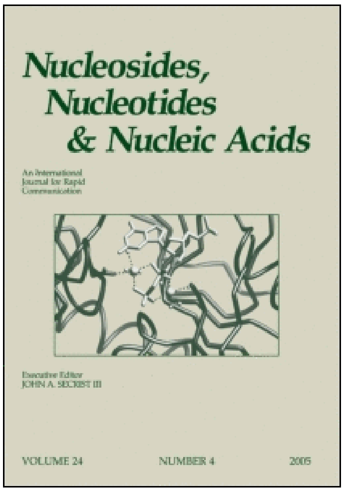

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Isolation of Oligodeoxynucleoside Phosphorothioate Diastereomers by the Combination of DEAE Ion-Exchange and Reversed-Phase Chromatography

Hideyuki Kanehara[a]; Masatsugu Mizuguchi[a]; Keisuke Makino[a]
[a] Department of Polymer Science and Engineering, Kyoto Institute of Technology, Kyoto, Japan

To cite this Article Kanehara, Hideyuki , Mizuguchi, Masatsugu and Makino, Keisuke(1996) 'Isolation of Oligodeoxynucleoside Phosphorothioate Diastereomers by the Combination of DEAE Ion-Exchange and Reversed-Phase Chromatography', Nucleosides, Nucleotides and Nucleic Acids, 15: 1, 399 — 406

To link to this Article: DOI: 10.1080/07328319608002393

URL: http://dx.doi.org/10.1080/07328319608002393

NUCLEOSIDES & NUCLEOTIDES, 15(1-3), 399-406 (1996)

ISOLATION OF OLIGODEOXYNUCLEOSIDE PHOSPHOROTHIOATE DIASTEREOMERS BY THE COMBINATION OF DEAE ION-EXCHANGE AND REVERSED-PHASE CHROMATOGRAPHY[a]

Hideyuki Kanehara, Masatsugu Mizuguchi and *Keisuke Makino
Department of Polymer Science and Engineering, Kyoto Institute of Technology,
Matsugasaki, Sakyo-ku, Kyoto 606, Japan

ABSTRACT: In antisense trials, oligodeoxynucleoside phosphorothioates (S-oligo) stereoisomers arise from the thiophosphate R/S configuration. We have explored effective separation of these isomers by the combination of DEAE ion-exchange (IELC) and reversed-phase (RPLC) liquid chromatography. Twenty-six S-oligos and some of the natural counterparts were examined. In the presence of DMTr at the 5'-end, isomers could be separated well by IELC, while RPLC gave good resolution in the absence of this protecting group. Combination of these two different modes leads to more effective isolation.

Oligodeoxynucleoside phosphorothioates (S-oligo) in which one of the two nonbridged oxygen atoms in each phosphate linkage is replaced by a sulfur atom have been applied as antisense medicine in clinical trials[1-3] as well as enzyme-specific inhibitors[4]. In antisense studies, S-oligo is one of the most promising molecules and has been utilized extensively.

Because of the introduction of the sulfur atom, R/S configuration is generated at each thiophosphate linkage so that n-mer S-oligo consists of $2^{(n-1)}$ diastereomers. S-oligos used in clinical trials, therefore, are mixtures of the isomers. Despite several attempts[5], fully stereoselective synthesis has not been achieved so far, although configurational influences of S-oligos on the specific regulation of gene expression have been considered important. At this moment, there is only one stereoselective synthesis employing 5'-*O*-DMTr-deoxynucleoside 3'-*O*-(2-thio-1,3,2-oxathiaphospholane) as a building block. This method, however, requires high skill and is difficult to attain a high coupling yield readily[5].

---

[a] Dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.

The most convenient method to prepare stereocontrolled S-oligos in the laboratory, therefore, is the HPLC separation of the isomer mixtures[6,7]. Several studies have been conducted by use of precisely controlled RPLC[8]. By measuring the melting temperature (Tm) of the duplexes formed between the isolated isomers and the complementary targets, it has been suggested that S-oligos with a Sp configuration give rise to higher Tm than Rp[9]. This observation implies that stereocontrolled S-oligos may have differential effects *in vivo* as antisense molecules. Therefore, stereoselective synthesis is highly essential to test this hypothesis. However, because of the lack of such method, establishment of the effective separation of the isomeric S-oligo would be useful for related fields. For example, it could provide stereocontrolled samples to the basic studies on the interaction of S-oligo isomers with the target as well as on the solution structure of the duplexes. These studies are extremely helpful for designing effective antisense S-oligos. However, a single mode of HPLC operation, for example RPLC, can separate only limited number of sequences and the combination with other chromatographic mode is needed.

