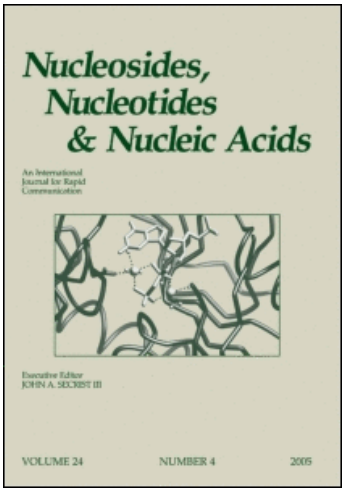

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Evaluation of Different Types of End-Capping Modifications on the Stability of Oligonucleotides Toward 3'- and 5'-Exonucleases

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EVALUATION OF DIFFERENT TYPES OF END-CAPPING MODIFICATIONS ON THE STABILITY OF OLIGONUCLEOTIDES TOWARD 3'- AND 5'-EXONUCLEASES

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ABSTRACT. Synthetic oligonucleotides are increasingly used because of their potential activity as regulators of gene expression. One of their major drawbacks is instability toward nucleases, in particular exonucleases. In this article, we studied some terminal modifications that can enhance exonuclease resistance, such as end-capping with alkylic chains (1,3-propanediol and 1,6-hexanediol), and with a modified nucleotide (2',3'-secouridine). These compounds were compared with the parent (natural) oligodeoxynucleotide and with different analogs containing a progressive number of phosphorothioate linkages. The resistance toward SVPDE and CSPDE (a 3'- and a 5'-exonuclease) was assessed, in vitro, by two independent techniques, UV and HPLC. Our results showed that the stability of all the modified oligonucleotides was at least 12 times that of the parent compound.

INTRODUCTION

Oligonucleotides are widely employed in molecular biology to switch down genes[1-5] and are increasingly used as antiviral and antitumoral agents, in animals as well as in humans[6-8]. Their applications span from antisense[9], to anti-genes[10-13], to ribozymes[14, 15] and aptamers[4, 16]. Independently of their use, natural oligonucleotides are degraded by nucleases present in several biological media. A great effort has been made to overcome this problem employing several kinds of chemical modifications in oligonucleotide

structure, ranging from modification of the phosphate linkages to modifications of the sugars or of the bases. So far, the most common used strategy is the substitution of the phosphates by phosphorothioate linkages. Although phosphorothioate oligonucleotides demonstrate a higher stability, they often increase non-antisense effects due to their tendency to bind proteins[17, 18].

As in several sera and cell cultures the greatest degradation can be ascribed to exonucleases, in particular to 3'-exonucleases, terminal protection can be considered a good compromise to confer to oligonucleotides a satisfying stability and minimize undesired side effects due to modifications. Examples of terminal protections are as old as the antisense methodology itself, being already employed in the early work of Zamecknic in 1978[1]. Since then, several methods have been described using, for instance, 3'-hairpins[19] or conjugation with lipophilic molecules like adamantane[20] just to cite a few.

In this paper we compare the protective effects of different terminal modifications (Table 1 and Figure 1), some of them original, against Snake Venom and Calf Spleen Phosphodiesterases.

RESULTS AND DISCUSSION

Aim of this work was to find simple ways to confer to oligonucleotides, designed to be used as antisense agents, a sufficient stability to easing protocols for their use. Although antisense agents require stabilization against both exo- and endonucleases for an *in vivo* use, during the preliminary screening for the most effective sequence and during the study of the biological response, phosphodiester oligonucleotides are still largely used. Their advantage over phosphorothioate analogs, for instance, is that they avoid toxicity and non-antisense specific effects, and that they use a more similar mechanism to that of natural antisense RNA oligonucleotides. Our rationale for this work was to look for simple modifications in terms of structure and synthetic procedure. The used oligonucleotides are listed in Table 1 and Figure 1.

As leading compound we chose a phosphodiester oligonucleotide, **AS8-N,** with a well balanced base composition, already employed, in a *tandem* system in a previous work[21].

Table 1

Sequence	Code
$^{5'}$TpCpGpApTpCpCpTpGpGpApTpGpApApApCpCpCpT$^{3'}$	**AS8-N**
p-p$^{5'}$TpCpGpApTpCpCpTpGpGpApTpGpApApApCpCpCpT$^{3'}$p-***p***	**AS8-P**
h-p$^{5'}$TpCpGpApTpCpCpTpGpGpApTpGpApApApCpCpCpT$^{3'}$p-***h***	**AS8-H**
$^{5'}$**u**pCpGpApTpCpCpTpGpGpApTpGpApApApCpCpCp**u**$^{3'}$	**AS8-U**
$^{5'}$**TsCsG**pApTpCpCpTpGpGpApTpGpApApApCpC**sCsT**$^{3'}$	**AS8-4PS**
$^{5'}$**TsCsG**pApT**sCsC**pTpGpGpApTpGpApApApC**sCsCsT**$^{3'}$	**AS8-7PS**
$^{5'}$**TsCsGsAsTsCsCsTsGsGsAsTsGsAsAsAsCsCsCsT**$^{3'}$	**AS8-All-PS**
$^{3'}$upgpcpapgpcpupapgpgpapcpcpupapcpupupupgpgpgpapcpu$^{5'}$	**S8-RNA**

