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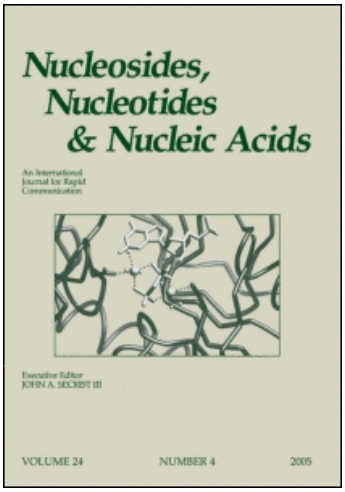

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C-5 Modified Nucleosides: Direct Insertion of Alkynyl-Thio Functionality in Pyrimidines

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C-5 Modified Nucleosides: Direct Insertion of Alkynyl-Thio Functionality in Pyrimidines

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ABSTRACT

A route is presented to append, in a single step, alkynyl thioesters to the 5-position of a pyrimidine ring of a nucleoside that is unprotected. These products should be useful to support in vitro selection experiments with functionalized DNA.

Key Words: Functionalized nucleoside; Thioester; Pd-mediated coupling; Chemical genetics; Synthetic biology.

INTRODUCTION

It has been two decades since the enzymic incorporation of functionalized nucleotides was first suggested to be useful to tag DNA with functionality that it did not intrinsically carry.[1,2] In the intervening years, enzymic incorporation of modified nucleotides has become the key to automated DNA sequencing.[3] Also, increasing the functionality of nucleic acids has been suggested to be a key to enhancing the power of nucleic acids as catalysts.[4] The consequential explosion of work using modified nucleotides in in vitro evolution experiments[5–15] has created a demand for routes to prepare these synthetically, in particular, those carrying functionality on the 5-position of a pyrimidine ring. In addition, some of these modified

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nucleosides possess interesting biological activities as antiviral agents,[14,16] and are important tools to study the conformation of nucleic acids.[17–19]

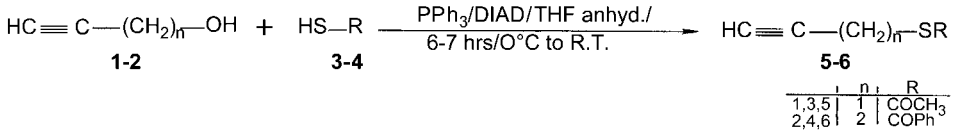
Recent efforts to expand the chemical functionality incorporatable into DNA by DNA polymerases have focused on pyrimidine nucleosides carrying a 5-position olefinic or acetylenic linker to which is attached a functional group. We have focused here on thiol functionalities, primarily because of their versatility, since thiols can be modified, or can form disulfide bridges.[20] Recent studies in these laboratories with polymerases[21] have found that these can accept the alkynyl linker holding these functionalities, and can be incorporated especially well.

The most common route to these structures involves the introduction of the linker (alkynol or allyl chloride) via palladium coupling to 5-iododeoxypyrimidines[22] or 5-chloromercuri-2′-deoxypyrimidine[23,24] followed by mesylation, displacement of the mesylate with the thiobenzoate anion, the replacement of the benzoyl by a *t*-butyl disulfide group (for more stable thiol protection), deprotection of the sugar and finally the protection of 5′-OH by a dimethoxytrityl group.[25] An alternative strategy involves the isomerization of uriedomethylene thiolactones to uracils derivatives, followed by protection of the thiol group by t-butyl disulfide, and converting the bases to nucleosides via a Hilbert-Johnson reaction.[26]

In both the methods, the sugar must be protected and deprotected, thus adding steps in the synthesis of the final compound. In this communication, we report a single step procedure for the synthesis of 5-position modified pyrimidine nucleosides carrying a protected thiol functionality. We also report the synthesis of the corresponding phosphoramidites and triphosphates.

RESULTS AND DISCUSSION

The formal displacement of hydroxyl group by a carboxylate is achieved for many carboxylic acids by a procedure that involves the reaction of the alcohol in the presence of diisopropylazodicarboxylate (DIAD) and triphenylphosphine.[27–32] Thus, Mitsunobu reaction of acetylenic alcohols **1, 2** with thioacetic acid (**3**) or thiobenzoic acid (**4**) in the presence of a pre-formed adduct from DIAD and triphenylphosphine yielded alkynylthioesters **5** and **6** as yellow, malodorous liquids in >90% yield (Sch. 1). Distillation of the yellow oils gave the pure products as colorless liquids. The benzoyl protecting group was selected in preference to the acetyl group because compound **6** absorbs UV light, facilitating its detection during column chromatography. Palladium-catalyzed coupling[33] of thioesters **5** and **6** in DMF with 5-iodo-2′-deoxy pyrimidines **7** and **10** yielded the C-5 thioalkynyl nucleoside derivatives **8**, **9** and **11**) in good yields (60–90%) (Sch. 2), even though the hydroxyl groups on the sugar were not protected.



Scheme 1.

