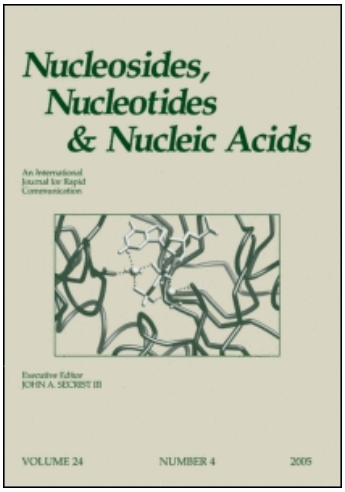


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

An Alternative Advantageous Protocol for Efficient Synthesis of Phosphorothioate Oligonucleotides Utilizing Phenylacetyl Disulfide (PADS)

R. Krishna Kumar[a]; Phil Olsen[a]; Vasulinga T. Ravikumar[a]
[a] Isis Pharmaceuticals, Inc., Carlsbad, California, USA

To cite this Article Kumar, R. Krishna , Olsen, Phil and Ravikumar, Vasulinga T.(2007) 'An Alternative Advantageous Protocol for Efficient Synthesis of Phosphorothioate Oligonucleotides Utilizing Phenylacetyl Disulfide (PADS)', Nucleosides, Nucleotides and Nucleic Acids, 26: 2, 181 — 188
To link to this Article: DOI: 10.1080/15257770601112739
URL: http://dx.doi.org/10.1080/15257770601112739

Nucleosides, Nucleotides, and Nucleic Acids, 26:181–188, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770601112739

Taylor & Francis
Taylor & Francis Group

AN ALTERNATIVE ADVANTAGEOUS PROTOCOL FOR EFFICIENT SYNTHESIS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES UTILIZING PHENYLACETYL DISULFIDE (PADS)

R. Krishna Kumar, Phil Olsen, and Vasulinga T. Ravikumar □
Isis Pharmaceuticals, Inc., Carlsbad, California, USA

□ *Phosphorothioate oligonucleotides could be synthesized using a 0.2 M solution of phenylacetyl disulfide (PADS) in a mixture of pyridine and acetonitrile (1:1, v/v) with > 99.9% step-wise efficiency. Unlike most other sulfurizing reagents that need to be stable in solution for performance, PADS needs to degrade and "age" in solution and hence performs efficiently even after storing the solution at room temperature for over a month. High yield and quality of oligonucleotides are produced thereby offering an alternative attractive protocol for use of this efficient sulfurizing reagent.*

INTRODUCTION

Syntheses of oligonucleotides and their analogs, for various applications, have reached a matured stage with scales as small as nanomolar to almost up to one mole. This has been made possible mainly due to few factors such as the development of phosphoramidite chemistry,[1] efforts to design new and more efficient automated synthesizers, and intense efforts aimed at lowering the cost and increasing the quality of oligonucleotides, both internally as well as from many other laboratories.[2] Due to increased stability over wild-type DNA, phosphorothioate oligonucleotides, where one of the non-bridging oxygens of the internucleotide phosphate is formally replaced by a sulfur atom, have become the modification of choice for design and development of therapeutic drugs.[3] To further increase the therapeutic value of these drugs, several sugar, backbone, and base modifications have been investigated and 2′-*O*-methoxyethyl sugar modified oligoribonucleotide chimera is of particular interest (at Isis Pharmaceuticals) and multiple drugs are in various stages of human clinical trials against a variety of diseases.[4,5]

The authors thank Mark Andrade and Anthony N. Scozzari for their valuable help.
Address correspondence to Vasulinga T. Ravikumar, Isis Pharmaceuticals, Inc., 2282 Faraday Avenue, Carlsbad, CA 92008. E-mail: vravikumar@isisph.com