In the oligonucleotide chemistry, ion-exchange chromatography has been used exclusively to purify oligomers with the same chain length[10]. It has been also reported that this mode produces only broad peaks in chromatograms[11,12]. This implies that each isomer has differential interactions with the stationary phase, due to the difference in their solution structures. Therefore, slight difference in retention times produce broad envelope peak shape. This suggests the possibility that S-oligo isomers may be separated by the aid of ion-exchange chromatography. In the present study, we have applied DEAE ion-exchange chromatography (IELC) to the isolation of the stereoisomers of S-oligo.

MATERIALS AND METHODS

Oligonucleotides

Both natural type oligodeoxynucleotides and S-oligos were synthesized on an automated synthesizer, Milligen/Biosearch Cyclone Plus DNA Synthesizer (Millipore, Burlington, MA), by the standard phosphoramidite methods. All reagents used were purchased from the same company. Synthesis was carried out on 1 µmol scale. Oligodeoxynucleoside phosphoramidite was treated either with iodine or with tetraethylthiuram disulfide to generate phosphodiester linkage and thiophosphate linkage, respectively. Deprotection of the oligomers was performed in conc. NH_3 at 55°C for 7 hr. After evaporation of NH_3, the oligomers were subjected to RPLC and IELC separation. The protecting group attached to the 5'-terminal, dimethoxytrityl group (DMTr), was removed in 80% CH_3COOH for 15 min at ambient temperature.

RPLC separation

An HPLC system (Shimadzu, Kyoto, Japan) consisted of an SCL-6B system controller, an SPD-M 6A photodiode-alley ultraviolet-visible wavelength absorbance detector, a pair of LC-6A pumps, and a PC-9801 RA51 host computer. Columns used were Superspher 100 RP-8(e) (4.6 X 250 mm) (Merck, Darmstadt, Germany) and Wakosil 5C18 (4.6 X 150 mm) (Wako Pure Chemical Industries, Osaka, Japan). Other chromatographic conditions are as follows: eluents, (A) 0.1 M triethylammonium acetate (pH 7.0) and (B) 0.1 M triethylammonium acetate (pH 7.0)/acetonitrile (50/50, v/v); elution mode, for DMTr-off oligos, 10-40% B for 25 min (linear gradient) and then 40-100% B for 10 min (linear gradient), or for DMTr-on oligos, 50% B (isocratic); flow rate, 0.7 mL/min; detection 254 nm; temperature, ambient.

IELC separation

An HPLC system used was same as that used for RPLC. Chromatographic conditions are as follows: column, TSK-GEL DEAE-2SW (4.6 X 250 mm) (Tosoh, Tokyo); eluent, (A) 50 mM CH_3COONH_4 and (B) 1.5 M CH_3COONH_4; gradient, 0-100 % B for 60 min (linear); flow rate, 1 mL/min; detection, 254 nm; temperature, ambient.

Enzymatic digestion for the configuration analysis of S-oligo

The absolute configuration of the separated S-oligo isomers was determined by the specific enzymatic digestion. Nuclease P1 (Wako) digests the Sp form specifically and snake venom phosphodiesterase (Pharmacia, Upsala, Sweden) the Rp form. For Nuclease P1 digestion, S-oligo was dissolved in the mixture of 50 mM acetate buffer (40 μL, pH 5.3), 10 mM $ZnSO_4$ (4 μL) and the enzyme (40 μL, 200 units/mL). For snake venom phosphodiesterase digestion, the same quantity of S-oligo was dissolved in the mixture of 100 mM Tris-HCl buffer (50 μL, pH 8.9), 20 mM $MgCl_2$ (5 μL) and the enzyme (20 μL, 250 units/mL). The reaction mixtures were incubated for 1 hr at 37°C. Digestion mixtures were analyzed by RPLC[13-15] employing the same conditions as used for the separation of S-oligo. Each chromatographic peak was identified by co-injecting authentic deoxynucleotides and oligodeoxynucleotides.