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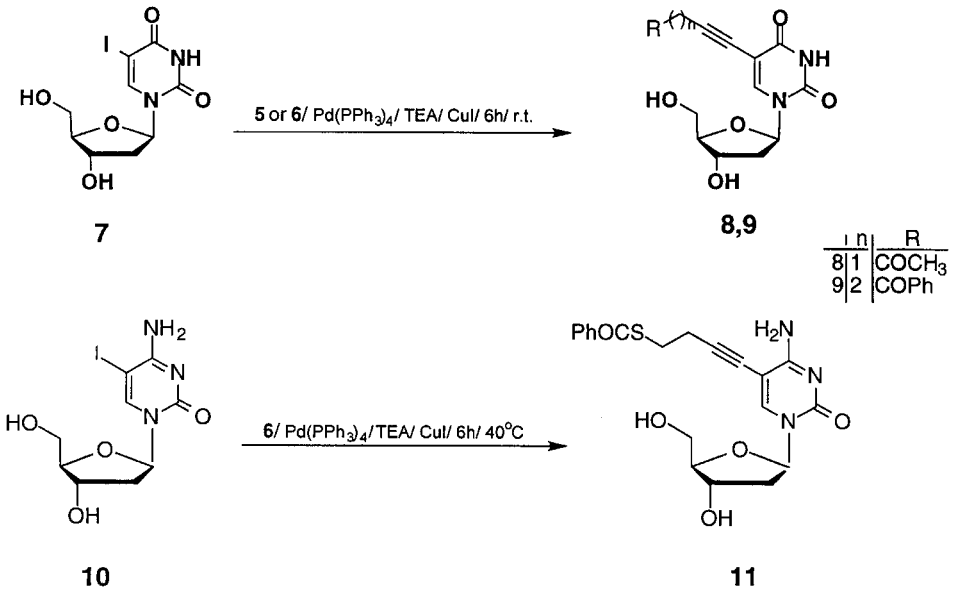

Scheme 2.

Treatment of the products with dimethoxytrityl chloride gave the corresponding 5′-OH protected nucleosides, which were then treated with di-*t*-butyl-1-(butylthio)-1,2-hydrazinedicarboxylate to replace the acetyl and benzoyl thiol protecting groups with more stable *t*-butyl disulfide group (Sch. 3). With compound **8**, the 5′-OH was protected after the displacement reaction to give compound **12**. Similarly, compound **11** yielded the 5′-OH protected *t*-butyldisulfide compound **14**.

Compounds **12** and **13**, upon acetylation, yielded the 3′-acetylated compounds, which upon treatment with trichloroacetic acid in CH_2Cl_2 gave the detritylated (free 5′-OH) compounds **17** and **18,** respectively. Using a standard procedure for triphosphate synthesis,[34] and subsequent deprotection of 3′-OH, compounds **19** and **20** were obtained as crude products. These triphosphates were purified by ion-exchange chromatography (**19**) or by RP-HPLC (**20**) (Sch. 4)

The oligonucleotide synthesis via standard solid phase chemistry requires 3′-*O*-N,N-diisopropyl-β-cyanoethyl phosphoramidites of the nucleoside. Thus compounds **12** and **13**, upon phosphitylation with N,N-diisopropyl-2 cyanoethylchloro phosphoramidite,[35] gave 3′-*O*-N,N-diisopropyl-β-cyanoethyl phosphoramidites (compounds **21** and **22**) (Sch. 5) in good yield.

CONCLUSION

This procedure offers a rapid and convenient way to introduce the thio-alkynyl group directly at the C-5 position of a pyrimidine nucleoside in a single step which does not require exhaustive protection and deprotection steps.

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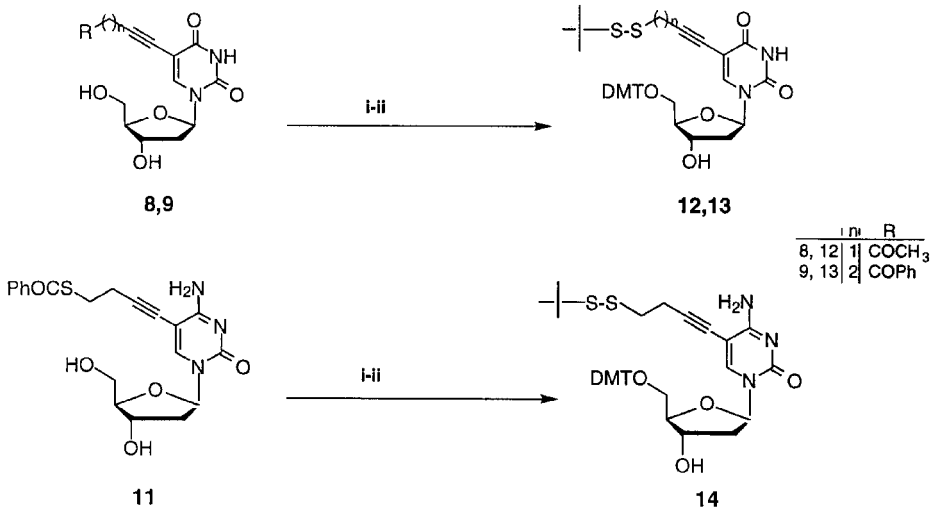
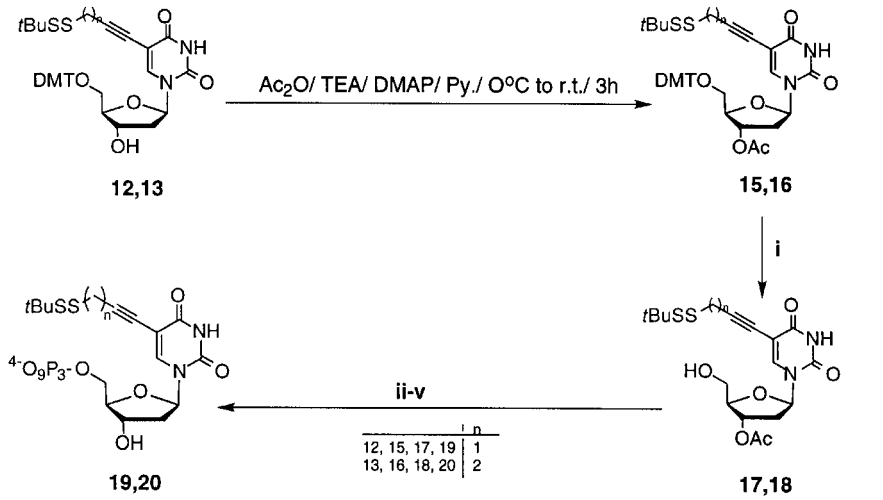


