

DNA Conjugation by the Staudinger Ligation: New Thymidine Analogues

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Dedicated to Prof. Dr. Wolfgang Pfeleiderer on the occasion of his 80th birthday

Abstract: Two novel modified 2'-deoxyuridine triphosphates carrying an azide functionality linked to the nucleobase were synthesized. For probing the sterical influence on enzymatic incorporation and Staudinger ligation, differently sized flexible linkers were chosen. Both nucleotides can completely replace natural thymidine in primer extension as well as polymerase chain reaction (PCR) using *Pyrococcus woesei* DNA polymerase. For PCR with larger gene fragments as template, however, the longer linker disturbs the DNA polymerase and yields less product. For azide-labeled primer extension products, subsequent conjugation of suitably functionalized phosphines via Staudinger ligation was achieved, for example for the conjugation of biotin as an affinity tag.

Key words: azides, conjugation, Staudinger ligation, DNA, nucleotides

To enable investigation of complex biological systems, efficient labeling techniques for the involved biopolymers are often required. Conjugation of functional molecules like dyes or affinity tags to these biopolymers allows further and better investigations of such systems. Ideally, the coupling reaction has to be site-specific, bioorthogonal, high yielding, and for in vivo experiments biocompatible and nontoxic. There is no standard conjugation protocol which fulfills all of these conditions so far.¹

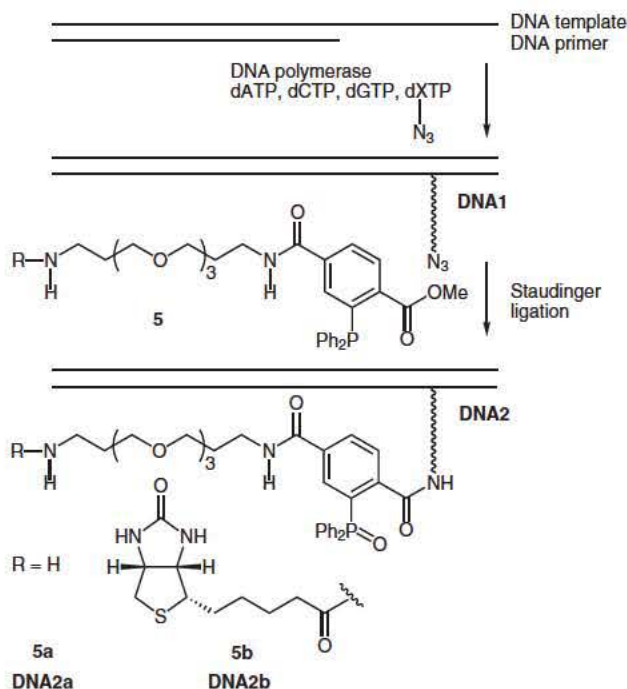
Recently, two promising reactions based on the reactivity of the azide moiety were reported.² One method makes use of the [2+3] cycloaddition reaction of an azide and an alkyne first reported by Huisgen.³ Copper catalysis promotes the reaction to proceed at room temperature⁴ and led to widespread applications especially in bioconjugate chemistry.⁵ Recently, the method has been extended for conjugation of DNA.^{6,7} On the other hand, the so-called Staudinger ligation developed by Bertozzi readily occurs between an azide and a phosphine without the need for further reagents.⁸ The intermediate aza-ylide gets thereby trapped by an acyl group to form a stable amide bond. This reaction has been applied, for example, in the conjugation of carbohydrates, protein and phage particles, peptide ligation, and immobilization.⁹ Few examples for the conjugation of DNA by Staudinger ligation are known. The conjugation of a fluorescence dye to the 5' terminus of single stranded DNA¹⁰ as well as the sequence specific conjugation of phenanthroline to DNA for subsequent Cu(I)-induced strand scission has been reported.^{11,12} Re-

cently, we presented the synthesis of 7-azide-modified 7-deaza-2'-deoxyadenosine, its incorporation in a growing DNA strand by enzymatic primer extension reaction, and subsequent biotin labeling by Staudinger ligation.¹³ Here, we report the synthesis of two new azide-modified thymidine analogues with different linker lengths and their conversion into triphosphates. Their incorporation into DNA was then investigated using primer extension reactions as well as PCR and the resulting DNA was subsequently conjugated with biotin using Staudinger ligation.

Our strategy to label DNA site-specifically involves two steps (Scheme 1): First the azide-modified triphosphate should replace the corresponding natural 2'-deoxynucleoside-5'-O-triphosphate (dNTP) in a DNA polymerase reaction. For this the polymerase has to accept the unnatural nucleoside and ideally can use it as a template for incorporation of the canonical base in PCR. Then the obtained azide-labeled DNA should serve as substrate for further modifications by Staudinger ligation with a suitably functionalized phosphine. Since it has been shown that 5-modified thymidine derivatives are accepted by DNA polymerases,^{7,14,15} we focused our synthesis on these kinds of analogues. A sterically encumbered moiety might disturb the acceptance of the DNA polymerase; however, a short linker might decrease the yield of the subsequent Staudinger ligation. So we designed two nucleotides bearing a flexible linker with different lengths towards the azide moiety to test which is best suited for our endeavor (**4a** and **4b**, Scheme 2).

The synthesis started with a Sonogashira reaction from fully TBS protected 5-iodo-2'-deoxyuridine **1** with two different unprotected alkynyl alcohols (pent-4-yn-1-ol or *O*-propargyl triethylene glycol) to afford the different linker lengths (Scheme 2).¹⁶ The alcohols **2** were then converted into the mesylates and afterwards substituted with NaN₃ to form the azides **3**. After deprotection of the hydroxyl groups of the sugar moiety, the corresponding 5' triphosphates **4** were obtained using the reported methods.¹⁷ The yields of each step were high in both cases, but overall slightly lower for the nucleoside bearing the triethylene glycol linker.

