Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 16 (2006) 2513–2517

Bioorganic & Medicinal Chemistry Letters

Development of siRNA for therapeutics: Efficient synthesis of phosphorothioate RNA utilizing phenylacetyl disulfide (PADS)

Vasulinga T. Ravikumar,* Mark Andrade, Recaldo L. Carty, Amy Dan and Steve Barone

Isis Pharmaceuticals, Inc., 2282 Faraday Avenue, Carlsbad, CA 92008, USA

Received 4 November 2005; revised 20 January 2006; accepted 20 January 2006

Abstract—Efficient synthesis of phosphorothioate RNA (PS-RNA) is demonstrated by using phenylacetyl disulfide (PADS) in a mixture of pyridine and acetonitrile (1:1, v/v) for 3 min. Sulfurization is achieved with >99.8% stepwise efficiency. This reagent also performs efficiently during synthesis of RNA containing PS:PO mixed backbone.

Use of short synthetic strands of oligoribonucleotides (siRNA) is of great interest as a novel mechanism (RNA interference) for specific and reversible modulation of gene expression.[1–5] Although there are conflicting reports on the nuclease stability of unmodified siRNA duplexes, there is growing evidence that many of them are degraded within minutes in mammalian serum.[6–8] Among the various analogs designed to make them stable for use as therapeutic candidates, phosphorothioate modification, where one of the non-bridging oxygens is formally replaced by a sulfur atom, is one of them.[9,10] However, synthesis of RNA and its analogs, in particular phosphorothioate RNA (PS-RNA) continues to pose a formidable challenge. In general, RNA synthesis strategies mimic the approaches developed for chemical synthesis of DNA and its analogs.[11–18] The synthesis is achieved using a repetitive, chemical procedure or cycle for each nucleoside addition performed on an insoluble solid support. Each cycle consists of a deprotection step, in which the 5′-hydroxyl of support-bound RNA chain is made available for elongation, a coupling step, in which this 5′-hydroxyl is condensed with an activated 3′-phosphoramidite to form phosphite triester linkage, a sulfurization step in which the unstable phosphite triester linkage is converted into a stable phosphorothioate triester linkage; and a capping step, where the uncoupled hydroxyl groups are blocked from further chain elongation.

The introduction of phosphorothioates into RNA can be achieved in different ways.[19–21] For oligoribonucleotides that are synthesized by automated, solid-phase phosphoramidite chemistry, sulfur can be introduced at any internucleotidic linkage with an efficient sulfur transfer reagent. One challenge in automated PS-RNA synthesis is to find reagents that are effective in the presence of bulky 2′-*O*-silyl-protecting group. Our initial efforts aimed at synthesizing high-quality PS-RNA were unsuccessful. Sulfur transfer reagents such as 3H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent),[22,23] dimethylthiarum disulfide (DTD),[24,25] and few other reagents[26–29] did not give satisfactory full-length product (<10%) and the quality was unacceptable due to high contamination of phosphate diester impurities. However, 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH)[30–32] gave acceptable results, but the reagent was expensive.[33] This necessitated the investigation of an alternative reagent that could be efficient, transfer sulfur quickly, inexpensive, and resulted in high quality of oligonucleotide.

Another challenge for efficient synthesis of phosphorothioate RNA is the choice of activator used. It is well known that RNA phosphoramidite coupling efficiency greatly depends on activator, activator concentration, and time. In general, due to steric hindrance of the 2′-O-protecting group, longer coupling time and acidic activators such as 5-ethylthio-1*H*-tetrazole (ETT)[13] or 5-benzylthio-1*H*-tetrazole (BTT) are preferred. In our

---

Keywords: siRNA; Oligonucleotide; Phosphorothioates; PADS; Sulfurization; Solid-support synthesis.

