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Efficient Synthesis of Antisense Phosphorothioate Oligonucleotides: Evaluation of Dichloroacetic Acid at Higher Concentration to Reduce Cycle Time

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Abstract:

Efficiency of phosphorothioate oligonucleotide syntheses could be improved by increasing the concentration of dichloroacetic acid in toluene to 10% from 3% (v/v) during the detritylation step. It was also found that dichloroacetic acid is better than dichloropropionic acid when compared at similar concentrations.

Introduction

Phosphorothioate oligonucleotide synthesis has achieved considerable progress.[1−7] Typically, synthesis at scales up to 600 mmol is performed in a cyclic manner on an automated solid-phase synthesizer using β-cyanoethyl-protected phosphoramidite derivatives of protected nucleosides, specifically 4,4′-dimethoxytrityl (DMT) protection of 5′-hydroxyl, benzoyl protection of adenine (dA^{bz}) and cytosine (dC^{bz}), and isobutyryl protection of guanine (dG^{ibu}). The synthesis is performed in a packed column solid-phase reactor, frequently on a Pharmacia OligoProcess DNA/RNA synthesizer. Deoxyribonucleoside phosphoramidite coupling is highly efficient, utilizing a low excess (1.75 mol equiv) of synthons, and coupling efficiency is very high (98.5−98.7%). The total synthesis run time is also remarkably low (<7 h) for a 20-mer phosphorothioate leading to more than 2 kg of purified active pharmaceutical ingredient. Thus, for pharmaceutical purposes the term *large scale is now redefined as oligonucleotide synthesis leading to one kilogram or more of purified active pharmaceutical ingredient* (API).

Routine manufacture of antisense drugs for market is still not optimal, however. As in all commercial drug manufacturing, the synthesis must always be made faster, better, and cheaper, and there are areas where this may be achieved. One such example is the detritylation step. Historically, removal of the DMT group was performed using a solution of 3% trichloroacetic acid in dichloromethane.[8] Later, the conditions were changed to 3% dichloroacetic acid (v/v) in dichloromethane.[8] For manufacture of antisense drugs, large volumes of dichloromethane were needed, and the solvent is not environmentally friendly. Recently, we reported[9−11] that halogenated solvent may be replaced with inexpensive and industrially acceptable solvents such as toluene without compromise in product quality. Herein, we report a further improvement, viz., replacement of 3% dichloroacetic acid with 10% dichloroacetic acid in toluene.

Results and Discussion

During routine synthesis, we noted that considerable time was required for detritylation step in each cycle. It is well-known that acids are retained by DNA as well by the solid support. Thus, we wanted to shorten net reaction time by saturating the solid support more quickly. This could be done in two ways, viz. ramping up pump speed to increase linear velocity or by using a higher acid concentration. The former choice was not preferred because as oligomers grow, more mass accumulates and increases back pressure. Thus, as a synthesis proceeds, high flow rates are difficult to maintain; when flow is slowed, depurination increases. Hence, higher concentration of acid is preferred. Initially, we tried 20% (v/v) dichloroacetic acid but found the solution to be viscous, leading to operational problems. After investigating all factors we concluded that a solution of 10% dichloroacetic acid in toluene (v/v) could be used to efficiently synthesize 20-mer phosphorothioate oligonucleotides at large scale.

To demonstrate this protocol, a 20-mer phosphorothioate oligodeoxyribonucleotide [PS-d(GTTCTCGCTGGTGA-GTTTCA)] was chosen as an example.[12] All syntheses were performed on a Pharmacia Akta OligoPilot DNA/RNA synthesizer using β-cyanoethyl phosphoramidite synthons (1.75 equivalents, 0.2 M in CH_3CN). 1*H*-Tetrazole (0.45 M in CH_3CN) was used as activator, and phenylacetyl disulfide (PADS) (0.2 M in 3-picoline-CH_3CN 1:1, v/v), as sulfur

