Two-Step Synthesis of a 5′-Azidothymidine Building Block for the Assembly of Oligonucleotides for Triazole-Forming Ligations

Hassan Said,[a] Claudia Guttroff,[a] Afaf H. El-Sagheer,[b,c] Clemens Richert*[a]

[a] Institut für Organische Chemie, Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany
Fax +49(0)71168564321; E-mail: lehrstuhl-2@oc.uni-stuttgart.de

[b] School of Chemistry, University of Southampton, Highfield, Southampton SO17 1BJ, UK

[c] Chemistry Branch, Department of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez Canal University, Suez 43721, Egypt

Received: 18.09.2012; Accepted after revision: 19.10.2012

Abstract: A two-step synthesis converting thymidine into a phosphotriester building block of 5′-azido-5′-deoxythymidine in 60% overall yield is presented. The building block was used to assemble an oligonucleotide with an azido group at its 5′-terminus, which underwent ligation–cycloaddition, producing a strand with PCR-compatible linkage in high yield.

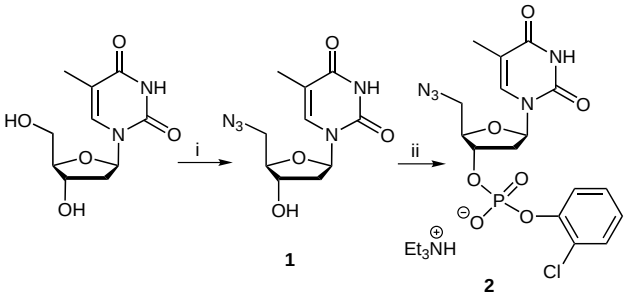
Key words: azides, phosphotriester chemistry, click reaction, oligonucleotide, ligation

Progress in the field of synthetic biology has been limited by the inability to synthesize DNA of great length.[1] The most common approach for the synthesis of long stretches of genetic information is the solid-phase synthesis of oligodeoxynucleotides and subsequent ligation of these oligonucleotides to the desired sequences, followed by polymerase-based formation of complementary strands and amplification.[2–5] Ligation reactions that rely on 1,3-dipolar cycloaddition between an azido-terminated oligonucleotide and a propargyl-terminated reaction partner have recently come into focus because they are robust, high yielding, and compatible with polymerase chain reaction (PCR) amplification.[6,7] This methodology has greater biological relevance than approaches involving other CLICK reactions.[8,9]

Unfortunately, azido-terminal oligonucleotides cannot be prepared via phosphoramidite building blocks because the potential for Staudinger reduction between the phosphorus(III) center and the azido group that makes such building blocks labile. Two approaches exist that overcome this complication rely on solid-phase synthesis for oligonucleotide chain assembly. One approach involves on-support, two-step conversion of the 5′-terminal hydroxyl group of the pre-assembled oligonucleotide into an azide.[10] On-support conversion has also been used for the preparation of 5-azido-2′-deoxyuridine, where the azido group is attached to the nucleobase.[11] The other approach uses a phosphorus(V) building block containing the azido group. Azido phosphodiester building blocks for phosphotriester-based chain extension[12,13] have been described for 2′-azido-2′-deoxynucleosides[14] and 3′-azido-2′,3′-deoxythymidine.[9] To the best of our knowledge, no building block of a 5′-azido-2′,5′-dideoxynucleoside for phosphotriester-based chain assembly has been reported. The lack of such a building block complicates preparation of the azido component for PCR-compatible triazole-linked ligation products.[6]

Here we report a convenient two-step synthesis of 5′-azidothymidine-3′-*O*-(2-chlorophenyl)monophosphate (**2**), starting from thymidine, and its use for the assembly of a 5′-azido-terminal oligonucleotide in high yield, based on a final phosphotriester coupling step. The deprotected oligonucleotide readily underwent a template-directed ligation to a 3′-propargyl-terminated strand via copper-catalyzed dipolar cycloaddition.