For Symbols explanation see Figure 1

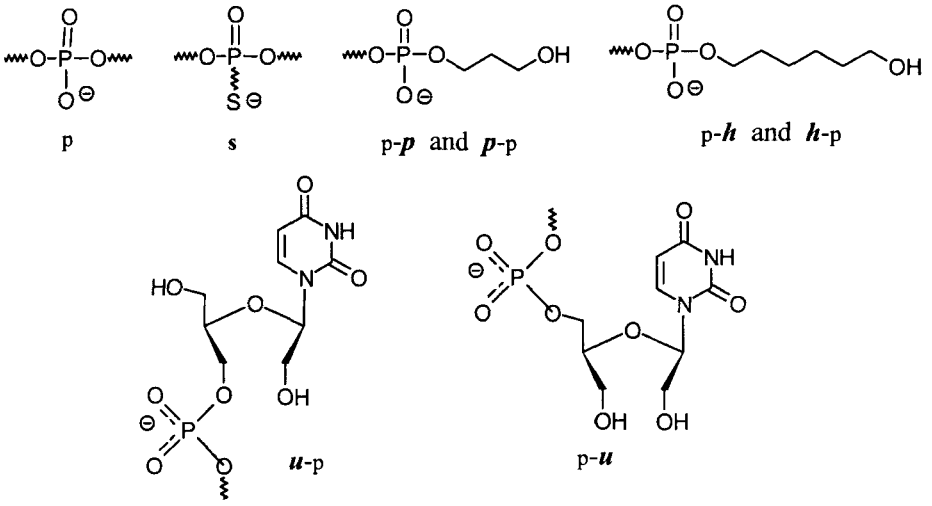


Figure 1: Explanation of the symbols used in Table 1.

The first modification was 5'- and 3'-capping with 1,3-propanediol, **AS8-P**. The use of propanediol as nucleoside substitute was described by Seela[22]. 1,3-propanediol mimics the C3-C5 carbon chain, as a 3'-tail it was found to confer resistance toward SVPDE degradation[23], furthermore a 5'-propanediol-oligonucletide was found to be processed with high yield but slow kinetics by T4-polynucleotide kinase[24].

The group of Hélène had already studied the influence of a dodecanol tail[25]; our original contribution was to study the influence of an intermediate tail made by 1,6-

hexanediol, **AS8-H**. It is interesting to note that while this work was in progress it was found that a 3'-dodecanol tail not only protected an oligonucleotide from nucleases but also enhanced the cleavage of the hybridized target by RNase H[26].

Next we chose to study the influence of a terminal capping with a simple modified nucleotide. Our group had already employed with satisfactory results L-deoxyribonucleosides for this purpose[27-29]. In this work we decided to prepare 2',3'-seconucleoside derivatives to get some insight into the importance of the sugar on the enzymatic recognition. We thought that such a modified nucleotide could still be effective in doing Watson-Crick base pair, so instead of adding the 2',3'-secouridine at 5' and 3' termini, we use it to replace the terminal thymidine nucleosides.

We chose to include in the list examples of the "minimal protection strategy" proposed by Peyman and Uhlmann[30] consisting in using a minimal number of phosphorothioate linkages. They found that the positions of the phosphorothioate bonds more effective to confer oligonucleotide protection were the terminal ones, and those near the pyrimidinic bases. So we designed **AS8-4PS** containing a double 5'- and 3'-end capping with phosphorothiate linkages and **AS8-7PS** containing three more phosphorothioate bonds adjacent to pyrimidinic bases.

We also prepared a full phosphorothiate oligonucleotide **AS8-All-PS** for comparison.

Synthesis of AS8-P and AS8-H

The preparation of the reagents needed for the synthesis of the propanediol tail was straightforward. First we prepared the 4,4'-dimethoxytrityl-propanediol using an excess of 1,3-propanediol as described by Seela[22]. Part of the compound was used to prepare the N,N-diisopropyl-(2-cyanoethyl)-phosphite by reaction with the corresponding chlorophosphite[22]. Another lot was used to prepare the succinic ester that was then used to derivatize a sample of aminopropyl-CPG[22,31] via activation with DCC and p-nitrophenol.

The hexanediol-loaded-CPG and the corresponding amidites were synthesized in analogy to what described for the propanediol compounds.

Before attending to the synthesis of the modified **AS8-P** and **AS8-H** we checked the reagent performance preparing, on the synthesizer, the symmetric propanediol and