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(1) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *24*, 2223 and references therein.
(2) Srivatsa, G. S.; Ravikumar, V. T.; Scozzari, A. N.; Cole, D. L. Special Issues for Synthetic Antisense Oligonucleotides. In *Biopharmaceutical Process Validation*; Sofer, G., Zabriskie, D. W., Eds.; Marcel Dekker: New York, 2000; Chapter 16, pp 309−327.
(3) Ravikumar, V. T.; Andrade, M.; Wyrzykiewicz, T. K.; Scozzari, A. N.; Cole, D. L. *Nucleosides Nucleotides* **1995**, *14*, 1219.
(4) Iyer, R. P.; Beaucage, S. L. In *Comprehensive Natural Product Chemistry*; Barton, D. H. R., Nakanishi, K., Eds.; DNA and Aspects of Molecular Biology, Vol. 7; Kool, E. T., Vol. Ed.; Pergamon Press: Elmsford, NY, 1999; p 105.
(5) Cole, D. L. in *Large-scale synthesis of therapeutic antisense oligonucleotides: cost reduction and manufacturing scale-up*. *International Round Table Conference* 1996, La Jolla, CA.
(6) Scozzari, A. N. *Large Scale Oligonucleotide Synthesis: Production, Purification and Analysis*; IBC Conference, October 28−29, 1997, San Diego, CA.
(7) Geiser, T. *Ann. N.Y. Acad. Sci*. **1990**, *616*, 173.
(8) Atkinson, T.; Smith, M. In *Solid-phase Synthesis of Oligodeoxyribonucleotides by the Phosphite-Triester Method in Oligonucleotide Synthesis: A Practical Approach*; Gait, M. J., Ed.; IRL Press: NY, 1984.
(9) Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem*. **1999**, *7*, 435.
(10) Krotz, A. H.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Green Chem*. **1999**, 277.
(11) Krotz, A. H.; Carty, R. L.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev*. **2000**, *4*, 190.

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Table 1. **Oligonucleotide Synthesized Using 10% Dichloroacetic Acid**

deblock acid	crude yield mg/μmol	crude full length (%) (RP HPLC)	purified full length[19] ($n-1$)(%) (CGE)	purified full length[20] ($n-1$)(%) (IP-LC−MS)	P═S:P═O (^{31}P NMR)	P═S:P═O (IP-LC−MS)
10% DCA (4 cv)	6.64	72	91.3 (1.7)	85.4 (1.5)	99.64:0.36	99.71:0.29

transfer reagent.[13,14] Capping reagents were made to recommended Pharmacia composition: Cap A: *N*-methylimidazole-CH_3CN (1:4 v/v), Cap B: acetic anhydride−pyridine−CH_3CN (2:3:5, v/v/v). Pharmacia PS200 dA Primer support was used in all experiments. Amidite and tetrazole solutions were prepared using anhydrous CH_3CN (ca. 10 ppm H_2O) and were dried further by addition of activated 4 Å molecular sieves (∼50 g/L). At the end of each synthesis, DMT-on support-bound oligo was first treated with a solution of triethylamine:CH_3CN (1:1, v/v) at room temperature for 8 h to remove the β-cyanoethyl protecting groups,[15] then treated with 30% aqueous ammonium hydroxide solution for 16 h at 55 °C to effect release from support and base deprotection. Yield (expressed in mg of oligonucleotide/μmol of support[16]), ^{31}P NMR, and analytical RP-HPLC (full-length determination) data were collected for each synthesis. In addition, a portion of crude material obtained from each synthesis was purified by C_{18} RP-HPLC; the final DMT group was removed and the final product analyzed by quantitative capillary gel electrophoresis (QCGE)[17,18] and by ion-pair liquid chromatography electrospray mass spectrometry (IP-LC−MS) (Table 1).

As can be seen from data presented above, a 10% solution of DCA in toluene can be used to efficiently synthesize phosphorothioate oligonucleotides. Use of this protocol has led to an approximate 20% reduction in run time compared to run time using 3% dichloroacetic acid in toluene, thereby providing a faster and cheaper method of manufacturing antisense oligonucleotide drugs.

Evaluation of Dichloropropionic Acid. Efficient removal of acid-labile 5′-*O*-DMT protecting groups from support-bound oligonucleotide is crucial to high product yield and quality. Although several reagents and conditions have been developed for removal of the DMT group from nucleosides,[21−46] many have limited applicability for automated oligonucleotide synthesis on solid supports. We wanted to evaluate dichloropropionic acid (DPA) (pK_a 2.06 vs 1.29 for DCA)[47] for detritylation because it has viscosity[48] similar to that of dichloroacetic acid but is less acidic. Acid exposure time during the detritylation step must be minimized to limit formation of depurinated oligonucleotide sequences.[49,50] IP-LC−MS was used to quantify depurinated species present in crude and purified oligonucleotide.