* Corresponding author. Tel.: +1 760 603 2412; fax: +1 760 603 4655; e-mail: vravikumar@isisph.com

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.01.081

Table 1. Synthesis parameters of cycle used on GE Amersham Biosciences Äkta 100 DNA/RNA synthesizer using 24 mL column

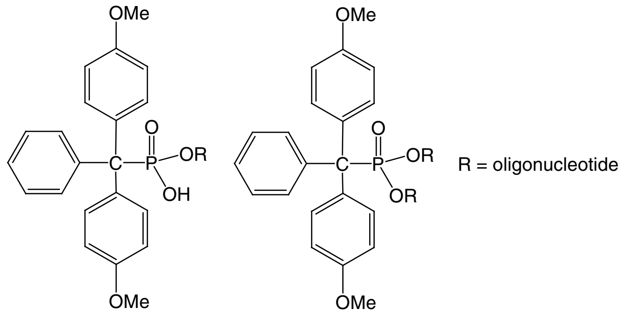
Step	Reagent	Time (min)
Detritylation	15% Dichloroacetic acid/toluene	3
Coupling	2′-*O*-TBDMS phosphoramidite (0.2 M) (4 equiv), 5-ethylthio-1*H*-tetrazole or 4,5-dicyanoimidazole (0.5 M) in acetonitrile	15
Sulfurization	PADS (0.2 M) in pyridine–CH_3CN (1:1, v/v)	3
Capping	Cap A: 10% acetic anhydride in toluene Cap B: 10% pyridine + 10% NMI in toluene	3

hands, using the conditions described in Table 1, we have found both ETT and 4,5-dicyanoimidazole (DCI)[34,35] gave comparable results (yield and quality based on ion-pair LC–MS).

This paper describes the use of phenylacetyl disulfide (PADS) to create PS-RNA using phosphoramidite chemistry, with superior results good enough for any application, in particular for therapeutics.

A fully modified phosphorothioate 20-mer of sequence 5′-UUU-GUC-UCU-GGU-CCU-UAC-UU-3′ targeted against PTEN was prepared on a 0.4 mmol scale on a GE Amersham Biosciences Äkta 100 DNA/RNA automated synthesizer using a 24 mL column and controlled pore glass loaded with standard succinate at 50 μmol/g.[36] The synthesis cycle used is shown in Table 1. 2′-*O*-TBDMS amidite monomers containing fast deprotecting groups (0.2 M solution in ACN; 4 equiv; 15 min contact time) and ETT or DCI (0.5 M solution in ACN) were used for coupling. 15% Dichloroacetic acid in toluene was used for detritylation.[37,38] Sulfurization was carried out using PADS (0.2 M solution in a mixture of pyridine/ACN 1:1, v/v) with a contact time of 3 min.[39,40] In the case of mixed backbone RNA synthesis, oxidation of the internucleotide phosphite was accomplished using iodine/tetrahydrofuran/pyridine/water for 2 min. At the end of synthesis, the support-bound oligonucleotide was treated with a solution of triethylamine and acetonitrile (1:1, v/v) for 2 h to remove acrylonitrile formed by deprotection of cyanoethyl group from phosphorothioate triester.[41] Subsequently, the solid support was incubated in a mixture of 30% aqueous ammonium hydroxide and ethanol (3:1, v/v) at 55 °C for 3 h to remove base-protecting groups. Removal of 2′-*O*-silyl group was effected using a solution of *N*-methylpyrrolidinone/triethylamine/TEA·3HF (1.5:0.75:1.0, v/v/v) at 65 °C for 3 h.[42,43] The purification of oligonucleotide was carried out by anion exchange chromatography and desalted to afford the oligonucleotide.