i = DMT-Cl/ DMAP/ TEA/ Py./ OoC to r.t./ 7h; ii = Di-*t*Butyl-1-(*t*Butylthio)-1,2-hydrazine-dicarboxylate/ LiOH/ MeOH-THF/ 90 min.

Scheme 3.



i = TCA 2% sol. in CH_2Cl_2/ 30min./ r.t./ aq. $NaHCO_3$-; ii = 2-Cl-4*H*-1,3,2-benzophosphorin-4-one/ Py. anhyd./ 1,4-Dioxane/ 10 min/ r.t.; iii = Tributylammonium pyrophosphate/ n-Bu_3N/ DMF/ 10 min./ r.t.; iv = I_2/ Py.-H_2O/ 15 min/ r.t./ Na_2SO_3/ H_2O/ r.t.; v = NH_4OH/ 1 h/ r.t.

Scheme 4.

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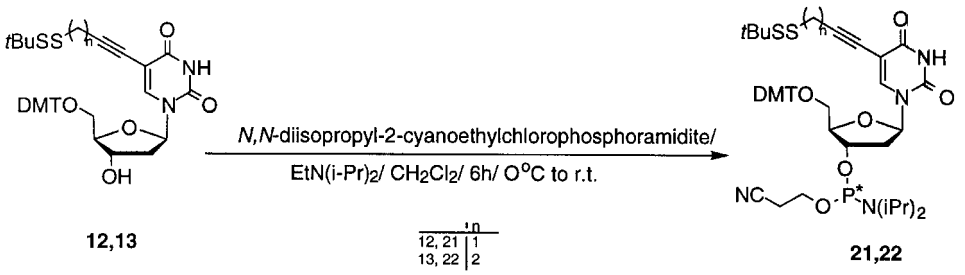

Scheme 5.

EXPERIMENTAL SECTION

Reagents were purchased from Acros, Aldrich, Fischer, Berry & Associates. Reactions were carried out under Ar. The stationary phase for TLC were Whatman (AL SIL G/UV) silica gel plates (250 μm layer) containing fluorescent indicator ($UV_{254\,nm}$). Organic compounds were visualized by UV light (254 nm) or by staining with a Ce/Mo reagent (2.5% phosphormolybdic acid, 1% $Ce^{IV}(SO_4)_2{\cdot}4H_2O$, 6% H_2SO_4 in water) and heating, generating 254 nm fluorescence. All reactions were carried out at room temperature (22–24°C), unless otherwised mentioned. Silica gel (230–425 mesh, Fisher) was used for column chromatography. AG 1-X8 resin (Bio-Rad) was purchased as the Cl^- form and converted to the HCO_3^- form by washing with 16 volumes of 1 M NH_4HCO_3 solution, by de-ionized water, and finally with 0.5 M NH_4HCO_3 solution; no Cl^- was detected. Ion-exchange chromatography was done with DEAE Sephadex (Sigma) equilibrated in 0.2 M $(Et_3NH)HCO_3$ (pH 7.0). NMR spectra were recorded on a Varian XL 300 spectrometer at 300 MHz for ^1H, using TMS as an internal reference, at 75.4 MHz for ^{13}C and 121.4 MHz for ^{31}P (which was referenced to H_3PO_4 as external standard). UV spectra were measured on a Varian Cary 1 Bio spectrophotometer. Mass spectrometry was performed by the Spectroscopy Services of the University of Florida Chemistry Department and by Dr. Lisa Lang at the University of Florida. For LSIMS, a Finnigan MAT-95Q apparatus was used; for HPLC/ESI-MS, a Finnigan MAT (San Jose, CA) was used. LCQ was done in electrospray ionization (ESI) mode using a Beckman Instruments (Fullerton, CA) System Gold, model 126 pump with Waters RP18 Symmetry Shield analytical column (2.1×150 mm + guard).

General Procedure for the Synthesis of Alkynylthioesters

Under an Ar atmosphere, diisopropyl azodicarboxylate (1.5 eq.) was added dropwise over 15 min to an ice-cooled solution of triphenylphosphine (1.5 eq.) in THF (33 mL). After having been stirred 30 min under ice cooling, the mixture was treated with a solution of thioacetic acid (**3**) or thiobenzoic acid (**4**) (1.5 eq.) and propargyl alcohol (**1**) or butin 4-ol (**2**), resp., (1.0 eq.) in THF (20 mL) over a period of 20 min. The mixture was stirred for another 6 h, during which it warmed to RT. The reaction was then quenched with MeOH and the solvents were removed in vacuo.

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The resulting oily residue was stored at −20°C overnight; a large amount of a white crystalline solid formed. The solid was removed by filtration and washed three times with petroleum ether under sonication. The combined filtrates were concentrated, and the oily residue was chromatographed on silica gel.