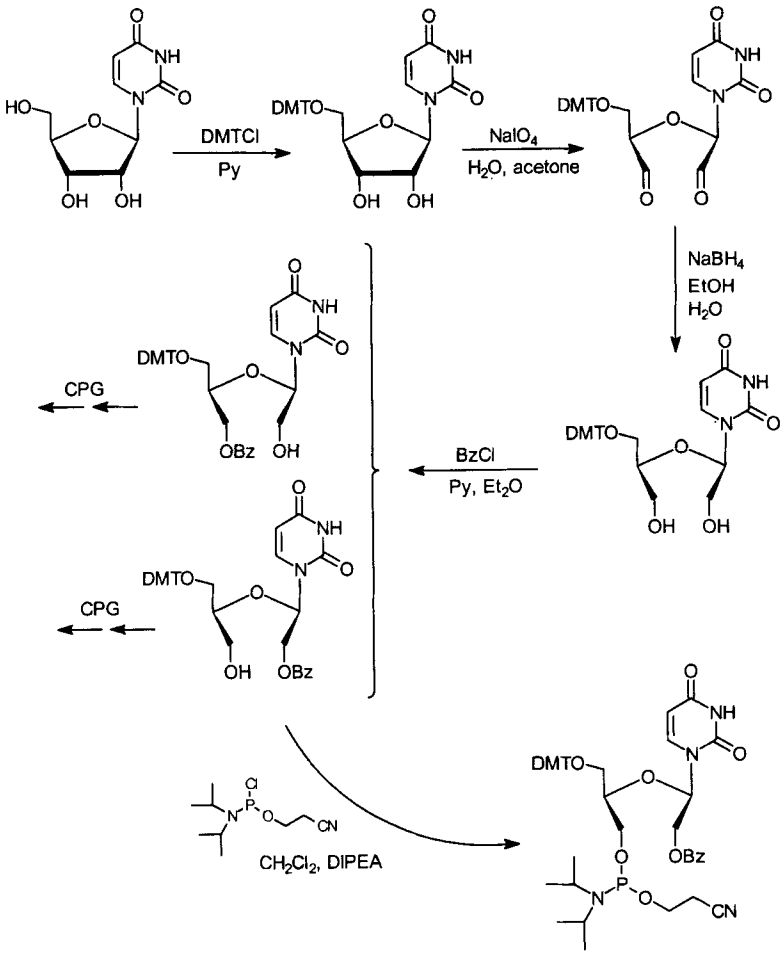


Figure 2: Synthesis of derivatives of 2'-3'-secouridine

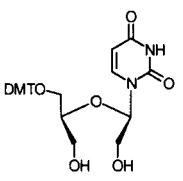
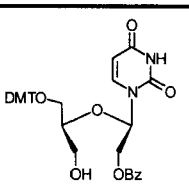
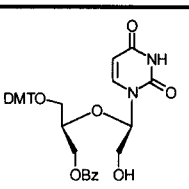
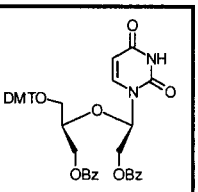
hexanenediol monophosphate dimers (***p***-p-***p*** and ***h***-p-***h***) without notice any change in the usual coupling efficiency. After deblocking the dimers gave satisfactory ^{31}P and ^{1}H-NMR spectra.

AS8-P and **AS8-H** were then prepared by solid phase synthesis starting from the modified supports using the manufacturer protocols.

Synthesis of AS8-U

The synthetic pathway[32,33] to the preparation of the 2',3'-secouridine amidite are depicted in Figure 2.

Table 2:
Products recovered after silica gel chromatography and relevant ^{1}H-NMR data

				
Entry #	**1**	**2**	**3**	**4**
Yield (%)	14	37	18	17
H1' (δ)	5.90	6.25	5.95	6.45
H2' (δ)	3.85-3.50	4.50	3.90-3.50	4.60-4.30
H3' (δ)	3.85-3.50	3.80-3.60	4.70-4.30	4.60-4.30
H4' (δ)	3.85-3.50	3.80-3.60	3.90-3.50	3.90

The 5'-O-DMT-2',3'-secouridine was obtained by following a simple procedure of oxidative scission followed by reduction of the dialdehyde with $NaBH_4$. The diol was obtained in a 85% yield. We thought that with a large protecting group we could have possibly directed the esterification toward the less crowded 2'-OH, so after a few attempts we found that treatment of the diol with one equivalent of benzoylchloride, in a 1 to 1 mixture of ether/pyridine at -10 °C, afforded a 2:1 mixture of 2'- and 3'-monobenzoylated derivatives, then resolved by silica gel chromatography. The obtained products are indicated in Table 2, together with the relevant ^{1}H-NMR chemical shifts.

The 2'-monobenzoylated compound was used to prepare the 3'-amidite, the other isomer and the unresolved mixture of 2'- and 3'-benzoyl derivatives were used to derivatize the aminohexyl-CPG following a procedure similar to that described for the propanediol derivatives.

Again we prepared the ***u***-p-***u*** dimer then **AS8-U**.

Synthesis of AS8-4PS, AS8-7PS, and AS8-All-PS

Phosphorothioated oligonucleotides were prepared applying the manufacturer protocols employing the Beaucage's sulfurizing agent[34].

The correct incorporation of the sulfur was confirmed by integral ratio in ^{31}P-NMR spectra after deblocking and purification of the oligonucleotides (22:80 and 44:79 for **AS8-4PS** and **AS8-7PS** respectively).

Synthesis and purification of S8-RNA

We chose to study the stability of the components of the series toward a two bases per side longer RNA, to take into account possible side effects due to the inserted modifications, (as extra bases we chose those found in the genomic sequence of human bax)[21], so a 24-mer complementary RNA, **S8-RNA,** was synthesized on a seco-uridine derivatized CPG using the protocol described by Sproat[35]. The crude RNA was then twice precipitated from an aqueous solution adding n-butanol. We recovered a pellet containing the precipitated RNA with an HPLC purity of about 80%. A fraction of higher purity (about 96%) was obtained by HPLC purification with $LiClO_4$ gradient elution and subsequent desalting[35]. Both fractions were used to measure the melting point of the modified oligonucleotides.

