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Antisense Phosphorothioate Oligodeoxyribonucleotide Targeted against ICAM-1: Use of I-Linker to Eliminate 3′-Terminal Phosphorothioate Monoester Formation

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Abstract:

Detritylation of a 5′-*O*-DMT-2′-deoxyadenosine moiety attached to solid support under acidic conditions (3% dichloroacetic acid/solvent) leads to depurination during oligonucleotide synthesis. Deprotection followed by reversed phase HPLC purification leads to desired oligonucleotide contaminated with significant levels of 3′-terminal phosphorothiaote (3′-TPT) monoester (*n* − 1)-mer. However, it is demonstrated that attachment of dA nucleoside through its exocyclic amino group to solid support leads to substantial reduction of 3′-TPT formation, thereby improving the quality of oligonucleotide synthesized.

Introduction

Antisense oligonucleotide as gene-expression modulators represent a new therapeutic paradigm. In recent years DNA analogues, especially phosphorothioate oligonucleotides in which a nonbridging oxygen of the internucleotide phosphate group is formally replaced by sulfur, have emerged as potential drugs for treatment of a variety of diseases through an RNAse H-mediated antisense mechanism of action.[1] With the first antisense drug (Vitravene) approved by the U.S. Food and Drug Administration for treatment of CMV retinitis in AIDS patients and several systemic drugs potentially reaching market in the next several years, development of economical and environmentally safe methods for synthesis of high-quality oligonucleotides has been a major focus of our research.[2] Typically, oligonucleotide synthesis on scales up to 300−600 mmol is performed in a cyclic manner on automated solid-phase synthesizers using phosphoramidite derivatives of protected nucleosides with 4,4′-dimethoxytrityl (DMT) protection of the 5′-hydroxyl group, benzoyl protection for adenine (dA^{bz}) and cytosine (dC^{bz}) and isobutyryl for guanine (dG^{ibu}).

ISIS 2302 (**1**) is a phosphorothioate oligodeoxyribonucleotide 20-mer [d(GCCCAAGCTGGCATCCGTCA) targeted to the 3′-untranslated region of human intercellular adhesion molecule-1 (ICAM-1) mRNA to selectively inhibit ICAM-1 gene expression. Clinical evaluation of oligonucleotide **1** in humans as potential therapy for a variety of diseases [Crohn's (phase III), ulcerative colitis (phase II) and psoriasis (phase II)] is currently underway.

In the synthesis of phosphorothioate oligodeoxyribonucleotides such as **1** through phosphoramidite coupling, removal of the acid-labile 4,4′-dimethoxytrityl 5′-protecting group (DMT) from support-bound nucleotide is crucial in each synthesis cycle for achieving high product yield and oligonucleotide quality. Although several reagents have been developed for this purpose, many have limited applicability to automated oligonucleotide synthesis on solid supports. The most commonly used reagents today are dilute solutions (2−15%) of an organic acid, typically trichloroacetic acid (TCA, pK_a 0.8) or dichloroacetic acid (DCA, pK_a, 1.5), in dichloromethane. Recently we have shown that toxic dichloromethane can be replaced by a relatively safe solvent such as toluene without compromising yield and quality of synthesized oligonucleotide.[3] This detritylating condition has been scaled to 300 mmol for routine synthesis of oligonucleotides such as **1**.

Depurination. Cleavage of the N-glycosyl bond between a base and its 2′-deoxyribose moiety in DNA generates an apurinic/apyrimidinic (abasic or AP) site. This phenomenon referred to as depurination or depyrimidination occurs spontaneously under physiologic conditions. Lindahl and Nyberg[4] have measured a rate constant for DNA depurination of 4×10^{-9}/s at 70 °C and pH 7.4 in a Mg^{2+}-containing buffer of physiologic ionic strength. Abasic sites may be generated chemically at random sites in DNA by acid or heat treatment and irradiation. AP sites can also be introduced

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(1) (a) Crooke, S. T. In *Antisense Therapeutics in Biotechnology & Genetic Engineering Reviews*; Intercept Ltd: Hampshire, UK, 1998; Vol. 15, pp 121−157. (b) Crooke, S. T. In *Basic Principles of Antisense Therapeutics in Handbook of Experimental Pharmacology: Antisense Research & Application*; Crooke, S. T., Ed.; Springer-Verlag: Berlin, Germany, 1998. (c) Crooke, S. T. *Antisense Nucleic Acid Drug Dev.* **1998**, *8*, 115. (d) Crooke, S. T. In *Burger's Medicinal Chemistry and Drug Discovery*; John Wiley: New York, 1995 and references therein.

(2) (a) Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem.* **1999**, *7*, 435. (b) Krotz, A. H.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Green Chem.* **1999**, 277. (c) Ravikumar, V. T.; Krotz, A. H.; Cole, D. L. *Tetrahedron Lett.* **1995**, *36*, 6587. (d) Turney, B. J.; Cheruvallath, Z. S.; Andrade, M.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 89. (e) Cheruvallath, Z. S.; Wheeler, P. D.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 485. (f) Eleuteri, A.; Capaldi, D. C.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 475. (g) Ravikumar, V. T.; Andrade, M.; Wyrzykiewicz, T. K.; Scozzari, A. N.; Cole, D. L. *Nucleosides Nucleotides* **1995**, *14*, 1219. (h) Krotz, A. H.; Klopchin, P.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 73. (i) Krotz, A. H.; Klopchin, P.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1997**, *16*, 1637. (j) Capaldi, D. C.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **1999**, *3*, 485. (k) Eleuteri, A.; Capaldi, D. C.; Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 182. (l) Eleuteri, A.; Cheruvallath, Z. S.; Capaldi, D. C.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 1803.