3-Acetylthio-1-propyne (5). Chromatography on silica gel with petroleum ether containing 0–5% (v/v) ether afforded **5** (0.577 g, 93%) ($R_f = 0.48$, n-pentane/ether 9.5:0.5). For elemental and MS analysis, the chromatography product was distilled at 51°C and 10 mbar to yield **5** as a colorless, malodorous liquid.

HRMS-EI: m/z (%) 114.0002 (100.00; M^+), 98.9863 (3.50), 96.0133 (6.07), 70.9837 (12.53), 43.0167 (27.40), 39.0264 (5.75). Anal. Calcd for C_5H_6OS: C, 52.60; H, 5.30; O, 14.01; S, 28.09. Found: C, 52.31; H, 5.32; O, 14.23; S, 28.24. ^{1}H NMR ($CDCl_3$): $\delta = 2.18$ (1H, t, $J = 2.7$ Hz, alkyne H), 2.37 (3H, s, CH_3), 3.64 (2H, d, $J = 2.7$ Hz, CH_2). ^{13}C NMR ($CDCl_3$): $\delta = 17.2$ (CH_3), 29.9 (CH_2), 70.6 (alkyne C), 78.5 (alkyne C), 193.5 (CO).

4-Benzoylthio-1-butyne (6). Elution with *n*-pentane containing 0.5–1.0% (v/v) ether yielded **6** (91%) ($R_f = 0.58$; *n*-pentane/ether 9.5:0.5) as a yellowish liquid.

HRMS-FAB (NBA): m/z (%) 191.053063 (100.00, $M^+ + 1$), 192.056240 (13.34), 193.050729 (5.46). $C_{11}H_{11}OS$

^{1}H NMR ($CDCl_3$): $\delta = 2.05$ (1H, t, $J = 2.6$ Hz; alkyne H), 2.59 (2H, dt, $J = 2.6$, 7.1 Hz, SCH_2CH_2), 3.24 (2H, *t*, J = 7.1 Hz; SCH_2), 7.42–7.48 (2H, m; ArH), 7.55–7.61 (1H, m; ArH), 7.95–7.99 (2H, m; ArH). ^{13}C NMR ($CDCl_3$): $\delta = 19.8$ (SCH_2CH_2), 28.2 (SCH_2), 69.9 (HC^*C), 82.4 (HC^*C), 127.5, 128.8, 133.7, 137 (Ar), 192 (CO).

General Procedure for Coupling Reactions

To a clear solution of 5-iodo-2′-deoxypyrimidine (**7** or **10**) (1.0 eq.) and tetrakis-(triphenyl) phosphine palladium (0) (0.1 eq.) in DMF (85 mL), TEA (2.0 eq.), thioester **5**/**6** (2.5 eq.) and copper (I) iodide (0.2 eq.) were added successively under argon in the order described. The flask was wrapped in aluminum foil to exclude light, and the solution was stirred for 5–12 h at room temperature. After removal of the solvent in vacuo, the remaining brown oil was chromatographed on silica gel.

5-(3-Acetylthio-1-propynyl)-2′-deoxyuridine (8). Elution with CH_2Cl_2/MeOH (9:1) (v/v) gave **8** (62%) ($R_f = 0.49$; CH_2Cl_2/MeOH 9:1) as a light yellow foam.

LRMS-FAB (NBA): m/z = 341 ($M^+ + 1$).

5-(4-Benzoylthio-1-butynyl)-2′-deoxyuridine (9). Elution with CH_2Cl_2/MeOH (93:7) afforded **9** (96%) ($R_f = 0.45$, CH_2Cl_2/MeOH 9:1) as a brown foam.

LRMS-FAB (NBA): m/z = 417 ($M^+ + 1$).

^{1}H NMR ($CDCl_3/CD_3OD$; 3:1): $\delta = 2.16$–2.25 (1H, m, 2′-Ha), 2.36 (1H, dd; 2′-Hb), 2.75–2.80 (2H, m, 9-Hab), 3.27–3.32 (2H, m, 10-Hab), 3.76 (1H, dd, 5′-Ha), 3.85 (1H, dd, 5′-Hb), 3.96–3.99 (1H, m, 4′-H), 4.42–4.46 (overlapped by CD_3OH signal, 3′-H), 6.25 (1H, pseudo t, $J = 6.4$ Hz, 1′-H), 7.45–7.50 (2H, m, ArH),

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7.59–7.64 (1H, m, ArH), 7.95–7.98 (2H, m, ArH), 8.17 (1H, s, 6-H). ^{13}C NMR ($CDCl_3/CD_3OD$ 3:1): $\delta = 20.2$ (9), 27.5 (10), 40.3 (2′), 61.1 (5′), 70.2, 72.3 (8, 3′), 85.4 (1′), 87.2 (4′), 91.6 (7), 99.6 (5), 126.8, 128.3, 133.3, 136.4 (Ar), 143.0 (6), 149.5 (2), 162.6 (4), 192.0 (PhCO).

5-(4-Benzoylthio-1-butynyl)-2′-deoxycytidine (11). Elution with $CHCl_3$/MeOH (93:7) gave **11** (72%) (R_f: 0.4; $CHCl_3$/MeOH 88:12) as a light yellow foam.

m/z = 416 ($M^+ + 1$).