With these triphosphates in hand, we next investigated the properties of **4a** and **4b** towards their action on DNA polymerases. In order to test the ability of DNA polymerases to accept the modified triphosphates and to incorporate the respective nucleotide into a nascent DNA strand, we set up a primer extension reaction. A 35-nucleotide (nt) template was designed in a way to contain a single A res-



Scheme 1 Schematic depiction for labeling of DNA with phosphines **5a** and **5b**.

idue, coding for insertion of a modified dTTP analogue after extending the 23nt primer strand by three residues (Figure 1A). The reactions were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). We tested *Thermus aquaticus* (*Taq*), *Thermococcus litoralis* [Vent (exo-)] and *Pyrococcus woesei* (*Pwo*) DNA polymerase, whereas *Pwo* DNA polymerase accepted the modified triphosphates best as has been reported by us¹³ and Famulok et al. for generation of highly functionalized DNA.¹⁴ Reactions lacking dTTP predominantly abort before the template A after extension by three nucleotides, while reactions including dTTP gave rise to full-length products (Figure 1B). In case of substitution of natural dTTP by **4a** or **4b** full-length product was also obtained in a similar fashion. However, the reaction product is somewhat shifted on the gel due to its higher molecular weight and therefore lower mobility in the polyacrylamide gel.

Next, we investigated whether the double-stranded enzymatically synthesized azide-labeled DNA is suitable for Staudinger ligation. First the temperature dependence was

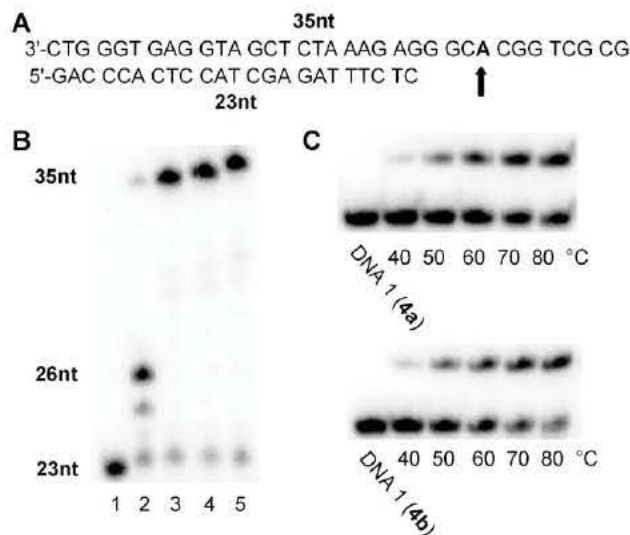
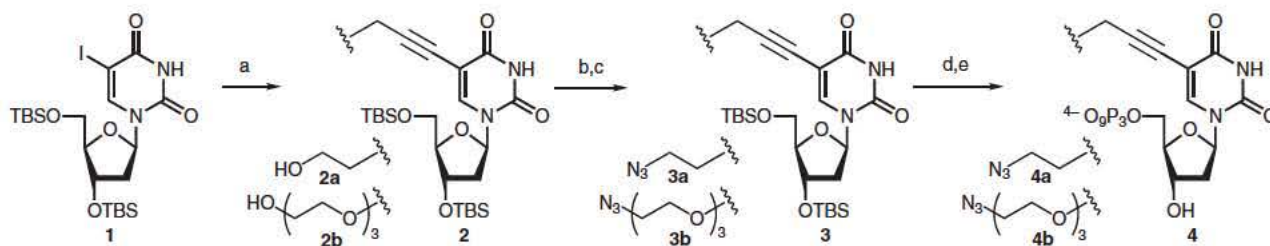


Figure 1 (A) Sequences of primer template complex used to study incorporation of modified triphosphates **4a** and **4b**. (B) Denaturing PAGE analysis of primer extension reactions. Lane 1: 5'-³²P-labeled 23nt primer strand; Lane 2: primer template complex including dATP, dCTP, dGTP, and *Pwo* DNA polymerase; Lane 3: same as in lane 2 including dTTP; Lane 4: same as in lane 2 including **4a**; Lane 5: same as in lane 2 including **4b**. (C) Staudinger ligation employing enzymatically synthesized double-stranded DNA (dsDNA) as shown in Figure 1B lane 4 (upper autoradiogram) or lane 5 (lower autoradiogram) with 25% DMF in aqueous buffer and phosphine **5a** at various temperatures as indicated. nt = nucleotide.

studied using phosphine **5a** as a model (Figure 1C).¹¹ The ligation reaction was carried out in aqueous buffer with 25% of DMF to increase the solubility of the phosphine compound. Increasing temperatures led to higher conversion within four hours reaction time. Interestingly, DNA derived from building block **4b** formed the reaction product in higher yields compared to **4a**. However, best results were obtained after increasing reaction time to 12 hours at 60 °C. For further ligation experiments we used phosphine **5b** bearing a biotin at its end. Biotin is often used in biochemistry for labeling issues and can be further conjugated with streptavidin functionalized materials.¹⁸ In this reaction, again, DNA with building block **4b** generated higher yields (70%) than **4a** (60%, Figure 2A). The yield was determined by quantifying the radioactivity of both the product and starting material DNA bands on the polyacrylamide gel by conventional phosphorimaging.



Scheme 2 Synthesis of azide modified triphosphates. *Reagents and conditions*: Et₃N, CuI, Pd(PPh₃)₄, DMF, 20 °C, **2a**: pent-4-yn-1-ol, 81%, **2b**: *O*-propargyl triethylene glycol, 83%; (b) MsCl, DIPEA, CH₂Cl₂, 0 °C; (c) NaN₃, DMF, 35 °C, **3a**: 70% over 2 steps, **3b**: 50% over 2 steps; (d) TBAF, THF, 0 °C; (e) proton sponge, POCl₃, PO(OMe)₃, 0 °C, then (Bu₃NH)₂H₂P₂O₇, Bu₃N then TEAB buffer, **4a**: 28% over 2 steps, **4b**: 23% over 2 steps.