(3) (a) Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem.* **1999**, *7*, 435. (b) Krotz, A. H.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Green Chem.* **1999**, 277. (c) Krotz, A. H.; Carty, R. L.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 190.

(4) Lindahl, T.; Nyberg, B. *Biochemistry* **1972**, *11*, 3610.

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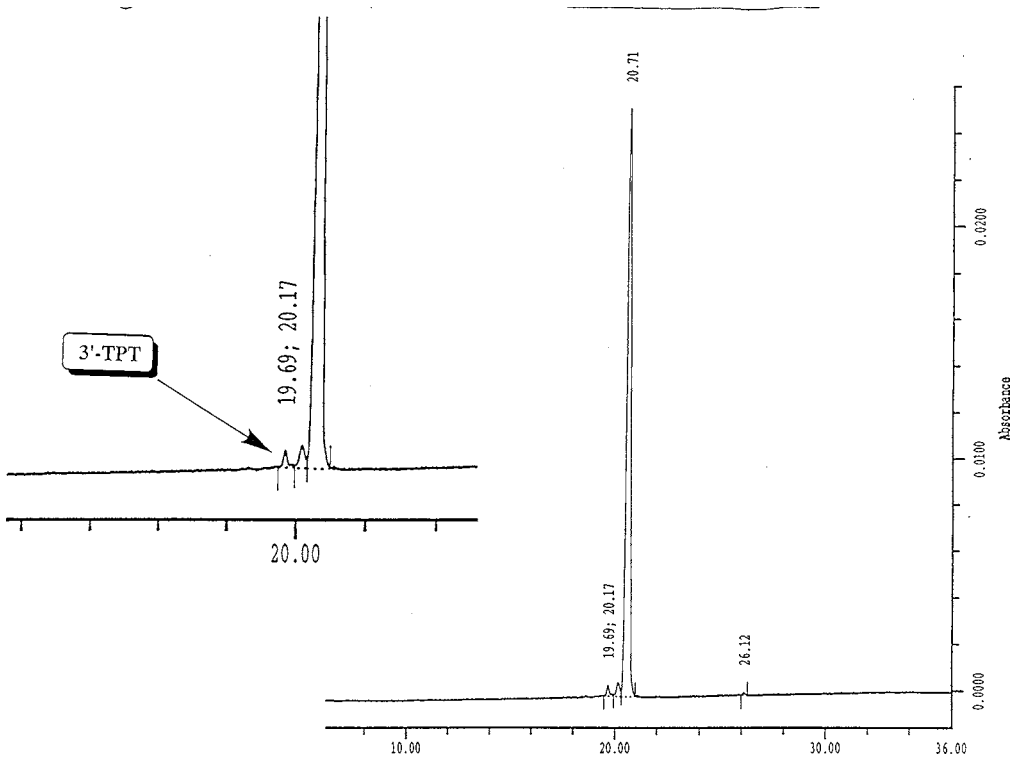


Figure 1. **CGE analysis of phosphorothioate oligonucleotide ISIS 2302 active pharmaceutical ingredient.**

in DNA by some antitumor antibiotics, including bleomycin which binds to DNA and causes breakage of thymine−deoxyribose bonds. The 2′-deoxyribose residue remaining after glycosidic bond hydrolysis exists in a ring-opened (aldehyde) and ring-closed (furanose) tautomeric equilibrium, with the former species highly base-labile. Cleavage of the sugar−phosphate backbone at such sites occurs readily via β-elimination. Support-bound oligonucleotides undergo cleavage of the N-glycosidic bond in N^6-benzoyl deoxyadenosine and to a lesser extent in N^2-isobutyryl deoxyguanosine.[5] At the nucleoside level, acidic hydrolysis of 2′-deoxyguanosine and 2′-deoxyadenosine involves rapid initial protonation of the base moiety followed by rate-limiting hydrolysis of the N-glysosidic bond of the resulting mono- and dication to give free purine bases and a resonance-stabilized oxonium ion which hydrolyzes to form a mixture of α− and β-2-deoxyribofuranoses. For adenine nucleosides the first protonation takes place at N1 and the second protonation at N7, and for guanine nucleosides the first protonation takes place at N7 and the second at N3. While this reaction proceeds more rapidly in acid than in neutral solution for all deoxyribonucleosides, the rates of hydrolysis of pyrimidine derivative are much less pH-dependent than those of purine derivatives. Depurination of short oligonucleotides with dA or dG located at terminal positions was found to be 3−4.5 times faster than that for internal residues. In contrast, chain breakage via β-elimination was 3.5−3.7 times faster at internal abasic sites. Electron-withdrawing phosphate groups on the 5′ side of an apurinic site facilitate chain breakage. From model studies on short DNA sequences bound to controlled-pore glass (CPG) support, it has been observed that depurination at dA occurs to the greatest extent when a dA residue is attached to solid support via the 3′-hydroxyl through an acyl linker. Once a subsequent nucleotide is added, glycosidic bond stability goes up markedly due to the electron-withdrawing effect of the added phosphotriester group. Depurination of dA^{bz} bound to CPG is ca. 5−6 times faster than dG^{ibu} bound to CPG. Thus, it is necessary to keep deblocking conditions as mild as possible (low acid strength, short exposure times) in order to minimize side depurination.[6]

