Oligonucleotides

DOI: 10.1002/ange.201400672

Efficient Solid-Phase Synthesis of pppRNA by Using Product-Specific Labeling**

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Abstract: *A novel solid-phase synthesis and purification strategy for 5′-triphosphate oligonucleotides by using lipophilic tagging of the triphosphate moiety is reported. This is based on triphosphate synthesis with 5′-O-cyclotriphosphate intermediates, whereby a lipophilic tag, such as decylamine, is introduced during the ring-opening reaction to give a linear gamma-phosphate-tagged species. This method enables the highly efficient synthesis of 5′-triphosphorylated RNA derivatives and their gamma-phosphate-substituted analogues and will especially facilitate the advancement of therapeutic approaches that make use of 5′-triphosphate oligonucleotides as potent activators of the cytosolic immune sensor RIG-I.*

It is well established that 5′-triphosphorylated viral RNA is a crucial pathogen-associated molecular pattern, which is detected by the cytosolic immune sensor RIG-I and triggers antiviral signaling, including the production of type I interferon.[1] Initial studies with well-defined synthetic 5′-triphosphate oligonucleotides resulted in the identification of a triphosphorylated blunt-ended duplex structure as the optimal RIG-I ligand[2] and provided essential insights into the binding of 5′-triphosphate to the RIG-I regulatory domain.[3] More recently, 3D structure determination of full length RIG-I[4] has provided a rational structural explanation for the recognition of the triphosphorylated blunt-ended duplex and the subsequent in vivo signal-generation steps that lead to interferon induction.

There is great interest in gaining a detailed understanding of the mechanism of this signaling pathway[5] and exploiting it to induce a controlled antiviral response for therapeutic purposes by using synthetic RIG-I agonists.[6] Moreover, RIG-I ligand-mediated selective apoptosis of cancer cells is the basis for the emerging field of RIG-I-based immunotherapy.[7] A flexible and efficient chemical synthesis of well-defined pppRNA derivatives is thus an essential requirement for providing sufficient pure material for structural and pharmacological studies.

Effective chemical synthesis of 5′-triphosphorylated RNA in the 20–50-mer range necessitates addressing the purification problem. The resolving power of anion-exchange chromatography for 5′-mono-, di- and triphosphorylated products of longer oligoribonucleotides is limited.[8] Moreover, the required denaturing and high-salt conditions make charge-based separations a tedious task.[9] Anion-exchange chromatography cannot cleanly resolve mono-, di- and triphosphates over 20nt in length. Given the sensitivity of RIG-I for terminal structural elements, charge-based purification methods are not optimal for providing unequivocally defined pppRNA ligands.

One of the most widely used methods for the synthesis of monomer nucleoside triphosphates is the cyclotriphosphate method,[10,11] which effectively combines 5′-phosphorylation with phosphate activation for the displacement reaction with pyrophosphate by using a single trifunctional reagent. This method is optimally suited for solid-phase applications because the first phosphitylation step is independent of the sequence and length of the controlled-pore glass (CPG)-bound protected oligoribonucleotide. Moreover, it enables the use of a large excess of the pyrophosphate nucleophile in the P–O–P-forming step, which ensures an essentially quantitative conversion of the initial active ester into the P2,P3-dioxo-5′-nucleosidyl cyclotriphosphite. The oxidation of this alpha-P^{III} intermediate is usually performed with aqueous iodine, thereby leading to alpha-P oxidation and concomitant ring opening in one step. Although several applications of this method for pppRNA synthesis have been reported,[12] in most cases, purification of the product mixtures requires gel electrophoresis or anion-exchange purification techniques.

We found that when the $P^{III} \rightarrow P^{V}$ conversion is performed under anhydrous conditions with an oxidation reagent such as *tert*-butyl hydroperoxide, the primary product is a stable solid-phase-bound RNA 5′-cyclotriphosphate. This intermediate is suitable for ring-opening reactions with nucleophiles with a wide range of nucleophilic reactivities[13] and enables selective tagging of the triphosphate moiety. Under anhydrous conditions, the cyclic intermediate enables essentially quantitative ring opening with aliphatic amines such as *n*-decylamine with reaction times in the range of a few minutes, thereby endowing the resulting substituted pppRNA derivatives with distinguishing chromatographic properties.