Reactor design and solid support are crucial to successful oligonucleotide synthesis. In contrast to small-scale synthesizers (e.g., ABI 394 RNA/DNA synthesizer) that typically use silica-based solid support, medium (1−300 mmol) and large-scale syntheses (>300 mmol) are most commonly performed in packed-bed reactors on polystyrene-based solid supports. Phosphorothioate oligonucleotide [PS-d(GTTCTC-GCTGGTGAGTTTCA)] was synthesized on an Amersham

(12) Affinitak (ISIS 3521) is targeted to the 3′-untranslated region of PKC-α mRNA selectively inhibiting PKC-α gene expression. This oligonucleotide inhibits the growth of a variety of tumor types in vivo using nude mouse tumor xenografts and sequence-specific antitumor activity supports an antisense mechanism of action in vivo. Clinical evaluation (pivotal Phase III) of this drug in humans as potential treatment for nonsmall cell lung cancer is currently ongoing. Geiger, T.; Muller, M.; Dean, N. M.; Fabbro, D. *Anticancer Drug Des*. **1998**, *13*, 35.

(13) Cheruvallath, Z. S.; Wheeler, P. D.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 484.

(14) 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.

(15) Capaldi, D. C.; Gaus, H.; Krotz, A. H.; Arnold, J.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Lowery, K.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev*. **2003**, *7*, 832−838.

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

(17) Srivatsa, G. S.; Batt, M.; Schuette, J.; Carlson, R. H.; Fitchett, J.; Lee, C.; Cole, D. L. *J. Chromatogr., Sect. A* **1994**, *680*, 469.

(18) Srivatsa, G. S.; Klopchin, P.; Batt, M.; Feldman, M.; Carlson, R. H.; Cole, D. L. *J. Pharm. Biomed. Anal*. **1997**, *16*, 619.

(19) The 91% full-length is typical when the entire DMT-on peak is collected. Much higher purity (∼96−97%% full-length) is easily achieved by efficient fractionation of the DMT-on peak. Fractionation of DMT-on peak results in less than a 5% loss of full-length material.

(20) The purified full-length by IP-LC−MS is less than CGE values since the former analytical tool is efficiently able to distinguish and quantify process-related species such as depurinated oligonucleotides that comigrate in CGE.

(21) Anderson, W.; Hayes, D. H.; Michelson, A. M.; Todd, A. R. *J. Chem. Soc*. **1954**, 1882.

(22) Lehrfeld, J. *J. Org. Chem*. **1967**, *32*, 2544.

(23) Kovac, P.; Bauer, S. *Tetrahedron Lett*. **1972**, *28*, 2349.

(24) Letsinger, R. L.; Finnan, J. L. *J. Am. Chem. Soc*. **1975**, *97*, 7197.

(25) Stawinski, J.; Hozumi, T.; Narang, S. A.; Bahl, C. P.; Wu, R. *Nucleic Acids Res*. **1977**, *4*, 353.

(26) Takaku, H.; Nomoto, T.; Sakamoto, Y.; Hata, T. *Chem. Lett*. **1979**, 1225.

(27) Engels, J. *Angew. Chem., Int. Ed. Engl*. **1979**, *18*, 148.

(28) Kohli, V.; Blocker, H.; Koster, H. *Tetrahedron Lett*. **1980**, *21*, 2683.

(29) Matteucci, M. D.; Caruthers, M. H. *Tetrahedron Lett*. **1980**, *21*, 3243.

(30) Kierzek, R.; Ito, H.; Bhatt, R.; Itakura, K. *Tetrahedron Lett*. **1981**, *22*, 3761.

(31) Koster, H.; Sinha, N. D. *Tetrahedron Lett*. **1982**, *23*, 2641.

(32) Chaudhuri, B.; Reese, C. B.; Weclawek, T. *Tetrahedron Lett*. **1984**, *25*, 4037.

(33) Bessodes, M.; Komiotis, D.; Antonakis, K. *Tetrahedron Lett*. **1986**, *27*, 579.