RESULTS AND DISCUSSION

Even though intense efforts have been made toward stereoselective synthesis of phosphorothioate oligonucleotides,[6] these approaches have not attained practical status for routine synthesis. Thus, the current preferred approach is a solid-phase synthesis involving acid-catalyzed phosphoramidite coupling of nucleoside monomers to form phosphite triester linkages which are made stable by sulfurization with a sulfur transfer reagent to form racemic phosphorothioate linkages. Phosphoramidite chemistry requires sulfurization after each coupling. It is crucial that the sulfur transfer step be highly efficient or else it could lead to formation of unnecessary side reactions.[7] In the last few years, sulfurization, both on solid phase and in solution have been reported by many groups.[8] Among these, phenylacetyl disulfide (PADS)[9] and 3 H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[10] are used preferentially, with the former reagent being well accepted in pharmaceutical industry for manufacture of phosphorothioate oligonucleotide drugs. Earlier, we reported that efficient synthesis could be achieved by using a 0.2 M solution of PADS in a mixture of 3-picoline and acetonitrile (1:1, v/v) with >99.9% step-wise efficiency.[9 a,b] However, this introduces use of another solvent viz., 3-picoline. Hence, we were interested to investigate if we could replace 3-picoline with pyridine for efficient synthesis. Pyridine is already being used during capping step and, if found suitable could eliminate use of one additional solvent. Elimination of any raw material is a good source of cost reduction in manufacture of therapeutic drugs. Besides cost of solvent/reagent, elimination leads to both direct and indirect labor savings which could be significant. Additionally, a solution of PADS in pyridine has less odor compared to a solution of PADS in 3-picoline, even though well ventilated hoods are recommended for use of both the solutions.

Investigation of PADS for Sulfurization

Encouraged by our initial small scale (1 μm) experiments on synthesis of homo-thymidine phosphorothioate 20-mer using 0.2 M solution of PADS in a mixture of pyridine and acetonitrile (1:1, v/v) with a contact time of 2 minutes, we tested the sulfurization efficiency in the solid-phase synthesis of mixed oligodeoxyribonucleotide sequence [PS-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)]. Synthesis was performed on a GE Amersham Biosciences Akta 100 DNA/RNA synthesizer closely resembling the production-scale synthesizer (OligoProcess, Sweden). Instead of using nucleoside succinate-loaded solid support, we used UnyLinker (Herbert Brown Research & Development, Dombivili, India), a universal linker molecule loaded on PS200 solid support (loading 200 μm/g) (Figure 1) (unpublished results).

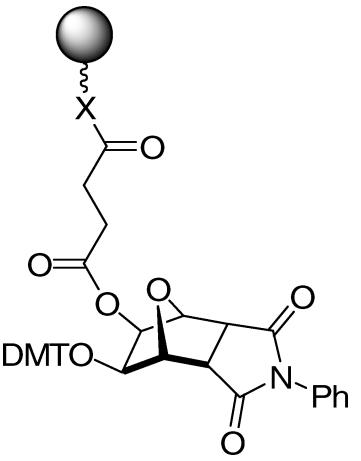


FIGURE 1 Universal linker attached solid support used for oligonucleotide synthesis.

The support was tightly packed in a steel column (volume 24 mL). Details of the synthesis cycle are given in Table 1. Ten percent dichloroacetic acid in toluene was used for deblocking of dimethoxytrityl (DMT) groups from 5′-hydroxyl group of the nucleotide. Extended detritylation condition (twice the column volume and contact time as the normal cycle) was used to remove the DMT group from the secondary hydroxyl group of the UnyLinker molecule. Standard commercially available β-cyanoethyl-protected phosphoramidites were used for synthesis. 4,5-Dicyanoimidazole (DCI) (0.5 M) in acetonitrile was used as activator during coupling step. Low-water acetonitrile (water content <10 ppm) was used for preparing phosphoramidite and activator solutions. 1.75 equivalents of amidites (both deoxy and 2′-*O*-methoxyethylribonucleosides) and a ratio of 1:1 (v/v) of amidite to activator solution were used during coupling step. At the end of synthesis, the support-bound DMT-on oligonucleotide was treated with a solution of triethylamine and acetonitrile (1:1, v/v) for 2 hours to remove acrylonitrile formed by deprotection of cyanoethyl group from phosphorothioate triester.[12] Subsequently, the solid support containing oligonucleotide was incubated with concentrated aqueous ammonium hydroxide at 55°C for 13 hours to complete cleavage from support, elimination of

TABLE 1 Synthesis parameters of cycle used on GE Amersham Biosciences Akta 100 DNA/RNA synthesizer at 1 mmole scale. Volume and time in parenthesis indicate the condition used for detritylation of UnyLinker molecule

