_m$XA$_n$**, the absolute configurations of α-**AXA$_6$** and β-**AXA$_6$** were also assigned to be R and S, respectively (data not shown).

DISCUSSION

Effect of the Configuration of X Residue on the Polarity of Modified ODN

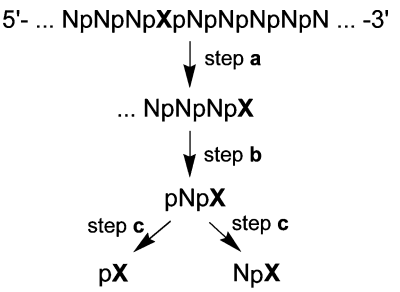
It is interesting that conventional ODS column can completely separate diastereomers of azobenzene-tethered ODNs within a large range of ODN length. The difference in retention time between two diastereomers can be explained by the polarity distinction caused by different interaction between hydrophobic azobenzene moiety and DNA bases. In comparison with β diastereomer, α diastereomer has higher polarity probably because the azobenzene can stack with the adjacent bases and escape from water more easily. For different sequences, the stronger stacking effect between azobenzene and the adjacent bases may cause larger difference in polarity between the two diastereomers (Table 1). Larger ΔRt was observed when purines are at both sides of azobenzene moiety probably because azobenzene is easier to stack with purine bases than with pyrimidine ones. Diastereomers of 5′-X$(pA)_{7}$-3′, 5′-X$(pT)_{7}$-3′, and 5′-$(Tp)_7$X-3′ could not be separated by all means, probably because of the too little polarity difference between them. But even for such sequences, the diastereomers could be separated when other intercalators were tethered instead of azobenzene: When the meta-aminoazobenzene or phenylazonaphthalene was attached to the same prochiral linker used here, the diastereomers of the corresponding modified ODNs also were completely separated (data not shown).[10,26]

Enzymatic Digestion of Modified ODN to Unnatural pX

As we expected, most of the nucleases could not digest azobenzene-tethered ODN to pX, because of the significant structure difference between X residue and natural deoxyribonucleoside. Interestingly, however, pX could be obtained when a large amount of Phosphodiesterase I was used (Figure 3). Phosphodiesterase I is a typical exonuclease that removes 5'-nucleotides one by one from the 3′-hydroxyl terminus of a natural ODN.[21,22] It recognizes the deoxyribonucleoside at the 3′-terminus and hydrolyzes the phosphodiester linkage next to it. The chemical structure at the 5′ side of the phosphodiester linkage to be hydrolyzed has no influence on the reaction.[27] Because of this nature, Phosphodiesterase I clips off the nucleotides from 3′-terminus rapidly and successively under normal conditions until the cleavage stops at the X residue. On the other hand, it was reported that a high concentration of Phoshphodiesterase I could work as an endonuclease and cleave the internal phosphodiester linkage.[28–32] Thus, it is reasonable that 5′-pNpX-3′ appeared as a main product after further digestion of 5′-NpNpNpX-3′ by use of the endonuclease activity.

Interestingly, the non-natural pX could be obtained from 5′-pNpX-3′ by Phosphodiesterase I, although the speed of digestion slowed down by more than 100 times, especially in the case of modified ODNs with S-configuration (Figure 2B). Probably, Phosphodiesterase I misidentified X residue as a natural nucleotide, and the phosphodiester between N and X was hydrolyzed by either the endonuclease or the exonuclease activity. Furthermore, Phosphodiesterase I also could remove monophosphate from 5′-pNpX-3′ to produce 5′-NpX-3′, which could not be hydrolyzed to pX at all by this enzyme. This is the first time that Phosphodiesterase I showed phosphatase activity. As a result, elongation of reaction time did not necessarily produce a high yield of pX, especially for the digestion of modified ODN with S-configuration (Figure 3B). In Scheme 2, the overall procedure for the digestion by Phosphodiesterase I is summarized: Phosphodiesterase I smoothly removes the 5′-nucleotide one by one from 3′-terminus of modified ODN to the X residue; Then it removes the nucleotides at 5′-side of the azobenzene moiety to give 5′-pNpX-3′; At the last step, pN was removed from 5′-pNpX-3′ to produce pX or the 5′-phosphate was removed to give 5′-NpX-3′ as a byproduct.

Modified ODN with R-configuration was more efficiently digested by Phosphodiesterase I than that with S-configuration, indicating that structure of the 2,2-bis(hydroxymethyl)-propion-(*p*-aminoazobenzene) in R-form might be more close to the natural ribose. NMR analysis revealed that R-configuration prefers clockwise winding so that it does not disturb right-handed ODN duplex whereas S-configuration disturbs it due to the tendency for counterclockwise winding.[18] Thus compatibility of R-configuration with natural ODN might allow its digestion by



SCHEME 2 Process of the digestion of azobenzene-tethered ODN by Phosphodiesterase I. Phosphodiesterase I first removes the 5′-nucleotides from 3′-terminus successively to the azobenzene moiety (step a), then nucleotides at 5′-side of NpNpNpX was removed to give pNpX (step b), finally 5′-nucleotide was removed to produce pX, or 5′-phosphate was removed to produce 5′-NpX-3′. Step a and step b proceed very much faster than step c.

Phosphodiesterase I. It should be noted that digestion rates of the diastereomers by Phosphodiesterase I also can be used for the absolute configuration assignment, because the S-configuration for all the sequences investigated here was more difficult to be digested than the R-one.

For almost all the sequences of modified ODNs, the α diastereomer, which was more efficient for photoregulating DNA hybridization, was determined as R-configuration.[16–18] For $\mathbf{T_6XT}$, however, α diastereomer has S-configuration. The two diastereomers of $\mathbf{T_6XT}$ showed similar photoregulation ability, although the retention time order was reverse on ODS column as compared with other modified ODNs. Detailed discussion of the photoregulation activity based on the configuration of the linker will be discussed in another article.

CONCLUSIONS

This work describes a facile enzyme-chromatographic method for determining the absolute configuration of modified ODNs. Diastereomers of azobenzene-tethered ODNs were successfully separated and digested to pX by a high concentration of Phosphodiesterase I. The enantiomers of obtained pX were separated by chiral HPLC using CHIRALCEL OJ-R chiral column. The absolute configuration was determined by comparing the retention time of pX with the authentic sample. It should be noted that application of natural enzyme to the digestion of non-natural nucleotides involving artificial scaffold has been scarcely reported. On the other hand, glycol nucleic acids (GNA), in which the riboses in the backbone are replaced by glycidols, have been reported recently.[33–35] This enzyme-chromatographic method might be used as a general approach for determination of the absolute configurations of those modified nucleic acids.

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24. Pure 5′-ApX-3′ could be obtained from the treatment of 5′-pApX-3′ with alkaline phosphatase. Further treatment of 5′-ApX-3′ with Phosphodiesterase I did not produce pX at all.
25. Isomers of TpX could not be separated on CHIRALCEL OJ-R column or ODS column under all the conditions we tried.
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