One of the crucial steps in PS-oligonucleotide synthesis is the incorporation of sulfur atom in the internucleotide linkage by oxidative sulfurization of P(III) phosphite triester intermediate to form a P(V) phosphorothioate triester linkage. Inefficient sulfurization during solid-phase synthesis leads to formation of two classes of process-related impurities **5** and **6** each containing a DMT-C-phosphonate moiety (Fig. 1).[44] Concentration of reagent, number of molar equivalents, and sulfurization contact time were optimized for efficient synthesis using PADS. Under this condition, no detectable levels of **5** or **6** were observed using our LC–MS analytical method. In addition, the low level (0.1% per linkage) of phosphate diester observed during use of PADS is probably not due to inefficient sulfurization but due to post-desulfurization occurring by currently unknown mechanism(s). ^{31}P NMR, strong anion exchange chromatography, and mass spectroscopy have been used for quantitative measurement of PO-content. In our laboratories, we use state-of-the-art ion-pair high-performance liquid chromatography/mass spectroscopy (IP-LC–MS) technique as a specific, accurate, and sensitive means of quantitating oligonucleotides containing PO-linkages within a matrix of PS-oligonucleotides. Table 2 and Figure 2 show the results of one of several phosphorothioate oligoribonucleotides synthesized using PADS. This represents >99.8% average stepwise sulfurization efficiency. The presence of cyanoethyl adduct on N3 of uracil base (CNET species) is not due to inefficient decyanoethylation protocol but due to the presence of cyanoethyl group already present (0.1%) as an adduct in uridine phosphoramidite as analyzed by IP-LC–MS.



DMT-C-phosphonate monoester **5**

DMT-C-phosphonate diester **6**

Figure 1. Two kinds of DMT-C-phosphonate formed due to inefficient sulfurization.

Tables 3 and 4 show the sequence and mass spectral analysis of various RNAs synthesized.

Table 2. PTEN targeted P═S RNA sequence 5′-UUU-GUC-UCU-GGU-CCU-UAC-UU-3′ was synthesized; MW = 6501.94 (calculated); 6501.30 (found)

Quality by IP-LC–MS	P═S	P═O	(*n* − 1)-mer	CNET
%	94.8	2.5	1.6	1.1

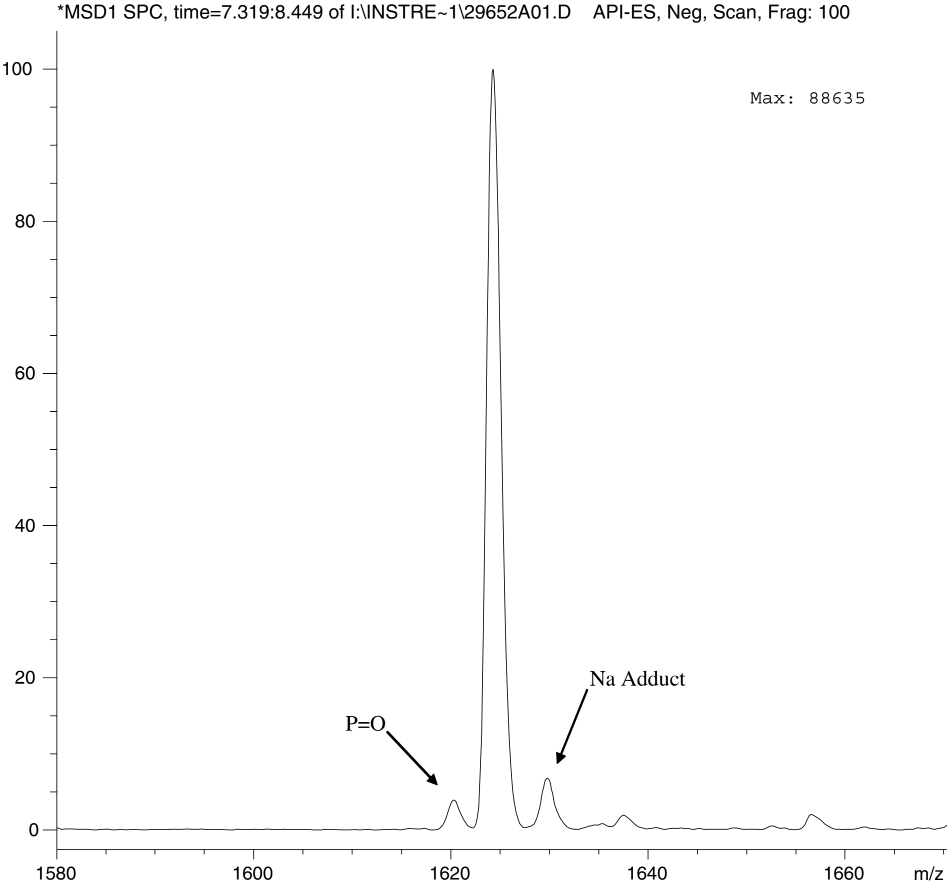


Figure 2. LC–MS analysis of crude P=S RNA synthesized.

