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2′-Deoxycytidines Carrying Amino and Thiol Functionality: Synthesis and Incorporation by Vent (Exo$^-$) Polymerase

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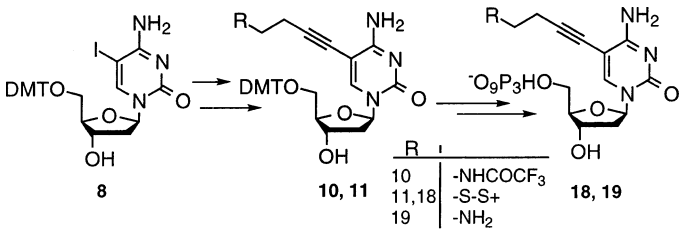
ABSTRACT



The synthesis of 2′-deoxycytidine nucleosides bearing amino and thiol groups appended to the 5-position of the nucleobase via a butynyl linker is described. The corresponding triphosphates were then synthesized from the nucleoside and incorporated into oligonucleotides by Vent (exo$^-$) DNA polymerase. The ability of Vent (exo$^-$) polymerase to amplify oligonucleotides containing these functionalized cytidine derivatives in a polymerase chain reaction (PCR) was demonstrated for the amino-functionalized derivative.

Deoxyribonucleosides carrying functionality appended at the 5-position of uracil were introduced three decades ago to complement the functionality that DNA carries intrinsically.[1] Fluorescent appendages attached to 2′,3′-dideoxynucleotides have been the key to automated DNA sequencing.[2] Functionalized appendages may also increase the power and versatility of nucleic acids as receptors, ligands, and catalysts.[3] Modified standard nucleotides have been incorporated into *in vitro* evolution experiments.[4]

The C5-position of the pyrimidine nucleobases is an appropriate place to introduce functionality, as the site lies in the major groove of the duplex, where appendages do not interfere with Watson−Crick pairing. Appendages at this site are also well tolerated by RNA and DNA polymerases, which do not interact with the nucleobases in the major groove.[4c,5,6]

Most of the previous work in this area has functionalized 2′-deoxyuridine, although some work has also appended functionality through the 5-position of 2′-deoxycytidine analogues.[7] We report here the synthesis of 2′-deoxycytidine nucleosides carrying amino or thiol terminal functionality appended at position C5 via a four-carbon alkynyl linker. We also report the successful incorporation of these as triphosphates into DNA using Vent (exo$^-$) DNA polymerase and PCR amplification.

Recent reports suggest that protected 2′-deoxycytidine nucleosides carrying a C-5 acetylene group readily suffer

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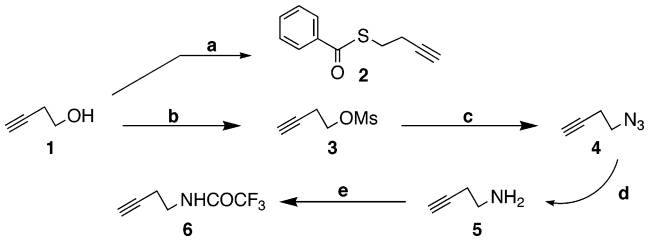
unwanted cyclization.[4b] Thus, we explored chemistry to attach substituents to the 5-position of an unprotected cytidine nucleoside.

But-1-yne-4-thiol (**2**) having its thiol group protected as the disulfide with *tert*-butylthiol was synthesized from but-3-yn-1-ol **1** as reported previously.[8] The corresponding 1-aminobut-3-yne, with the amino group protected as the trifluoroacetyl amide, was synthesized from but-3-yn-1-ol via a route adapted from the literature.[9] Here, the free hydroxyl group of **1** was converted to the corresponding azide via mesylation. The azide was then reduced to the corresponding amine with PPh_3. The reaction mixture was acidified, the side products were extracted, and the free amine (**5**) was isolated by the neutralization of its acid salt. Reaction with trifluoroacetic anhydride gave **6** in 51% yield (Scheme 1).

Scheme 1[a]

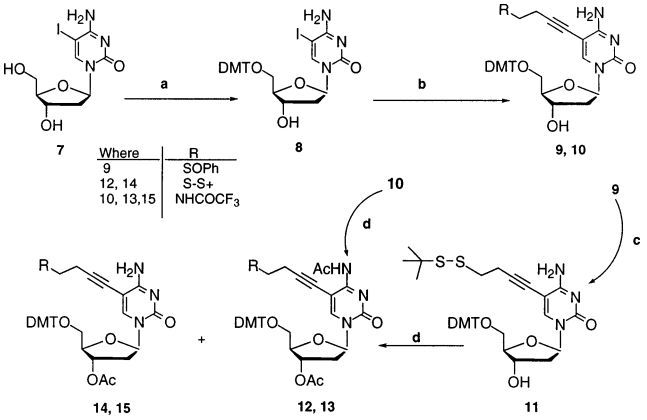


[a] Conditions: (a) PPh_3/DIAD/THF/TEA/14−18 h/rt; (b) MsCl/anhydrous ether/3 h/0 °C/H_2O; (c) NaN_3/anhydrous DMF/3.5 h/67 °C/H_2O; (d) PPh_3/anhydrous ether/H_2O/18 h/0 °C to rt/10% HCl (aq); 10% NaOH (aq); (e) anhydrous MeOH/$CF_3CO_2CH_3$/18 h/0 °C to rt.