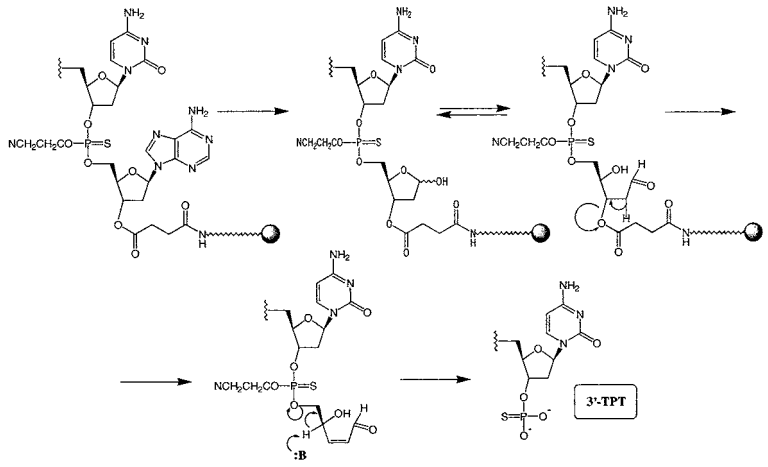
3′-Terminal Phosphorothioate Monoester. During the synthesis and analytical control of phosphorothioate oligonucleotide **1** for clinical evaluation, we observed by capillary gel electrophoresis (CGE) of purified active pharmaceutical ingredient (API) a peak almost equal in area % to (n − 1)-mer but migrating faster (Figure 1). We reasoned that it could not be an (n − 2)- or an (n − 3)-mer.

Isolation of the unknown, following slab-gel electrophoresis and characterization by LC−MS revealed a species with a mass of 6438.5, consistent with a 3′-terminal phosphorothioate (3′-TPT) monoester of (n − 1)-mer of **1**. A reasonable hypothesis is that, during the first detritylation step, dA nucleoside attached to solid support undergoes significant depurination leading to an abasic site that on incubation with concentrated aqueous ammonium hydroxide at 55 °C undergoes fragmentation of the abasic site, leading to 3′-TPT monoester (see Scheme 1).

(5) (a) Zoltewicz, J. A.; Clark, D. F.; Sharpless, T. W.; Grahe, G. *J. Am. Chem. Soc*. **1970**, *92*, 1741. (b) Zoltewicz, J. A.; Clark, D. F. *J. Org. Chem*. **1972**, *37*, 1193. (c) Panzica, R. P.; Rousseau, R. J.; Robins, R. K.; Townsend, L. B. *J. Am. Chem. Soc*. **1972**, *94*, 4708. (d) Hevesi, L.; Wolfson-Davidson, E.; Nagy, J. B.; Nagy, O. B.; Bruylants, A. *J. Am. Chem. Soc*. **1972**, *94*, 4715. (e) Lonnberg, H.; Heikkinen, E. *Acta Chem. Scand*. **1984**, *B38*, 673. (f) Zielonacka-Lis, E. *Nucleosides Nucleotides* **1989**, *8*, 383.

(6) (a) Septak, M. *Nucleic Acids Res*. **1996**, *24*, 3053. (b) Paul, C. H.; Royappa, A. T. *Nucleic Acids Res*. **1996**, *24*, 3048.

Scheme 1. **Mechanism of formation of 3′-TPT**



Results and Discussion

Testing the Hypothesis. To test the hypothesis, a phosphorothioate oligodeoxyribonucleotide (T_{19}dA) was first prepared. Three syntheses were performed on a Pharmacia OligoPilot II DNA/RNA synthesizer using standard cyanoethyl phosphoramidite synthon (0.2 M solution in acetonitrile) and 1*H*-tetrazole (0.45 M solution in acetonitrile) for activation. Commercially available Pharmacia HL 30 dA Primer Support (90 μmol/g loading) was used. During the first synthesis, a standard 2-min detritylation cycle was used (control experiment, oligo A). In the second synthesis, the first detritylation was extended to 30 min while keeping all subsequent detritylation conditions similar to those of control (oligo B). In the third synthesis, the first detritylation was further extended to 60 min while keeping all subsequent detritylation conditions similar to those of control (oligo C). Three percent dichloroacetic acid in toluene was used for removing dimethoxytrityl groups from the 5′-hydroxyl group. Unlike dichloromethane, which permits conductivity-based or UV-based DMT cation yields, no conductivity-based detritylation measurement of yields was available when toluene was used as the deblocking solvent. For sulfurization, phenylacetyl disulfide (PADS) in 3-picoline/acetonitrile (1:1, v/v, 1.25 column volume, 2-min contact time) was used.[7] At the end of a synthesis, oligonucleotide was deprotected, purified in the usual manner by C18 reversed phase HPLC, and all DMT-bearing oligonucleotide fractions were collected, detritylated, and analyzed by LC−MS and CGE analysis. Figures 2 and 3 show the CGE and LC−MS analyses of the three synthesized oligonucleotides, respectively. The data clearly demonstrate that depurination increases with increased acid contact time, as evidenced by increased levels of 3′-TPT oligomers in the product.

Currently, C18 reversed phase HPLC is used for purification of phosphorothioate oligonucleotides because it allows facile separation of 5′-DMT oligonucleotide from acyl-capped failure sequences. However, since 3′-TPT oligomer also possesses a 5′-DMT group, reversed phase HPLC does not readily discriminate between it and full-length oligomer. This is shown clearly in the case of phosphorothioate oligonucleotide **1** (Figure 1). Hence, we reasoned that the problem should be circumvented during synthesis and not during purification. We next hypothesized that if we eliminate the presence of the first depurinated residue, TPT formation should be controlled. Herein we report a convenient and general method for synthesis of oligodeoxyribonucleotides containing a dA residue at the 3′ terminus with very substantially reduced levels of 3′-TPT monoester impurities.