Table 3. Synthesis of RNA thioates using PADS

Sequence	Mass	
	Calcd	Found
$5'\text{-X-}\underline{\mathrm{UUUGUCUCUGGUCCUUACUU}}\text{-}3'$	6581.92	6580.25
$5'\text{-X-}\underline{\mathrm{AGGCUGGUGGCACCAGGGAA}}\text{-}3'$	6909.31	6908.84
$5'\text{-}\underline{\mathrm{UUGUCUCUGGUCCUUACUU}}\text{-}3'$	6179.71	6178.92
$5'\text{-X-}\underline{\mathrm{GGCGAAUGAGACUUCUCUUA}}\text{-}3'$	6753.15	6754.08
$5'\text{-}\mathrm{UUUUGUCUCUG}\underline{\mathrm{GUCCUUAC}}\text{-}3'$	6002.97	6001.73
$5'\text{-}\mathrm{UUGUCUCUGGU}\underline{\mathrm{CCUUACUU}}\text{-}3'$	6002.97	6003.11
$5'\text{-}\mathrm{UUGU}\underline{\mathrm{CU}}\mathrm{CUGGUCCUUA}\underline{\mathrm{CUU}}\text{-}3'$	5970.83	5971.87

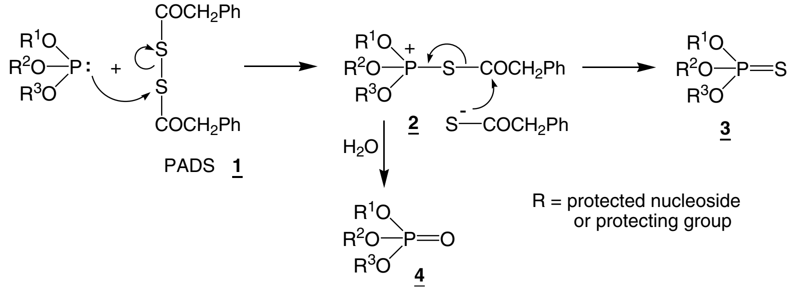
Underlined nucleotides are phosphorothioates; X denotes terminal phosphate monoester.

A generally accepted hypothesis for sulfur transfer reaction involves initial attack of phosphite triester on sulfur transfer reagent (**1**) to form a phosphonium salt (**2**) as an intermediate which then leads to formation of phosphorothioate triester (**3**). This intermediate (**2**) is sensitive to moisture and, if attacked by water molecule leads to eventual formation of phosphate triester (**4**) as the undesirable by-product (Scheme 1). However, we have observed that addition of water (up to 1200 ppm) in PADS solution does not lead to increased level of PO formation besides an obviously expected result of decreased yield.[45] This clearly indicates that sulfurization by PADS leads to a different intermediate as compared to other reagents. The exact mechanism is still under investigation.

In summary, we have demonstrated that efficient synthesis of phosphorothioate oligoribonucleotides could be achieved using PADS in pyridine/ACN, (>99.8% stepwise efficiency). This reagent is inexpensive and can be used in a variety of synthesizers thereby making it versatile. Multiple phosphorothioate oligoribonucleotides at various scales and different synthesizers have been synthesized in our laboratories using PADS for in vivo studies.