The general procedure for the pppRNA synthesis is outlined in Scheme 1. After the last coupling step and 5′-detritylation of the full-length synthesis product, the CPG loaded with the nucleobase-, ribose-, and phosphate-pro-

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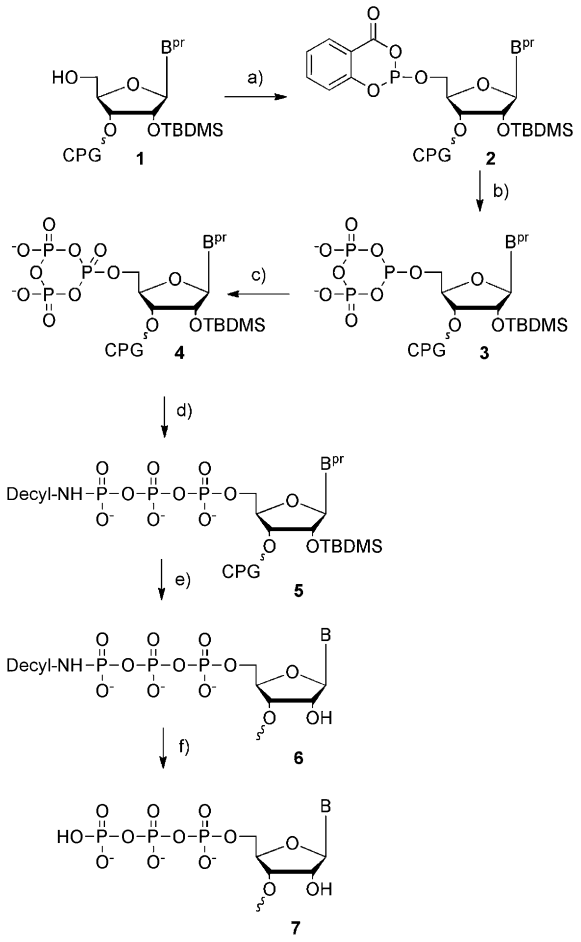
[**] This research was supported by grants from the German Research Foundation (SFB670, SFB704) to G.H. M.G., G.H., and J.L. are funded by the excellence cluster Immunosensation. We thank Dr. I. Roehl and S. Seiffert (Axolabs GmbH, Analytics) for measurement of the LC–MS spectra and helpful discussions. pppRNA = 5′-triphosphate RNA.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201400672.



Scheme 1. Synthesis of 5′-triphosphate RNA. a) 50 mM salicyl phosphorochloridite in anhydrous dioxane/pyridine 3:1 v/v; 30 min; b) 0.5 M bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF, 10 min, then CH_3CN wash; c) 1.1 M *t*BuOOH in dry CH_3CN, 15 min, then CH_3CN wash; d) 5 M *n*-decylamine in dry CH_3CN, 3 min, then CH_3CN wash; e) AMA, 30 min RT, then AMA 10 min at 65 °C; 1 M TBAF in THF, 16 h; combined yield 73.7%; f) pH 3.8 buffer, 60 °C, 70 min. B^{pr} = protected nucleobase, TBDMS = *tert*-butyldimethylsilyl, DMF = *N*,*N*-dimethylformamide, AMA = 40% aqueous methylamine/concentrated aqueous ammonia 1:1 v/v, TBAF = tetra-*n*-butylammonium fluoride, THF = tetrahydrofuran.

tected oligoribonucleotide **1** is treated with a 50 mM solution of salicylphosphochloridite in dioxane/pyridine 3:1 v/v for 30 min under strict exclusion of moisture (see the Supporting Information). The immobilized active ester **2**[10] is then reacted with a 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in DMF. The large excess of pyrophosphate enables a virtually quantitative conversion of intermediate **2** into the P^{III}-P^{V} cyclic anhydride **3** within 10 min. After oxidation with *t*BuOOH and washing with anhydrous acetonitrile, the resulting solid-phase-bound RNA cyclotriphosphate **4** is reacted with a 1 M *n*-decylamine solution in acetonitrile for 3 min to give the immobilized gamma amidate **5** without cleaving the 3′-succinate linkage. The oligoribonucleotide is then released from the support and nucleobase- and 2′-OH-deprotected with AMA and Bu_4NF under standard conditions.