Sonogashira coupling[10] of the compounds with the 5′-tritylated 5-iodo-2′-deoxycytidine (**8**) in the presence of palladium(0) catalyst gave the desired products (**9** and **10**) (Scheme 2).

The 5′-tritylated nucleoside was used in preference to the 5′-hydroxy nucleoside because the former is easier to handle (with respect to monitoring and its greater solubility) and saves solvents during column chromatography purification.

Scheme 2[a]

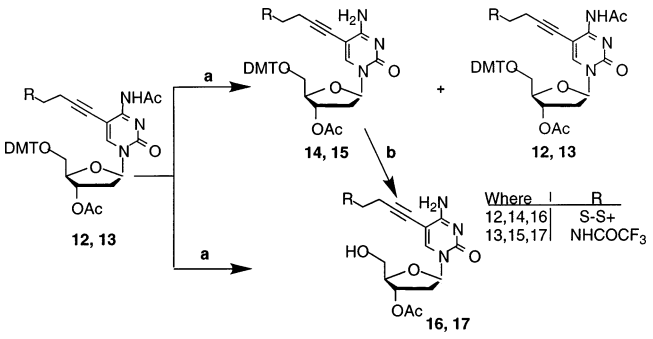


[a] Conditions: (a) DMTCl/TEA/DMAP/Py anhydrous/6 h/0 °C to rt; (b) **2** or **6**/CuI/TEA/$Pd(PPh_3)_4$/DMF anhydrous/10−14 h/rt; (c) Di-*t*-butyl-1-(*t*-butylthio)-1,2-hydrazine-dicarboxylate/LiOH/MeOH−THF/90 min anhydrous. (d) Py/DMAP/TEA/Ac_2O/4 h/0 °C to rt.

The benzoyl-protecting group on **9** was replaced by the more stable *tert*-butylthio group[8] in a one-step reaction with di-*t*-butyl-1-(*t*-butylthio)-1,2-hydrazine-dicarboxylate in the presence of LiOH to give **11**. Acetylation of **10** and **11** yielded mixtures of both di- and monoacetylated products, **12** and **14**, **13** and **15**, respectively, with the major products being the desired **14** and **15**.

We attempted to selectively deacetylate **12** and **13** using $ZnBr_2$.[11] An equimolar amount of the Lewis acid gave the desired **14** and **15**, together with the unreacted starting compound **12** and **13**, in approximately equal amounts. Treatment with 2 equiv of $ZnBr_2$ yielded the N-4 deacetylated and deprotected 5′-OH compounds **16** and **17** only. Compounds **16** and **17** were also prepared from **14** and **15** using the commercial deprotecting mixture (2.5% trichloroacetic acid in CH_2Cl_2) for oligonucleotide synthesis via solid-phase chemistry (Scheme 3).

Scheme 3[a]



[a] Conditions: (a) $ZnBr_2$/MeOH/$CHCl_3$/2 h/rt; (b) TCA in CH_2Cl_2/30 min/rt.

Triphosphate synthesis was carried out following literature procedures.[12] Thus, reaction of **16** and **17** with $POCl_3$ in

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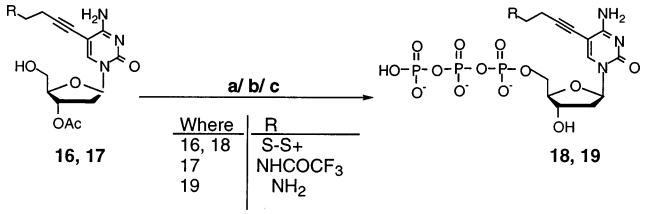
trimethyl phosphate and proton sponge gave the phosphorodichloridates as intermediates, which were converted *in situ* with pyrophosphate to the corresponding triphosphates.

Hydrolysis with NH_4OH gave the desired nucleotide triphosphates, **18** and **19** (Scheme 4).

Scheme 4[a]



[a] Conditions: (a) $POCl_3$/$(MeO)_3P(O)$/proton sponge/2.5 h/0 °C; (b) Tri-*n*-butylammonium pyrophosphate/*n*-tributylamine/DMF/TEAB/2 min/0 °C to rt; (c) NH_3/rt/18 h.

The ability of the functionalized dCTP analogues (**18** and **19**) to serve as substrates for thermostable DNA polymerases under PCR conditions was then studied. Since different polymerases often behave differently with unnatural nucleosides,[4h] representatives of two evolutionary families of DNA polymerases[13] were examined. These were the Taq DNA polymerase from *Thermus aquaticus* (representing Family A) and Vent (exo$^-$) DNA polymerase from *Thermococcus litoralis* (representing Family B).