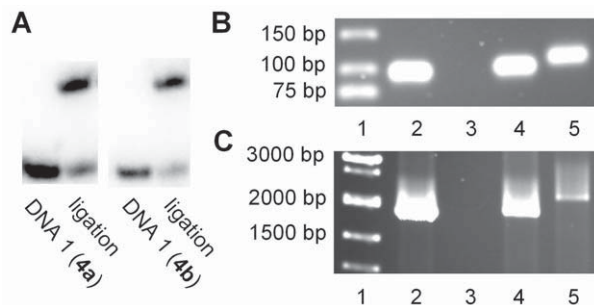


Figure 2 (A) Denaturing PAGE analysis of Staudinger ligation with phosphine **5b** with 25% DMF for 12 h at 60 °C employing dsDNA, which contains **4a** (left gel) or **4b** (right gel). (B) 2.5% agarose gel stained with ethidium bromide showing PCR products of 98nt template. Lane 1: Marker; Lane 2: PCR with dTTP; Lane 3: PCR without TTP; Lane 4: PCR with **4a** instead of dTTP; Lane 5: PCR with **4b** instead of dTTP. (C) 0.8% agarose gel stained with ethidium bromide showing PCR products of 1.9k nt template. Lanes as in Figure 2B. nt = nucleotide; bp = base pair.

Finally, we tested the suitability of building blocks **4a** and **4b** for substituting dTTP in polymerase chain reactions (PCR). In this case we only tested *Pwo* DNA polymerase, first with a short template of 98nt in size. As with the primer extension tests we conducted one control reaction with all four natural dNTPs leading to a single PCR product with the desired length. In another control we performed the same reaction in the absence of dTTP leading to no observable PCR product formation (Figure 2B). Using standard PCR conditions the DNA polymerase was able to substitute compounds **4a** and **4b** for TTP and amplify the modified DNA in a similar fashion as with all four natural dNTPs. Again a small shift of the product bands to lower mobility was observed for the modified analogues, which is more pronounced with the larger modification of **4b**. Encouraged by these results we switched to a larger genomic DNA fragment as template for the PCR. Here, compound usage of **4a** resulted in similar amounts of PCR product compared to the PCR with all natural dNTPs. PCR including compound **4b** instead of dTTP produced less but observable product. Presumably the larger modification interfered with the DNA polymerase. Nevertheless only one PCR product was observed, and so we were able to generate large azide-labeled DNA fragments with both compounds.

In conclusion, a straightforward synthesis of azide modified thymidines was developed. The corresponding triphosphates were tested with different DNA polymerases indicating that *Pwo* DNA polymerase was most suited for our endeavor. Even amplification of large DNA fragments could be accomplished by PCR using this DNA polymerase and the modified triphosphates. The azide-labeled DNA in turn can be conjugated with phosphines that bear several functional groups, such as biotin. For Staudinger ligation reactions DNA generated by building block **4b** seems to be superior over DNA derived from **4a** due to smaller sterical hindrance. On the other hand, **4b** leads to smaller PCR product formation, when amplifying large

gene fragments with **4b** instead of dTTP under standard reaction conditions. Further development of the reaction conditions might lead to improvements along these lines. However, Staudinger ligation with large azide-labeled DNA fragments could lead to some new applications in nanobiotechnology as well as material science.

All melting and boiling point values quoted are uncorrected. All reagents are commercially available and were used without further purification. TBS protected nucleoside **1**,¹⁹ *O*-tosyl triethylene glycol,²⁰ and phosphines **5a** and **5b**¹¹ were prepared according to literature. Solvents were stored over molecular sieves (Fluka) and used directly without further purification, unless otherwise noted. All reactions were conducted under rigorous exclusion of air and moisture. Elemental analyses were carried out by the microanalysis facility of the University of Konstanz. NMR spectra were recorded on a Jeol JNA-LA-400 (¹H: 400 MHz, ¹³C: 101 MHz, ³²P: 162 MHz), a Bruker Avance DRX 600 (¹H: 600 MHz, ¹³C: 151 MHz). The solvent signals were used as references and the chemical shifts converted to the TMS scale and are given in ppm (δ). ESI-IT mass spectra were recorded on a Bruker Daltonics esquire 3000+. Flash chromatography was done using Merck silica gel G60 (230–400 mesh), and Merck precoated plates (silica gel 60 F₂₅₄) were used for TLC. Both technical solvents were distilled prior to use. Petroleum ether (PE) used refers to the fraction boiling in the range 35–80 °C.

O-Propargyl Triethylene Glycol

To a suspension of NaH (1.5 equiv, 0.69 g, 17.2 mmol) in THF (5 mL), was added propargyl alcohol (1.5 equiv, 1.0 mL, 17.2 mmol) at 0 °C. After 30 min, a solution of *O*-tosyl triethylene glycol (1.0 equiv, 3.5 g, 11.5 mmol) in THF (10 mL) was added and the mixture stirred for 3 h at 0 °C. Then it was combined with sat. aq NaHCO₃ (15 mL) and extracted with EtOAc (6 × 10 mL). The combined organic layers were dried (MgSO₄), and concentrated in vacuo. The residue was purified by flash chromatography [silica gel (EtOAc-PE, 2:3 to 5:1); *R_f* = 0.3 (EtOAc-PE, 3:1)]; yield: 1.0 g (46%).

¹H NMR (400 MHz, CDCl₃): δ = 2.43 (t, ⁴*J* = 2.2 Hz, 1 H, CH), 3.57–3.64 (m, 2 H, CH₂OH), 3.64–3.75 (m, 10 H, OCH₂CH₂O), 4.19 (d, ⁴*J* = 2.2 Hz, 2 H, CCH₂O).