Analysis of a typical reaction mixture of a 24-mer RNA synthesis with ion-pair reversed-phase chromatography coupled to ESI mass spectroscopy (IP-RP LC–MS) revealed the product composition shown in Figure 1 a. The efficiency of the phosphitylation step can be estimated from the amounts of residual 5′-OH 24-mer and the desired product **7**. The results confirm a greater than 95% conversion of the CPG-bound protected 5′-OH oligoribonucleotide. During the following steps, an essentially side-product-free conversion of **2** into **3**, **4**, and **5** is obtained. Identification of minor side products within the 24nt fraction reveals only trace amounts of H-phosphonate RNA and the pppRNA hydrolysis product. The minor peaks marked with an asterisk in Figure 1 a result from shorter 5′-capped oligonucleotide synthesis failure products. This triphosphorylation procedure, which comprises pyrophosphate exchange, oxidation, and ring opening, is comparable in efficiency to the standard amidite coupling reactions and results in a reaction mixture containing 74% triphosphorylated product **6**.

The DecNHpppRNA 24-mer (**6**) was isolated by using preparative reversed-phase HPLC. Using *n*-decylamine for derivatization of the triphosphate product causes sufficient lipophilic shift to enable a clean and convenient separation from all nontagged components of the reaction mixture on a PRP-1 column (Figure 1 b). IP-RP LC–MS analysis of the isolated preparative HPLC product fraction (Figure 1 c, d) confirms efficient isolation of **6** in greater than 97% purity.

Compound **6** could be quantitatively converted into pppRNA (**7**) by acid catalyzed P–N bond hydrolysis at pH 3.8 at 60 °C (Figure 1 e, f). The hydrolysis reaction is completed after 70 min (Figure S1 in the Supporting Information) and occurs without degradation of the triphosphate moiety. These mild conditions are identical to the conditions used for the removal of 2′-ACE protecting groups and do not lead to hydrolysis or isomerization of the inter-ribonucleotide bonds.[14]

IP-RP LC-MS analyses of intermediate **6** (Figure 1 c, d) and end product **7** (Figure 1 e, f) show no detectable base modifications resulting from the triphosphorylation reaction. Analysis of the end product (Figure 1 e, f) resolves small amounts of cyanoethyl-containing base modifications resulting from the Michael addition of acrylonitrile and transamination products formed during AMA treatment, both of which are well known from standard RNA synthesis.

Potential side products resulting from phosphitylation at uridine O-4 or guanosine O-6 are likely reverted to the parent nucleoside by a mechanism similar to the acetate-mediated base regeneration in the coupling–capping–oxidation cycle of standard oligonucleotide synthesis.[15] The nucleophile that attacks the P^{III} of the nucleobase-phosphitylated product is in this case the excess pyrophosphate, which is applied as a 0.5 M solution in the reaction.

To demonstrate the general utility of these synthesis and purification conditions, we synthesized a series of pppRNA derivatives in the 10–52nt range (Table 1). One characteristic of this method is a nearly sequence-independent elution pattern since the lipophilic tag alone determines the product retention time. Table 1 shows examples prepared for 2′-OMe modification interference analysis (Table 1, entries e–g) and