Melting experiments

Equivalent amounts (2 μM per strand) of DNAs and RNA were mixed in an UV cell containing 0.1 M NaCl, 0.1 M Tris-HCl, at pH 7.0. We followed the UV absorbance at 260 nm from 18 to 80 °C heating the cells at a rate of 0.5 °C per min. We did not observe any difference using the two lots of RNA in determining the melting temperature (we only noticed a slight enhancement of cooperativity with the purer RNA data not shown). The observed temperature are reported in Table 3.

We can say that the propanediol and hexanediol modifications did not change the stability of the duplex and the secouridine lowered the T_m of 2 °C as did the double PS capping. The increase in the number of phosphorothioate linkages correlates with a decrease in the stability, as well documented in literature.

Resistance toward SVPDE

The UV cuvettes containing the oligonucleotides and 0.1 U/OD/ml of Snake Venom Phosphodiesterase (SVPDE) were thermostatted at 37 °C in the UV spectrophotometer. We followed the degradation registering the absorbance value at 260 nm. We withdrew samples at 90 min and at 22 h. The samples contained in micro-tubes were rapidly

Table 3: Observed T_m
a) reproducibility in measurement is ± 0.5 °C

oligonucleotide	T_m (°C)[a]
AS8-N	65
AS8-P	66
AS8-H	66
AS8-U	64
AS8-4PS	64
AS8-7PS	63
AS8-All-PS	59

warmed by immersion in a water bath at 80 °C for 2 min then stored at -18 °C. The samples were then used to perform the HPLC analysis on an anion exchange column. The UV results are shown in Figure 3.

We considered **AS8-N** completely digested after 24 h and we used that value of the absorbance as the reference for the complete degradation of all the samples. We used also HPLC analysis to assess the amount of oligonucleotide degradation. We found a fair accordance with the UV hyperchromic analysis (Table 4). From HPLC chromatograms we saw the formation of two main products assigned to unresolved purines and pyrimidines monophosphates. The absence in the chromatograms of products at intermediate retention times (observed only in the **AS8-7PS** degradation) revealed that the initial attack of the oligonucleotides by the enzyme was the slowest step, once the terminal group was digested the degradation proceeded rapidly to the terminal products (monophosphate nucleotides). We did not observe interference from the nuclease. We used the changes in absorbance from oligonucleotide to mononucleotides to correct the ratio of the area of the peaks in the chromatograms. The chromatograms of **AS8-H** at 90 min and 22 h are reported as example in Figure 4.

UV and HPLC data demonstrated that for the modified sequences the degradation never reached 100%, that was attributed to a slow deactivation of the nuclease at 37 °C. A new analysis with a 10 times higher SVPDE concentration showed the same relative

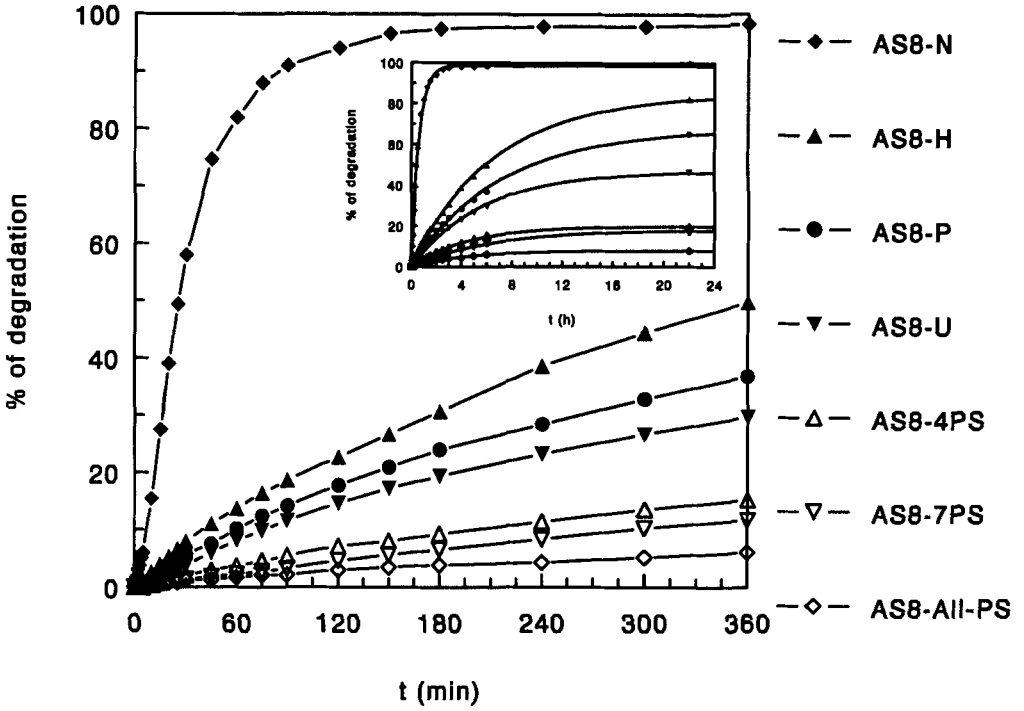
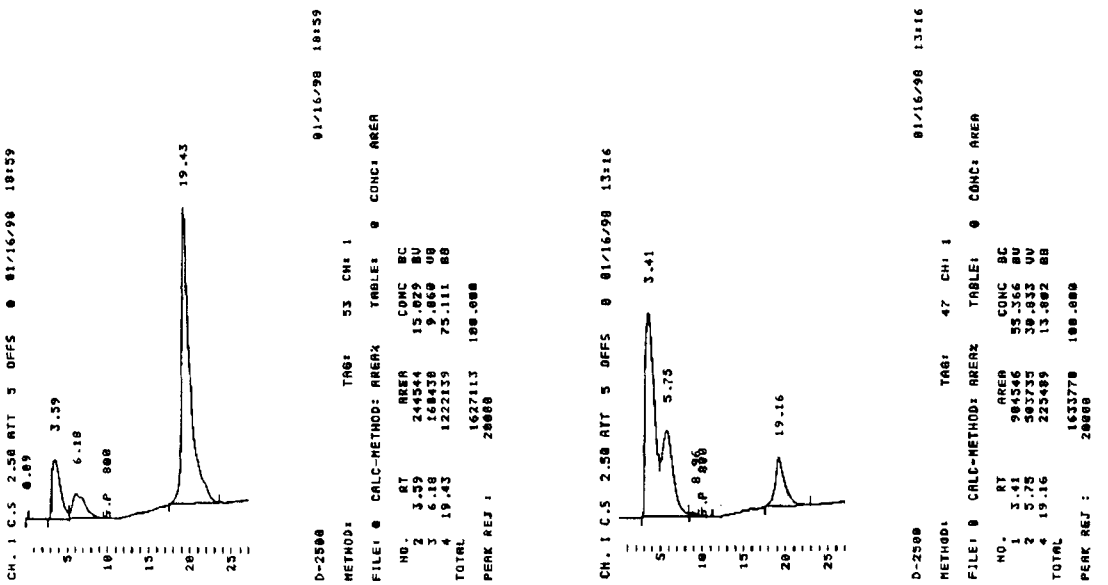