Attachment of Protected dA to Solid Support. Conventionally, 5′-DMT-protected deoxynucleosides are attached to solid supports such as CPG, Tentagel, or Primer Support through their 3′-hydroxyl groups via a succinyl or other linker. During our investigation of new base-protecting groups to reduce levels of depurination, we envisioned that 3′-TPT formation caused by depurination of dA residue attached to solid support could be eliminated or minimized by attaching the nucleoside to solid support through its exocyclic amino group. Any depurination caused by detritylation of the DMT group would lead to cleavage of the glycosidic bond and the sugar residue would be washed away. The heterocyclic moiety still attached to solid support is then incapable of elongation, thereby leading to overall synthesis of higher-quality oligonucleotides (see Scheme 2).

Recently, Pfleiderer et al. reported[8] a novel linker for oligonucleotide synthesis incorporating cyclic diacyl protection of exocyclic amino groups. However, our experience has shown that diacyl-protected bases are not stable to oligomerization conditions and hence cannot be used for efficient synthesis of high quality oligonucleotides. Hence, we devised an acyclic acyl-protecting group capable of attachment to solid support. Treatment of commercially available 3′-*O*-acetyl-5′-*O*-dimethoxytrityl-2′-deoxyadenosine with activated succinyl or glutaryl or adipoyl chloride led to cyclized diacyl compound instead of an open-chain molecule. Instead, use of activated dodecanedioyl dichloride led to the desired open-chain compound in moderate yields (50−55%) as the triethylammonium salt. Loading of dA nucleoside through this linker to the amino-derivatized

(7) Cheruvallath, Z. S.; Carty, R. L.; Moore, M. N.; Capaldi, D. C.; Krotz, A. H.; Wheeler, P. D.; Turney, B. J.; Craig, S. R.; Gaus, H. J.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 199.

(8) Waldvogel, S. R.; Pfleiderer, W. *Helv. Chim. Acta* **1998**, *81*, 46.

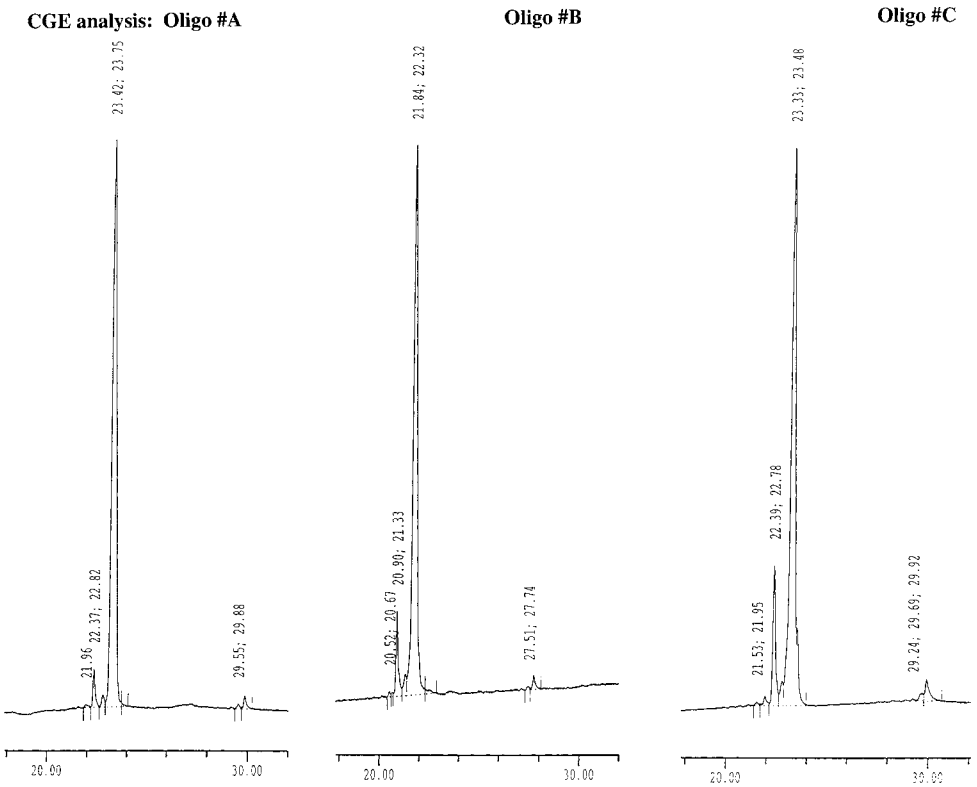


Figure 2. **CGE analysis of oligonucleotides synthesized using standard solid support.**