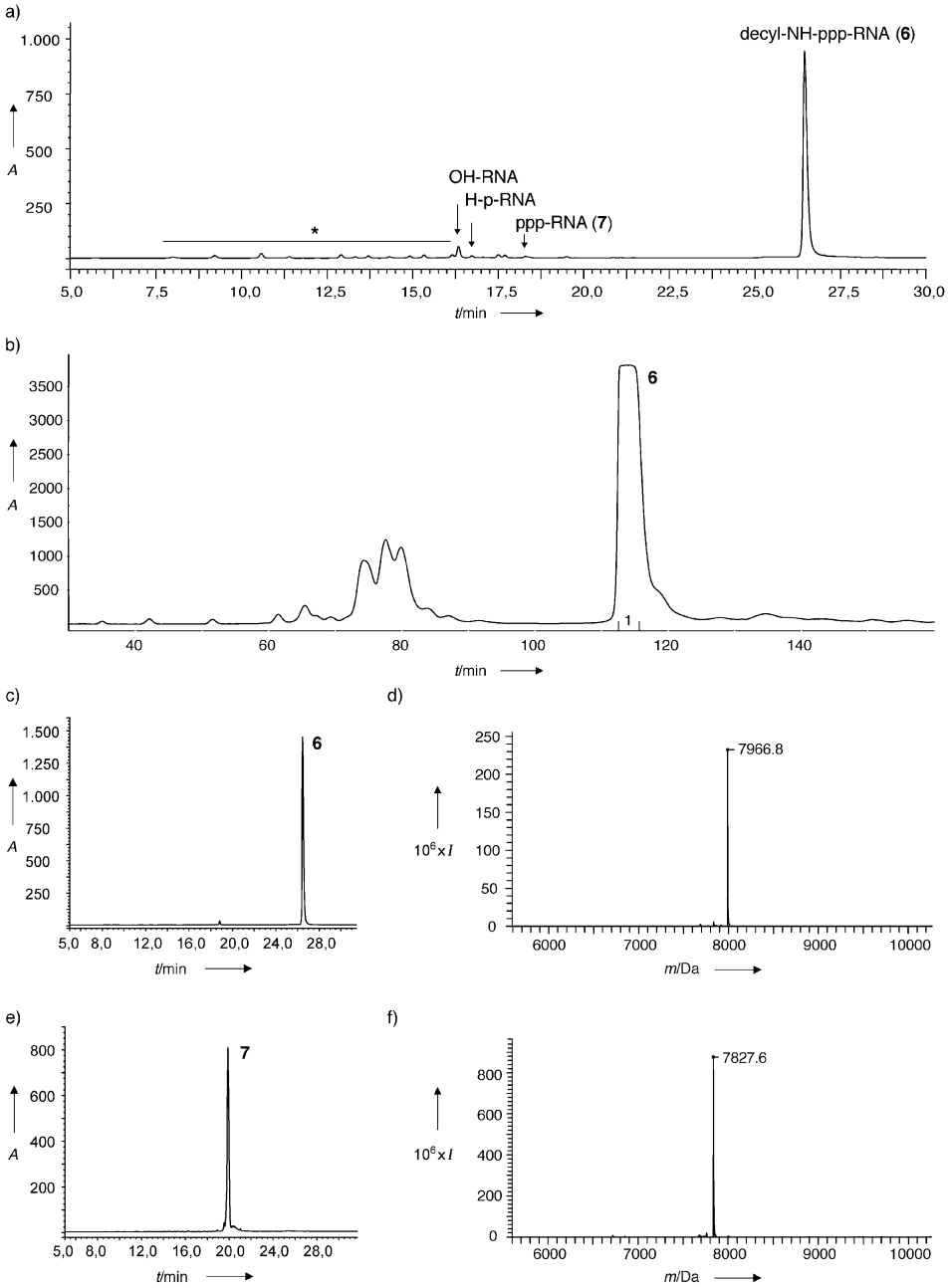


Figure 1. a) IP-RP LC–MS profile of a crude 5′-decyl-NH-pppRNA reaction mixture (sequence: 5′-GACGCUGACCCUGAAGUUCAUCUU): 5′-OH-RNA (3.1%; MW calcd: 7589.6 Da, found: 7587.6 Da); 5′-H-phosphonate-RNA (0.7%; MW calcd: 7653.6 Da, found: 7651.6 Da); 5′-pppRNA (0.4%; MW calcd: 7829.5 Da; found: 7827.6 Da), and 5′-decyl-NH-pppRNA (73.7%; MW calcd: 7969.6 Da; found: 7966.8 Da). Oligonucleotide synthesis failure products are marked with *. b) RP-HPLC purification of a 1 μmol scale reaction of 5′-Decyl-NH-pppRNA. c, d) IP-RP LC profile (c) and deconvoluted ESI-MS spectrum (d) of the isolated product peak from (b): 5′-decyl-NH-pppRNA (**6**; 96.0%; MW calcd: 7969.6 Da; MW found: 7966.8 Da). e, f) IP-RP LC profile (e) and deconvoluted ESI-MS spectrum (f) of the 5′-pppRNA end product (**7**; 83.2%, MW calcd: 7829.5 Da; MW found: 7827.6 Da). *A* = absorbance at 260 nm.

for the comparison of length variants (Table 1, entries a, b, h–m; Figure S2 in the Supporting Information). This feature enables a clean separation of tagged and nontagged products even with a 52-mer sequence (Table 1, entry m) or with sequences containing GC-rich stretches (Table 1, entries c, d).

As verified by MALDI-TOF analysis (Table 1, Figure S2), the modification scheme clearly selects for triphosphate derivatives over ppRNA or monophosphates in all cases, and the pppRNA can be obtained without contamination by shorter sequences or mono- and diphosphate side products. Furthermore, the method is also compatible with the use of modified CPG supports to generate 3′-modified pppRNA derivatives, and polar 3′-residues such as biotin or fluorescein (Table 1, entry n, o) do not interfere with the elution pattern.

