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An orthogonal photolabile linker for the complete “on-support” synthesis/fast deprotection/hybridization of RNA†

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The preparation of a polystyrene solid support decorated with a photolabile linker is described. The entire post-synthetic processing of RNA can be carried out in the solid phase in a minimum amount of time. The deprotected RNA is available for “on-support” hybridization and photolysis releases siRNA duplexes under mild, neutral conditions.

The scientific breakthroughs in gene silencing have triggered an onset of interest for small interfering RNA (siRNA) in loss-of-function experiments^{1,2} and as potential therapeutics.^{3,4} As therapeutics, their final outcome crucially depends on the design and the fine-tuning of their chemical and biophysical properties,^{5,6} which can complicate RNA synthesis, deprotection and purification procedures. Loss-of-function experiments, on the other hand, rely on gene screening with large siRNA libraries.^{7–10} The preparation of such libraries is costly and time-consuming, and consequently, the demand for faster, efficient and more affordable RNA synthesis methods is escalating.^{11,12}

In many ways, the deprotection of RNA can be regarded as the most time-consuming step. In particular, fluoride-mediated deprotection of *tert*-butyldimethylsilyl (TBDMS), widely used as a protecting group for the 2'-OH function,^{13,14} typically relies on long treatments with TBAF or triethylamine-trihydrofluoride (TREAT-HF). While shorter alternatives exist, such as mixtures of *N*-methylpyrrolidone in TREAT-HF,¹⁵ the subsequent isolation of RNA and the required removal of fluoride ions before purification on silica-based HPLC columns contribute heavily to the processing time. A fast and practical desilylation method is therefore highly desired.

Performing deprotection in the solid phase, whereby excess reagents and prematurely cleaved shortmers can simply be washed away, is an attractive idea to eliminate work-up procedures.

However, it supposes that the attachment between RNA and support is chemically resistant (orthogonal) to the deprotection reagents. Examples of such strategies with orthogonal linkers on solid supports include allyl moieties,^{16,17} disulfide bonds^{18,19} and hydroquinone-*O,O'*-diacetic acid (“Q-linker”²⁰) when used in combination with levulinyl-type of 2'-protecting groups.^{21,22} An additional orthogonal linker is derived from the photolabile (2-nitrophenyl)propyloxycarbonyl (NPPOC²³). When covalently bound to controlled-pore glass (CPG) supports, NPPOC-based linkers are cleaved under mild, photolytic conditions^{24–26} and have been applied to RNA synthesis with 2'-base labile protecting groups.^{27–29} Alternatively, photolabile linkers in conjunction with biotin have been introduced into ribonucleosides to facilitate the isolation of RNA after elongation on CPG and deprotection.³⁰ However, clearly, the chemical properties of CPG preclude any “on-support” desilylation with fluoride-containing reagents.

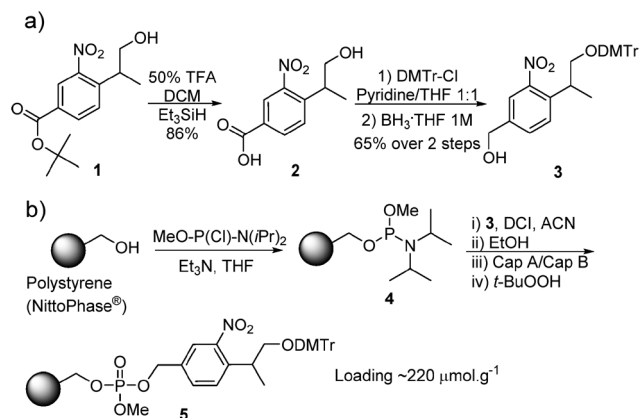
An ideal and truly orthogonal linker would be amenable to RNA synthesis and deprotection with standard 2'-*O*-TBDMS phosphoramidites and reagents. We surmised that a polystyrene (PS) support coupled to a photolabile NPPOC linker would resist HF treatment and therefore bypass the need for work-up and desalting procedures. In addition, a strategy involving the use of microwaves was explored in order to accelerate TBDMS removal with TREAT-HF.³¹ We then reasoned that the resulting deprotected RNA would be available for “on-support” hybridization to its complementary strand,^{25,32} thereby providing a swift method for the preparation of siRNA duplexes. The ability to prepare duplexes in the solid phase after deprotection participates in our efforts to access siRNA libraries in a minimum amount of time (no HPLC purification) and enables a control over the precise ratio of complementary strands (excess RNA is washed away). A final photodeprotection would release the double-stranded RNA into an appropriate buffered solution.