¹³C NMR (101 MHz, CDCl₃): δ = 58.2, 61.5, 68.9, 70.1, 70.2, 70.5, 72.4, 74.6, 79.5.

ESI-MS: *m/z* = 211.0 [M + Na]⁺.

Compounds **2a** and **2b**; General Procedure

To a solution of **1** (1.0 equiv) and CuI (0.2 equiv) in degassed DMF, were added with stirring the corresponding alkynol (**2a**: pent-4-yn-1-ol, **2b**: *O*-propargyl triethylene glycol, 3.0 equiv), (PPh₃)₄Pd (0.1 equiv) and Et₃N (2.0 equiv). The mixture was protected from light and stirred at r.t. (for **2a**, 16 h; for **2b**, 18 h). The mixture was combined with sat. aq NaHCO₃ (10 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (10 mL), dried (MgSO₄), and concentrated in vacuo. The residue was purified by flash chromatography.

3',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-5-(5-hydroxypent-1-ynyl)-2'-deoxyuridine (**2a**)

The reaction was carried out with deoxyuridine **1** (1.0 g, 1.72 mmol), CuI (65 mg, 0.34 mmol), pent-4-yn-1-ol (476 μ L, 5.16 mmol), (PPh₃)₄Pd (27 mg, 0.17 mmol), and Et₃N (482 μ L, 3.43 mmol) in DMF (5 mL). Purification: silica gel (EtOAc-PE, 1:1 to 3:2); *R_f* = 0.3 (EtOAc-PE, 3:2); yield: 750 mg (81%).

¹H NMR (600 MHz, CDCl₃): δ = 0.09 (s, 3 H, CH₃Si), 0.1 (s, 3 H, CH₃Si), 0.15 (s, 3 H, CH₃Si), 0.16 (s, 3 H, CH₃Si), 0.91 (s, 9 H, *t*-

C_4H_9Si), 0.95 (s, 9 H, $t-C_4H_9Si$), 1.77–1.86 (m, 2 H, $CH_2CH_2CH_2OH$), 2.00–2.07 (m, 1 H, H-2'a), 2.31 (ddd, $^2J_{2'b,2'a} = 13.1$ Hz, $^3J_{2'b,1'} = 5.7$ Hz, $^3J_{2'b,3'} = 2.5$ Hz, 1 H, H-2'b), 2.51 (t, $^3J = 6.8$ Hz, 2 H, $CH_2CH_2CH_2OH$), 3.76–3.81 (m, 3 H, H-5'b, $CH_2CH_2CH_2OH$), 3.91 (dd, $^2J_{5'a,5'b} = 11.4$ Hz, $^3J_{5'a,4'} = 2.0$ Hz, 1 H, H-5'a), 3.97–3.99 (m, 1 H, H-4'), 4.40–4.43 (m, 1 H, H-3'), 6.30 (t, $^3J_{1',2'a} = ^3J_{1',2'b} = 5.7$ Hz, 1 H, H-1'), 7.93 (s, 1 H, H-6), 8.28 (br s, 1 H, NH).

^{13}C NMR (151 MHz, $CDCl_3$): $\delta = -4.5, -4.4, -3.8, -3.6, 17.4, 19.0, 19.5, 26.7, 27.0, 32.8, 43.0, 62.7, 64.0, 73.0, 73.3, 86.7, 89.3, 95.6, 101.6, 142.5, 150.0, 162.7$.

ESI-MS: $m/z = 561.5$ [M + Na] $^+$.

Anal. Calcd for $C_{26}H_{46}N_2O_6Si_2$: C, 57.96; H, 8.60; N, 5.20. Found: C, 57.98; H, 8.62; N, 5.14.

3',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-5-[12-hydroxy-(4,7,10-trioxadodec-1-ynyl)]-2'-deoxyuridine (**2b**)

The reaction was carried out with deoxyuridine **1** (0.5 g, 0.86 mmol), CuI (33 mg, 0.17 mmol), *O*-propargyl triethylene glycol (0.48 g, 2.50 mmol), $(PPh_3)_4Pd$ (13 mg, 0.09 mmol), and Et_3N (242 μ L, 1.72 mmol) in DMF (5 mL). Purification: silica gel (CH_2Cl_2 -MeOH, 20:1); $R_f = 0.4$ (CH_2Cl_2 -MeOH, 20:1); yield: 460 mg (83%).

1H NMR (400 MHz, $CDCl_3$): $\delta = 0.05$ (s, 3 H, CH_3Si), 0.06 (s, 3 H, CH_3Si), 0.10 (s, 3 H, CH_3Si), 0.11 (s, 3 H, CH_3Si), 0.86 (s, 9 H, $t-C_4H_9Si$), 0.90 (s, 9 H, $t-C_4H_9Si$), 1.99 (ddd, $^2J_{2'a,2'b} = 13.2$ Hz, $^3J_{2'a,1'} = 7.5$ Hz, $^3J_{2'a,3'} = 5.9$ Hz, 1 H, H-2'a), 2.31 (ddd, $^2J_{2'b,2'a} = 13.0$ Hz, $^3J_{2'b,1'} = 5.9$ Hz, $^3J_{2'b,3'} = 2.5$ Hz, 1 H, H-2'b), 3.57–3.77 (m, 13 H, H-5'b, OCH_2CH_2O), 3.87 (dd, $^2J_{5'a,5'b} = 11.4$ Hz, $^3J_{5'a,4'} = 2.5$ Hz, 1 H, H-5'a), 3.94–3.96 (m, 1 H, H-4'), 4.34–4.39 (m, 3 H, H-3', CH_2O), 6.27 (t, $^3J_{1',2'a} = 7.5$ Hz, $^3J_{1',2'b} = 5.9$ Hz, 1 H, H-1'), 8.00 (s, 1 H, H-6), 8.50 (br s, 1 H, NH).