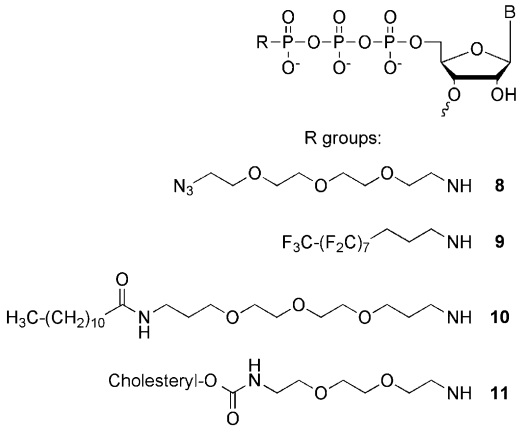
The immobilized RNA cyclotriphosphate **4** is suitable for ring-opening reactions with a broad range of substituted amines, thereby giving access to the gamma substituted derivatives as summarized in Scheme 2. Derivatization using azidoalkylamine **8**, perfluoroalkylamine **9**, or the monoacetylated lipophilic diamine **10** was performed with a 0.1M solution of the amine in acetonitrile with an increased reaction time of 3 h to provide the substituted triphosphates in good yields (Scheme 2; Table 2, **8**–**10**; Figure S3 in the Supporting Information). In this way, terminal azide functionalities suitable for further derivatization by click chemistry and perfluoroalkyl residues suitable for fluorous affinity chromatography can be easily incorporated into the pppRNA products. Ring opening of **4** with excess 2,2′-(ethylenedioxy)diethylamine gives a pppRNA analogue with a free terminal amino group, which may be further derivatized on-column with electrophilic reagents as exemplified with cholesteryl chloroformate (Table 2, **11**).

In summary, we report a novel synthesis strategy that enables the convenient, high-yield conversion of immobilized protected oligoribonucleotides into 5′-cyclotriphosphates and their application in a product-specific labeling strategy for efficient purification of 5′-triphosphate oligoribonucleotides.

Table 1: Examples of pppRNA synthesis.