Our initial plan of action concerned the aromatic amide functionality that usually connects the NPPOC moiety to the solid support.²⁷ Because of its relative sensitivity to ammonolysis, we wished to replace the amide group with a stable *O*-methylphosphate instead. The synthesis of photolabile linker **3** is described

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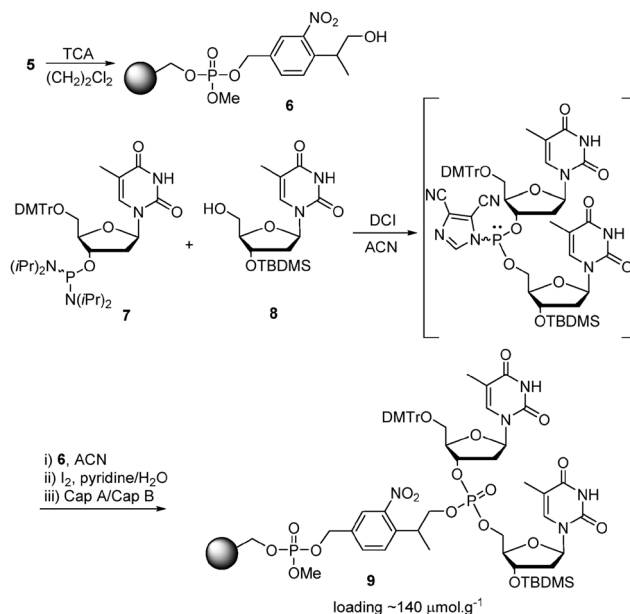
Scheme 1 (a) Synthesis of the photocleavable linker **3**. (b) Immobilization of the photocleavable NPPOC derivative **3** onto the PS solid support (NittoPhase[®]).

in Scheme 1a. Starting from the known compound **1**,^{33–35} hydrolysis of the *tert*-butyl ester in TFA led to carboxylic acid **2**. Next, the primary alcohol function was protected with a dimethoxytrityl (DMTr) and then subjected to a borane-mediated reduction yielding alcohol **3**.

To immobilize our photolabile linker, we chose a high-loaded PS-based solid support (NittoPhase[®]) and transformed hydroxyl groups to *O*-methylphosphoramidites **4** using a suitable chlorophosphine (Scheme 1b). Alcohol **3**, pre-mixed with dicyanoimidazole (DCI) as the activator, was then coupled to amidite **4** for 1 h. Unreacted amidite functions were quenched upon the addition of ethanol and the remaining hydroxyl groups were capped using a mixture of Ac_2O and *N*-methylimidazole. Finally, oxidation of the phosphite triester linkage with *tert*-butylhydroperoxide^{36,37} afforded the photocleavable solid support **5**. The determination of loading density as described in the literature³⁸ revealed a loading of $\sim 220 \mu\text{mol g}^{-1}$.

To investigate the cleavage efficiency by photolysis as well as the stability of the linker under basic conditions, we set out to synthesize a model dT₁₀ oligonucleotide. The deprotection was performed in 40% MeNH₂ in water and the recovered supernatant was shown to contain no UV-active material, demonstrating the stability of the photolabile linker under those conditions. The supported oligonucleotide was then released by UV irradiation (centered at 350 nm) in a triethylammonium acetate buffer and 35 O.D. units of oligonucleotide were collected after 2 h, which corresponds to 42% cleavage efficiency (33% after 1 h, Fig. S1a, ESI[†]). Under these conditions, literature precedents suggest that less than 3% photodimerized thymidine are expected to be produced.³⁹ The crude, 3'-phosphorylated dT₁₀ oligonucleotide was then analyzed using RP-HPLC (Fig. S1b, ESI[†]) and compared to a crude sample prepared according to standard methods. A clear main product and very few impurities were detected in the HPLC trace of the photocleaved dT₁₀, indicating that solid-phase synthesis of **5** is effective.