Figure 3: Degradation of oligonucleotides by SVPDE, (the insert shows readings until 22 h).

Table 4: correspondence between UV and HPLC at 90 min and 22 h

Oligonucleotide	% of intact oligo after 90 min		% of intact oligo after 22 h	
	UV data	HPLC data	UV data	HPLC data
AS8-N	9.1		0	0
AS8-H	81.3	79.1	17.8	16.7
AS8-P	85.8	86.3	36.7	32.6
AS8-U	88.4	87.1	53.4	50.7
AS8-4PS	94.5	95.6	80.5	90.0
AS8-7PS	96.8	96.9	82.6	88.4
AS8-All-PS	97.8	93.6	92.2	90.1



AS8-H + SVPDE after 90 min **AS8-H + SVPDE after 22 h**

Figure 4: HPLC chromatograms of AS8-H after 90 min and 22 h of incubation with SVPDE

stability of the derivatives with complete digestion of all the sequences but **AS8-All-PS** (data not shown).

Resistance toward CSPDE

The Calf Spleen Phosphodiesterase (CSPDE) proved to be less active in the used conditions, despite the higher formal concentration employed (0.566 U/OD/ml). The solutions containing the oligonucleotide and the enzyme were thus thermostatted at 37 °C in sealed microtubes. Periodically samples were transferred in a 0.6 ml cuvette for the UV reading, then reintroduced in the microtubes. Samples were withdrawn at 21, 42 and 76 h and treated as previously described for the HPLC measurements. Figure 5 shows the findings of the UV hyperchromic analysis.

In this experiment the differences between phosphorothioates and the other kind of protections are less sensible. The HPLC produced a different profile of degradation compared with the previous analysis, indeed we could only see a unique fast eluting

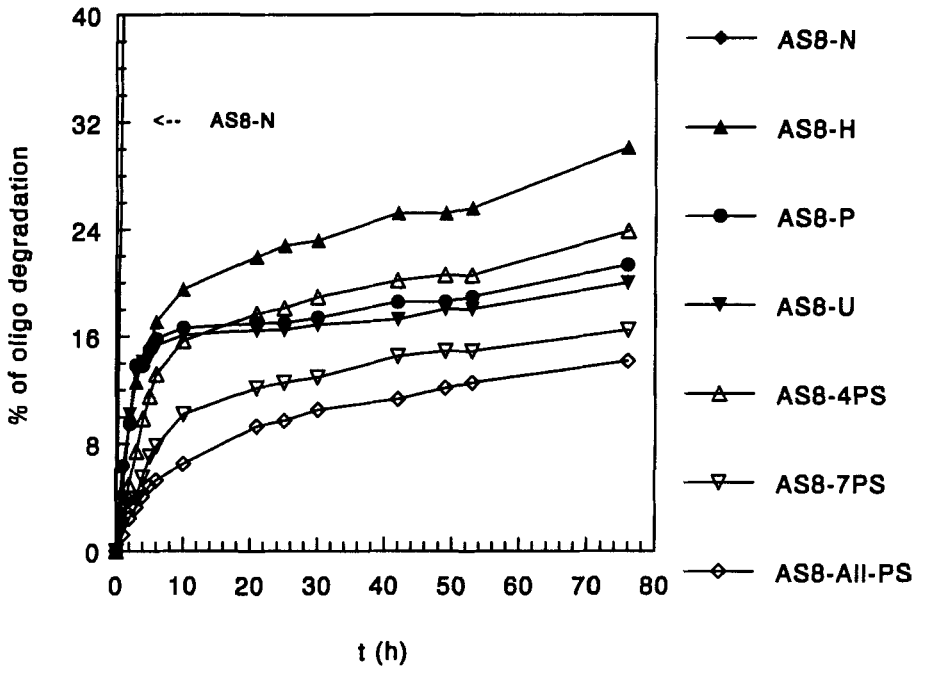


Figure 5:

