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Sequencing of oligonucleotide phosphorothioates based on solid-supported desulfurization

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ABSTRACT

We described a solid-supported desulfurization procedure allowing easy access to the sequence analysis of oligonucleotide phosphorothioates. The described method is based upon selective removal of the 2-cyanoethyl phosphate protecting groups, followed by iodine-promoted desulfurization of the resulting phosphorothioate diesters. Automatic oxidation of oligonucleotide phosphorothioates, anchored via an ester linkage to a standard solid support (LCAA/CPG), is combined with Maxam-Gilbert solid-support sequencing. The overall procedure allows rapid simultaneous sequence analysis of several oligonucleotide analogs.

INTRODUCTION

Investigation of oligonucleotide phosphorothioates as antisense therapeutic agents (1) has created a challenge associated with their large-scale synthesis, purification and analysis. Improvements in synthesis methods have increased efficiency (2), but the highly automated synthetic process must be monitored to ensure correct sequence of the products. This is especially crucial from a therapeutic point of view, where sequence fidelity is a key regulated parameter. Phosphorothiodiester resistance to nucleases and increased stability to the base-catalyzed hydrolysis (3) prevent effective use of the standard enzymatic sequence analysis methods (4) or use of direct chemical sequencing methods (5). Mass spectrometric sequencing of small oligonucleotides and their analogs appears a promising technique, but low abundance of fragment ions in the 500–4000 m/z range represents a severe limitation for objectives other then molecular weight determination (6).

In this report, we show that these problems can be overcome by conversion of the oligonucleotide phosphorothioate to the corresponding phosphodiester, followed by Maxam-Gilbert chemical sequencing.

RESULTS AND DISCUSSION

Several laboratories have studied oxidation of a range of phosphorothioate diesters (2). The methods described were applied mainly to oligonucleotides containing one or two phosphorothioate linkages. All reported procedures were carried out in solution, and required tedious, small scale purification of the oxidation products, prior to further analysis. In our opinion, existing desulfurization methods are unsatisfactory for routine sequencing analysis.

As an alternative we investigated oxidation of the oligomer while still attached to a synthesis solid support (*LCAA/CPG* (7)). We felt this would allow quick separation of desulfurized products from the reaction mixture, and should minimize a reported problem with their degradation (8,9). Iodine-promoted oxidation of phosphorothioate esters occurs under mild conditions and we chose this reaction as well suited to a solid support approach.

The protection strategy typically used with phosphoramidite chemistry employs base-labile, permanent protecting groups for phosphate (2-cyanoethyl) and aglycone residues (N-acyl) in combination with a succinate ester linkage, anchoring oligonucleotide to the solid support, cleavable also under basic conditions (2).

The phosphorothioate triesters, as opposed to the corresponding diesters, cannot be desulfurized to the phosphodiesters when oxidation is performed using iodine (10,11). Thus desulfurization of the oligonucleotide phosphorothioate triesters on the support required the phosphate protecting groups to be removed prior to desulfurization, in a selective fashion, without hydrolysis of the ester linkages. We found that a mixture of tert-butylamine in pyridine (1;9, v/v), previously shown selective for 2-cyanoethyl group removal from phosphotriesters (12), also works for phosphorothioate triesters. Selective removal of the 2-cyanoethyl groups was verified by ^{31}P NMR, following cleavage from support and was found to be complete after 90 minutes at 25°C. Stability of the succinyl ester linkage of the support was confirmed by monitoring the total amount of DMT groups present on the support (4 hours deprotection time did not result in detectable linker cleavage).

The CPG-bound oligonucleotide phosphorothioates, used in the described studies, S-d(TTGCTTCCATCTTCCTCGTC) (**1**), S-d(GCGTTTGCTCTTCTTCTTGCG) (**2**) and their corresponding phosphodiester analogs d(TTGCTTCCATCTTCCTCGTC) (**3**) and d(GCGTTTGCTCTTCTTCTTGCG) (**4**), were synthesized using standard phosphoramidite chemistry (2). 3*H*-1,2-Benzodithiol-3-one1,1-dioxide sulfurizing agent was used (13).