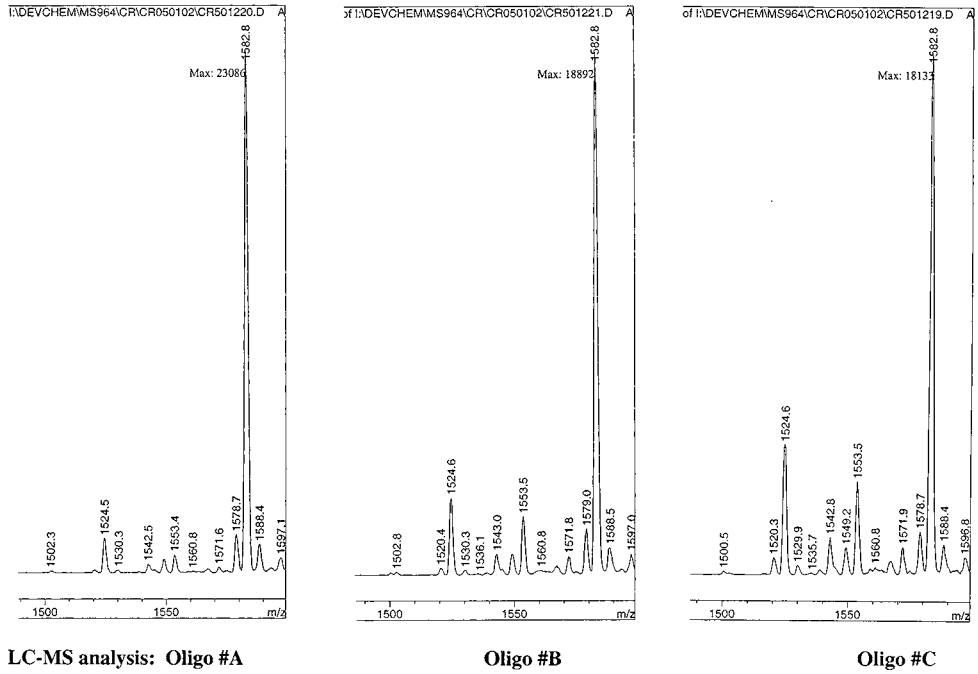
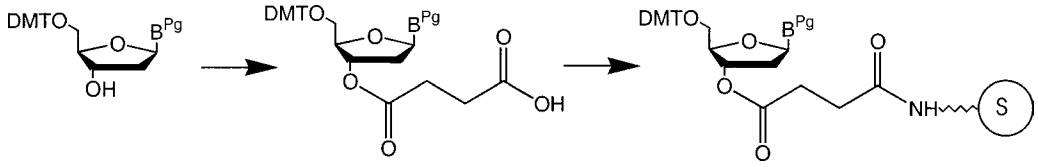


Figure 3. **LC−MS analysis of oligonucleotides synthesized using standard solid support.**

Scheme 2. **Standard method of oligonucleotide synthesis**



Primer Support under standard conditions afforded the desired dA-loaded support in 85−90 μmol/g loading. No further optimization of I-linker length was undertaken since we felt that the side chain should be stable to various

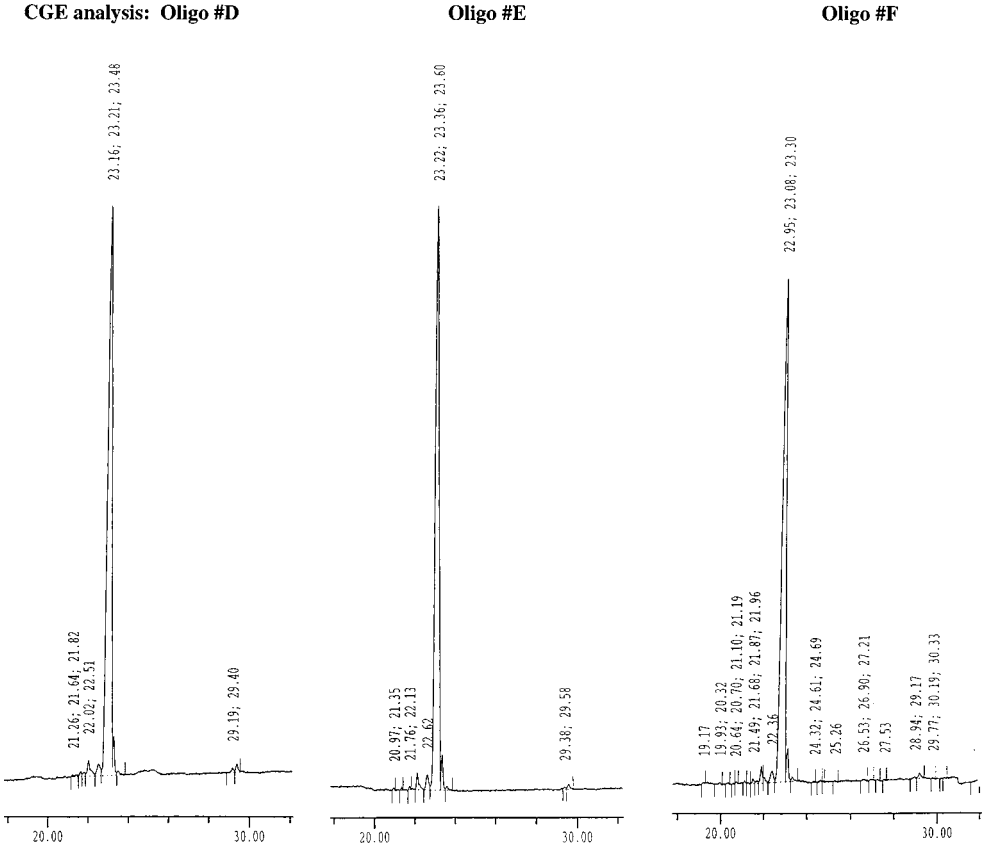
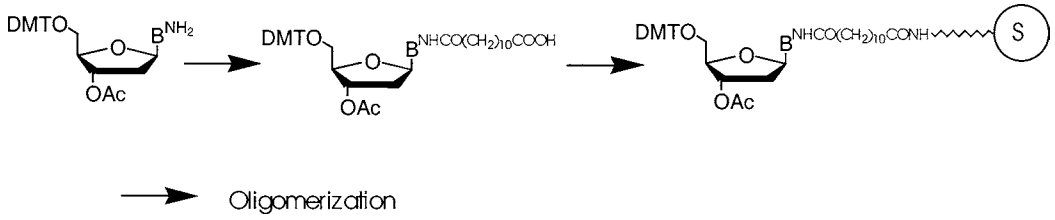


Figure 4. **CGE analysis of oligonucleotides synthesized using I-linker solid support.**

Scheme 3. **Oligonucleotide synthesis using I-linker solid support**



detritylating agents and would not influence the level of depurination (see Scheme 3).