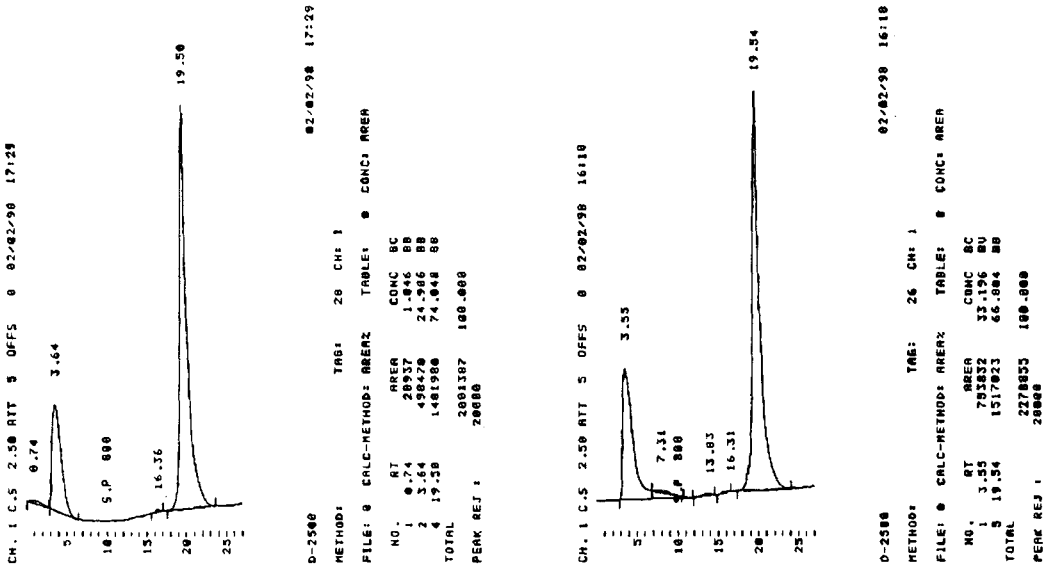
Degradation of oligonucleotides by CSPDE. AS8-N is completely digested in less than 6 h.

compound, increasing with time, corresponding to unresolved 3'-nucleotides (Figure 6). The beginning of the digestion was the rate determining step, as in the case with SVPDE.

Again the correlation between UV and HPLC was fair as shown in Table 5.

CONCLUSIONS

In this work we compared the enhancement of 3'- and 5'-exonuclease resistance conferred to oligodeoxyribonucleotides by end capping with propanediol, hexanediol, and 2'-3'-secouridine, by double end capping with phosphorothioate linkages, and by examples of "minimal protection" strategy. We found that although the complete phosphorothioate is the most stable oligonucleotide, the chimeric oligonucleotides still retain a good stability (more than 12 times the natural phosphodiester oligonucleotide) and could find useful applications in antisense experiments, especially those carried out



AS8-H + CSPDE after 21 h

AS8-H + CSPDE after76 h

Figure 6: HPLC chromatograms of AS8-E after 21 and 76 h of incubation with CSPDE

Table 5: Correspondence between UV and HPLC at 21, 42 and 76 h

Oligonucleotide	% of intact oligo after 21 h		% of intact oligo after 42 h		% of intact oligo after 76 h	
	UV	HPLC	UV	HPLC	UV	HPLC
AS8-N	43.5	40.0	0	0	-	-
AS8-P	83.0	83.3	81.4	82.4	87.0	80.9
AS8-H	78.0	78.2	74.8	78.5	69.9	71.6
AS8-U	83.5	84.8	82.7	84.0	80.0	82.1
AS8-4PS	82.3	82.2	79.8	79.6	76.1	76.3
AS8-7PS	87.8	87.8	85.5	85.4	83.5	83.6
AS8-All-PS	90.7	90.6	88.7	88.5	85.8	86.0

in cell cultures, where exonucleases are supposed to be the major responsible for oligonucleotide degradation, and in cases in which phosphorothioates are suspected to alter the "true antisense" response.

MATERIALS AND METHODS

Oligonucleotides were synthesized on a Pharmacia Gene Assembler II plus using commercial amidites. Aminohexyl-CPG was purchased from ChemGenes. Triethylammonium bicarbonate (TEAB) was prepared by passing CO_2 (g) through a 2 M solution of triethylamine (Fluka) in HPLC grade water at 0 °C until pH 7.4 was reached. DEAE Sephacel was purchased by Pharmacia, Dowex-50 W8 resin from Fluka. HPLC were performed with a Waters 600E instrument equipped with DEAE 5-PW column (Toso-Haas). NMR spectra were recorded whit a Varian VXR 200 MHz spectrometer. UV measurement were done with a Perkin Elmer 554 spectrophotometer equipped with a MGW Lauda RC5 thermostat and a MGW Lauda R40/2 digital thermometer; an electronic device was used to generate a linear gradient of temperature.