Step	Reagent	Volume (ml)	Time (min)
Detritylation	10% dichloroacetic acid/toluene	72 (144)	3 (6)
Coupling	Phosphoramidite (0.2M), 4,5-dicyanoimidazole (0.5 M) in acetonitrile	8.8, 8.8	3
Sulfurization	PADS (0.2M) in pyridine-CH_3CN (1:1, v/v)	43	3
Capping	Ac_2 O/pyridine/CH_3CN, NMI/CH_3CN	30, 30	2.5

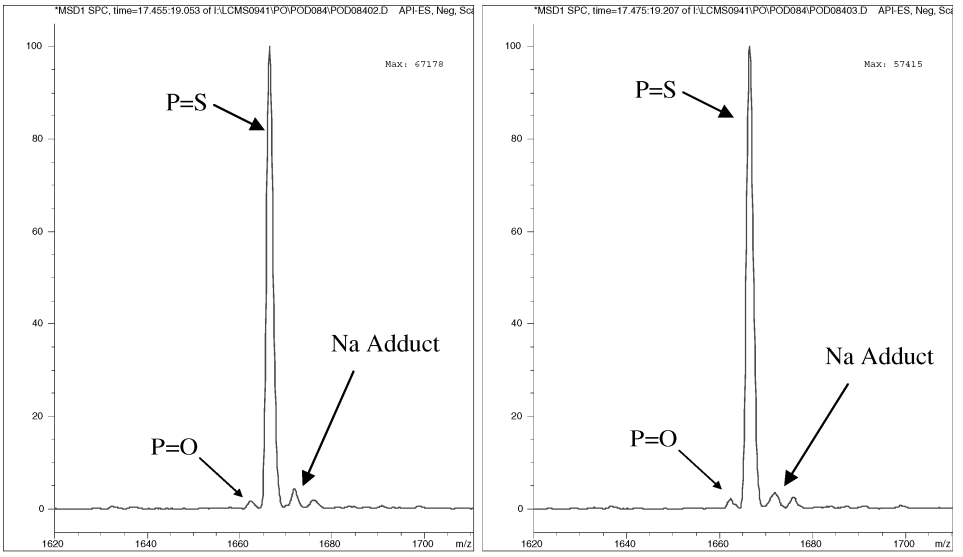


FIGURE 2 Ion-pair LC-MS analysis of oligonucleotide synthesized.

UnyLinker molecule to liberate 3′-hydroxy group of oligonucleotide and deprotection of base-protecting groups. The crude oligonucleotide was analyzed by ion-pair LC-MS (Figure 2). The results are summarized in Table 2. The oligonucleotide was purified in the usual manner using C18 reversed phase HPLC; all fractions containing DMT group were collected, detritylated, precipitated, and lyophilized.

Synthesis of 2′-Modified Phosphorothioate Oligoribonucleotides

Several 2′-*O*-methoxyethyl modified chimera are being evaluated against a variety of diseases and are in various stages of human clinical trials potentially requiring large quantities of active pharmaceutical ingredient (API) per year. As part of this overall objective, we were interested in investigating the performance of PADS under above

TABLE 2 Comparison of oligonucleotides synthesized using PADS under different conditions. Depurinated species include n-G, n-A/n-G+H_2 O, n-A+H_2 O, 3′-terminal phosphorothioate monoester (TPT); PADS solution was used on Day 6 after preparation

Condition used for PADS sulfurization	Crude yield (mg/μm)	Crude full length (%) (IP-LC-MS)	Total (n-1)-mer (%) (IP-LC-MS)	Total depurinated species (%) (IP-LC-MS)	PS:PO (%) (IP-LC-MS)
3-Picoline:ACN (1044–138)	7.4	72	1.7	1.05	99.9:0.1
Pyridine:ACN (1044–139)	7.5	72	1.2	1.06	99.9:0.1