RESULTS AND DISCUSSION

Separation of S-oligo (dC)

Using RPLC and IELC, as a preliminary trial, we examined S-oligo(dC) ranging from dimer to pentamer which carry thiophosphate linkage at each unit. In this case, DMTr was removed. Chromatograms obtained for the samples by IELC are depicted in FIG.1. In IELC chromatogram, CsC (s: thiophosphate) eluted at 9.2 min in slightly split two peaks. Similarly trimer, tetramer and pentamer eluted, respectively, in the three, three and four peaks although they are mixtures of four, eight and sixteen isomers,

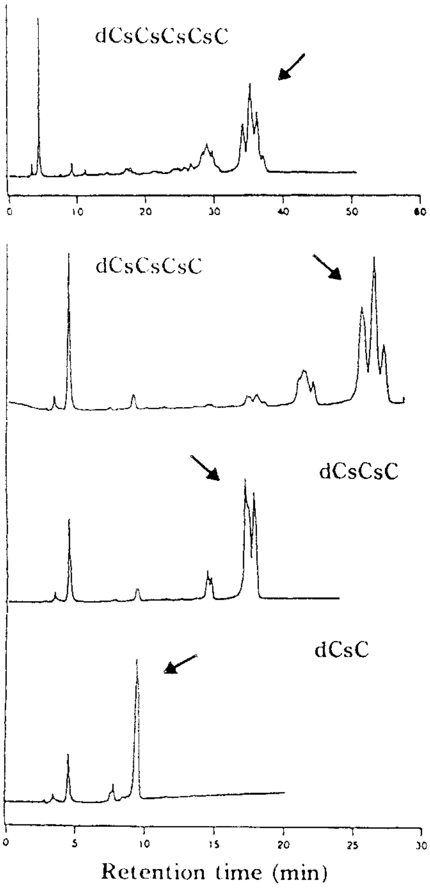

FIG. 1 HPLC chromatograms of phosphorothioate oligo(dC)$_n$ [n=2~6] by IELC.

respectively: The major envelope peaks are indicated by the arrow. This result suggests that isomers of these simple homopolymers have configuration-dependent interaction with the stationary phase, possibly with the DEAE cation.

On the other hand, as demonstrated previously[6)], RPLC produced well separated peaks, implying also the configuration-specific hydrophobic interaction of the isomers with the stationary phase, the octadecyl chain. In some chromatograms, however, the number of the chromatographic peaks observed are also less than the expected number of the isomers.

The above results suggest that if these two modes are combined for the recognition of the difference of isomers, it will provide more powerful technique than the single use of conventional RPLC.

Separation of dimers

It has been reported that particularly for S-oligos with DMTr off, RPLC separates many isomeric dimers in two well separated peaks[7]. In the present study, DMTr-off dimers such as dTsG, dGsT, dCsC, dAsT and dTsT were examined and base-line separation of the isomers was attained. By the specific enzymic digestion, it was determined that Rp forms of S-oligo elute faster than Sp forms: This result was consistent with the data reported previously[7].

On the other hand, IELC showed poor resolution for the dimers. For convenient evaluation of the results, peak separation is indicated tentatively by a parameter of resolution (Rs) which is usually applied for isocratic separation. As summarized in TABLE 1, the Rs values are <1. In addition to the phosphorothioate dimers, corresponding phosphodiester dimers were also examined under the same IELC conditions and found to elute faster than S-oligo dimers, as represented in TABLE 1. This trend is similar to the retention order in RPLC.

Separation of DMTr-off deoxyoligonucleotides which carry one thiophosphate

The above experiments indicate that the difference in the solution structure of S-oligo isomers is recognized by IELC. To see the extent of such recognizing ability more clearly, we applied the IELC mode to the deoxyoligonucleotides which carry one thiophosphate linkage and would have similar solution structures. The samples tested are 5'-dAsTCG-3', 5'-dCAsTCG-3', 5'-dGCAsTC-3', 5'-dGCsATCG-3', 5'-dGATsTCC-3', 5'-dGCTsCCG-3', and 5'-dGCAsTCG-3'. For comparison, RPLC was also carried out. As listed in TABLE 2, these samples showed unsatisfactory resolution in IELC. For some samples, however, IELC gave rise to better resolution than RPLC, indicating the potential for isomer separation by the combined modes.

