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DNA Interstrand Cross-Linking Upon Irradiation of Aryl Halide C-Nucleotides

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Abstract

γ-Radiolysis kills cells by damaging DNA via radical processes. Many of the radical pathways are O_2 dependent, which results in a reduction in the cytotoxicity of ionizing radiation in hypoxic tumor cells. Consequently, there is a need for chemical agents that increase DNA damage by ionizing radiation under O_2 deficient conditions. Modified nucleotides that are incorporated in DNA and produce highly reactive σ-radicals are useful as radiosensitizing agents. Aryl halide C-nucleotides (**4**–**6**) were incorporated into oligonucleotides by solid phase synthesis. Duplex DNA containing **4**–**6** forms interstrand cross-links upon γ-radiolysis under anaerobic conditions or UV-irradiation. Deep Vent (exo$^-$) DNA polymerase accepted the nucleotide triphosphate of C-nucleotide **6** as a substrate and preferentially incorporated it opposite pyrimidines but no further extension was detected. Incorporation of **6** in extended products by Deep Vent (exo$^-$) during PCR or by Sequenase during copying of single stranded DNA plasmid was undetectable. Aryl halide nucleotide analogues that produce DNA interstrand cross-links under anaerobic conditions upon irradiation are potentially useful as radiosensitizing agents but further research is needed to identify molecules that are incorporated by DNA polymerases and do not block further polymerization for this approach to be useful in cells.

Keywords

nucleic acid oxidation; DNA damage; nucleotide analogues; interstrand cross-links; radiosensitization

Introduction

Radical mediated DNA damage is the source of the cytotoxic effects of ionizing radiation. Ionizing radiation's effects are enhanced by O_2, which competes with thiols that can restore DNA to its native structure. Some tumors are deficient in O_2 (hypoxic), resulting in a decrease in radiation efficiency. Radiosensitizing agents have been developed to overcome the limitations imposed by hypoxia. Some of the most well studied radiosensitizing agents are nucleotides that are incorporated into cellular DNA by polymerases. 5-Bromo- (BrdU) and 5-iodo-2′-deoxyuridine (IdU) are incorporated in DNA in place of thymidine and

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Supporting Information Available.
Experimental procedures for PCR and Sequenase experiments. Representative hydroxyl radical cleavage autoradiogram for cross-link identification. Sample kinetic plots for incorporation of dG and **6** opposite dC in **20**. Spectral data for previously unreported compounds. MALDI-TOF-MS for oligonucleotides containing C-nucleotides and cross-linked products. This material is available free of charge via the Internet at http://pubs.acs.org.

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sensitize the biopolymer to ionizing radiation by scavenging solvated electrons produced from the ionization of water and/or that are released from other portions of the DNA and producing a highly reactive σ-radical (**1**, Scheme 1).[1,2] The σ-radical abstracts hydrogen atoms from adjacent nucleotides producing strand breaks and alkali-labile lesions.[3–8] Recently, it was discovered that **1** also yields interstrand cross-links (ICLs), but only in non-base paired regions of duplex DNA.[9–11] ICLs are a very deleterious form of DNA damage that are absolute blocks to replication and transcription and are repaired by nucleotide excision repair (NER). The possible importance of cross-linking by **1** is magnified by recent examples in which ICLs are converted ("misrepaired") during NER to double strand breaks, the most deleterious form of DNA damage.[12–14] These observations inspired us to design radiosensitizing agents that produce ICLs in base paired regions of DNA.