^{13}C NMR (101 MHz, $CDCl_3$): $\delta = -5.3, -5.2, -4.6, -4.4, 18.1, 18.6, 25.8, 26.1, 42.0, 59.0, 61.6, 62.9, 68.9, 70.2, 70.3, 70.5, 72.2, 72.5, 77.5, 85.7, 88.2, 89.7, 99.3, 142.7, 148.9, 161.3$.

ESI-MS: $m/z = 665.5$ [M + Na] $^+$.

Compounds **3a** and **3b**; General Procedure

To a solution of **2** (1.0 equiv) in CH_2Cl_2 at 0 °C was added DIPEA (**2a**: 1.8 equiv, **2b**: 1.5 equiv) and stirred for **2a**: 30 min, **2b**: 10 min, then MsCl (1.2 equiv) was added slowly and the mixture stirred; for **2a**: 40 min, **2b**: 20 min at 0 °C (in the case of **2b**, the mixture was evaporated and the crude product was used directly without purification). For **2a**, the mixture was combined with sat. aq $NaHCO_3$ (10 mL) and extracted with Et_2O (3×15 mL). The combined organic layers were washed with brine (15 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by flash chromatography [silica gel (EtOAc-PE, 1:2); $R_f = 0.8$ (EtOAc-PE, 3:1)]. To a solution of the respective mesylate (1.0 equiv) in DMF was added NaN_3 (**2a**: 6.5 equiv, **2b**: 5.0 equiv). The mixture was warmed to 35 °C for 20 h, then combined with sat. aq $NaHCO_3$ (5 mL) and extracted with EtOAc (3×5 mL). The combined organic layers were dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by flash chromatography.

3',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-5-(5-methanesulfonate-pent-1-ynyl)-2'-deoxyuridine

Prepared from **2a** (0.32 g, 0.59 mmol), DIPEA (182 μ L, 1.07 mmol), and MsCl (55 μ L, 0.71 mmol) in CH_2Cl_2 (5 mL); yield: 270 mg (74%).

1H NMR (600 MHz, $CDCl_3$): $\delta = 0.07$ (s, 3 H, CH_3Si), 0.08 (s, 3 H, CH_3Si), 0.13 (s, 3 H, CH_3Si), 0.14 (s, 3 H, CH_3Si), 0.89 (s, 9 H, $t-C_4H_9Si$), 0.93 (s, 9 H, $t-C_4H_9Si$), 1.97–2.05 (m, 3 H, H-2'a, $CH_2CH_2CH_2OMs$), 2.31 (ddd, $^2J_{2'b,2'a} = 13.1$ Hz, $^3J_{2'b,1'} = 6.0$ Hz, $^3J_{2'b,3'} = 2.7$ Hz, 1 H, H-2'b), 2.55 (dd, $^3J = 6.8$ Hz, $^3J = 6.8$ Hz, 2 H,

$CH_2CH_2CH_2OMs$), 3.07 (s, 3 H, OSO_2CH_3), 3.77 (dd, $^2J_{5'b,5'a} = 11.5$ Hz, $^3J_{5'b,4'} = 2.1$ Hz, 1 H, H-5'b), 3.89 (dd, $^2J_{5'a,5'b} = 11.5$ Hz, $^3J_{5'a,4'} = 2.4$ Hz, 1 H, H-5'a), 3.96–3.98 (m, 1 H, H-4'), 4.37–4.41 (m, 3 H, H-3', $CH_2CH_2CH_2OMs$), 6.27 (dd, $^3J_{1',2'a} = 7.3$ Hz, $^3J_{1',2'b} = 6.0$ Hz, 1 H, H-1'), 7.94 (s, 1 H, H-6), 8.05 (br s, 1 H, NH).

^{13}C NMR (151 MHz, $CDCl_3$): $\delta = -4.5, -4.4, -3.8, -3.6, 16.9, 19.0, 19.5, 26.7, 27.0, 28.8, 38.2, 42.9, 49.3, 64.0, 69.5, 73.3, 86.7, 89.4, 93.4, 101.1, 143.0, 150.0, 162.6$.

ESI-MS: $m/z = 639.4$ [M + Na] $^+$.

Anal. Calcd for $C_{27}H_{48}N_2O_8SSi_2$: C, 52.57; H, 7.84; N, 4.54. Found: C, 52.62; H, 7.77; N, 4.50.

3',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-5-(5-azidopent-1-ynyl)-2'-deoxyuridine (**3a**)

Prepared from mesylate derived from **2a** (70 mg, 0.11 mmol), and NaN_3 (46 mg, 0.71 mmol) in DMF (2 mL). Purification: silica gel (EtOAc-PE, 1:3 to 1:1); $R_f = 0.8$ (EtOAc-PE, 1:1); yield: 61 mg (95%).