After the separation, enzymic digestion analysis was also performed for the isomers separated by both modes, showing the same retention order as that obtained for the dimers described above.

Separation of S-oligo and S-oligo analog isomers with DMTr on

The influence of the DMTr group on the separation was studied using 5'-DMTr-dTsG-3', 5'-DMTr-dGsT-3', 5'-DMTr-dCAsTCG-3', 5'-DMTr-dGCAsTC-3', 5'-DMTr-dGCsATCG-3', and 5'-DMTr-dGATsTCC-3'.

For RPLC chromatography of oligonucleotides, it has been demonstrated that sufficient separation and peak shape are obtained when the sample has DMTr at the 5'-terminal. This is why DMTr is retained in regular purification. However, this was not the case in the separation of oligodeoxynucleotides with one thiophosphate linkage. As shown in TABLE 3, three of four samples produced the Rs value of zero.

TABLE 1 Chromatographic analysis of S-oligo and natural type dimers by IELC.

Oligomer (5'→3')	Retention time (min)		Rs
dTsG	9.1	9.3	0.80
dToG	7.4		-
dGsT	9.3	9.4	0.36
dGoT	7.7		-
dCsC	9.2	9.3	0.30
dCoC	7.4		-
dAsT	9.5	9.7	0.58
dTsT	8.4		0

Rs: obtained under the gradient condition which is usually applied to isocratic separation.

TABLE 2 R/S separation of S-oligo having one phosphorothioate linkage by IELC and RPLC.

Oligomer (5'→3')	Retention time (min)				Rs	
	IELC		RPLC		IELC	RPLC
dAsTCG	19.5	19.9	14.7	15.1	0.84	2.74
dCAsTCG	21.4	22.0	18.0	18.2	1.19	0.93
dGCAsTC	21.2	21.6	17.4		1.00	0
dGCsATCG	24.4	24.9	17.2	18.0	0.97	3.19
dGCTsCCG	20.4	21.4	17.0	18.6	2.18	6.69
dGATsTCC	22.7	23.0	19.0	20.1	0.46	5.00
dGCTsTCG	23.4	23.8	18.6	19.5	0.82	4.17
dGCAsTCG	24.2	24.7	21.2		0.88	0

Rs: obtained under the gradient condition which is usually applied to isocratic separation.

TABLE 3 R/S separation of S-oligo having 5'-DMTr group by IELC and RPLC.

Oligomer (5'→3')	Retention time (min)				Rs	
	IELC		RPLC		IELC	RPLC
DMTr-dTsG	29.8	45.2	-		3.58	-
DMTr-dGsT	46.8	51.2	-		1.52	-
DMTr-dCAsTCG	51.2	52.9	11.3	11.9	0.78	1.24
DMTr-dGCAsTC	46.0	48.4	9.4		1.54	0
DMTr-dGCsATCG	52.4	55.4	8.8		1.50	0
DMTr-dGATsTCC	50.5		9.9		0	0

Rs: obtained under the gradient condition which is usually applied to isocratic separation.

On the other hand, by IELC, all the samples except 5'-DMTr-dGATsTCC-3' resulted in improved resolution. For example, Rs of the dimers 5'-TsG-3' and 5'-dGsT-3' whose Rs are 0.80 and 0.36 without 5'-DMTr, became 3.58 and 1.52, respectively: It should be noted that base-line separation is achieved if Rs>1.2. The introduction of DMTr at the 5'-terminal also led to the improved resolution of the isomers of oligodeoxynucleotides carrying one thiophosphate.

These data suggest that first separation should be carried out for S-oligo with DMTr by IELC. If the separation is incomplete with IELC, RPLC should be used after the deprotection of DMTr.

Mechanism of IELC separation of S-oligo isomers

Generally, in the separation of oligonucleotides by ion-exchange chromatography, negatively charged phosphate group interacts with the cationic DEAE group of the stationary phase and subsequently other interaction between the oligonucleotides and the stationary phase is induced. Thus the sample is retained in a column according to the strength of the overall interaction. In the case of separation of S-oligos by IELC, however, the latter interaction should be negligible because the stationary phase used in this study has only DEAE cation directly bonded to the silica matrix. Interaction between this cation and the solute, therefore, is responsible for the separation.