Multiple, iodine-based desulfurization conditions were investigated. The model phosphorothioate (**1**) was oxidized with:

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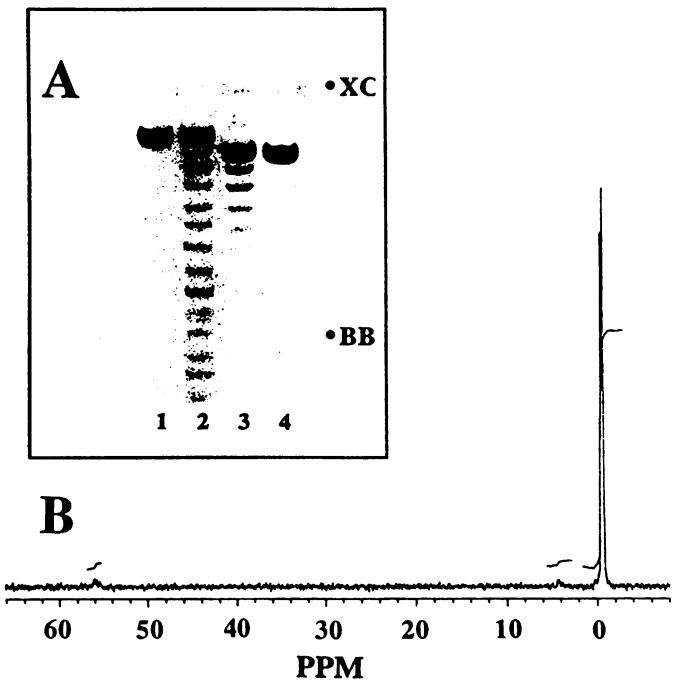

Figure 1. Analysis of the automatically desulfurized oligonucleotide phosphorothioate S-d(TTGCTTCCATCTTCCTCGTC) (**1**), analyzed by PAGE (20% gel, 7.5 urea) (**A**) and ^{31}P NMR (**B**). **A.1.**—purified (**1**); **A.2.**—crude (**1**), **A.3.**—crude desulfurized (**1**) and **A.4.**—purified phosphorodiester analog of (**1**). **B**—crude desulfurized (**1**). Oligonucleotides were visualized with Stains-All. The positions of the length markers, xylene cyanol FF dye (XC) and bromophenol blue dye (BB) are indicated.

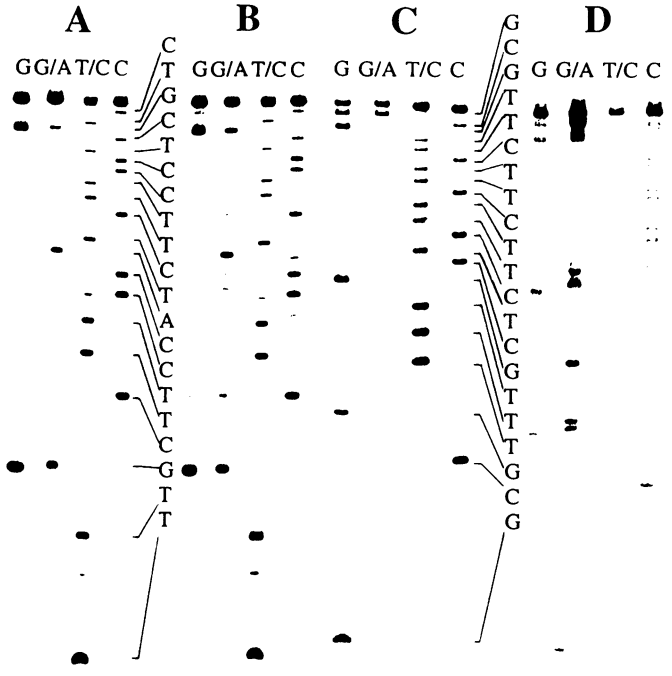

Figure 2. Computer-derived images of the cleavage patterns from sequencing of the oligonucleotide phosphorothioates S-d(TTGCTTCCATCTTCCTCGTC) (**1**) and S-d(GCGTTTGCTCTTCTTCTTGCG) (**2**). **A**—desulfurized (**1**); **B**—phosphodiester analog of (**1**); **C**—desulfurized (**2**); **D**—direct sequencing of the oligonucleotide phosphorothioate (**2**). Analyzed oligonucleotide phosphorothioates were desulfurized automatically on the DNA synthesizer and sequenced by Maxam–Gilbert method on the Hybond™ M&G paper (16).