Based upon cross-linking resulting from the exposure of DNA containing BrdU and other nucleotides to ionizing radiation (or UV irradiation), we rationalized that the rotational barrier around the glycosidic bond, coupled with the high reactivity of **1** prevented it from producing ICls in base paired regions.[11,15–17] We hypothesized that non-hydrogen bonding nucleotide analogues would be well suited for producing ICLs because the absence of stabilizing interactions with the opposite strand would reduce barriers for adopting a conformation that is conducive to cross-link formation. In choosing molecules that might be expected to display this reactivity we benefited from the significant advances over the past two decades in developing nonnative nucleotides to probe polymerase mechanism and to expand the genetic code. These molecules avoid hydrogen bonding during selective recognition of native and other nonnative nucleotides.[18–22] Our objective is less challenging in this regard because nonselective incorporation opposite native nucleotides is desirable, provided cross-linking is inducible. Furthermore, high incorporation levels are unnecessary due to the high impact that DNA interstrand cross-links have on biochemical processes. Using the work of Kool as a guide, a series of oligonucleotides containing aryl iodide C-nucleotides (**2**, **3**, Scheme 2) were synthesized by solid phase synthesis.[23–26] The molecules produced ICLs in duplexes containing any of the 4 native nucleotides opposite the nucleotide analogues when exposed to UV-irradiation. O_2 had little effect on UV-induced cross-linking. Cross-links were formed with the opposing nucleotide and to varying extents with flanking thymidines depending upon the nucleotide opposite the radical precursor. ICLs were also produced when the duplexes were exposed to γ-radiolysis under anaerobic conditions. The presence of a hydroxyl radical quencher (*t*-BuOH) had no effect on ICL formation, ruling out the involvement of this reactive oxygen species. In contrast to UV-irradiation O_2 quenched cross-linking when DNA was exposed to γ-radiolysis, suggesting that the nucleotides would selectively sensitize hypoxic cells. The dioxygen effect also suggested that solvated electrons, which are scavenged by O_2, react with the aryl iodide C-nucleotide analogues to produce σ-radicals that are directly responsible for cross-linking. These experiments established that halogenated aromatic nucleotide analogues could produce ICLs but the respective nucleotide triphosphates of these first generation molecules were not expected to be good substrates for DNA polymerases. Herein, we describe our efforts to design nucleotide analogues that selectively cross-link the opposing strand of DNA when exposed to ionizing radiation under O_2 deficient conditions, but whose nucleotide triphosphates are also accepted as substrates by DNA polymerase(s).

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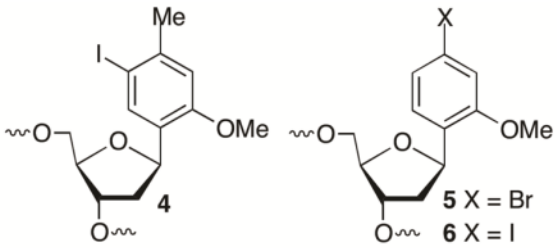
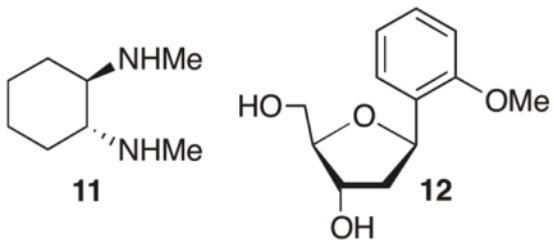


Results and Discussion

The molecules described in this study were based on a combination of our own cross-linking results using aryl halide C-nucleotides (e.g. **2**, **3**) and investigations that revealed the importance of a hydrogen bond acceptor in the minor groove for polymerase interactions.[27,28] Consequently, oligonucleotides containing **4**–**6** were synthesized and evaluated for ICL formation upon UV-photolysis and γ-radiolysis. Compound **4** was previously reported by Romesberg and is most closely related structurally to BrdU and Idu.[29] Aryl halides **5** and **6** were chosen based upon the successful cross-linking by **3**.[26] (Please note that for simplicity the aryl halides are identified to by the same numerical descriptor whether they are present as the monomer or as a component within an oligonucleotide.)

Synthesis of C-aryl halide nucleosides and their incorporation into oligonucleotides

Compound **4** was previously incorporated into oligonucleotides via its respective phosphoramidite.[29] This synthesis was repeated as described and the general approach was used to prepare the requisite phosphoramidites (**10a**,**b**) for synthesizing oligonucleotides containing **5** and **6** (Scheme 3). Consequently, the 5-bromo-2-iodoanisole (**8**) was coupled with **7** and the 3′-keto-nucleoside analogue (**9**) was partially purified following desilylation. The nucleoside (**5**) was obtained via directed reduction and carried on to phosphoramidite **10a** using standard methods. The iodine analogue (**6**) was prepared from the **5** by displacing the bromide via a previously reported method using a mixture of NaI/CuI and *trans*-*N*,*N*′-dimethyl-1,2-cyclohexanediamine (**11**) in a pressure bottle in a manner similar to that previously described by Kool.[30,31] The reaction must be followed closely by ^{1}H NMR to avoid forming the reduction product (**12**). The iodide (**6**) was also carried on to **10b** via standard methods.