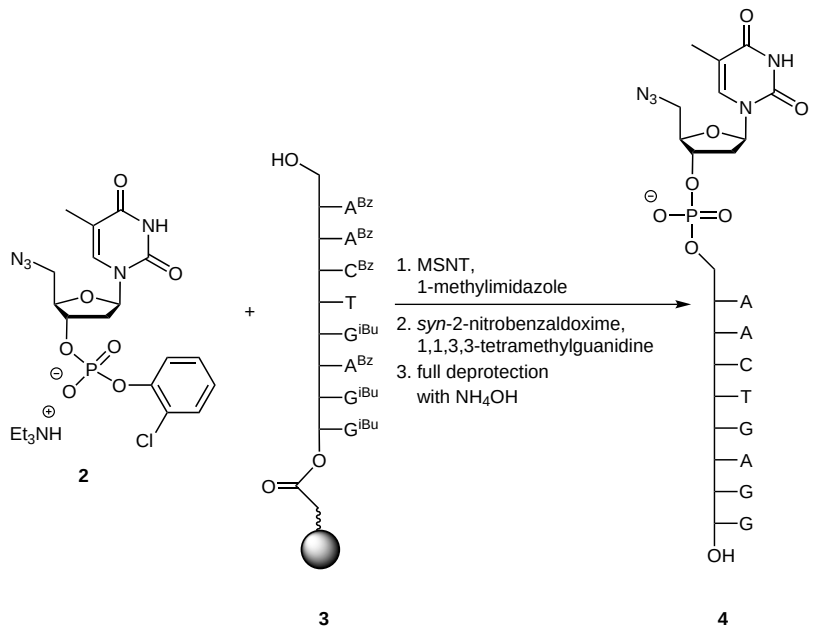
Scheme 1 shows the synthesis of **2**. One-step regioselective conversion of the 5′-hydroxyl group into an azide[15] was followed by phosphorylation under conditions similar to those used by Sproat and Gait[13] and more recently by Micura and co-workers.[14] After column chromatography, **2** was obtained in 60% overall yield. Coupling of **2** to support-bound oligonucleotide **3** gave full length oligonucleotide **4** that was released from the support and fully deprotected after removal of the chlorophenyl protecting group (Scheme 2).



Scheme 1 Synthesis of 5′-azidothymidine building block **2**. *Reagents and conditions*: (i) Ph_3P, NaN_3, CBr_4, DMF, 24 h, 78%; (ii) *N*-methylimidazole, 1,2,4-triazole, Et_3N, 2-chlorophenyl phosphorodichloridate, in THF, 77%.

Figure 1 shows MALDI–TOF mass spectra of crude oligonucleotide **5** prepared by the conventional, two-step derivatization of the full length oligonucleotide, as described

SYNLETT 2012, 23, 2923–2926
Advanced online publication: 16.11.2012
DOI: 10.1055/s-0032-1317605; Art ID: ST-2012-B0788-L



Scheme 2 Coupling of chlorophosphate building block **2** to oligonucleotide **3** on controlled pore glass, followed by two-stage deprotection

in reference 10, and of slightly longer oligonucleotide **4** prepared by the present methodology, using **2**. The phosphotriester-based coupling, followed by two-step deprotection proceeded smoothly, producing **4** with minimal amounts of side products.

With azido-terminal oligonucleotide **4** in hand, we then proceeded to ligating this oligonucleotide to 3′-propargyl-terminated oligonucleotide **6** on DNA 23-mer **7** as template (Figure S3 in the Supporting Information). As expected, the 1,3-dipolar cycloaddition proceeded quickly, giving ligation product **8** in near-quantitative yield, as determined by gel electrophoresis (Figure 2).

In conclusion, we have presented a convenient two-step synthesis of a thymidine building block for solid-phase synthesis of 5′-azido-terminal oligonucleotides, together with evidence for the successful ligation of such an oligonucleotide.[16–19] We expect that a similar approach can be adopted to prepare the 5′-azido building blocks with the remaining three nucleobases (A, C, and G). Syntheses of 5′-azido-2′,5′-dideoxynucleosides are known.[15]

Acknowledgment

The authors thank Prof. T. Brown for a review of the manuscript. This work was supported by DFG (grant No. RI 1063/8-2 to C.R.) and BBSRC (sLOLA grant No BB/J001694/1 ‘Extending the boundaries of nucleic acid chemistry’).