	Sequence (5′-3′)[a]	Yield [%][b]	Calcd [M−H]	Found [*M*−H][c]
a	pppAGAAAUUAUUCAUGGCAGACUU	46.7	7250.2	7250.2
b	pppAAGAUGAACUUCAGGGUCAGCGUC	76.1	7954.6	7957.4
c	pppGCGGCGUCGAGAAGUAUUUGACUUTT	61.5	8558.0	8563.0
d	pppGCGGCGCUCAACUUGCAUUAAUUCTT	68.6	8437.9	8442.6
e	pppG(Me)ACGCUGACCCUGAAGUUCAUCUU	72.7	7843.6	7848.7
f	pppGA(Me)CGCUGACCCUGAAGUUCAUCUU	75.1	7843.6	7845.1
g	pppGAC(Me)GCUGACCCUGAAGUUCAUCUU	59.0	7843.6	7843.6
h	pppGACGCUGACCCU	80.0	4009.2	4010.0
i	pppGACGCUGACCCUGAAGU	76.4	5664.2	5664.4
j	pppGACGCUGACCCUGAAGUUCAU	78.3	6911.0	6912.8
k	pppGACGCUGACCCUGAAGUUCAUCUU	69.0	7828.5	7828.8
l	pppGACGCUGACCCUGAAGUUCAUCUUACG	46.1	8808.1	8809.3
m	pppGACGCUGACCCUGAAGUUCAUCUUGAAAAAGAUGAACUUCAGGGUCAGCGUC	76.5	16939.0	16939.7
n	pppGACGCUGACCCUGAAGUUCAUCUU-FAM	71.8	8397.6	8399.2
o	pppGACGCUGACCCUGAAGUUCAUCUU-biotin	65.1	8398.1	8403.5

[a] ppp = 5′-triphosphate, N = ribonucleotide, N(Me) = 2′-*O*-methyl nucleotide, FAM = 6-carboxyfluorescein (Figure S2). [b] Percentage yield in the crude reaction mixtures as calculated from integration of the reversed phase chromatograms. [c] *m*/*z* measured by MALDI-TOF MS (Figure S2 in the Supporting Information).



Scheme 2. Synthesis of gamma-substituted pppRNA analogues.

Table 2: Examples of R–pppRNA synthesis.[a]

R	Yield [%][b]	Calcd [*M*−H]	Found [*M*−H][c]
8	49	8028.6	8030.9
9	76	8287.5	8290.3
10	68	8212.8	8214.8
11	21	8370.9	8370.6

[a] RNA sequence (5′-3′): GACGCUGACCCUGAAGUUCAUCUU. [b] Percentage yield in the crude mixture as calculated from integration of the reversed phase chromatograms. [c] *m*/*z* measured by MALDI-TOF MS (Figure S3 in the Supporting Information).

This synthesis route gives pure pppRNA independent of both the sequence length and the efficacy of the initial phosphorylation reaction and oligonucleotide coupling steps. The synthesis procedure is straightforward and can be carried out on standard automated oligonucleotide synthesizers. Efforts are currently underway to adapt the solvent systems for large-scale synthesizer compatibility to facilitate the application of this method to the development of pppRNA based therapies.

Received: January 21, 2014
Published online: March 25, 2014

Keywords: immunology · medicinal chemistry · oligonucleotides · phosphorylation · RNA