The phosphoramidites of the halogenated nucleotide analogues were incorporated into oligonucleotides (**13**–**15**) via automated solid phase synthesis using standard procedures and reagents, with the exception that an extended (15 min) time was used for coupling the modified phosphoramidites. Oligonucleotides containing **13**–**15** were deprotected using “AMA” conditions (1:1 aqueous methylamine and concentrated NH_4OH) at 65 °C.32 The oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis and characterized by MALDI-TOF-MS following desalting.[33]

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5'-d(CGA GTA CTG CAA **X**AA CGT GTA CAG C)

13 X = **4**; **14** X = **5**; **15** X = **6**

5'-d(CGA GTA CTG C A A **X** AA CGT GTA CAG C)
3'-d(GCT CAT GAC $GT_{15}T_{14}$ $\mathbf{Y}_{13}$TT GCA CAT GTC G)

16a-d X = **4**
17a-d X = **5**
18a-d X = **6**
a Y = A; **b** Y = C; **c** Y = G; **d** Y = T

Interstrand cross-link formation upon UV-irradiation of halogenated C-nucleotides

Cross-link formation upon UV-irradiation (30 min) under aerobic conditions in a Rayonet photoreactor (λ_{max} = 300 nm) of duplexes containing modified nucleotides **4**–**6** was determined using 5′-^{32}P-**16a**–**d** - 5′-^{32}P-**18a**–**d** (Table 1) in which the strand containing the C-nucleotide was radiolabeled. ICL yields from **4** were consistently lower, by at least 50%, than the respective duplexes containing either **5** or **6**. UV-induced cross-linking yields obtained from **5** (5′-^{32}P-**17a**–**d**) and **6** (5′-^{32}P-**18a**–**d**) are more similar to one another, with the exception when aryl bromide **5** was opposite dG (5′-^{32}P-**17c)** when the yield reached 65%. Although average ICL yields are (with the exception of when dG is opposite the modification) slightly higher for the aryl iodide (**6**, 5′-^{32}P-**18**) than **5**, the variations are such that they are within experimental error of one another. It is not known why the ICL yield for **5** opposite dG is so much greater than in all other duplexes examined, nor can we rule out different photochemical efficiencies from substrate to substrate to explain the lower ICL yields from duplexes containing **4**.

It is tempting to ascribe the large difference in ICL yields between **4** and the other C-nucleotides to differences in the molecules' conformations. If **4**–**6** adopt conformations (as drawn) equivalent to that of a native nucleotide in its *anti* form, the halide in **4** lies in the major groove (Scheme 4) in a position equivalent to the bromine or iodine in BrdU and IdU respectively. Cross-link formation would require rotation about the glycosidic bond into the *syn*-equivalent conformation. C-nucleotides **5** and **6** contain the halide at the position analogous to C4 in a native pyrimidine and the orientation of the halide (and subsequent radical center) with respect to the opposing strand will be relatively insensitive to the conformation about the pseudo-glycosidic bond. However, if responsible for the observed selectivity these factors should also affect cross-linking induced by γ-radiolysis, which does not exhibit the same preference (see below). Alternatively, the differences in UV-induced cross-linking yields may be due to the involvement of a mechanism other than direct excited state homolysis of the aryl halide bond. For instance, the 5-halopyrimidines are converted into **1** (Scheme 1) via photoinduced electron transfer.[34,35] Such a mechanism cannot be discounted for these molecules, nor is it certain whether **4**–**6** would behave differently in this type of process. However, it would be consistent with differences in cross-linking yields between UV- and γ-irradiation, provided that σ-radical yields from a photoinduced electron transfer process were different for **4**–**6**.

Despite the significant difference in ICL yields between **4** and the C4-halogenated nucleotides (**5** and **6**) their preferred cross-linking site(s), as determined by reaction with hydroxyl radical were quite similar.[36] The major site of cross-linking was T_{14} in all 3 duplexes containing dC opposite the C-nucleotide (**16b**–**18b**).[33] However, the cross-linking preferences for **4**–**6** were different than those of **2** and **3**. The latter formed the majority of cross-links with an opposing dC.[26]

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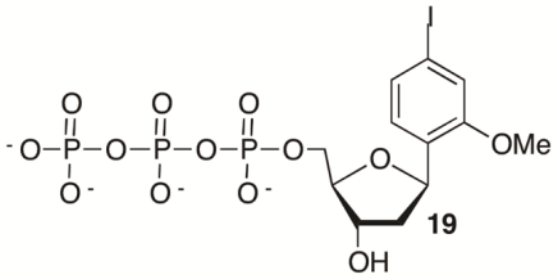
Interstrand cross-link formation upon ^{137}Cs-irradiation of halogenated C-nucleotides