To further extend the usefulness of this approach, we wished to develop and offer an alternative leading to the formation of 3'-unmodified strands. To do so, and inspired by recent attempts,²⁷ we chose to append the NPPOC linker to the internucleotidic



Scheme 2 Derivatization of the photocleavable NPPOC-linked solid support **5** with a 3'-O-TBDMS dTdT dimer.

linkage between two dT monomers (Scheme 2). It was expected that photocleavage would trigger the release of a dTdT dimer 3'-OH, a common 3'-overhang in siRNA design. Following a published procedure,⁴⁰ we prepared dT phosphorodiamidite **7** and 3'-O-TBDMS protected dT **8**. Upon activation with DCI, the mixed monomers **7** and **8** reacted to a phosphorimidazolide intermediate which was directly added to detritylated solid support **6**. After a coupling time of 20 min, the excess reagents were removed and the resulting phosphite triester oxidized. Finally, capping of unreacted hydroxyl groups gave the derivatized photolabile solid support **9** in good yield ($\sim 140 \mu\text{mol g}^{-1}$).

With our photolabile supports **5** and **9**, we moved on to RNA synthesis and considered the use of microwaves to accelerate desilylation times. We first synthesized a 12 mer RNA sequence containing all four bases on the solid support **5** using conventional 2'-silyl protected phosphoramidites (see ESI[†]). The decyanoethylation and base deprotection step was effected using MeNH₂ for 30 min at 60 °C and the supported, 2'-protected RNA then underwent TREAT-HF-mediated desilylation at 60 °C for 10 min under microwave (MW) irradiation. The solid support was found to be relatively stable under those conditions (6% cleavage). After a final photolysis for 1 h at room temperature in RP buffer, the deprotected RNA was analyzed by RP-HPLC and the chromatogram showed little to no trace of 2'-protected material, suggesting that desilylation is complete in as little as 10 min under MW (Fig. S2, ESI[†]). To further validate our method, we prepared a 21mer sense strand (*luc1*) of the siRNA targeting the firefly luciferase mRNA. The RP-HPLC trace after on-support deprotection and subsequent photolysis is shown in Fig. 1a and compared to a crude sample of the same sequence obtained following standard procedures. Synthesis on our light-labile support yielded a crude 21mer RNA of excellent quality ($\sim 73\%$ of full-length product in the crude) and the identity of the purified product was confirmed by ESI-MS (Fig. 1a-inset).

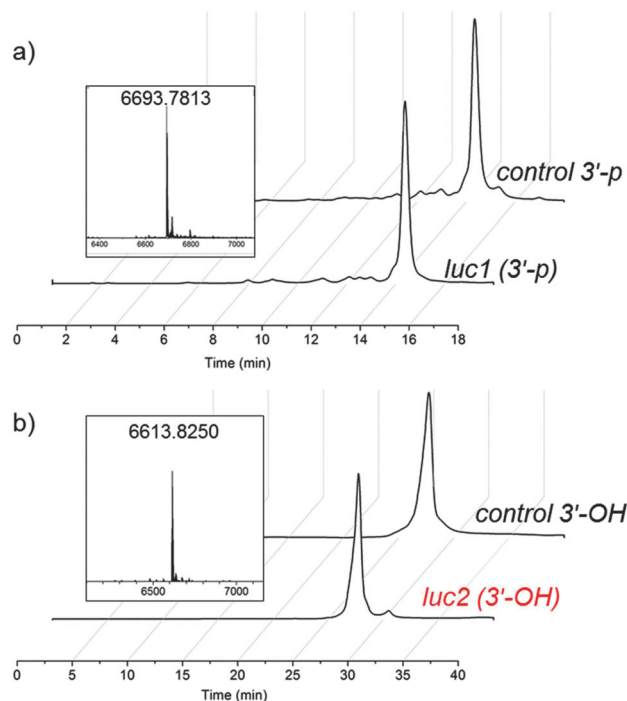


Fig. 1 RP-HPLC traces of the sense strand of the siRNA targeting firefly luciferase mRNA. Sequence (5' to 3'; t = dT): GCUUGAAGUCUUUAAUUAAtt. (a) Analytical RP traces of crude *luc1* synthesized (front) on the solid support **5** and (back) on the standard LCAA-CPG solid support with standard deprotection. (b) Preparative RP traces of crude *luc2* synthesized (front) on the solid support **9** and (back) on the standard LCAA-CPG solid support with standard deprotection. Left insets: ESI⁺-MS analysis of purified strands. $M_{\text{calcd}} = 6693.85$ Da (*luc1*) and 6613.75 Da (*luc2*).