SVPDE was supplied from Pharmacia, CSPDE from Sigma.

TLC analysis was conducted on Merck 5719 silica gel (230-400 mesh) plates, flash chromatography was performed on Merck 9385 silica gel (230-400 mesh). Pyridine was distilled from CaH_2 and stored over 4 Å molecular sieves. All other chemicals were of commercial grade and used as received.

The following abbreviations are used through the text or figures: DMT = 4,4'-dimethoxytrityl, Bz = benzoyl, Py = pyridine, Et = ethyl, Ac = acetyl, Me = methyl, DIPEA = ethyldiisopropyl amine.

Benzoylation of 5'-O-(4,4'-dimethoxytrityl)-2',3'-secouridine (compounds **2-4** in Table 2)

3.61 g of compound **1**[32,33] (Table 2), were co-evaporated three times with anhydrous pyridine, then dissolved, under nitrogen, in 33 ml of a 1/1 mixture of Py and Et_2O. To this mixture cooled to -10 °C were added, under stirring, 0.728 ml of BzCl (1 equivalent). After 4 h the reaction was quenched with few drops of MeOH. We

observed the formation of three new compounds in a 2/1/1 ratio corresponding to **2**, **3** and **4** (Table 2). The reaction was stopped when a 10-15% of the starting material was still present. The mixture was concentrated till 2-3 ml of volume then diluted with CH_2Cl_2 and washed with a 10% w/v aqueous citric acid solution, then with a 5% w/v $NaHCO_3$ aq. solution then with water. The organic phase was dried with Na_2SO_4 concentrated and triturated with cyclohexane/Et_2O 8/2. The precipitate was purified on a silica gel column eluting with CH_2Cl_2/MeOH 95/5. We obtained 0.80 g of **4** R_f= 0.75; 1.50 g of **2** (46% of the converted material, 36.8% total yield) R_f= 0.4; 0.77g of **3** R_f= 0.2; and 0.5g of **1** R_f= 0.1.

2:

^{1}H-NMR in $CDCl_3$, δ(ppm): 7.95 m 2H (H-6, $^3J_{H-H}$ = 8.05 Hz, H-p of Bz), 7.6-7.1 m 13H (Ar-H of DMT, Ar-H of Bz), 6.75 m 4H (Ar-H in o to OMe), 6.25 t 1H (H-1'), 5.6 d 1H (H-5, $^3J_{H-H}$ = 8.05 Hz), 4.5 m 2H (H-2'), 3.8-3.6 m 9H (6 OCH_3, 2H-3', H-4'), 3.2 d 2H (H-5').

^{13}C-APT in $CDCl_3$ (77ppm). δ(ppm): 165.9 (-COO-), 162.9 (C-2), 158.6 (C-4), 150.6 (C-4 e C-4' of DMT), 144.4 (C-1'' of DMT), 139.6 (C-6), 135.5 (C-1 e C-1' of DMT), 133.5-129.7 (Ar C-H), 129.0 (C-1 of Bz), 128.6-113.2 (Ar C-H), 102.8 (C-5), 86.7 (C quat of DMT), 82.0 (C-1'), 80.4 (C-4'), 64.0 (C-2'), 63.4 (C-3'), 62.4 (C-5'), 55.2 (OCH_3).

3:

^{1}H-NMR in $CDCl_3$, δ(ppm): 7.94 m 2H (H-6, $^3J_{H-H}$ = 8.05 Hz, H-p of Bz), 7.6-7.1 m 13H (Ar-H of DMT, Ar-H of Bz), 6.75 m 4H (Ar-H in o to OMe), 5.95 t 1H (H-1'), 5.5 d 1H (H-5, $^3J_{H-H}$ = 8.05 Hz), 4.7-4.3 m 2H (H-3'), 3.9-3.5 m 9H (6 OCH_3, 2H-2', H-4'), 3.28 d 2H (H-5').

^{13}C-APT in $CDCl_3$ (77ppm). δ(ppm): 166.2 (-COO-), 163.6 (C-2), 158.4 (C-4), 151.4 (C-4 e C-4' of DMT), 144.3 (C-1'' of DMT), 140.1 (C-6), 135.5 (C-1 e C-1' of DMT), 133.2-129.6 (Ar C-H), 129.4 (C-1 of Bz), 128.3-113.1 (Ar C-H), 102.5 (C-5), 86.6 (C quat of DMT), 83.8 (C-1'), 76.6 (C-4'), 63.2 (C-3'), 63.0 (C-2'), 62.8 (C-5'), 55.1 (OCH_3).