1H NMR (600 MHz, $CDCl_3$): $\delta = 0.09$ (s, 3 H, CH_3Si), 0.1 (s, 3 H, CH_3Si), 0.15 (s, 3 H, CH_3Si), 0.16 (s, 3 H, CH_3Si), 0.91 (s, 9 H, $t-C_4H_9Si$), 0.95 (s, 9 H, $t-C_4H_9Si$), 1.85 (dddd, $^3J = 6.8, 6.8, 6.8, 6.8$ Hz, 2 H, $CH_2CH_2CH_2N_3$), 2.03 (ddd, $^2J_{2'a,2'b} = 13.3$ Hz, $^3J_{2'a,1'} = 7.7$ Hz, $^3J_{2'a,3'} = 6.3$ Hz, 1 H, H-2'a), 2.31 (ddd, $^2J_{2'b,2'a} = 13.3$ Hz, $^3J_{2'b,1'} = 5.9$ Hz, $^3J_{2'b,3'} = 2.7$ Hz, 1 H, H-2'b), 2.51 (t, $^3J = 6.8$ Hz, 2 H, $CH_2CH_2CH_2N_3$), 3.45 (t, $^3J = 6.8$ Hz, 2 H, $CH_2CH_2CH_2N_3$), 3.78 (dd, $^2J_{5'b,5'a} = 11.5$ Hz, $^3J_{5'b,4'} = 2.2$ Hz, 1 H, H-5'b), 3.91 (dd, $^2J_{5'a,5'b} = 11.5$ Hz, $^3J_{5'a,4'} = 2.3$ Hz, 1 H, H-5'a), 3.98 (ddd, $^3J_{4',5'b} = 2.2$ Hz, $^3J_{4',5'a} = 2.3$ Hz, $^3J_{4',3'} = 2.2$ Hz, 1 H, H-4'), 4.40–4.43 (m, 1 H, H-3'), 6.3 (dd, $^3J_{1',2'a} = 7.7$ Hz, $^3J_{1',2'b} = 5.9$ Hz, 1 H, H-1'), 7.93 (s, 1 H, H-6), 8.27 (br s, 1 H, NH).

^{13}C NMR (151 MHz, $CDCl_3$): $\delta = -4.5, -4.4, -3.8, -3.6, 18.0, 19.0, 19.5, 26.8, 27.0, 28.7, 43.0, 51.3, 64.0, 73.4, 73.5, 86.7, 89.4, 94.1, 101.4, 142.9, 150.0, 162.5$.

ESI-MS: $m/z = 586.6$ [M + Na] $^+$.

3',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-5-[12-azido-(4,7,10-trioxadodec-1-ynyl)]-2'-deoxyuridine (**3b**)

Prepared from **2b** (0.1 g, 0.16 mmol), DIPEA (40 μ L, 0.23 mmol), MsCl (38 μ L, 0.18 mmol) in CH_2Cl_2 (5 mL) followed by the addition of NaN_3 (50 mg, 0.78 mmol) and DMF (2 mL). Purification: silica gel (CH_2Cl_2 -MeOH, 20:1); $R_f = 0.8$ (CH_2Cl_2 -MeOH, 20:1); yield: 53 mg (50% over two steps).

1H NMR (600 MHz, $CDCl_3$): $\delta = 0.08$ (s, 3 H, CH_3Si), 0.09 (s, 3 H, CH_3Si), 0.14 (s, 3 H, CH_3Si), 0.15 (s, 3 H, CH_3Si), 0.90 (s, 9 H, $t-C_4H_9Si$), 0.94 (s, 9 H, $t-C_4H_9Si$), 2.03 (ddd, $^2J_{2'a,2'b} = 13.2$ Hz, $^3J_{2'a,1'} = 7.6$ Hz, $^3J_{2'a,3'} = 6.0$ Hz, 1 H, H-2'a), 2.33 (ddd, $^2J_{2'b,2'a} = 13.2$ Hz, $^3J_{2'b,1'} = 5.9$ Hz, $^3J_{2'b,3'} = 2.6$ Hz, 1 H, H-2'b), 3.40 (t, $^3J = 5.1$ Hz, 2 H, $OCH_2CH_2N_3$), 3.67–3.71 (m, 8 H, OCH_2CH_2O), 3.72–3.75 (m, 2 H, $OCH_2CH_2N_3$), 3.77 (dd, $^2J_{5'b,5'a} = 11.4$ Hz, $^3J_{5'b,4'} = 2$ Hz, 1 H, H-5'b), 3.90 (dd, $^2J_{5'a,5'b} = 11.4$ Hz, $^3J_{5'a,4'} = 2.4$ Hz, 1 H, H-5'a), 3.97–4.00 (m, 1 H, H-4'), 4.39 (s, 2 H, CH_2O), 4.40–4.43 (m, 1 H, H-3'), 6.29 (dd, $^3J_{1',2'a} = 7.6$ Hz, $^3J_{1',2'b} = 5.9$ Hz, 1 H, H-1'), 8.02 (s, 1 H, H-6), 8.40 (br s, 1 H, NH).

^{13}C NMR (151 MHz, $CDCl_3$): $\delta = -4.5, -4.4, -3.8, -3.6, 19.0, 19.4, 26.7, 27.0, 43.0, 51.7, 60.0, 64.0, 70.2, 71.0, 71.5, 71.6, 71.7, 73.4, 78.4, 86.9, 89.4, 90.6, 100.5, 144.1, 150.0, 162.2$.

ESI-MS: $m/z = 690.3$ [M + Na] $^+$.

Compounds **4a** and **4b**; General Procedure

To a solution of **3** (1.0 equiv) in THF, was added TBAF (1 M solution in THF; 2.2 equiv) at 0 °C. The mixture was allowed to warm to r.t. and stirred; for **3a**: 16 h, **3b**: 20 h, and evaporated in vacuo to give the crude unprotected sugar, which was purified by flash chromatography. This product (1.0 equiv) and proton sponge [1.8-

bis(dimethylaminonaphthalene)] (1.5 equiv) were dried overnight protected from light in vacuo, dissolved in trimethyl phosphate, and cooled to 0 °C. Freshly distilled POCl₃ (**4a**: 1.3 equiv, **4b**: 2.2 equiv) was added to the mixture and stirred; for **4a**: 3.5 h, **4b**: 4.5 h. A 0.5 M soln of (Bu₃NH)₂H₂P₂O₇ in anhyd DMF (**4a**: 5 equiv, **4b**: 10 equiv) and Bu₃N (**4a**: 10 equiv, **4b**: 20 equiv) were added simultaneously to the mixture. After 5 min, 1 M aq triethylammonium bicarbonate (TEAB buffer, pH 7.5) was added and the aqueous layer was washed with EtOAc (2 × 10 mL). The aqueous layer was lyophilized and the resulting residue purified by ion-exchange chromatography [DEAE-Sephadex A-25, linear gradient of TEAB buffer (0.1 M to 1 M, 1000 mL), flow 2 mL/min] and further purified by RP-MPLC (RP-18, 40–63 μm) using a gradient of 5% (200 mL), 20% (200 mL) and 40% (200 mL) MeCN in 50 mM aq triethylammonium acetate (TEAA buffer, pH 7.0). The triphosphates **4a** and **4b** were eluted with 20% MeCN in 50 mM aq TEAA buffer.