For S-oligo, it has been demonstrated that the negative charge is delocalized between the sulfur and oxygen atoms16,17). This characteristic charge delocalization seems not to influence the electrostatic interaction mechanism of S-oligo because it is evident that ionic strength dominating the electrostatic interaction is stronger for phosphodiester than S-oligo. It is likely that some other interaction with DEAE groups accounts for the longer retention: For instance, it has been reported that thiophosphate groups interact with amino groups through hydrogen bonding18,19). In the present chromatographic system, the interaction of S-oligo with the acetate in the eluent should also be considered for the retention mechanism. Thus the balance of the strength of the interactions between S-oligo and DEAE cation and between S-oligo and acetate causes the longer retention. In the present study, it was also found that the difference in the configuration of S-oligo isomers leads to the differential interaction with DEAE cation. These combined effects need to be considered to account for the characteristic retention behavior of S-oligo in the IELC separation, although the marked effect of DMTr on the peak resolution has remained here to be explained.

In summary, we have demonstrated that the combination of IELC and RPLC is a convenient and potentially useful method to prepare stereocontrolled short S-oligos for the basic research of antisense studies, such as investigations of the structural characteristics of S-oligo isomers with different chirality. This will be greatly helpful for designing ideal antisense molecules. This method will also be useful in eliminating impurities in antisense S-oligos, which might cause undesirable side effects *in vivo*.

ACKNOWLEDGEMENT

This work was supported by Rational Drug Design Laboratories.

REFERENCES

1. Eckstein, F. *Annu. Rev. Biochem.* **54**, 367-402 (1985).
2. Zamecnik, P.C.; Goodchild, J.; Taguchi, Y. and Sarin, P. S. *Proc. Natl. Acad. Sci. USA* **83**, 4143-4146 (1986).
3. Matsukura, M.; Shinozuka, K.; Zon, G.; Mitsuya, H.; Reitz, M.; Cohen, J.S. and Broder, S. *Proc. Natl. Acad. Sci. USA* **84**, 7706-7710 (1987).
4. Eckstein, F. *Angew. Chem. Int. Ed. Engl.* **22**, 423-439 (1983).
5. Stec, W.J.; Grajkowski, A.; Koziolkiewicz, M. and Uznanski, B. *Nucleic Acids Res.* **19**, 5883-5888 (1991).
6. Wilk, A. and Stec, W. J. *Nucleic Acids Res.* **23**, 530-534(1995)
7. Stec, W. J. and Zon, G. *J. Chromatogr.* **326**, 263-280 (1985).
8. Murakami, A.; Tamura, Y.; Wada, H. and Makino, K. *Anal. Biochem.* **223**, 285-290 (1994).
9. LaPlanche, L. A.; James, T. L.; Powell, C.; Wilson, W. D.; Uznanski, B.; Stec, W. J.; Summers, M. F. and Zon, G. *Nucleic Acids Res.* **14**, 9081-9093 (1986).
10. Zon, G. and Thompson, J. A. *Biochromatogr.* **1**, 22-32 (1986).
11. Metelev, V. and Agrawal, S. *Anal. Biochem.* **200**, 342-346 (1992).
12. Agrawal, S.; Tang, J. Y. and Brown, D. M. *J. Chromatogr.* **509**, 396-399 (1990).
13. Bryant, F. R. and Benkovic, S. J. *Biochemistry* **18**, 2825-2828 (1979).
14. Potter, B. V. L.; Connolly, B. A. and Eckstein, F. *Biochemistry* **22**, 1369-1377 (1983).
15. Burgers, P. M. J. and Eckstein, F. *Proc. Natl. Acad. Sci. USA* **75**, 4798-4800 (1978).
16. Frey, P. A. and Sammons, R. D. *Science* **228**, 541-545 (1985).
17. Iyengar, R.; Eckstein, F. and Frey, P. A. *J. Am. Chem. Soc.* **106**, 8309-8310 (1984).
18. Donohue, J. *J. Mol. Biol.* **45**, 231-235 (1969).
19. Makino *et al.*, Unpublished data.