Next, the same sequence was synthesized on the derivatized support **9** and then deprotected and photolyzed as described above. The resulting crude material was analyzed on RP-HPLC (Fig. 1b) and the main product (*luc2*) was characterized using MS, which confirmed the presence of a 3'-OH species (Fig. 1b-inset). The photocleavage efficiency after 1 h at r.t. was 31%, similar to that of the original solid support **5** but when performed in ACN spiked with 1% piperidine, a maximum of ~50% cleavage was obtained. DMSO, a solvent of choice for photocleavage in a particular case,²⁸ was in our hands a poorer alternative (22% cleavage after 1 h).

Overall, with only two hours required to fully deprotect and to release RNA from the support, our method provides a fast route towards high-quality oligonucleotides. In addition, RNA isolation is greatly facilitated since the supported oligomer is recovered by simple filtration and no desalting is needed before HPLC purification.

With the supported sense strands in hand, yielding either a 3'-phosphate or a 3'-OH upon photocleavage, we were keen to attempt "on-bead" hybridization experiments. The complementary antisense strand (*luc3*) was obtained following conventional procedures and added in ten-fold excess to a mixture of supported-*luc1* or *luc2* in a concentrated sodium citrate (20× SSC) buffer. We found it necessary to use high salt buffers to counteract the high-density of charged phosphodiester on PS beads, negatively affecting the ability of the sense strands to

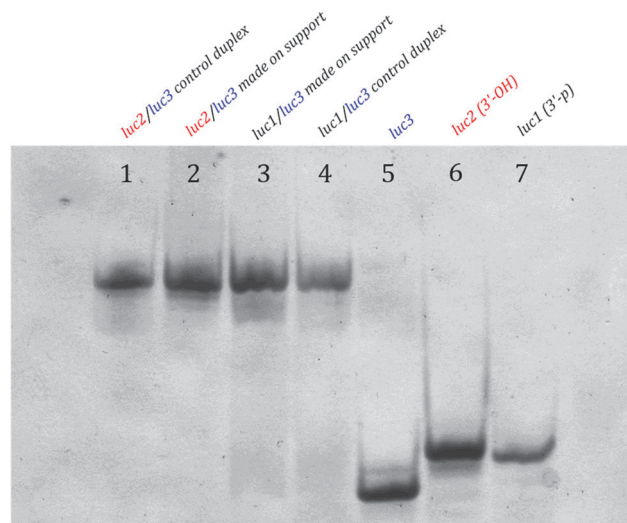


Fig. 2 Evaluation of duplex formation and stoichiometry on 24% non-denaturing PAGE. Sense strands (*luc1* and *luc2*) are loaded as crudes after photolysis. The antisense strand (*luc3*) was synthesized, deprotected and purified according to standard methods. Duplexes made on support (*luc1/luc3* and *luc2/luc3*) are loaded after photolysis. Gel was visualized by staining with Stains-all.

pair with their complement. After 3 h of hybridization at 45 °C, the excess *luc3* was removed and the solid support washed with buffers of increasing stringency (see ESI[†]). We observed only a limited release of *luc3* during these washing steps. Non-complementary strands did not significantly bind under those conditions. Next, the beads were filtered, washed and photo-deprotected in buffer. The duplexes were analyzed by MS and were identified as single strands only (Fig. S3, ESI[†]), which dissipates the possibility of UV-mediated crosslinking. The recovered RNA duplexes (*luc1/luc3* and *luc2/luc3*) were then loaded onto 24% non-denaturing PAGE and visualized by staining. As shown in Fig. 2, on-support hybridization experiments (lanes 2 and 3) yielded duplexes whose mobility in gel was similar to that of authentic samples (lanes 1 and 4). Bands moving slightly faster than those of the duplexes (lanes 2 and 3) may possibly correlate with hybrids of *luc3* and shortmers of the photocleaved *luc1* and *luc2*. Interestingly, no excess of either single strand was detected; this technique thus allows for the preparation of siRNAs of rigorous stoichiometry. It also facilitates the preparation of siRNA libraries since no precise quantitation of either strand is required before mixing, and a known excess of the solution strand suffices.