^{1}H NMR (DMSO-d_6): $\delta = 1.92$–2.08 & 2.12–2.19 (m, 2H, 2′H), 2.74 (t, 2H, $J = 2.6$, -CH_2), 3.19 (t, 2H, $J = 2.6$, -CH_2) 3.55–3.81 (m, 2H-5′H), 3.57 (t, 2H, -CH_2), 3.78–3.81 (m, 1H, 4′), 4.19–4.22 (m, 1H, 3′), 5.03 (t, 1H,5′-OH), 5.17 (d, 1H, 3′-OH), 6.11 (t, 1H, H'), 6.72 (bs, 1H, -NH), 7.52–7.57 (m, 2H, ArH), 7.65–7.68 (m, H, ArH), 7.92–7.95 (m, 2H, ArH), 8.11 (s, 1H, H-6), 8.21 (bs, 1H, NH).

General Procedures for the 5′-OH Protection and Displacement Reactions

5′-*O*-(4,4′-Dimethoxytrityl)-5-(propynyl)-2′-deoxyuridine *tert*-butyl disulfide (12). To a suspension of **8** (1.635 g, 4.805 mmol) in MeOH (50 mL, anhyd.) under Ar, di-tert-butyl 1-(tert-butylthio)-1,2-hydrazine dicarboxylate (1.848 g, 5.766 mmol, 1.2 eq.) and LiOH.H_2O (0.303 g, 7.21 mmol, 1.5 eq.) were added simultaneously. The reaction mixture was allowed to stir for 1.5 h at rt. Thereafter, the solvent was removed in vacuo and the crude residue was purified over silica gel column. Elution with CH_2Cl_2/CH_3OH (95:5 v/v) yielded the desired product (1.091 g, 59%). The compound obtained above (1.091 g, 2.824 mmol) was coevaporated with pyridine (6 mL) and redissolved in pyridine (15 mL) under Ar. The solution was cooled in an ice bath, and subsequently DMAP (0.086 g, 0.706 mmol, 0.25 eq.), DMTCl (1.435 g, 4.236 mmol, 1.5 eq.) and TEA (0.787 mL, 5.648 mmol, 2.0 eq.) were added. After 5 min the ice bath was removed, and stirring of the reaction mixture was continued at room temperature. The reaction was quenched after 3.5 h by addition of MeOH (1.6 mL). The solvents were removed in vacuo, and the residue was chromatographed on silica gel (CH_2Cl_2/MeOH 98:2) to yield **12** (1.484 g, 76%) ($R_f = 0.5$; CH_2Cl_2/MeOH 95:5) as a yellow solid.

^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 1.28$ (9H, s; C(CH_3)$_3$), 2.25–2.30 (1H, m; 2′-H_a), 2.48–2.52 (1H, m; 2′-H_b), 3.35–3.42 (4H, m; 9-H_{ab}, 5′-H_{ab}), 3.79 (6H, s; 2 OCH_3), 4.08–4.10 (1H, m; 4′-*H*), 4.53–4.55 (1H, m; 3′-*H*), 6.28 (1H, dd, J = 6.0, 7.5 Hz; 1′-*H*), 6.84–6.87 (4H, m; Ar*H*), 7.21–7.36 (m; Ar*H* + C*H*Cl_3), 7.42–7.44 (2H, m; Ar*H*), 8.01 (1H, s; 6-*H*), 8.81 (1H, br s; N*H*). ^{13}C NMR ($CDCl_3$): $\delta = 30.0$, 30.5 (9, C(CH_3)$_3$), 41.5 (2′), 47.8 (*C*(CH_3)$_3$), 55.2 (O*C*H_3), 63.5 (5′), 72.6, 75.2 (8, 3′), 85.6 (1′), 86.4 (4′), 87.0 (O*C*(Ph)$_3$), 89.5 (7), 100.0 (5), 113.4 (Ar), 127.0, 127.8, 128.0, 130.0, 135.5 (Ar), 142.6 (6), 144.4 (Ar), 149.1 (2), 158.6 (Ar), 161.2 (4).

5′-*O*-(4,4′-Dimethoxytrityl)-5-(butynyl)-2′-deoxyuridine *tert*-butyl disulfide (13). Nucleoside **9** (0.801 g, 1.923 mmol) was coevaporated with pyridine (5 mL) and redissolved in pyridine (12.5 mL). DMAP (0.059 g, 0.481 mmol, 0.25 eq.) and DMTCl (0.912 g, 2.692 mmol, 1.4 eq.) were added. After 6.5 h stirring at RT, the reaction

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was quenched with MeOH (2 mL). And the solvents were removed in vacuo. Chromatography of the crude residue on silica gel (CH_2Cl_2/MeOH 98:2) yielded the 5′-OH protected compound (1.079 g, 78%) ($R_f = 0.45$; CH_2Cl_2/MeOH 95:5) as an orange foam.

To a solution of above 5′-OH protected compound (1.112 g, 1.547 mmol) in deaerated THF/MeOH (3:1, 14 mL), di-*tert*-butyl 1-(*tert*-butylthio)-1,2-hydrazinedicarboxylate (0.645 g, 2.01 mmol, 1.3 eq.) and $LiOH \cdot H_2O$ (0.097 g, 2.3 mmol, 1.5 eq.) were added simultaneously under argon. The reaction mixture was then stirred for 1 h 20 min, diluted with Et_2O and washed with NaCl (aq.). The middle layer formed was kept separately, diluted with CH_2Cl_2 and washed with NaCl (aq.). The combined organic phases were reduced to dryness in vacuo. After chromatography on silica gel (CH_2Cl_2/CH_3OH 97:3), **13** (0.736 g, 68%) was obtained as a yellow foam ($R_f = 0.4$; petroleum ether/acetone 3:2).

m/z = 703 ($M^+ + 1$).