Table 4. Synthesis of alternating phosphate/thioates RNA using PADS

Sequence	Mass	
	Calcd	Found
$5'\text{-}\mathrm{A_{po}A_{ps}G_{po}U_{ps}A_{po}A_{ps}G_{po}G_{ps}A_{po}C_{ps}C_{po}A_{ps}G_{po}A_{ps}G_{po}A_{ps}C_{po}A_{ps}A}\text{-}3'$	6322.5	6322.1
$5'\text{-}\mathrm{U_{ps}U_{po}G_{ps}U_{po}C_{ps}U_{po}C_{ps}U_{po}G_{ps}G_{po}U_{ps}C_{po}C_{ps}U_{po}U_{ps}A_{po}C_{ps}U_{po}U}\text{-}3'$	6035.1	6034.4
$5'\text{-}\mathrm{U_{ps}U_{po}U_{ps}G_{po}A_{ps}A_{po}A_{ps}A_{po}U_{ps}G_{po}U_{ps}U_{po}G_{ps}A_{po}U_{ps}C_{po}U_{ps}C_{po}C}\text{-}3'$	6129.2	6128.9



Scheme 1. General mechanism of sulfurization to form phosphorothioate triesters.

Acknowledgments

The authors thank Phil Olsen and Hans Gaus for their valuable help.

References and notes

1. Crooke, S. T. *Biochim. Biophys. Acta* **1999**, *1489*, 31.
2. Zamore, P. D.; Tuschl, T.; Sharp, P. A.; Bartel, D. P. *Cell* **2000**, *101*, 25.
3. Elbashir, S. M.; Harborth, J.; Lendecker, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Genes Dev.* **2001**, *15*, 188.
4. Manoharan, M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 570, and references cited therein.
5. Soutschek, J.; Akinc, A.; Bramlage, B.; Charisse, K.; Constien, R.; Donoghue, M.; Elbashir, S.; Geick, A.; Hadwiger, P.; Harborth, J.; John, M.; Kesavan, V.; Lavine, G.; Pandey, R. K.; Racie, T.; Rajeev, K. G.; Roehl, I.; Toudjarska, I.; Wang, G.; Wuschko, S.; Bumcrot, D.; Koteliansky, V.; Limmer, S.; Manoharan, M.; Vornlocher, H. P. *Nature (London)* **2004**, *432*, 173.
6. Braasch, D. A.; Paroo, Z.; Constantinescu, A.; Ren, G.; Oz, O. K.; Mason, R. P.; Cory, D. R. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1139.
7. Chiu, Y. L.; Rana, T. M. *RNA* **2003**, *9*, 1034.
8. Braasch, D. A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; White, M. A.; Corey, D. R. *Biochemistry* **2003**, *42*, 7967.
9. Heidenreich, O.; Pieken, W.; Eckstein, F. *FASEB J.* **1993**, *7*, 90.
10. Allerson, C. K.; Sioufi, N.; Jarres, R.; Prakash, T. P.; Naik, N.; Berdeja, A.; Wanders, L.; Griffey, R. H.; Swayze, E. E.; Bhat, B. *J. Med. Chem.* **2005**, *48*, 901.
11. Marshall, W. S.; Kaiser, R. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 222.
12. Komatsu, Y.; Ohtsuka, E. *Comp. Nat. Prod. Chem.* **1999**, *6*, 81.
13. Wincott, F.; DiRenzo, A.; Shaffer, C.; Grimm, S.; Tracz, D.; Workman, C.; Sweedler, D.; Gonzalez, C.; Scaringe, S.; Usman, N. *Nucleic Acids Res.* **1995**, *23*, 2677.
14. Davis, R. H. *Curr. Opin. Biotechnol.* **1995**, *6*, 213.
15. Micura, R. *Angew. Chem., Int. Ed.* **2002**, *41*, 2265.
16. Vinayak, R.; Andrus, A.; Mullah, B.; Tsou, D. *Nucleic Acids Symp. Ser.* **1995**, 123.
17. Damha, M. J.; Ogilvie, K. K. In *Methods in Molecular Biology Protocols for Oligonucleotides and Analogs*; Agrawal, S., Ed.; Humana: Totowa, NJ, 1993; Vol. 20, p 81.
18. Sproat, B. S. In *Methods in Molecular Biology Oligonucleotide Synthesis: Methods and Applications*; Herdewijn, P., Ed.; Humana: Totowa, NJ, 2005; Vol. 288, p 17.
19. Eckstein, F. *Annu. Rev. Biochem.* **1985**, *54*, 367.
20. Agrawal, S.; Tang, J.-Y. *Tetrahedron Lett.* **1990**, *31*, 7541.