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

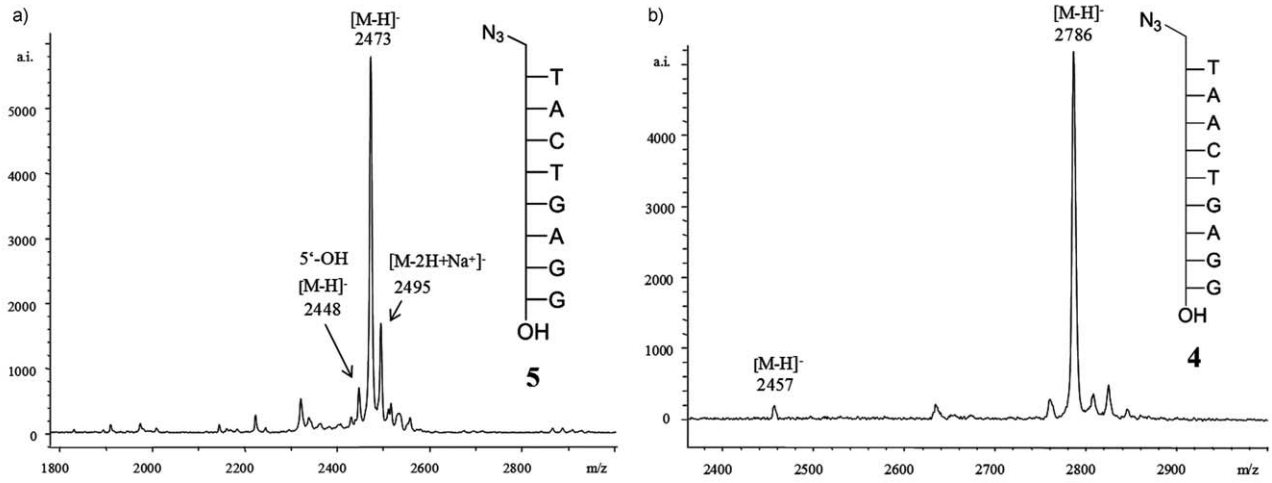


Figure 1 MALDI–TOF mass spectra of crude 5′-azido-terminal oligonucleotides. (a) Sequence **5**, as prepared by on-support conversion of the 5′-terminal alcohol into the corresponding azide,[10] followed by deprotection, and (b) sequence **4**, prepared by chain extension with **2**, followed by two-stage deprotection.

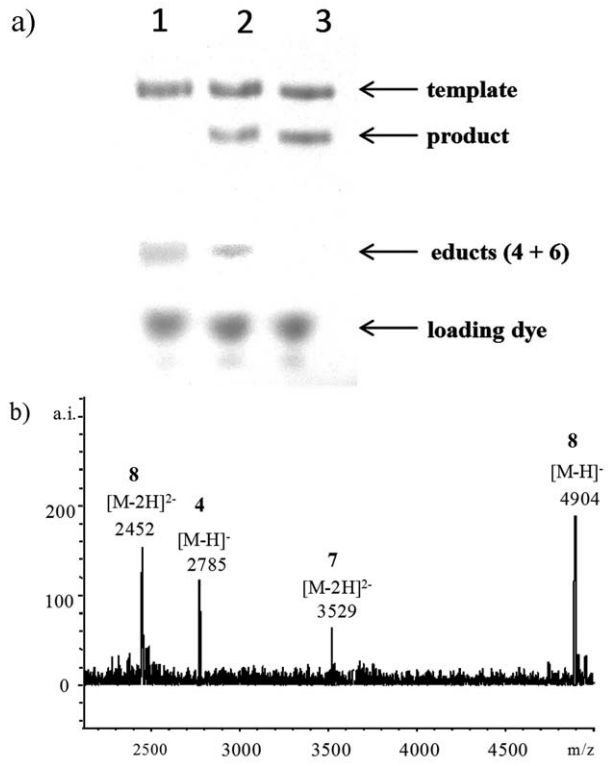


Figure 2 Results of ligation assays. (a) Denaturing polyacrylamide gel (20% PAGE) of samples of the ligation, as imaged by UV-shadowing. Lane 1: 200 pmol of each of oligonucleotides **4** and **6** (educts), and template strand **7**; lane 2: 200 pmol of ligation mixture after 1 h; lane 3: 200 pmol of ligation mixture after 2 h; (b) MALDI–TOF mass spectrum of reaction mixture of the template-directed cycloaddition between 5′-azide oligonucleotide **4** with oligonucleotide **6** on template **7** to produce ligation product **8**. Assignment: $[M - H]^-$ of **4** = 2785 g/mol and $[M - 2\ H]^{2-}$ of template strand **7** = 3529 g/mol; ligation product **8** (M = 4904 g/mol) and $[M - 2\ H]^{2-}$ of ligation product at 2452 g/mol.