(34) Krainer, E.; Naider, F.; Becker, J. *Tetrahedron Lett*. **1993**, *34*, 1713.

(35) Fuentes, J.; Cuevas, T.; Pradera, M. A. *Synth. Commun*. **1994**, *24*, 2237.

(36) Sekine, M. *Nucleosides Nucleotides* **1994**, *13*, 1397.

(37) Patil, S. V.; Mane, R. B.; Salunkhe, M. M. *Synth. Commun*. **1994**, *24*, 2423.

(38) Leonard, N. J.; Neelima *Tetrahedron Lett*. **1995**, *36*, 7833.

(39) Hwu, J. R.; Jain, M. L.; Tsay, S. C.; Hakimelahi, G. H. *Chem. Commun*. **1996**, 545.

(40) Asakura, J.; Robins, M. J.; Asaka, Y.; Kim, T. H. *J. Org. Chem*. **1996**, *61*, 9026.

(41) Wang, Y.; McGuigan, C. *Synth. Commun*. **1997**, *27*, 3829−3833.

(42) Yang, S. G.; Lee, D. H.; Kim, Y. H. *Heteroat. Chem*. **1997**, *8*, 435.

(43) Wahlstrom, J. L.; Ronald, R. C. *J. Org. Chem*. **1998**, *63*, 6021.

(44) Lu, R. J.; Liu, D.; Giese, R. W. *Tetrahedron Lett*. **2000**, *41*, 2817.

(45) Jones, G. B.; Hynd, G.; Wright, J. M.; Sharma, A. *J. Org. Chem*. **2000**, *65*, 263.

(46) Pathak, A. K.; Pathak, V.; Seitz, L. E.; Tiwari, K. N.; Akhtar, M. S.; Reynolds, R. C. *Tetrahedron Lett*. **2001**, *42*, 7755.

(47) Reinhardt, L. A.; Sacksteder, K. A.; Cleland, W. W. *J. Am. Chem. Soc*. **1998**, *120*, 13366.

(48) DPA has viscosity of 1.4540 vs 1.4660 for DCA.

(49) Paul, C. H.; Royappa, A. T. *Nucleic Acids Res*. **1996**, *24*, 3048.

(50) Septak, M. *Nucleic Acids Res*. **1996**, *24*, 3053.

Table 2. **Synthesis Parameters of Cycle Used on Pharmacia Akta OligoPilot synthesizer**

step	reagent	volume (mL)	time (min)
detritylation	10% acid/toluene (3 cv or 4 cv)	72 or 96	1.5 or 2
coupling	phosphoramidite (0.2 M), 1*H*-tetrazole (0.45 M) in acetonitrile	10, 15	5
sulfurization	PADS (0.2 M) in 3-picoline-CH_3CN (1:1, v/v)	36	3
capping	Ac_2O/pyridine/CH_3CN, NMI/CH_3CN	24, 24	2

Table 3. **Comparison of oligonucleotide synthesized using dichloropropionic acid (DPA)**

deblock acid	crude yield mg/μmol	crude full length (%) (RP HPLC)	purified full length[16] (%) (IP-LC−MS)	(n − 1) (%) (IP-LC−MS)	P═S:P═O (^{31}P NMR)	P═S:P═O (IP-LC−MS)
10% DPA (3 cv)	6.56	72	79.1	11.2	99.78:0.22	99.81:0.19
10% DPA (4 cv)	6.60	73	80.6	7.7	99.74:0.26	99.77:0.23
10% DPA (4 cv)	6.62	74	81.3	7.6	99.74:0.26	99.80:0.20
10% DCA (4 cv) (control expt)	6.58	73	85.5	1.4	99.70:0.30	99.75:0.25

Pharmacia Biotech Akta OligoPilot DNA/RNA synthesizer closely resembling a production-scale synthesizer (e.g., APB OligoProcess). Primer Support PS 200 (1 mmol scale; 5.18 g, loading 193 μmol/g; dA support from APB) was tightly packed in a glass column (volume 24 mL). Details of the synthesis cycle are given in Table 2.