21. Morvon, F.; Rayner, B.; Imbach, J. L. *Tetrahedron Lett.* **1990**, *31*, 7149.
22. Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253.
23. Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693.
24. Wang, Z.; Song, Q.; Sanghvi, Y. S. In *Methods in Molecular Biology Oligonucleotide Synthesis Methods and Applications*; Herdewijn, P., Ed.; Humana: Totowa, NJ, 2005; Vol. 288, p 51.
25. Song, Q.; Wang, Z.; Sanghvi, Y. S. *Nucleoside Nucleotide Nucleic Acids* **2003**, *22*, 629.
26. Effimov, V. A.; Kalinkina, A. L.; Chakhmakhcheva, O. G.; Hill, T. S.; Jayaraman, K. *Nucleic Acids Res.* **1995**, *23*, 4029.
27. Stec, W. J.; Uznanski, B.; Wilk, A.; Hirschbein, B. L.; Fearon, K. L.; Bergot, B. J. *Tetrahedron Lett.* **1993**, *34*, 5317.
28. Tang, J.-Y.; Han, Y.; Tang, J. X.; Zhang, Z. *Org. Process Res. Dev.* **2000**, *4*, 194.
29. Rao, M. V.; Reese, C. B.; Zhengyun, Z. *Tetrahedron Lett.* **1992**, *33*, 4839.
30. Xu, Q.; Barany, G.; Hammer, R. P.; Musier-Forsyth, K. *Nucleic Acids Res.* **1996**, *24*, 3643.
31. Xu, Q.; Musier-Forsyth, K.; Hammer, R. P.; Barany, G. *Nucleic Acids Res.* **1996**, *24*, 1602.
32. Ma, M. Y.-X.; Dignam, J. C.; Fong, G. W.; Li, L.; Gray, S. H.; Samuel, B. J.; George, S. T. *Nucleic Acids Res.* **1997**, *25*, 3590.
33. The cost of EDITH reagent at multi-kilogram quantities is \$8 per gram.
34. Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic. Acids Res.* **1998**, *26*, 1046.
35. Wang, Z.; Siwkowski, A.; Lima, W. F.; Olsen, O.; Ravikumar, V. T. *Bioorg. Med. Chem.*, Submitted for publication.
36. Reagents and solvents were obtained from commercial suppliers and were used without further purification. Phenylacetyl disulfide was obtained from Acharya Chemicals, Dombivili, India. 2′-*O*-TBDMS-protected ribonucleoside phosphoramidites were obtained from Pierce Co., Milwaukee, WI. Low-water acetonitrile was purchased from Burdicken Jackson. Controlled pore glass loaded with RNA monomer (50 μmol/g) was purchased from Pierce, Milwaukee, WI. 4,5-Dicyanoimidazole (DCI) was purchased from American International Company (AIC). 5-Ethylthio-1*H*-tetrazole (ETT) was obtained from ChemGenes, MA.
37. Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem.* **1999**, *7*, 435.
38. Krotz, A. H.; Carty, R. L.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2004**, *4*, 190.

39. 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.
40. Krotz, A. H.; Gorman, D.; Mataruse, P.; Foster, C.; Godbout, J. D.; Coffin, C. C.; Scozzari, A. N. *Org. Process Res. Dev.* **2004**, *8*, 852.
41. 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.
42. Gasparutto, D.; Livache, T.; Bazin, H.; Duplaa, A. M.; Guy, A.; Khorlin, A.; Molko, D.; Roget, A.; Teoule, R. *Nucleic Acids Res.* **1992**, *20*, 5159.
43. Westman, E.; Stromberg, R. *Nucleic Acids Res.* **1994**, *22*, 2430.
44. Capaldi, D. C.; Gaus, H. J.; Carty, R. L.; Moore, M. N.; Turney, B. J.; Decottignies, S. D.; McArdle, J. V.; Scozzari, A. N.; Ravikumar, V. T.; Krotz, A. H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4683.
45. Capaldi, D. C.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **1999**, *3*, 485.