References

(1) Reese, C. *Org. Biomol. Chem.* **2005**, *3*, 3851.
(2) Dillo, P. J.; Rosen, C. A. *BioTechniques* **1990**, *9*, 298.
(3) Stemmer, W. P. C.; Crameri, A.; Ha, D.; Brennan, T. M.; Heyneker, L. *Gene* **1995**, *164*, 49.
(4) Engels, J. W. *Angew. Chem. Int. Ed.* **2005**, *44*, 7166.
(5) Gibson, D. G.; Young, L.; Chuang, R. Y.; Venter, J. C.; Hutchison, C. A.; Smith, H. O. *Nat. Methods* **2009**, *6*, 343.
(6) El-Sagheer, A. H.; Sanzone, A. P.; Gao, R.; Tavassoli, A.; Brown, T. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 11343.
(7) El-Sagheer, A. H.; Brown, T. *Chem. Commun.* **2011**, *47*, 12057.
(8) Kumar, R.; El-Sagheer, A.; Tumpane, J.; Lincoln, P.; Wilhelmsson, L. M.; Brown, T. *J. Am. Chem. Soc.* **2007**, *129*, 6859.
(9) El-Sagheer, A. H.; Brown, T. *J. Am. Chem. Soc.* **2009**, *131*, 3958.
(10) (a) Miller, G. P.; Kool, E. T. *Org. Lett.* **2002**, *4*, 3599. (b) Miller, G. P.; Kool, E. T. *J. Org. Chem.* **2004**, *69*, 2404.
(11) Bayer, C.; Wagenknecht, H. A. *Chem. Commun.* **2010**, *46*, 2230.
(12) Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 767.
(13) Sproat, B. S.; Gait, M. *J. Am. Chem. Soc.* **1976**, *98*, 3655.
(14) (a) Aigner, M.; Hartl, M.; Fauster, K.; Steger, J.; Bister, K.; Micura, R. *ChemBioChem* **2011**, *12*, 47. (b) Fauster, K.; Hartl, M.; Santner, T.; Aigner, M.; Kreutz, C.; Bister, K.; Ennifar, E.; Micura, R. *ACS Chem. Biol.* **2012**, *7*, 581.
(15) (a) Bannwarth, W. *Helv. Chim. Acta* **1988**, *71*, 1517. (b) Mag, M.; Engels, J. W. *Nucleic Acids Res.* **1989**, *17*, 5973.
(16) **5′-Azido-5′-deoxythymidine (1)**: The preparation of compound **1** used a slight modification of the protocol described in reference 15, using NaN_3 instead of LiN_3. Briefly, triphenylphosphine (3.21 g, 12.24 mmol, 1.2 equiv), NaN_3 (1.95 g, 30.04 mmol, 3 equiv) and CBr_4 (4.01 g, 12.09 mmol, 1.2 equiv) were added to a solution of thymidine (2.48 g, 10.23 mmol, 1 equiv) in anhyd DMF (40 mL). The reaction mixture was stirred at r.t. for 24 h under argon. When TLC (CH_2Cl_2–MeOH, 9:1) showed complete conversion, the reaction mixture was treated with sat. $NaHCO_3$ solution (50 mL). After extracting with $CHCl_3$ (3 × 50 mL), the organic solution was washed once with H_2O and twice with brine (2 × 100 mL), followed by drying over Na_2SO_4. The reaction mixture was filtered and the solvents were evaporated. The crude product was purified by column chromatography on silica, eluting with a step gradient of MeOH in CH_2Cl_2 (0–6%) to give compound **1** (2.14 g, 78%). The spectroscopic data were in agreement with the literature.[15]
5′-Azidothymidine-3′-*O*-(2-chlorophenyl)monophosphate (2): 1,2,4-Triazole (0.757 g, 10.3 mmol, 5.5 equiv) was dissolved in anhyd THF (23 mL) and treated with Et_3N (1.3 mL, 9.3 mmol, 5 equiv). To this, 2-chlorophenyl phosphorodichloridate (800 µL, 4.62 mmol, 2.5 equiv) was added, immediately leading to a white precipitate. The suspension was stirred at 20 °C for 30 min. Then, a solution of 5′-azido-5′-deoxythymidine (0.5 g, 1.87 mmol, 1 equiv) and 1-methylimidazole (600 µL, 7.42 mmol, 4 equiv) in anhyd THF (8 mL) was added, and the reaction mixture was stirred at r.t. for 45 min under Ar. When TLC (CH_2Cl_2–MeOH, 9:1) showed complete conversion, the reaction was quenched with H_2O (400 µL) and Et_3N (1.5 mL). The solvents were evaporated, and the residue was dissolved in CH_2Cl_2 (50 mL) and sat. $NaHCO_3$ (50 mL). The product was extracted in CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with brine (3 × 100 mL) and dried over Na_2SO_4. After evaporation of the solvents, the crude product was purified by column chromatography on silica (CH_2Cl_2–MeOH–Et_3N, 98:1:1 for packing the column), eluting with a step gradient of 0% to 9% MeOH–0.5% Et_3N to give **2** (0.803 g, 77%) as an orange-colored foam.
^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.15 (t, ^3J = 7.3 Hz, 9 H, CH_3CH_2NH), 1.79 (s, 3 H, thymidine-Me), 2.23 (m, 2 H, 2′-H, 2″-H), 3.03 (q, ^3J = 7.3 Hz, 6 H, CH_3CH_2NH), 3.54 (m, 2 H, 5′-H, 5″-H), 4.04 (m, 1 H, 4′-H), 4.62 (m, 1 H, 3′-H), 6.14 (t, ^3J = 6.6 Hz, 1 H, 1′-H), 6.93 (t, ^3J = 7.8 Hz, 1 H, ArH), 7.18 (t, ^3J = 7.2 Hz, 1 H, ArH), 7.35 (d, ^3J = 7.9 Hz, 1 H, ArH), 7.51 (s, 1 H, 6-H), 7.58 (d, ^3J = 7.8 Hz, 1 H, ArH), 9.5 (br s, 1 H, CH_3CH_2NH), 11.35 (s, 1 H, NH). ^{31}P NMR (121.5 MHz, DMSO-d_6): δ = –6.87. MS (ESI$^-$; MeOH–H_2O, 1:1): *m/z* calcd for $C_{16}H_{16}ClN_5O_7P$ $[M–H]^-$: 456.05, found: 456.02.
(17) **Synthesis of 5′-Azide Oligonucleotide (4)**: Azido building block **2** was coupled to the 5′-terminus of the fully protected oligonucleotide attached on controlled pore glass. The immobilized octamer oligodeoxynucleotide on cpg (**3**, 10 mg, approx. 0.2 µmol loading, DMT-off state), had been purchased from Biomers Inc. (Ulm, Germany), where it had been assembled via conventional automated DNA synthesis.