Next, we investigated the ability of the siRNAs hybridized on-support to reduce the expression of the firefly luciferase gene by targeting its corresponding mRNA in an *in vitro* assay (Fig. 3). These duplexes exhibited an RNAi activity with IC₅₀ values of 19 nM and 9 nM for *luc1/luc3* and *luc2/luc3* respectively, similar to that of control duplexes (17 nM and 13 nM, in the same respective order), thus providing an additional evidence of duplex integrity after photocleavage. It also shows that, in our hands, the sense strand needs not be purified for the duplex to be equally potent. Work is underway to extend the method to

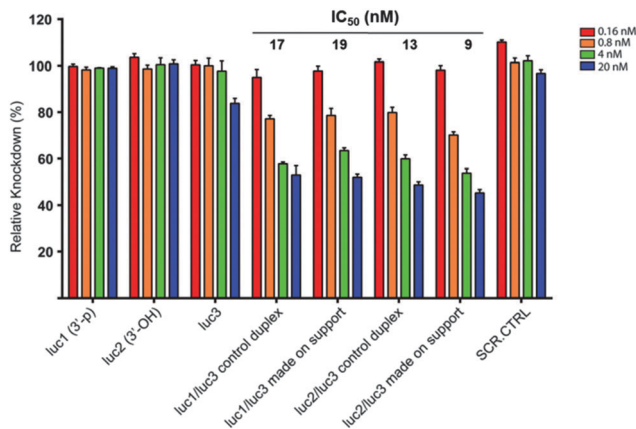


Fig. 3 Firefly luciferase gene knockdown assays in HeLa cells with siRNAs made in solution or on-support and carrying either a 3'-phosphate (*luc1*) or a 3'-OH (*luc2*) at the end of the sense strand and tested at different duplex concentrations (20 nM; 4 nM; 0.8 nM and 0.16 nM). Error bars indicate standard deviation. SCR.CTRL: scrambled siRNA for control purposes.

the preparation of siRNAs with single strands synthesized exclusively on photolabile supports.

In summary, a photosensitive linker grafted onto PS beads was prepared and employed in a new "on-support" method for the synthesis, deprotection and hybridization of RNA. A chief asset is its orthogonality to the deprotection reagents commonly used in nucleic acid chemistry, allowing for a fast and simple post-synthetic processing of DNA and RNA. In addition, the ability to form duplexes on a support without the need for HPLC purification could greatly simplify the preparation of siRNA libraries. Our approach also paves the way for a mild and practical assembly of RNA containing base-sensitive or biolabile groups ("prodrugs"), a matter of increasing importance to the design of potent siRNAs.^{27,41}