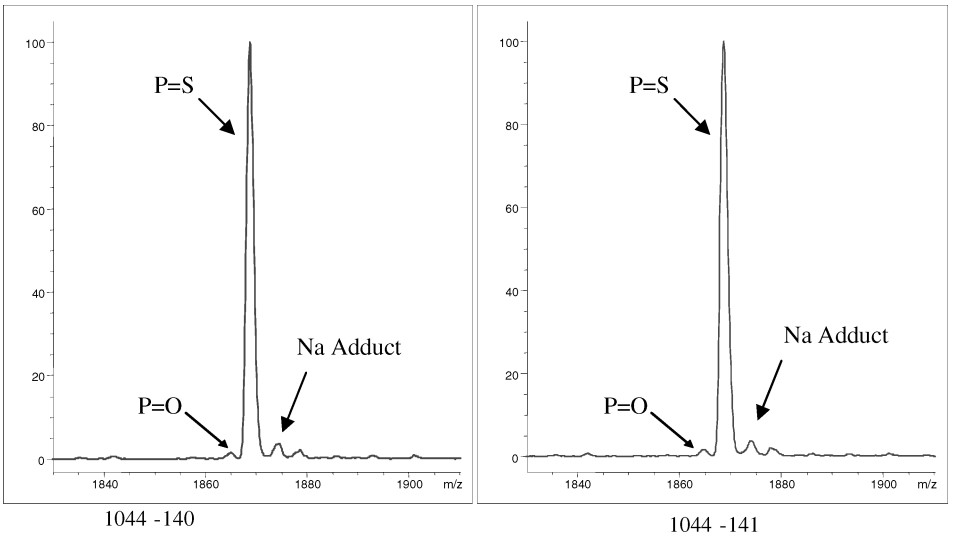


FIGURE 3 Ion-pair LC-MS analysis of oligonucleotide synthesized.

experimental conditions. A 20-mer 2′-*O*-methoxyethyl modified phosphorothioate oligonucleotide [5′-PS-[$G^{me}C^{me}C^{me}U$]-d[AGT^{me}CTG^{me}CTT^{me}C]-[$G^{me}CA^{me}C^{me}C$]] (nucleotides in italics indicate 2′-*O*-methoxyethyl modified ribonucleotides; ^{me}C = 5-methylcytidine; ^{me}U = 5-methyluridine) was chosen as an example. Synthetic conditions were exactly the same (scale and cycle) except that PADS solution was used on day seven after preparation. The crude oligonucleotide was analyzed by ion-pair LC-MS (Figure 3). The results are summarized in Table 3.

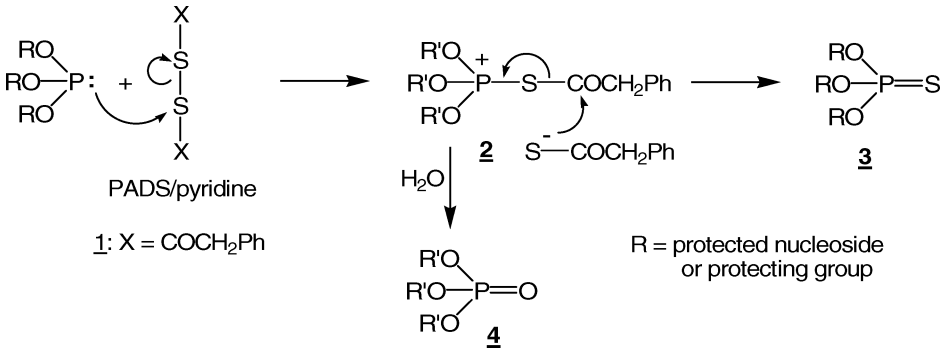
Similar results were obtained when PADS solution was used on day forty after preparation (data not shown).

Mechanism of Sulfurization

A generally accepted hypothesis for sulfur transfer reaction involves initial attack of the phosphite triester on sulfur transfer reagent (**1**) to form a

TABLE 3 Comparison of oligonucleotides synthesized using PADS under different conditions. Depurinated species include n-G, n-A/n-G+H_2 O, n-A+H_2 O, 3′-terminal phosphorothioate monoester (TPT); PADS solution was used on Day 7 after preparation

Condition used for PADS sulfurization	Crude yield (mg/μm)	Crude full length (%) (IP-LC-MS)	Total (n-1)-mer (%) (IP-LC-MS)	Total depurinated species (%) (IP-LC-MS)	PS:PO (%) (IP-LC-MS)
3-Picoline:ACN (1044–140)	7.26	71	0.8	0.44	99.93 : 0.07
Pyridine:ACN (1044–141)	7.14	72	0.8	0.42	99.92 : 0.08



SCHEME 1 General mechanism of sulfurization to form phosphorothioate triesters.

phosphonium salt (**2**) as an intermediate which then leads to the formation of phosphorothioate triester (**3**) (Scheme 1). This intermediate is sensitive to moisture (attack by water molecule) and leads to eventual formation of phosphate diester (**4**) as the undesirable by-product. However, we have observed[11 a] that addition of water (up to 1200 ppm) in PADS solution does not lead to increased level of PO formation besides obvious expected result of decreased yield. This clearly indicates that sulfurization by PADS leads to a different intermediate as compared to other reagents. The exact mechanism is still under investigation.