^{1}H NMR ($CDCl_3$): $\delta = 1.25$ (9H, s, C $(CH_3)_3$), 2.24–2.53 (6H, m, 2′-Hab, 9-Hab and 10-Hab), 3.31 (1H, dd, $J = 3.3$, 10.8 Hz, 5′-Ha), 3.43 (1H, dd, $J = 2.8$, 10.8 Hz, 5′-Hb), 3.79 (6H, s, 2 × OCH_3), 4.07–4.10 (1H, m, 4′-H), 4.51–4.56 (1H, m, 3′-H), 6.33 (1H, t, $J = 6.0$ Hz, 1′-H), 6.83–6.86 (4H, m, ArH), 7.20–7.35 (9H, m, ArH),7.42–7.44 (2H, m, ArH), 8.08 (1H, s, 6-H), 8.84 (1H, bs, NH). ^{13}C NMR ($CDCl_3$): $\delta = 20.3$ (9), 29.8 (C(CH_3)$_3$), 37.7 (10), 41.5 (2′), 47.8 (C $(CH_3)_3$), 55.2 (OCH_3), 63.4 (5′), 71.6, 72.3 (8, 3′), 85.6 (1′), 86.5 (4′),87.0 (OC(Ph)$_3$), 92.9 (7), 100.7 (5), 113.3 (Ar), 127.0, 127.9, 128.1, 129.9, 135.5 (Ar), 142.1 (6), 144.5 (Ar),149.2 (2), 158.6 (Ar), 161.6 (4).

5′-*O*-(4,4′-Dimethoxytrityl)-5-(butynyl)-2′-deoxycytidine *tert*-butyl disulfide (14). For the 5′-OH protection step, elution with $CHCl_3$/MeOH (97.5:2.5 v/v) gave the compound as a yellow solid (74%) ($R_f = 0.5$; $CHCl_3$/MeOH 86:14). For displacement reaction, elution with $CHCl_3$/MeOH (98:2 v/v) ($R_f = 0.55$; $CHCl_3$/MeOH 86:14) gave compound **14** (52%) as a yellow foam.

m/z = 416 ($M^+ + 1$).

^{1}H NMR ($CDCl_3$): $\delta = 1.29$ (s, 9H, C $(CH_3)_3$), 2.78 (bs, 1H, NH), 2.15–2.2 & 2.71–2.78 (m, 2H, 2′H), 2.54–2.57 (m, 4H, -CH_2), 3.31–3.35 (m, 2H, -5′H), 3.57 (t, 2H, -CH_2), 3.78 (s, 6H, 2× OCH_3), 4.14–4.25 (m, 1H, 4′), 4.48–4.56 (m, 1H, 3′H), 5.03 (t, 1H, 5′ -OH), 5.85 (bs, 1H, NH), 6.33 (t, 1H, t, J = 6.0 Hz, 1′-H'), 6.48 (bs, 1H, -NH), 6.81–6.84 (m, 4H, ArH), 7.18–7.28 (m, 7H, ArH), 7.42–7.45 (m, 2H, ArH), 8.13 (s, 1H, H-6); ^{13}C NMR ($CDCl_3$, 300 MHz): d = 20.1 (9), 30.0 (C (CH3)3), 37.7 (10), 42.5 (2′), 48.1 (C $(CH_3)_3$), 55.8 (OCH_3), 63.8 (5′), 72.1, 72.3 (3′), 85.6 (1′), 86.5 (4′), 87.0 (OC $(Ph)_3$), 92.2 (7), 99.5 (5), 113.3 (Ar), 127.0, 128.0, 128.3, 129.9, 136.0 (Ar),142.1 (6), 144.5 (Ar),149.2 (2), 159.0 (Ar), 163.0 (4).

General Procedure Acetylation and Detritylation Reaction

Dimethoxytritylated nucleoside **12**/**13** (1.0 eq.) was coevaporated with pyridine (3 mL) and redissolved in pyridine (5 mL) under Ar. Acetic acid anhydride (10 eq.) was added dropwise, and the reaction mixture was stirred for 1.5 h. The solution was then cooled in an ice bath, and the reaction was quenched with MeOH (5 mL). The solvents were removed in vacuo. The remaining yellow foam was coevaporated

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with toluene (3 mL) and subsequently dissolved in a solution of trifluoro acetic acid in CH_2Cl_2 (2% (v/v), 50 mL). The bright red reaction mixture was stirred for 30 min. It was neutralized with saturated $NaHCO_3$ solution (24 mL) and extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo to yield a crude yellow foam which was further purified by chromatography on silica gel.

3′-*O*-(Acetyl)-5-(1-propynyl)-2′-deoxyuridine *tert*-butyl disulfide (17). Elution with CH_2Cl_2/MeOH (98:2 v/v) yielded **17** (0.296 g, 70%) as a yellowish foam ($R_f = 0.35$; CH_2Cl_2/MeOH 97:3).

3′-*O*-(Acetyl)-5-(1-butynyl)-2′-deoxyuridine *tert*-butyl disulfide (18). Elution with CH_2Cl_2/MeOH (98:2 v/v) yielded **18** (0.057 g, 64%) as a yellowish foam ($R_f = 0.32$, CH_2Cl_2/MeOH 97:3).LRMS-FAB (NBA): m/z 443 ($M^+ + 1$).