[1] a) V. Hornung et al., *Science* **2006**, *314*, 994–997; b) M. Schlee, *Immunobiology* **2013**, *218*, 1322–1335.
[2] a) M. Schlee et al., *Immunity* **2009**, *31*, 25–34; b) A. Schmidt et al., *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12067–12072.
[3] a) Y. Wang, et al., *Nat. Struct. Mol. Biol.* **2010**, *17*, 781–787; b) C. Lu, H. Xu, C. T. Ranjith-Kumar, M. T. Brooks, T. Y. Hou, F. Hu, A. B. Herr, R. K. Strong, C. C. Kao, P. Li, *Structure* **2010**, *18*, 1032–1043.
[4] a) D. Luo, S. C. Ding, A. Vela, A. Kohlway, B. D. Lindenbach, A. M. Pyle, *Cell* **2011**, *147*, 409–422; b) D. Luo, A. Kohlway, A. Vela, A. M. Pyle, *Structure* **2012**, *20*, 1983; c) E. Kowalinski, T. Lunardi, A. A. McCarthy, J. Louber, J. Brunel, B. Grigorov, D. Gerlier, S. Cusack, *Cell* **2011**, *147*, 423–435; d) F. Jiang, A. Ramanathan, M. T. Miller, G.-Q. Tang, M. Gale, S. S. Patel, J. Marcotrigiano, *Nature* **2011**, *479*, 423–427.
[5] D. Kolakofsky, E. Kowalinski, S. Cusack, *RNA* **2012**, *18*, 2118–2127.
[6] a) L. Martinez-Gil, P. H. Goff, R. Hai, A. Garcia-Sastre, M. L. Shaw, P. Palese, *J. Virol.* **2013**, *87*, 1290–1300; b) H. Poeck, et al., *Nat. Med.* **2008**, *14*, 1256–1263.
[7] a) J. G. van den Boorn, G. Hartmann, *Immunity* **2013**, *39*, 27–37; b) R. Besch, et al., *J. Clin. Invest.* **2009**, *119*, 2399–2411.
[8] I. Zlatev, T. Lavergne, F. Debart, J. J. Vasseur, M. Manoharan, F. Morvan, *Org. Lett.* **2010**, *12*, 2190–2193.
[9] B. S. Sproat, T. Rupp, N. Menhardt, D. Keane, B. Beijer, *Nucleic Acids Res.* **1999**, *27*, 1950–1955.
[10] J. Ludwig, F. Eckstein, *J. Org. Chem.* **1989**, *54*, 631–635.
[11] a) K. Burgess, D. Cook, *Chem. Rev.* **2000**, *100*, 2047–2059; b) M. Hollenstein, *Molecules* **2012**, *17*, 13569–13591.
[12] a) S. Nagata, K. Takagaki, T. Wada, *Chem. Pharm. Bull.* **2012**, *60*, 1212–1215; b) N. Paul, G. Springsteen, G. F. Joyce, *Chem. Biol.* **2006**, *13*, 329–338; c) D. B. Olsen, F. Benseler, J. L. Cole, M. W.

Stahlhut, R. E. Dempski, P. L. Darke, L. C. Kuo, *J. Biol. Chem.* **1996**, *271*, 7435 – 7439; d) K. Kossen, N. K. Vaish, V. R. Jadhav, C. Pasko, H. Wang, R. Jenison, J. A. McSwiggen, B. Polisky, S. D. Seiwert, *Chem. Biol.* **2004**, *11*, 807 – 815; e) R. K. Gaur, B. S. Sproat, G. Krupp, *Tetrahedron Lett.* **1992**, *33*, 3301 – 3304; f) F. Civril, M. Bennett, M. Moldt, T. Deimling, G. Witte, S. Schiesser, T. Carell, K.-P. Hopfner, *EMBO Rep.* **2011**, *12*, 1127; g) S. S. Carroll, F. Benseler, D. B. Olsen, *Methods Enzymol.* **1996**, *275*, 365 – 382; h) G. G. Brownlee, E. Fodor, D. C. Pritlove, K. G. Gould, J. J. Dalluge, *Nucleic Acids Res.* **1995**, *23*, 2641 – 2647; i) A. V. Lebedev, Koukhareva II, T. Beck, M. M. Vaghefi, *Nucleosides Nucleotides Nucleic Acids* **2001**, *20*, 1403 – 1409.

[13] D. G. Knorre, V. A. Kurbatov, V. V. Samukov, *FEBS Lett.* **1976**, *70*, 105 – 108.

[14] a) S. A. Scaringe, F. E. Wincott, M. H. Caruthers, *J. Am. Chem. Soc.* **1998**, *120*, 11820 – 11821; b) S. A. Scaringe, *Methods* **2001**, *23*, 206 – 217.

[15] R. T. Pon, N. Usman, M. J. Damha, K. K. Ogilvie, *Nucleic Acids Res.* **1986**, *14*, 6453 – 6470.