Recently, we have shown that aging of PADS in a base (3-picoline) is crucial for efficient performance.[9b] When pyridine was used as base we observed that aging takes longer (as shown by higher levels of phosphate diester when used within 48 hours of preparation) and a minimum of 2 days is needed to achieve maximum performance efficiency. Also, contrary to 3-picoline, a solution of 0.2 M solution of PADS in acetonitrile/pyridine does not undergo multiple color changes and remains as a pale brown solution even after several days. Use of this formulation in efficient synthesis even after storing at room temperature for forty days is a clear advantage over many other sulfurizing reagents.

In conclusion, we have demonstrated that efficient synthesis of phosphorothioate oligonucleotides could be achieved using PADS in pyridine:ACN, (>99.9% step-wise efficiency) thereby eliminating use of one additional solvent.

REFERENCES AND NOTES

1. (a) Beaucage, S.L.; Iyer, R.P. Advances in the synthesis of oligonucleotides by the phosphoramidite Approach. *Tetrahedron* **1992**, 48, 2223–2311; (b) Beaucage, S.L.; Iyer, R.P. Phosphoramidite derivatives and their synthetic applications. *Tetrahedron* **1993**, 49, 1925–1963; (c) Beaucage, S.L. Oligonucleotide Synthesis. In *Comprehensive Natural Product Chemistry*, Barton, D.H.R., Nakanishi, K., eds., Pergamon Press, New York, 1999; vol. 7.05, p. 105.