The support was dried at 0.1 mbar for 1 h. A solution of 5′-azido nucleotide **2** (8.5 mg, 15 μmol), previously co-evaporated twice from anhydrous pyridine and dried at 0.1 mbar, was treated with 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT; 22.2 mg, 75 μmol) in anhydrous pyridine (100 μL) at r.t. for 15 min. Then, 1-methylimidazole (10 μL, 126 μmol) was added and the mixture was transferred to a polypropylene cup containing the DNA-bearing cpg. After 50 min, the supernatant was carefully aspirated, and the cpg was washed with anhyd pyridine (3 × 200 μL) and MeCN (5 × 200 μL). The support was treated with a solution of *syn*-2-nitrobenzaldoxime (18 mg, 10 mmol) and 1,1,3,3-tetramethylguanidine (20 μL) in dioxane–H_2O (250 μL, 1:1). The mixture was left at r.t. for 16 h and the support was then washed with dioxane–H_2O (1:1; 5 × 200 μL). After drying at 0.1 mbar, the cpg was treated with NH_4OH (25% aq NH_3, 500 μL) for 5 h at 55 °C. The mixture was cooled to r.t. and excess NH_3 was removed by gently blowing a stream of nitrogen onto the surface until the solution was odorless. The solution was lyophilized and the oligonucleotide was purified by reversed-phase HPLC with a step gradient of MeCN (0% for 5 min to 25% in 35 min, t_R = 24 min) in 0.1 M triethylammonium acetate buffer (pH 7.0). Elution was monitored by UV-absorption at λ = 260 nm.

(18) **Synthesis of 3′-Alkyne Oligonucleotide 6**: The oligonucleotide was synthesized via standard phosphoramidite synthesis. The first nucleoside 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-propargyl-5-methyldeoxycytidine was attached to the solid support (33 μmol/g loading, AM polystyrene, Applied Biosystems) according to the method reported in reference 20. The support was transferred into a DNA synthesizer column and the assembly of the sequence was performed in the 3′- to 5′-direction, followed by cleavage, deprotection, and HPLC purification.

(19) **Template-Directed Ligation of Oligonucleotides**: Oligonucleotides **4**, **6** and template strand **7** (0.5 nmol each) were mixed, lyophilized and dissolved in 0.2 M NaCl (125 μL). For annealing, the solution was heated to 85 °C for 5 min and cooled to 4 °C in the course of 2 h. A solution of *tris*-hydroxypropyltriazole[21] (2.8 μmol in 0.2 M NaCl, 38 μL), sodium ascorbate (4 μmol in 0.2 M NaCl, 8 μL) and $CuSO_4 \cdot 5H_2O$ (0.04 μmol in 0.2 M NaCl, 4 μL) was added to the oligonucleotides, and the reaction mixture was kept at 0 °C for 1 h and at r.t. for 1 h. Then, the sample was desalted by gel filtration (Sephadex G-25 column) and analyzed by MALDI–TOF–MS and 20% denaturing polyacrylamide gel electrophoresis.

(20) El-Sagheer, A. H.; Brown, T. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 15329.

(21) Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853.