Deblock solution (either 10% dichloroacetic acid or 10% dichloropropionic acid in toluene) was passed through the column at each deblock step. Conductivity-based or UV-based DMT yields were obtained when halogenated solvents (dichloromethane or trifluorotoluene) were used. In contrast, UV-based but not conductivity-based detritylation yield information was available using solvents such as toluene or xylene. A total of four syntheses were performed, duplicate syntheses with 4 column volumes (cv) of dichloropropionic acid, one synthesis with 3 column volumes of DPA, and one synthesis with 3 column volumes of DCA as control. The relative performance of deblock solutions was judged by comparing both quantity and quality of oligonucleotide product. For DMT-on/DMT-off purification of crude oligonucleotide, a C_{18} reversed-phase high-performance liquid chromatography (RP-HPLC) method was used that allowed facile separation of 5′-*O*-DMT-on oligonucleotide from capped failure sequences. The total DMT-on peak was collected for analysis without further fractionation.

During initial experimentation we noticed that >1 cv of deblock solution must be passed through the synthesis column before the color of DMT cation is first observed in the eluent. The first fractions of deblock solution that pass through the synthesis column are largely depleted of acid (due to retention by DNA and support) but contain a significant concentration of DMT groups.[51] Occurrence of orange color (DMT cation) is associated with delayed elution of dichloroacetic acid. A plot of time when dimethoxytrityl cation absorption is first detected by UV versus cycle number (position in the growing chain) shows a direct increase in DMT cation front elution time with increasing number of nucleotides bound to support (Figure 1). From the difference in delay between the first and 19th detritylation (the 20th nucleotide was synthesized as DMT-on), it may be estimated that, on average, ca. 2 mmol of acid per mmol of nucleotide on support are retained. Thus, from data presented in Table 3, dichloropropionic acid is not strong enough to remove the DMT groups efficiently as shown by increased levels of (n − 1)-mers. The level of (n − 1)-mers is reduced with increase in column volumes of acid, but dichloropropionic acid does not give acceptable oligo quality when compared to dichloroacetic acid.

(51) It was found that 40−60% of total eluted DMT groups are in the "colorless" initial eluent.

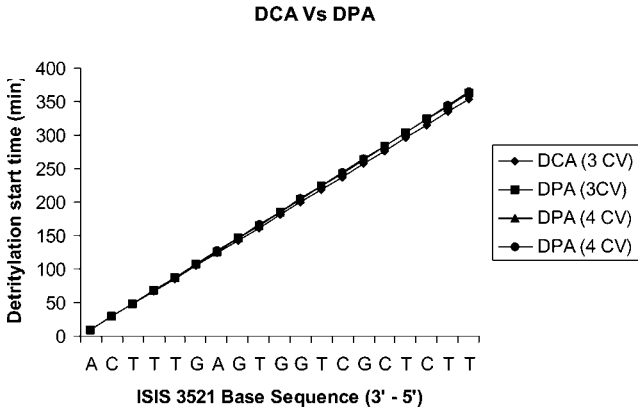

Figure 1. **Detritylation start time versus nucleotide position in oligonucleotide.**

Conclusions

In summary, we have demonstrated that it is possible to use 10% dichloroacetic acid in toluene for efficient synthesis of phosphorothioate oligonucleotides. This protocol was successfully transferred to manufacturing, and currently all first-generation drugs (except Vitravene) as well as second-generation APIs, viz., 2′-*O*-methoxyethyloligoribonucleotide phosphorothioates, are being manufactured using this condition.

Experimental Section

General Procedures. All solvents were dried prior to use. Chemical reagents were purchased from Aldrich. ^{31}P NMR spectra were recorded on a Varian Unity-400 instrument operating at 162 MHz. RP-HPLC was performed on a Waters system (600E System Controller, 996 Photodiode Array

Detector, 717 Autosampler). Mass spectra were acquired using a LCQ quadrupole ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA). For general synthesis of oligonucleotides refer to our earlier publication.[52]

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

HPLC Analysis and Purification. Analysis and purification of oligonucleotides by RP-HPLC was performed on a Waters Nova-Pak C_{18} column (3.9 mm × 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 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 evaporated in a vacuum and redissolved in 50 μL of water; the DMT group 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.

(52) Ravikumar, V. T.; Krishna Kumar, R.; Capaldi, D. C.; Turney, B.; Rentel, C.; Cole, D. L. *Org. Process Res. Dev.* **2003**, *7*, 259.

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