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Notes and references

- V. Mittal, *Nat. Rev. Genet.*, 2004, **5**, 355–365.
- A. T. Willingham, Q. L. Deveraux, G. M. Hampton and P. Aza-Blanc, *Oncogene*, 2004, **23**, 8392–8400.
- G. R. Rettig and M. A. Behlke, *Mol. Ther.*, 2012, **20**, 483–512.
- J. C. Burnett and J. J. Rossi, *Chem. Biol.*, 2012, **19**, 60–71.
- G. F. Deleavey and M. J. Damha, *Chem. Biol.*, 2012, **19**, 937–954.
- J. B. Bramsen, A. Grünweller, R. K. Hartmann and J. Kjems, *Handbook of RNA Biochemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, 2014, pp. 1243–1278.
- S. E. Mohr and N. Perrimon, *Wiley Interdiscip. Rev.: RNA*, 2012, **3**, 145–158.
- S. Sharma and A. Rao, *Nat. Immunol.*, 2009, **10**, 799–804.
- C. Falschlehner, S. Steinbrink, G. Erdmann and M. Boutros, *Biotechnol. J.*, 2010, **5**, 368–376.
- Q. Pan, L. J. W. van der Laan, H. L. A. Janssen and M. P. Peppelenbosch, *Trends Biotechnol.*, 2012, **30**, 206–215.
- R. I. Hogrefe, B. Midthune and A. Lebedev, *Isr. J. Chem.*, 2013, **53**, 326–349.
- S. L. Beaucage and C. B. Reese, *Current Protocols in Nucleic Acid Chemistry*, John Wiley & Sons, Inc., 2009, vol. 38, pp. 2.16.11–2.16.31.
- K. K. Ogilvie, K. L. Sadana, E. A. Thompson, M. A. Quilliam and J. B. Westmore, *Tetrahedron Lett.*, 1974, **15**, 2861–2863.
- K. K. Ogilvie, N. Usman, K. Nicoghossian and R. J. Cedergren, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 5764–5768.
- F. Wincott, A. DiRenzo, C. Shaffer, S. Grimm, D. Tracz, C. Workman, D. Sweedler, C. Gonzalez, S. Scaringe and N. Usman, *Nucleic Acids Res.*, 1995, **23**, 2677–2684.
- X. Zhang, B. L. Gaffney and R. A. Jones, *Nucleic Acids Res.*, 1997, **25**, 3980–3983.
- T. J. Matray, D. J. Yoo, D. L. McMinn and M. M. Greenberg, *Bioconjugate Chem.*, 1997, **8**, 99–102.
- J. Jemielity, P. Heinonen, H. Lönnberg and E. Darzynkiewicz, *Nucleosides Nucleic Acids*, 2005, **24**, 601–605.
- H. Salo, A. Guzaev and H. Lönnberg, *Bioconjugate Chem.*, 1998, **9**, 365–371.
- R. T. Pon and S. Yu, *Nucleic Acids Res.*, 1997, **25**, 3629–3635.
- J. G. Lackey, D. Sabatino and M. J. Damha, *Org. Lett.*, 2007, **9**, 789–792.
- J. G. Lackey, D. Mitra, M. M. Somoza, F. Cerrina and M. J. Damha, *J. Am. Chem. Soc.*, 2009, **131**, 8496–8502.
- S. Bühler, I. Lagoja, H. Giegrich, K.-P. Stengele and W. Pfeleiderer, *Helv. Chim. Acta*, 2004, **87**, 620–659.
- C. Dell'Aquila, J.-L. Imbach and B. Rayner, *Tetrahedron Lett.*, 1997, **38**, 5289–5292.
- D. L. McMinn, R. Hirsch and M. M. Greenberg, *Tetrahedron Lett.*, 1998, **39**, 4155–4158.
- M. Su, J. Wang and X. J. Tang, *Chem. – Eur. J.*, 2012, **18**, 9628–9637.
- R. Johnsson, J. G. Lackey, J. J. Bogojoski and M. J. Damha, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 3721–3725.
- J. Xu, C. D. Duffy, C. K. W. Chan and J. D. Sutherland, *J. Org. Chem.*, 2014, **79**, 3311–3326.
- R. A. Johnsson, J. J. Bogojoski and M. J. Damha, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 2146–2149.
- K. Tomaya, M. Takahashi, N. Minakawa and A. Matsuda, *Org. Lett.*, 2010, **12**, 3836–3839.
- M. Manoharan, M. E. Jung, K. Rajeev, R. K. Pandey and G. Wang, *WO2005097817 A2*, 2005.
- L. J. Brown, J. Cummins, A. Hamilton and T. Brown, *Chem. Commun.*, 2000, 621–622.
- J. Smirnova, D. Wöll, W. Pfeleiderer and U. E. Steiner, *Helv. Chim. Acta*, 2005, **88**, 891–904.
- H. A. Fahim and A. M. Fleifel, *J. Chem. Soc.*, 1952, 4519–4521.
- M. J. Damha, M. Hassler, T. H. Chan, N. M. Reddy and R. A. Donga, *WO2013026142 A1*, 2013.
- Y. Hayakawa, M. Uchiyama and R. Noyori, *Tetrahedron Lett.*, 1986, **27**, 4191–4194.
- Y. Hayakawa and M. Kataoka, *J. Am. Chem. Soc.*, 1998, **120**, 12395–12401.
- M. J. Damha, P. A. Giannaris and S. V. Zabarylo, *Nucleic Acids Res.*, 1990, **18**, 3813–3821.
- H. Venkatesan and M. M. Greenberg, *J. Org. Chem.*, 1996, **61**, 525–529.
- A. Grajkowski, J. Cieślak, J. S. Kauffman, R. J. Duff, S. Norris, D. n. I. Freedberg and S. L. Beaucage, *Bioconjugate Chem.*, 2008, **19**, 1696–1706.
- T. Laverne, C. Baragney, C. Dupouy, N. Parey, W. Wuensche, G. Szakiel, J.-J. Vasseur and F. Debart, *J. Org. Chem.*, 2011, **76**, 5719–5731.