To prove the hypothesis, the same phosphorothioate oligodeoxyribonucleotide (viz., T_{19}dA) was chosen. Three syntheses were performed on a Pharmacia OligoPilot II DNA/RNA synthesizer using the standard cyanoethyl phosphoramidite synthon (0.2 M solution in acetonitrile; 2 equiv) and 1*H*-tetrazole (0.45 M solution in acetonitrile) for activation. During the first synthesis, standard 2-min detritylation was used (control experiment, oligo D). In the second synthesis, the first detritylation was extended to 30 min while keeping all subsequent detritylation conditions similar to those of the control (oligo E). In the third synthesis, the first detritylation was further extended to 60 min while keeping all subsequent detritylation conditions similar to those of the control (oligo F). At end of synthesis, the oligonucleotide was deprotected and purified in the usual manner using C18 reversed phase HPLC; all DMT-bearing fractions were collected, detritylated, and analyzed by LC−MS and CGE analysis. No significant difference in the relative efficiency of cleavage of final oligomer product from solid support for the N-linked vs standard succinoyl attachment was observed. Figures 4 and 5 show CGE and LC−MS analyses of three synthesized oligonucleotides, respectively. Data show that the formation of 3′-TPT oligonucleotide could be eliminated using the I-linker solid support. We feel that the presence of negligible levels of 3′-TPT is due to inefficient capping of the solid support. Sites that have not been loaded with nucleoside and not capped completely would react with incoming phosphoramidite to form a P−N bond which, upon heating with ammonium hydroxide, would lead to 3′-TPT.

Syntheses of ISIS 2302. Finally, phosphorothioate oligodeoxyribonucleotide 20-mer [PS(GCC-CAA-GCT-GGC-ATC-CGT-CA), ISIS 2302] was synthesized using I-linker solid support as well as standard solid support (HL30 Primer). Figure 6 shows CGE analysis of oligonucleotides synthesized. No detectable difference in yield between the two experiments was observed as measured by weighing of the thoroughly dried solid support.[9]

(9) Experience has taught us that yields expressed in terms of weight/μmol of support are more reliable than those expressed in terms of optical density/μmol.

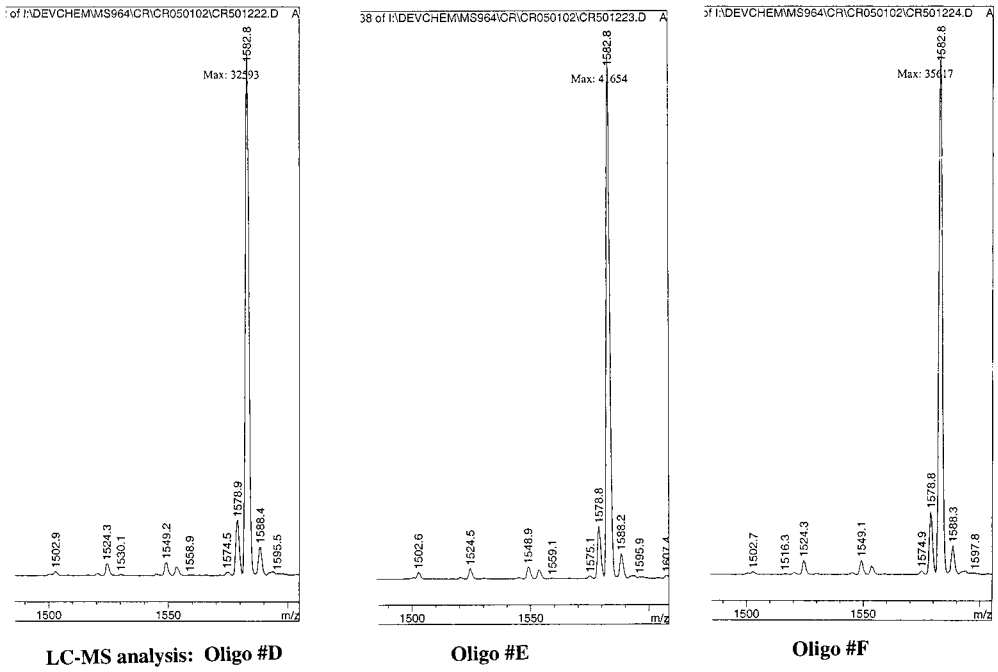


Figure 5. **LC−MS analysis of oligonucleotides synthesized using I-linker solid support.**