1) 0.24 M solution of iodine in a mixture of ethanol/5% sodium bicarbonate in water (0.125;3, v/v) (14), 2) pyridine/water (1;2, v/v) (10) and 3) THF/water/1-methylimidazole (16;3;1; v/v/v) (15). After 60 minutes iodine solutions were removed by washing. After final deprotection, the products of oxidation were analyzed by polyacrylamide gel electrophoresis (PAGE), ^{31}P NMR and ion-exchange HPLC (data not shown). Conditions 1) and 2) removed only 37% and 88% of sulfur, respectively . The best result was obtained using conditions 3), giving a conversion yield of 96.0%. Further optimization of these conditions to increase water content and oxidation time (0.24 M I_2 in THF/water/1-methylimidazole; 16;6;1, v/v/v, 130 min.), gave conversion yields of 99.6–99.8%.

The desulfurization procedure has been automated and can be directly performed using a standard DNA synthesizer. After completion of the synthesis, sample of a CPG-bound fully-protected oligonucleotide phosphorothioate (**1**) and (**2**) (1 μmol of each) was placed in two disposable columns (as used for DNA synthesis in 1 μmol scale) and subjected to desulfurization. The automatic cycle included the following steps:

1) Wash. Acetonitrile (1 ml). *2) Removal of 2-cyanoethyl protecting groups.* CPG was washed with a 10% solution of tert-butylamine in pyridine (10 washes, 0.8 ml each) was passed through the column during 110 minutes. *3) Wash.* Acetonitrile (1 ml). *4) Desulfurization.* The CPG was washed with a 0.24M solution of iodine in THF/water/1-methylimidazole (13 portions of the oxidizing solution (0.77 ml each) and passed through the column for 130 minutes. The desulfurization mixture was prepared and stored for use as separate solutions: (0.48 M solution of I_2 in the mixture of THF/water; 16;6, v/v) and (1-methylimidazole/ THF/water (2;16;6, v/v/v). The solutions were premixed during delivery to the column. This procedure was necessary to ensure successful oxidation. *5) Wash.* Acetonitrile (1 ml).

Automated oxidation of oligonucleotide phosphorothioates (**1**) and (**2**) yielded oligonucleotide phosphodiesters d(TTGCTTC-CATCTTCCTCGTC) (**5**) and d(GCGTTTGCTCTTCTTCTT-GCG) (**6**), with desulfurization yield equal to 99.4% and 99.6%, respectively (^{31}P NMR) (Figure 1). 5′-O-Dimethoxytrityl (DMT) protecting group was removed on the solid support prior to final deprotection. Oligomers (**1**)–(**6**) were deprotected and cleaved from support under standard conditions (30% ammonium hydroxide, 16 hours at 55°C). Oligonucleotides (**2**), (**3**), (**4**) and (**5**) (1 O.D. of each) were ^{32}P-labeled in a standard kination buffer (50 mM Tris–HCl pH9.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM spermidine–HCl, 0.05 mM EDTA) (20μl), containing [γ-^{32}P] ATP (60 μCi) and T4 polynucleotide kinase (2 units).

Labeled oligomers were purified using PAGE, and sequenced on an ion-exchange Hybond™ M&G paper (Amersham), by the Maxam-Gilbert method, according to manufacturer's procedure (16). Hybond™ M&G allowed, simultaneous sequencing of several oligonucleotides. Oligonucleotide fragments generated by the sequencing procedure were fractionated by PAGE (20% gel, 7.5 M urea). The computer-derived images of the sequencing gels (Figure 2) were obtained with the aid of a phosphorimager (Molecular Dynamics) (17). The cleavage patterns obtained for

desulfurized oligonucleotide phosphorothioates (**1**) and (**2**) (Figure 2.A,C) were identical with patterns generated for their corresponding phosphodiester analogs (**3**) (Figure 2.B) and (**4**) (data not shown). Obtained cleavage patterns proved that desulfurization process does not affect the results of analysis. Attempted Maxam–Gilbert direct sequencing of the fully thioated oligonucleotide (2) (without prior oxidation) failed, leading to inconclusive results shown at Figure 2.D.

In summary, we have established a rapid and effective method for sequence analysis of oligodeoxynucleotide phosphorothioates. The procedure utilizes automated desulfurization of a small sample of the product. After deprotection, labeling and purification, oxidized oligomers are immobilized on an ion-exchange carrier and sequenced by the Maxam-Gilbert method. The described method allows determination of the sequence and detection of the incorrectly incorporated nucleotide syntons. This is a vital step in the analysis of therapeutic oligonucleotides (18). The described method is not intended to be a measure of purity due to a low sensitivity of chemical sequencing. The iodine-mediated mechanism of oxidation should be similar for phosphorothioate, phosphorodithioate (8,15) and phosphoroselenoate diesters (19). Thus, the described procedure could possibly be extended to sequencing of these oligonucleotide classes.

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