5-(5-Azidopent-1-ynyl)-2'-deoxyuridine

Prepared from **3a** (58 mg, 0.10 mmol), TBAF soln (226 μL, 0.23 mmol) in THF (2 mL). Purification: silica gel (CH₂Cl₂-MeOH, 20:1); *R_f* = 0.6 (CH₂Cl₂-MeOH, 20:1); yield: 32 mg (93%).

¹H NMR (400 MHz, MeOD): δ = 1.75 (dddd, ³*J* = 6.8, 6.8, 6.8, 6.8 Hz, 2 H, CH₂CH₂CH₂N₃), 2.10–2.26 (m, 2 H, H-2'a, H-2'b), 2.43 (t, ³*J* = 6.8 Hz, 2 H, CH₂CH₂CH₂N₃), 3.40 (t, ³*J* = 6.8 Hz, 2 H, CH₂CH₂CH₂N₃), 3.67 (dd, ²*J*_{5'b,5'a} = 12.0 Hz, ³*J*_{5'b,4'} = 3.4 Hz, 1 H, H-5'b), 3.75 (dd, ²*J*_{5'a,5'b} = 12.0 Hz, ³*J*_{5'a,4'} = 3.0 Hz, 1 H, H-5'a), 3.84–3.88 (m, 1 H, H-4'), 4.31–4.36 (m, 1 H, H-3'), 6.18 (t, ³*J*_{1',2'a} = ³*J*_{1',2'b} = 6.6 Hz, 1 H, H-1'), 8.19 (s, 1 H, H-6).

¹³C NMR (101 MHz, CDCl₃): δ = 17.3, 28.8, 41.6, 51.3, 62.7, 72.2, 73.7, 87.1, 89.4, 93.8, 101.3, 145.1, 151.9, 165.5.

ESI-MS: *m/z* = 357.9 [M + Na]⁺.

5-(5-Azidopent-1-ynyl)-2'-deoxyuridine-5'-triphosphate (**4a**)

Prepared from 5-(5-azidopent-1-ynyl)-2'-deoxyuridine (29 mg, 86 μmol), proton sponge (28 mg, 0.13 mmol), POCl₃ (10.5 μL, 0.11 mmol), (Bu₃NH)₂H₂P₂O₇ solution (0.86 mL, 0.43 mmol), Bu₃N (229 μL, 0.86 mmol), and trimethyl phosphate (1 mL); yield: 25.7 mg (30%).

¹H NMR (400 MHz, MeOD): δ = 1.20–1.35 [m, 36 H, (HNEt₃)⁺₄], 1.8–1.93 (m, 2 H, CH₂CH₂CH₂N₃), 2.30–2.40 (m, 2 H, H-2'b, H-2'a), 2.47–2.55 (m, 2 H, CH₂CH₂CH₂N₃), 3.13–3.25 [m, H-5'a, H-5'b, (HNEt₃)⁺₄], 3.25–3.46 (m, 2 H, CH₂CH₂CH₂N₃), 4.1–4.2 (m, 1 H, H-3'), 4.56–4.62 (m, 1 H, H-4'), 6.26 (m, 1 H, H-1'), 8.0 (s, 1 H, H-6).

³¹P NMR (162 MHz, MeOD): δ = -22.5 (m, 1 P, P_β), -10.6 (d, ²*J*_{α,β} = 20.2 Hz, 1 P, P_α), -9.3 (d, ²*J*_{γ,β} = 20.2 Hz, 1 P, P_γ).

ESI-MS: *m/z* = 575.0 [M - H]⁻.

5-[12-Azido-(4,7,10-trioxadodec-1-ynyl)]-2'-deoxyuridine

Prepared from **3b** (43 mg, 64 μmol), TBAF soln (142 μL, 0.14 mmol) in THF (2 mL). Purification: silica gel (CH₂Cl₂-MeOH, 20:1); *R_f* = 0.5 (CH₂Cl₂-MeOH, 10:1); yield: 26.5 mg (94%).

¹H NMR (600 MHz, MeOD): δ = 2.23 (ddd, ²*J*_{2'a,2'b} = 13.5 Hz, ³*J*_{2'a,1'} = 6.3 Hz, ³*J*_{2'a,3'} = 6.5 Hz, 1 H, H-2'a), 2.32 (ddd, ²*J*_{2'b,2'a} = 13.5 Hz, ³*J*_{2'b,1'} = 6.3 Hz, ³*J*_{2'b,3'} = 3.7 Hz, 1 H, H-2'b), 3.38 (t, ³*J* = 4.9 Hz, 2 H, OCH₂CH₂N₃), 3.65–3.70 (m, 8 H, OCH₂CH₂O), 3.72–3.76 (m, 3 H, H-5'b, OCH₂CH₂N₃), 3.90 (dd, ²*J*_{5'a,5'b} = 12.0 Hz, ³*J*_{5'a,4'} = 3.0 Hz, 1 H, H-5'a), 3.92–4.00 (m, 1 H, H-4'), 4.38 (s, 2 H, CH₂O), 4.39–4.42 (m, 1 H, H-3'), 6.24 (t, ³*J*_{1',2'a} = ³*J*_{1',2'b} = 6.3 Hz, 1 H, H-1'), 8.35 (s, 1 H, H-6).