Synthesis of ISIS 2302

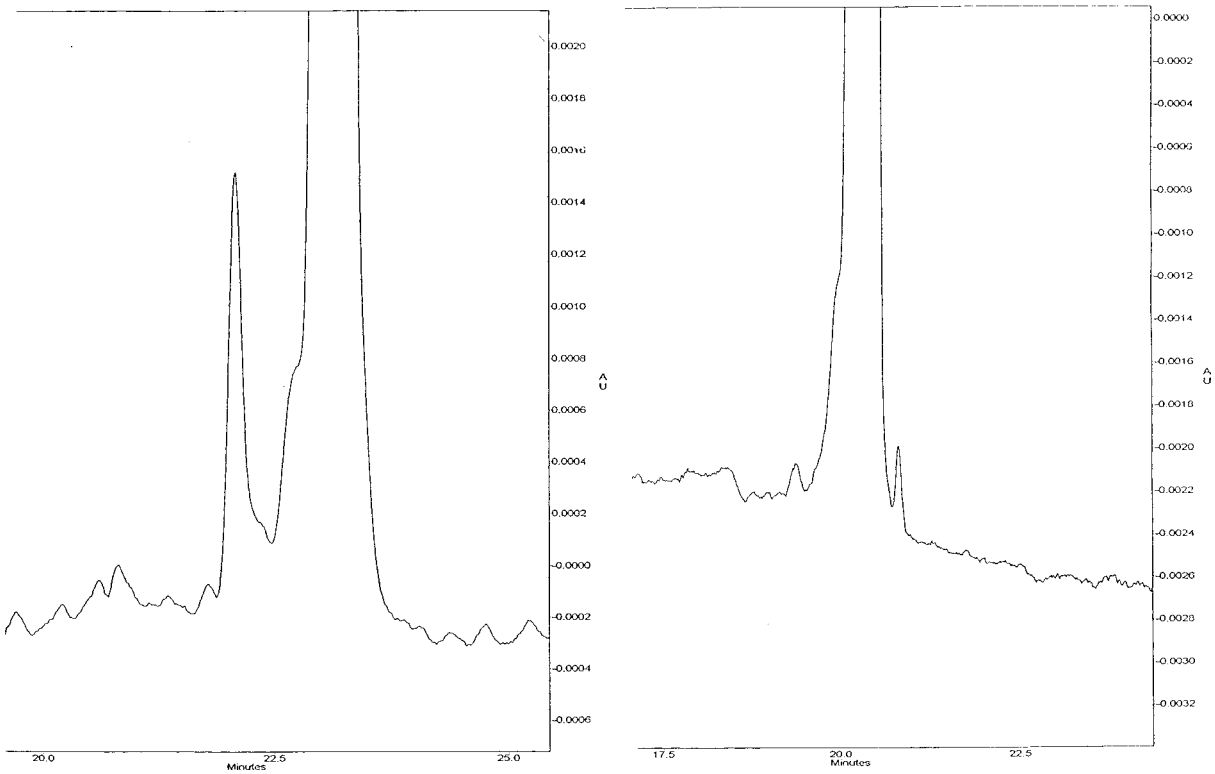


Figure 6. **CGE analysis of phosphorothioate oligonucleotide ISIS 2302 synthesized using I-linker solid support.**

Conclusions

As evident from the data presented in this work, high-quality oligonucleotides having dA at the 3′ end may be synthesized using this method. Similar 3′-TPT oligonucleotides of different length and base composition were also synthesized by using I-linker solid support and analyzed by LC−MS and found to be consistent with the above observation.

Experimental Section

General. All solvents were dried prior to use. Chemical reagents were purchased from Aldrich. NMR spectra were

recorded on a Varian Unity-200 instrument with TMS as internal standard. Capillary gel electrophoresis was performed on a eCAP ssDNA 100 gel capillary (47 cm) on a P/ACE System 5000 using the following conditions: tris/borate/7 M urea buffer (all Beckman), running voltage 14.1 kV, temperature 40 °C. Electrospray ionization mass spectrometry was performed using a Hewlett-Packard 59987A electrospray quadrupole mass analyzer using negative polarity.

5′-*O*-Dimethoxytrityl-3′-*O*-acetyl-2′-deoxyadenosine. The above compound was purchased from ChemGenes Inc. as a colorless amorphous solid.

^{1}H NMR (200 MHz, $CDCl_3$): δ 8.3 (s, 1H), 8.0 (1H), 7.2−7.5 (m, 9H), 6.8 (dd, 4H), 6.5 (dd, 1H), 6.1 (bs, 2H), 5.5 (bd, 1H), 4.3 (m, 1H), 3.8 (s, 6H), 3.4 (d, 2H), 2.9 (m, 1H), 2.7 (m, 1H), 2.1 (s, 3H).

^{13}C NMR (200 MHz, $CDCl_3$): δ 170.6, 158.8, 156.3, 153.3, 149.9, 144.7, 138.7, 135.8, 130.3, 128.4, 127.2, 120.1, 113.5, 86.9, 84.5, 75.5, 63.9, 55.4, 38.3, 21.2.

FAB-HRMS *m*/*z*: 595.25. Calculated for $C_{33}H_{33}N_5O_6$: 595.26.

Reaction of 5′-*O*-Dimethoxytrityl-3′-*O*-acetyl-2′-deoxyadenosine with Dodecanedioyl Dichloride. To a stirred solution of dodecanedioyl dichloride (2 mmol) in anhydrous tetrahydrofuran (50 mL) under argon was added 1*H*-tetrazole (6 mmol) followed by triethylamine (20 mmol). Stirring at room temperature for 1−2 h afforded the ditetrazolide compound. The reaction mixture was filtered under anhydrous conditions into another round-bottom flask, and to it was added a solution of 5′-*O*-DMT-3′-*O*-acetyl-2′-deoxyadenosine (1 mmol) in anhydrous tetrahydrofuran (50 mL). After stirring for 6−8 h, the reaction was quenched with triethylammonium bicarbonate solution (50 mL). The reaction mixture was concentrated, taken up in ethyl acetate (200 mL), and washed with brine (50 mL). Drying (Na_2SO_4) followed by concentration and purification by flash silica gel chromatography ($CHCl_3$−MeOH−TEA, 97.5:1.5:1, v/v/v) afforded the desired product as a colorless amorphous solid. Yield = 3.2 g (3.5 mmol, 42%).