^{1}H NMR ($CDCl_3$): $\delta = 1.34$ (9H, s; C(C*H*$_3$)$_3$), 2.11 (3H, s; C*H*$_3$), 2.32–2.49 (3H, m; 2′-*H*$_{ab}$ and 5′-O*H*), 2.75–2.91 (4H, m; 9-*H*$_{ab}$ and 10-*H*$_{ab}$), 3.90–4.01 (2H, dd; 5′-*H*$_{ab}$), 4.12–4.14 (1H, m; 4′-*H*), 5.34–5.37 (1H, m; 3′-*H*), 6.28 (1H, dd, $J = 6.1$, 8.1; 1′-*H*), 8.04 (1H, s; 6-*H*), 8.66 (1H, br s; N*H*).^{13}C NMR ($CDCl_3$): $\delta = 20.4$, 21.0 (9, CH_3CO), 29.9 ($C(CH_3)_3$), 37.8, 38.6 (10, 2′), 48.0 ($C(CH_3)_3$), 62.6 (5′), 72.4 (8), 74.8 (3′), 85.4 (1′), 86.1 (4′), 92.7 (7), 100.8 (5), 142.9 (6), 149.2 (2), 161.3 (4), 170.6 (CH_3CO).

General Procedure for Triphosphate Synthesis: 19 and 20

3′-Acetylated nucleoside **17** (0.070 g, 0.162 mmol)/**18** (0.54 g, 0.122 mmol) was coevaporated with pyridine (1 mL). The nucleoside was then redissolved in pyridine (0.162 mL/0.122 mL) and 1,4-dioxane (0.486 mL/0.366 mL) under argon. A freshly prepared solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in 1,4-dioxane (1 M, 0.162 mL/0.122 mL, 1.0 eq.) was added to the well stirred reaction mixture. A white precipitate formed immediately. After 10 min, a well vortexed emulsion from tributylammonium pyrophosphate (110 mg/83 mg) in DMF/tri-*n*-butylamine (3:1, 648 μL/488 μL)was added quickly. The precipitate dissolved immediately. After 10 min, a 1% solution of iodine in pyridine/water (98:2, 3.24 mL/2.24 mL) was added dropwise. After 15 min, the solvents were removed at 40°C under reduced pressure. The remaining brown oil was dissolved in water/MeOH (1:1, 10 mL). After 30 min, conc. ammonia solution (20 mL) was added and the suspension was stirred for 1 h. The solvents were then removed at 40 °C under reduced pressure. The remaining light brown oil was coevaporated with water. Water/MeCN (95:5, 2 mL) was added to yield a light brown suspension. The insoluble components were removed by filtration through a 0.2 mm cellulose acetate membrane, yielding a clear yellow filtrate.

5′-*O*-Triphosphate-5-(1-propynyl)-2′-deoxyuridine *tert*-butyl disulfide (19). 19 was isolated from the filtrate by chromatography on DEAE sephadex ion exchange resin (30 mL, 1.5×18 cm; TEAB 0.1 M to 0.8 M (linear gradient) in the presence of 10% MeCN). The eluate fraction containing **19** was lyophilized to dryness, and

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the residue was redissolved in water (D_2O for NMR sample) and lyophilized three more times to remove residual TEAOAc.

^{1}H NMR (D_2O): $\delta = 1.20$ (9H, s; C(CH_3)$_3$), 2.20–2.25 (2H, 2′-H_{ab}), 2.80–2.84 (2H, 9-CH_2), 3.62 (1H, m; 4′-*H*), 4.02–4.08 (2H, m; 5′-H_{ab}), 4.46–4.50 (1H, m; 3′-*H*), 6.11 (1H, t; 1′-*H*), 7.97 (1H, s; 6-*H*) ppm. ^{31}P NMR (D_2O): $\delta = -8.9$ (d, J = 19.4 Hz), −9.7 (d, J = 19.4 Hz), −21.5 (m) ppm.

5′-*O*-Triphosphate-5-(1-butynyl)-2′-deoxyuridine *tert*-butyl disulfide (20). **20** was recovered from the filtrate by RP-HPLC (Nova-Pak HR® C18 cartridge (Waters), 6 μm60 Å, 25 × 100 mm. Solvent A: TEAOAc (25 mM, pH 7.0); solvent B: ACN. Flow rate: 5.5 mL/min. 0–1 min 100% A; 10 min 14% B (linear); 55 min 19% B (linear). $R_t \approx 37$ min). The eluate fraction containing **20** was lyophilized to dryness, and the residue was re-dissolved in water (D_2O for NMR sample) and lyophilized three more times to remove residual TEAOAc. **20** (ca. 10 mmol, 8%) was stored at −20°C as 5 mM solution in water. The yield was estimated based on UV absorbance, with an assumed extinction coefficient $\varepsilon_{260} = 3{,}200\ M^{-1}cm^{-1}$ for **20**.[18]

UV (water) $\lambda_{max} = 233$, 293 nm. $\lambda_{min} = 258$ nm.HRMS-FAB (Glycerol): Calcd for $C_{17}H_{26}N_2O_{14}P_3S_2$ (monoanion): m/z 639.0038 (M^- −1). Found: 639.0083.

^{1}H NMR (D_2O): $\delta = 1.34$ (9H, s; C(CH_3)$_3$), 2.36–2.40 (2H), 2.80–2.84 (2H), 2.95–3.00 (2H), 4.15–4.27 (3H, m; 4′-*H*, 5′-H_{ab}), 4.64 (1H, m; 3′-*H*), 6.26 (1H, t; 1′-*H*), 8.05 (1H, s; 6-*H*). ^{31}P NMR (D_2O): $\delta = -8.4$ (d, $J = 19.8$ Hz), −9.3 (d, $J = 19.8$ Hz), −21.1 (t, $J = 19.8$ Hz).