2. (a) Ravikumar, V.T.; Kumar, R.K.; Capaldi, D.C.; Turney, B.; Rentel, C.; Cole, D.L. Antisense phosphorothioate oligodeoxyribonucleotide targeted against ICAM-1: Use of I-linker to eliminate 3′-terminal phosphorothioate monoester formation. *Org. Proc. Res. Dev.* **2003**, 7, 259–266 and references cited therein; (b) Krotz, A.H.; McElroy, B.; Scozzari, A.N.; Cole, D.L; Ravikumar, V.T. Controlled detritylation of antisense oligonucleotides. *Org. Proc. Res. Dev.* **2003**, 7, 47–52; (c) Zhang, Z.; Tang, J-Y. Recent development in scale up of modified oligodeoxynucleotides. *Current Op. Drug Disc. Develop.* **1998**, 1, 304–313 and references cited therein.
3. (a) Lima, W.F.; Wu, H.; Nichols, J.G.; Prakash, T.P.; Ravikumar, V.; Crooke, S.T. Human RNase H1 uses one tryptophan and two lysines to position the enzyme at the 3′-DNA/5′-RNA terminus of the heteroduplex substrate. *J. Biol. Chem.* **2003**, 278, 49860–49867; (b) Agrawal, S.; Iyer, R.P. Perspectives in antisense therapeutics. *Pharmacol. Ther.* **1997**, 76, 151–160; (c) Dias, N.; Stein, C.A. Antisense oligonucleotides: Basic concepts and mechanisms. *Mol. Cancer Ther.* **2002**, 1, 347–355; (d) Bennett, C.F. Efficiency of antisense oligonucleotide drug discovery. *Antisense Nucleic Acid Drug Dev.* **2002**, 12, 215–224.
4. Altmann, K.-H.; Dean, N.; Fabbro, D.; Freier, S.; Geiger, T.; Haner, R.; Husken, D.; Martin, P.; Monia, B.; Muller, M.; Natt, F.; Nicklin, P.; Phillips, J.; Pieles, U.; Sasmor, H.; Moser, H. Second generation of antisense oligonucleotides: From nuclease resistance to biological efficacy in animals. *Chimia* **1996**, 50, 168–176.
5. A number of second-generation phosphorothioate oligonucleotides are in various stages of preclinical and clinical trials against APOB-100, PTP-1B, VLA4, TRPM2, survivin, STAT-3. eIF-4E, etc. for the treatment of a variety of diseases such as cancer, psoriasis, diabetes, asthma, arthritis, multiple sclerosis, etc.
6. (a) Suska, A.; Grajkowski, A.; Wilk, A.; Uznanski, B.; Blaszczyk, J.; Wieczorek, M.; Stec, W.J. Antisense oligonucleotides: Stereocontrolled synthesis of phosphorothioate oligonucleotides. *Pure & Appl. Chem.* **1993**, 4, 707–714 and references cited therein; (b) Stec, W.J.; Wilk, A. Stereocontrolled synthesis of oligo(nucleoside phosphorothioate)s. *Angew. Chem. Int. Ed. Engl.* **1994**, 33, 709–722 and references cited therein; (c) Lesnikowski, Z.J. Stereocontrolled synthesis of P-chiral analogues of oligonucleotides. *Bioorg. Chem.* **1993**, 21, 127–155.
7. Capaldi, D.C.; Gaus, H.J.; Carty, R.L.; Moore, M.N.; Turney, B.J.; Decottignies, S.; MaArdle, J.V.; Scozzari, A.N.; Ravikumar, V.T.; Krotz, A.H. Formation of 4,4′-dimethoxytrityl-C-phosphonate oligonucleotides. *Bioorg. Med. Chem. Lett.* **2004**, 14, 4683–4690.
8. (a) Cheruvallath, Z.S.; Kumar, R.K.; Rentel, C.; Cole, D.L.; Ravikumar, V.T. Solid phase synthesis of phosphorothioate oligonucleotides utilizing diethyldithiocarbonate disulfide (DDD) as an efficient sulfur transfer reagent. *Nucleosides Nucleotides & Nucleic Acids* **2003**, 22, 461–468 and references cited therein.
9. (a) 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. Synthesis of antisense oligonucleotides: Replacement of 3 H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) with phenylacetyl disulfide (PADS) as efficient sulfurization reagent: From bench to bulk manufacture of active pharmaceutical ingredient. *Org. Proc. Res. Dev.* **2000**, 4, 199–204; (b) Krotz, A.H.; Gorman, D.; Mataruse, P.; Foster, C.; Godbout, J.D.; Coffin, C.C.; Scozzari, A.N. Phosphorothioate oligonucleotides with low phosphate diester content: Greater than 99.9% sulfurization efficiency with "aged" solutions of phenylacetyl disulfide (PADS). *Org. Proc. Res. Dev.* **2004**, 8, 852–858; (c) Roelen, H.C.P.F.; Kamer, P.C.J.; van den Eist, H.; van der Marel, G.A.; van Boom, J.H. A study on the use of phenylacetyl disulfide in the solid-phase synthesis of oligodeoxynucleoside phosphorothioates. *Recl. Trav. Chim. Pays-Bas* **1991**, 110, 325–331; (d) Kamer, P.C.J.; Roelen, H.C.P. F.; van den Eist, H.; van der Marel, G.A.; van Boom, J.H. An efficient approach toward the synthesis of phosphorothioate diesters via the Schonberg reaction. *Tetrahedron Lett.* **1989**, 30, 6757–6760.
10. (a) Iyer, R.P.; Egan, W.; Regan, J.B.; Beaucage, S.L. 3 H-1,2-Benzodithiole-3-one 1,1-dioxide as an improved sulfuring reagent in the solid phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.* **1990**, 112, 1253–1254; (b) Iyer, R.P.; Phillips, L.R.; Egan, W.; Regan, J.B.; Beaucage, S.L. The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3 H-1,2-benzodithiol-3-one 1,1-dioxide as a sulfur-transfer reagent. *J. Org. Chem.* **1990**, 55, 4693–4699.
11. (a) Capaldi, D.C.; Scozzari, A.N.; Cole, D.L.; Ravikumar, V.T. Is it essential to use anhydrous acetonitrile in the manufacture of phosphorothioate oligonucleotides. *Org. Process Res. Dev.* **1999**, 3, 485–487; (b) Hayakawa, Y.; Hirata, A.; Sugimoto, J.; Kawai, R.; Sakakura, A.; Kataoka, M. Effect

of molecular sieves in the liquid-phase synthesis of nucleotide via the phosphoramidite method. *Tetrahedron* **2001**, 57, 8823–8826.

12. 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. Synthesis of high-quality antisense drugs. Addition of acrylonitrile to phosphorothioate oligonucleotides: Adduct characterization and avoidance. *Org. Proc. Res. Dev.* **2003**, 7, 832–838.