Preparation of amidites

The amidites of the DMT-propanediol, -hexanediol and **2** were prepared in the following way: the corresponding DMT-derivative was coevaporated three times with

anhydrous Py, then dissolved under nitrogen atmosphere in 5 ml per mmol of CH_2Cl_2 and 5 equivalents of DIPEA and 1.8-2.0 equivalents of N,N-diisopropyl-(2-cyanoethyl)-chlorophosphite (prepared as described by Koster[36]) were added. The progress of the reaction was followed by TLC analysis, then the reaction was quenched with few drops of water, diluted with CH_2Cl_2 and washed with 5% w/v aq $NaHCO_3$ then chromatographed on silica gel with EtOAc/cyclohexane/Et_3N 6/3/1 v/v/v. The solvent was then evaporated to obtain the pure amidites as judged by ^1H and ^{31}P-NMR spectra.

Preparation of CPG derivatives

The corresponding DMT derivatives of propanediol, hexanediol and benzoyl-2',3'-secouridine were linked to the aminohexyl- (or aminopropyl-) CPG preparing the corresponding succinyl p-nitrophenol-derivative, using DCC as condensing reagent, according to the described preparation of CPG-propanediol[22, 31]. After the synthesis and extensive washing of unreacted reagent, the CPG was capped with Ac_2O, DMAP in Py (0.6 ml, 30 mg 3 ml per g of CPG respectively) for 2-3 h. (To obtain a good loading with the hexanediol we had to carry out the reaction overnight). The CPG was then washed again and checked with the ninhydrine test. The final loading was measured through the absorbance of the released DMT group after treatment with 3% DCA in CH_2Cl_2 (v/v).

Starting with an initial loading of 90 μM/g of free NH_2, we obtain after coupling, and capping with Ac_2O and DIPEA, a final load of 54, 51 and 41 μmol/g, for CPG-***p***, -***h***, and -***u*** respectively).

Synthesis and purification of oligonucleotides

The natural, the end-capped and the phosphorothioate oligonucleotides were synthesized on a Pharmacia gene assembler II-plus using the manufacturer protocols on a 1.3 μmol scale with commercially available amidites. After deblocking, the oligodeoxynucleotides were purified on an anion exchange chromatographic column on DEAE Sephacell (Pharmacia) using a gradient of TEAB. A 10% CH_3CN was added for the phosphorothioate containing compounds. Fractions more than 96% pure at 260 nm by HPLC analysis on (DEAE 5-PW Toso-Haas) were pooled, coevaporated with water to remove the excess of TEAB, converted to sodium salt by using a Dowex-50 W8

resin, and finally lyophilized. The RNA was purified as, described in the text, applying the protocol developed by Sproat[35].

UV experiments

UV measurements were done with a Perkin Elmer 554 spectrophotometer equipped with a MGW Lauda RC5 thermostat and a MGW Lauda R40/2 digital thermometer; an electronic device was used to generate a linear gradient of temperature. Equimolar amounts of each strand were mixed in 0.1M Tris·HCl, 0.1M NaCl, pH 7.0; the final concentration was approximately 3.0 μM per strand (assuming an ε_M = 187000 for every oligonucleotide[37]. The cells were heated to 80 °C for 15 minutes, then slowly cooled down. The thermal profile was registered using a gradient of temperature of 0.5 °C/min, from 18 to 80 °C.

Degradation with SVPDE and HPLC analysis

UV measurements were performed dissolving each oligonucleotide in a buffer containing: 50 mM NaCl, 50 mM Tris-HCl, 7 mM $MgCl_2$, pH 8.6 until a concentration of 1 OD/ml. To this solution was added the enzyme (Pharmacia 0.1 U/OD/ml) previously dissolved in 110 mM NaCl, 110 mM Tris-HCl, 15 mM $MgCl_2$, pH 8.9 and an equal volume of glycerol[38]. Absorbance values were followed maintaining the cuvettes at 37 °C. HPLC analysis were performed on a TosoHaas DEAE 5PW using UV detection at 260 nm. As eluent were used the following solutions: **A** 0.02 M $NaClO_4$, 0.02M NH_4OAc, in H_2O/CH_3CN 9/1, **B** 0.6 M $NaClO_4$, 0.02M NH_4OAc, in H_2O/CH_3CN 9/1. We used the following gradient 100% **A** for 2 min then to 40% **B** in 20 min then isochratic. **AS8-7PS** and **AS8-All-PS** were analyzed whith the gradient 100% **A** to 54% **B** in 31 min then isochratic.

For the UV reading we used the following formula: molar fraction of degraded oligo at time t = $(A_t-A_0)/(A_\infty-A_0)$. As we worked with substantially the same base sequence we used the value of the absorbance of AS8-N after 24 h of treatment with SVPDE as value of A_∞ for all the compounds. We used the ratio A_∞/A_0 found to be 1.24 to correct the integral ratios obtained from the HPLC analys in the following way: ratio of intact oligo = (area of the intact oligo) / (area of the intact oligo + (area of degraded peaks)/ 1.24).

Degradation with CSPDE

Each oligonucleotides was dissolved in 1.2 ml of buffer containing 0.125 mM sodium-citrate-HCl pH 6.5[39,40] until a concentration of 1 OD/ml then 30.0 µl of enzyme ((10 U in 140 µl) diluted with other 300 µl of water) were added and the sealed microtubes were kept at 37 °C. Periodically 500 µl were transferred in a microcuvette (1 cm × 0.2 cm × 3 cm) quantified at 260 nm then reintroduced in the in the thermostatted tubes. UV and HPLC calculation were performed as above described.

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