¹³C NMR (151 MHz, CDCl₃): δ = 41.8, 51.8, 59.7, 62.5, 70.2, 71.1, 71.4, 71.5, 71.6, 71.9, 78.8, 87.1, 89.2, 90.2, 100.0, 145.7, 151.2, 164.3.

ESI-MS: *m/z* = 463.2 [M + Na]⁺.

5-[12-Azido-(4,7,10-trioxa-1-dodecynyl)]-2'-deoxyuridine-5'-triphosphate (**4b**)

Prepared from 5-[12-azido-(4,7,10-trioxadodec-1-ynyl)]-2'-deoxyuridine (26.5 mg, 60 μmol), proton sponge (19.4 mg, 90 μmol), POCl₃ (17 μL, 0.19 mmol), (Bu₃NH)₂H₂P₂O₇ solution (1.2 mL, 0.6 mmol), Bu₃N (0.32 mL, 1.2 mmol), and trimethyl phosphate (1 mL); yield: 15.4 mg (24%).

¹H NMR (600 MHz, MeOD): δ = 1.27 [t, ³*J* = 7.0 Hz, (HNEt₃)⁺₄], 2.27 (m, 2 H, H-2'b, H-2'a), 3.07–3.12 [m, (HNEt₃)⁺₄], 3.37 (t, ³*J* = 4.9 Hz, 2 H, OCH₂CH₂N₃), 3.64–3.69 (m, 8 H, OCH₂CH₂O), 3.71–3.74 (m, 2 H, OCH₂CH₂N₃), 4.06–4.10 (m, 1 H, H-4'), 4.14–4.19 (m, 1 H, H-5'b), 4.24–4.3 (m, 1 H, H-5'a), 4.40 (s, 2 H, CH₂O), 4.58–4.61 (m, 1 H, H-3'), 6.22 (t, ³*J*_{1',2'a} = ³*J*_{1',2'b} = 6.8 Hz, 1 H, H-1'), 8.08 (s, 1 H, H-6).

³¹P NMR (162 MHz, MeOD): δ = -21.6 (m, 1 P, P_β), -10.4 (d, ²*J*_{α,β} = 21.6 Hz, 1 P, P_α), -8.6 (d, ²*J*_{γ,β} = 20.8 Hz, 1 P, P_γ).

ESI-MS: *m/z* = 677.8 [M - H]⁻.

Primer Extension

DNA primer strands were purchased from IBA, dNTPs were from Roche, *Pwo* DNA polymerase, 10 × *Pwo* reaction buffer (100 mM Tris-HCl (pH 8.8), 250 mM KCl, 20 mM MgSO₄) were from Peqlab. A typical primer extension reaction (20 μL) contained 1 × *Pwo* reaction buffer, 150 nM ³²P-labeled primer, 200 nM template, 100 μM each of **4a** or **4b**, 100 μM each of dATP, dCTP, dGTP, and 0.12 U/20 μL *Pwo* DNA polymerase. First primer and template were annealed in 1 × *Pwo* reaction buffer by heating the probes to 95 °C and allowing to cool down to 20 °C. Afterwards the primer template complex, nucleotides, and *Pwo* DNA polymerase were incubated at 72 °C for 30 min. The reactions were quenched by addition of 20 μL PAGE gel loading buffer (80% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanole FF) and the product mixtures were analyzed by 12% denaturing polyacrylamide gel, and subjected to autoradiography.

Staudinger Ligation on DNA 1

The primer extension product was used for the Staudinger ligation. The modified DNA was prepared according to the procedure mentioned above. The concentrations of dNTPs and *Pwo* DNA polymerase were adjusted to 200 μM and to 0.5 U/20 μL, respectively. After incubation at 72 °C for 60 min, the 20 μL reactions were quenched with 1 μL of 210 mM EDTA solution. The reaction product was purified using Sephadex G-25 spin columns. For the Staudinger ligation, 2.5 μL of 1 M NaHCO₃/Na₂CO₃ buffer (pH 9.0), 2.5 μL of **5a** or **5b** (10 mM in DMF) and 5 μL of the primer extension product (~1 μM) were mixed and incubated either at 60 °C for 12 h or at various temperatures for 4 h in the case of temperature-dependence experiments. The reactions were stopped by adding 10 μL of PAGE-gel-loading buffer and the product mixtures were analyzed by 12% denaturing polyacrylamide gel.

PCR

Typical PCR reactions (20 μL) contained 1 × *Pwo* reaction buffer, 200 μM each of **4a** or **4b**, 200 μM each of dATP, dCTP, dGTP, 500 nM primer I, 500 nM primer II, 1.2 pg/μL template (98 bp), and 0.02 U/μL *Pwo* DNA polymerase. A typical PCR cycling protocol began with an initial denaturation at 95 °C for 60 s and was followed by 35 cycles of 95 °C, 30 s; 55 °C, 35 s; 72 °C, 40 s. The reactions were quenched by addition of 4 μL agarose-gel-loading buffer (10 mM Tris-HCl, 60 mM EDTA, 30% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanole FF) and the product mixtures were analyzed by agarose gel electrophoresis.

The PCR reaction using the 1.9 kB plasmid as template was done according to the procedure mentioned above. The concentrations of plasmid DNA and *Pwo* DNA polymerase were adjusted to 0.4 ng/μL and 0.04 U/μL, respectively. The PCR cycling protocol was de-

natured at 95 °C for 120 s, followed by 35 cycles of 95 °C, 60 s; 63 °C, 60 s; and 72 °C, 240 s. The reactions were quenched by the addition of 4 µL agarose-gel-loading buffer and the product mixtures were analyzed by agarose gel electrophoresis.

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