Primer extension experiments were done using PAGE-purified 5′-[γ-^{32}P]-radiolabeled primer (5′-GCG TAA TAC GAC TCA CTA TAG-3′) and template (5′-GAC ACG CGC TAT AGT GAG TCG TAT TAC GC-3′) (both from Integrated DNA Technologies, Coralville, IA).

Taq incorporated neither **18** nor **19** with acceptable efficiency (data not shown). Vent (exo$^-$) DNA polymerase incorporated both **18** and **19**. Thus, Vent polymerase incorporated **19** (Figure 1; Lane 6) opposite G in a single base extension. Full-length product was obtained upon addition of a complete set of natural triphosphates excluding dCTP (Figure 1; Lane 5).

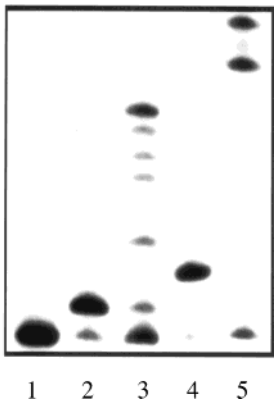


Figure 1. Primer extension experiment using amino-functionalized 2′-deoxycytidine triphosphate **19**. Denaturing 20% PAGE−urea gel was used. 5′-Radiolabeled primer was visualized by a phosphoimager. All lanes contain annealed primer and template, buffer, and water. Lane 1: All natural dNTPs (0.1 mM each), no polymerase (negative control). Lane 2: Vent (exo$^-$) DNA polymerase and dCTP only showing pausing after incorporation of dC. Lane 3: Vent (exo$^-$) polymerase and natural dNTPs showing formation of full-length product (positive control). Lane 4: Vent (exo$^-$) polymerase and **19** only, showing incorporation of a single **19**; lower mobility due to positively charged functionality. Lane 5: Vent (exo$^-$) polymerase and **19**, dTTP, dGTP, dATP, expecting incorporation of three **19**s.

Similarly successful incorporation was observed for analogue **18** with protected thiol functionality (data in Supporting Information).

Compound **19** carrying a free amino group successfully replaced dCTP in PCR amplification using Vent (exo$^-$) polymerase (Figure 2; Lane 6). This PCR required incorporation of 22 and 31 functionalized cytidine analogues per strand in the forward and reverse reactions.

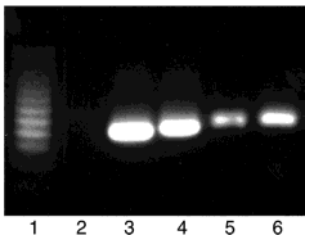


Figure 2. PCR amplification with **19** replacing dCTP. Agarose gel (2%) was used and stained with ethidium bromide. A total of 25 PCR cycles were run, with 2 min each incubation. Lane 1: Promega DNA ladder 25−300 nts. Lane 2: Negative control, lacking polymerase. Lane 3: Positive control with standard dNTPs. Lane 4: Positive control including both 2′-dCTP and **19** (1:1 ratio) and standard dNTPs. Lane 5: With 2 μM **19** and standard dNTPs. Lane 6: With 4 μM **19** and standard dNTPs. Template (100mer): 5′-CGC ATT ATG CTG AGT GAT ATC TAT CCA GAC CTA GAA AGA GTG CAC TGA TGC TGT TCG AGC GCA CGG CCT CCA ACA TGC CGT CCA TGC ACC ACT AGA CCT C-3′. Primer (24mer): 5′-GAG GTC TAG TGG TGC ATG GAC GGC-3′. Reverse primer (24mer): 5′-CGC ATT ATG CTG AGT GAT ATC TAT-3′.

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Relatively low concentrations of triphosphate (2 μM) were used in the PCR amplification. Compound **19** alone supported the synthesis of full-length products at these concentrations (Figure 2; lane 5), although doubling the concentration of **19** increased the amount of full-length product formed. This suggested that **19** was able to support PCR amplification, but at modestly lower efficiency. Consistent with this conclusion, a 1:1 mixture of **19** and dCTP in a 1:1 mixture showed reduction in the amount of full-length product observed relative to dCTP alone. As a caveat, it is important to note that these results are consistent with, but do not absolutely prove, the presence of the functionalized cytidine derivative in the product.

Two features of these results are particularly noteworthy. First, in primer extension experiments, DNA carrying 3 equiv of **19** showed a double band.

Polymerases frequently add a nontemplated nucleotide to the product, and this is, we believe the simplest explanation for this observation.[14]

Further, although **19** supported the PCR amplification of DNA, the yield appeared to be lower than with standard dCTP. We considered the possibility that the positively charged amine groups may disrupt the intercalation by the similarly cationic ethidium bromide. As the intensity of the band increases when the concentration of **19** is doubled, we rejected this hypothesis. Rather, it seems that the replacement of dCTP by **19** simply lowers the yield of full-length product.

The accessibility of these functionalized nucleoside derivatives and polymerases that accept their triphosphates sufficiently well to support the polymerase chain reaction gives two new tools to those wishing to perform molecular biology with DNA containing thiol and amino functionalities. This is another step toward the development of a synthetic biology.[15]

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Supporting Information Available: Experimental procedures and characterization of all new compounds along with NMR spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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