^{1}H NMR (200 MHz, $CDCl_3$): δ 9.5 (bs, 1H), 8.7 (s, 1H), 8.2 (s, 1H), 7.4−7.2 (m, 9 H), 6.8 (d, 4H), 6.5 (m, 1H), 5.5 (bd, 1H), 4.3 (bs, 1H), 3.8 (s, 6H), 3.4 (d, 2H), 2.8 (m, 1H), 2.3 (m, 1H), 2.1 (s, 3H), 1.2−1.8 (m, 20H).

^{13}C NMR (200 MHz, $CDCl_3$): δ 179.26, 173.43, 170.51, 158.83, 152.73, 151.31, 149.83, 144.66, 141.45, 135.73, 130.25, 128.13, 127.2, 122.43, 113.43, 86,95, 84.72, 75.31, 63.82, 55.43, 45.04, 43.01, 38.2, 36.1, 29.63, 25.84, 25.2, 24.18, 21.25, 8.98.

FAB-HRMS *m*/*z*: 908.53. Calculated for $C_{51}H_{68}N_6O_9$: 908.54

Loading of N-Protected-5′-*O*-DMT-3′-*O*-acetyl-2′-deoxyadenosine on HL 30 Primer Solid Support. Loading of protected nucleoside was performed under standard conditions using amino-derivatized primer support purchased from Amhersham-Pharmacia Biotech. Amino-derivatized HL 30 primer support, Hunig's base (12 equiv), HBTU activator (4 equiv), the DMT dA nucleoside with the linker synthesized as above, and anhydrous acetonitrile (100 mL) were taken in a round-bottom flask and closed and shaken mechanically at room temperature for 4 h. The support was then washed with acetonitrile (100 mL) and dried. Then a mixture of Cap A and Cap B solution used for oligomerization (20 mL each) was added to the support followed by a catalytic amount of 4-(dimethylamino)pyridine and shaken overnight mechanically. The support was washed with acetonitrile (200 mL), anhydrous DMF (100 mL), methanol (100 mL), and finally with anhydrous ether (200 mL) The support was then dried thoroughly and tested for loading (loading = 90 μmol/g).

Phosphorothioate Oligodeoxyribonucleotide Synthesis. All syntheses were performed on a Pharmacia OligoPilot II DNA/RNA synthesizer[10] using β-cyanoethyl phosphoramidite synthon (1.75 equiv/coupling, 0.2 M in MeCN). 1*H*-Tetrazole (0.45 M in MeCN) was used as activator, and phenylacetyl disulfide (PADS) (0.2 M in 3-picoline:MeCN, 1:1 v/v), as sulfur transfer reagent. Capping reagents were made according to the recommended Pharmacia recipe: Cap A = *N*-methylimidazole−MeCN (1:4 v/v), Cap B = acetic anhydride−pyridine−MeCN (2:3:5, v/v/v). Pharmacia HL 30 Primer Support was used in all experiments. Amidite and tetrazole solutions were prepared by using anhydrous MeCN (ca. 10 ppm) and were dried further by addition of activated 4 Å molecular sieves (ca. 50 g/L). The Pharmacia OligoPilot II (and OligoProcess) synthesizers use a reactor design in which the solid support (polystyrene:dextran) is packed into a vessel resembling a preparative chromatography column. Reagents pass through the column as bands, thus minimizing solvent and reagent usage (this is in contrast to the earlier Milligen and ABI synthesizers in which the solid support was agitated while suspended in solvent). The coupling step in each cycle comprises alternate deliveries of activator and phosphoramidite solutions, the first and last deliveries in an individual coupling step being tetrazole. At the end of each synthesis, DMT-on support-bound oligonucleotide was treated with 30% aqueous ammonium hydroxide solution for 16 h at 55 °C to effect release from the support and base and phosphate deprotection. Yield (expressed in mg of oligonucleotide/μmol of support) and ^{31}P NMR and capillary gel electrophoresis (CGE) data were collected for each synthesis. In addition, a portion of crude material obtained from each synthesis was purified by reversed phase HPLC and the purified material examined by CGE and LC−MS.

Workup. Primer support (ca. 100 mg) was treated with 2 mL og NH_4OH (30%) for 15 h at 55 °C, filtered, and rinsed with ethanol/water (1/1, v/v), and the combined solutions were evaporated to dryness under vacuum. The residue was dissolved in 200 μL of water.

HPLC Analysis and Purification. Analysis and purification of oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Nova-Pak C_{18} column (3.9 m × 300 mm) using a Waters HPLC system (600E System Controller, 996 photodiode array detector, 717 autosampler). For analysis an acetonitrile (A)/0.1M triethylammonium acetate gradient was used: 5% to 35% A from 0 to 10 min, then 35% to 40% A from 10 to 20 min, then 40% to 95% A from 20 to 25 min, flow rate = 10 mL/min/50% A from 8 to 9 min, 9 to 26

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min at 50%, flow rate = 1.0 mL/min, t_R(DMT-off) 10−11 min, t_R(DMT-on) 14−16 min. The DMT-on fraction was collected and was evaporated in a vacuum and redissolved in 50 μL water; the DMT group was removed as described below.

Dedimethoxytritylation. An aliquot (30 μL) was transferred into an Eppendorff tube (1.5 mL), and acetic acid (50%, 30 μL) was added. After 30 min at room temperature sodium acetate (2.5 M, 20 μL) was added, followed by cold ethanol (1.2 mL). The mixture was vortexed and cooled in dry ice for 20 min. The precipitate was spun down with a centrifuge, the supernatant was discarded, and the precipitate was rinsed with ethanol and dried under vacuum.

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