3′-*O*-(N,N-Diisopropyl-β-cyanoethylphosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-5-(propynyl)-2′-deoxy-uridine *tert*-butyl disulfide (21). Dimethoxytritylated nucleoside **12** (0.560 g, 0.813 mmol) was dissolved in CH_2Cl_2 (freshly distilled over CaH_2, 4 mL) under argon. The solution was cooled in an ice bath, and N,N-diisopropylethylamine (“Huenig’s base”, 779 μL, 4.472 mmol, 5.5 equiv) and chloro-N,N-diisopropylamino-β-cyanoethylphosphine (199 μL, 0.894 mmol, 1.1 equiv) were added dropwise. After a few minutes, the ice bath was removed, and stirring was continued at ambient temperature for 3.5 h. The volume of the reaction mixtures was reduced to half the volume under reduced pressure. The concentrated reaction mixture was chromatographed on silica gel ($CHCl_3$/EtOAc/TEA; 72:25:3). The obtained yellowish foam (0.728 g, 101%) was dissolved in CH_2Cl_2 (1.4 mL). The solution was dropped very slowly into vigorously stirred hexane (32 mL). A fluffy precipitation formed. After 1.5 h, the supernatant was decanted. The off-white precipitate (0.640 g) contained the diastereomers of **21** (ca.0.627 g, 89% yield) and a contaminant (presumably hydrolysed phosphitylation reagent, ca. 8 mol% = 13 mg as judged by ^{31}P NMR).

($R_f = 0.49$; $CHCl_3$/EtOAc/TEA 70:25:5).

^{1}H NMR ($CDCl_3$), 2 diastereomers: $\delta = 1.08$–1.19 (24H, m; 4 NCH(CH_3)$_2$), 1.27, 1.28 (2 × H, 2 s; 2 C(CH_3)$_3$), 2.24–2.35 (2H, m; 2 2′-*H*), 2.44–2.48 (2H, m; OCH_2CH_2CN), 2.52–2.65 (4H, m; 2 2′-*H*, OCH_2CH_2CN), 3.24–3.90 (16H, m; 2 OCH_2CH_2CN, 4 NC*H*(CH_3)$_2$, 2 9-H_{ab}, 2 5′-H_{ab}), 3.79, 3.80 (2 × 6H, 2 s; 2 OCH_3), 4.16–4.23 (2H, m; 2 4′-*H*), 4.59–4.65 (2H, m; 2 3′-*H*), 6.24–6.30 (2H, m; 2 1′-*H*), 6.83–6.88 (8H, m; Ar*H*), 7.20–7.37 (m; Ar*H* + C*H*Cl_3), 7.42–7.46 (4H, m; Ar*H*), 8.04, 8.08 (2 × 1H, 2 s; 2 6-*H*). ^{13}C NMR ($CDCl_3$), 2 diastereomers:

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$\delta = 20.3$ (2 signals, OCH_2CH_2CN), 24.5, 24.6 ($NCH(CH_3)_2$), 29.9, 30.4 (9, $C(CH_3)_3$), 40.6 (2′), 43.1, 43.3 ($NCH(CH_3)_2$), 48.1 ($C(CH_3)_3$), 55.2 (OCH_3), 58.1, 58.4 (OCH_2CH_2CN), 63.2 (5′), 74.0, 75.1 (8, 3′), 85.6, 85.7 (1′, 4′), 87.0 ($OC(Ph)_3$), 89.4 (7), 100.0 (5), 113.3 (Ar), 126.9, 127.8, 128.0, 129.9, 130.0, 135.2, 135.4 (Ar), 142.6 (2 signals, 6), 144.4 (Ar), 149.0 (2), 158.6 (Ar), 161.1 (4). ^{31}P NMR ($CDCl_3$), 2 diastereomers: $\delta = 154.16$, 154.56.

3′-*O*-(N,N-Diisopropyl-β-cyanoethylphosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-5-(1-butynyl)-2′-deoxyuridine *tert*-butyl disulfide (22). Dimethoxytritylated nucleoside **13** (0.140 g, 0.199 mmol) was dissolved in CH_2Cl_2 (freshly distilled over CaH_2, 1 mL) in an Ar atmosphere. The solution was cooled in an ice bath, and N,N-diisopropylethylamine (''Huenig's base'', 191 μL, 1.095 mmol, 5.5 equiv) and chloro-N,N-diisopropylamino-β-cyanoethylphosphine (49 μL, 0.219 mmol, 1.1 equiv) were added. The mixture was stirred for 6 h while gradually warming to rt. The reaction mixture was directly loaded onto a silica gel column and chromatographed ($CHCl_3$/EtOAc/TEA; 75:25:1). The obtained product was subjected to a second chromatography on silica gel (petroleum ether/EtOAc/TEA/MeOH 5:2:1:0.2). The obtained product was dissolved in CH_2Cl_2 (0.5 mL) and dropped into vigorously stirred hexane (10 mL). A white, flaky precipitate formed. After 2 h the supernatant was decanted. The precipitate was dried under reduced pressure to afford the diastereomers of **22** (0.70 mg, 39%) as a white foam. The integrity of **22** was verified indirectly after incorporation into oligonucleotides.

ESI MS (iPrOH/water 1:1, TEA (30 mM)): CCCGXATTT m/z calcd 2823. Found 2821. CCCGXXATTT m/z calcd 3285. Found 3283.

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