^{137}Cs irradiation (315 Gy) of duplexes containing C-nucleotides **4**–**6** under anaerobic conditions also produced interstrand cross-links (Table 2). In comparison to UV-irradiation, γ-radiolysis produced much more similar yields of ICLs amongst the 12 duplexes examined. Cross-link formation was slightly less efficient in duplexes containing the aryl bromide (**5**). This was true regardless of the identity of the nucleotide opposite the C-nucleotide. Although the difference is less than 2-fold within any one family of duplexes containing the same C-nucleotide, ICL formation was least efficient when dG was opposite the modified nucleotide. Exposing 5′-^{32}P-**16a**–**d** - 5′-^{32}P-**18a**–**d** to the same dose of radiation under aerobic conditions produced less than 2% ICLs and adding *t*-BuOH (10 mM) prior to irradiation had no effect on cross-link yield (data not shown). These effects are consistent with generation of the respective σ-radicals by loss of halide ion from the radical anions following reaction of the aryl halides with a solvated electron. Cross-link formation by **4**–**6** was more efficient than the previous aryl iodides (e.g. **2**, **3**), which also produce ICLs via σ-radicals, despite being exposed to less than one-half the dose.[26]

DNA polymerase incorporation of 6 via its C-nucleotide triphosphate (19)

The above experiments indicate that **4**–**6** will function as radiosensitizing agents when present in DNA. To be a complete radiosensitizing agent, the triphosphate of such molecules must be incorporated into cellular DNA by polymerase(s) and the same molecule or an appropriate precursor must pass through the cell membrane. Romesberg reported on the incorporation of **4** into DNA, as well as its effect on polymerase activity when present in a DNA template.[29] The Klenow fragment of *E. coli* DNA polymerase I (Klenow), a model polymerase, incorporated 3 of the 4 native 2′-deoxynucleotides opposite **4**, albeit only dA was introduced with moderate efficiency. This was desirable for the authors' goals but averse to our own. As a proof of principle we chose to examine the incorporation of **6** by DNA polymerase instead of **5** because it provided higher ICL yields when exposed to ^{137}Cs. Choosing a model polymerase was difficult, as there are more than a dozen DNA polymerases in human cells, several of which have evolved to be promiscuous, error prone. Any one of these might achieve our goal and incorporate low levels of **6** opposite native nucleotides in a DNA template. Deep Vent (exo$^-$) was selected as a model polymerase because it tolerates other nonnative nucleotide triphosphates and backbone modifications.[37,38]



The nucleotide triphosphate of **6** (**19**) was synthesized by standard methods and purified by ion-exchange and C_{18}-reverse phase HPLC. The kinetics of its incorporation opposite dC in **20** was examined quantitatively and compared to that of dGTP because a duplex containing this nucleotide opposite **6** yielded the highest yield of radiolytically induced ICLs (Table 2). Under steady-state conditions dG was incorporated ~1,300-times more efficiently than **6** (Table 3).[39] The predominant source of this selectivity was an ~650-fold lower apparent K_m ($K_{m(app)}$) for dGTP. The ability of Deep Vent (exo$^-$) to accept **19** and incorporate **6** opposite

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the other 3 native nucleotides was examined qualitatively at 70 μM (the $K_{m(app)}$ opposite dC). At this single concentration of **19**, the rate of incorporation opposite T was approximately the same as when dC was in the template. In contrast, Deep Vent (exo$^-$) incorporated **6** very weakly opposite dA and not at all when dG was in the template under these conditions. A direct comparison to data in the literature is not available. However, Romesberg found that of the 4 native nucleotides, dA incorporation opposite **12** was most efficient.[27] Incorporation of the other 3 native nucleotides was too slow to measure. In contrast, Klenow exhibited the same order of nucleotide incorporation opposite **4** (dC > T > dA > dG) as observed here for incorporation of **6** opposite native nucleotides by Deep Vent (exo)$^-$.[29]

Extension of the nascent strand is typically even more challenging than nonnative nucleotide incorporation.[27,29] The effect of **6** in the growing strand on polymerization was qualitatively examined in two ways. Full-length extension of **20** was examined in the presence of all 4 native dNTPs (200 μM each) and compared to extension in which **19** (200 μM) was substituted for dGTP. While Deep Vent (exo$^-$) produced full-length product within 5 min when all 4 native nucleotide triphosphates were present, yet the reaction containing **19** gave no full-length material after 1 h (Figure 1). Multiple extension products were formed, some of which based upon their length could contain as many as 3 molecules of **6** it were the only nucleotide inserted opposite dC. Since extension products alone do not distinguish between incorporation of **6** or any of the native nucleotides. Consequently, the ability of Deep Vent (exo$^-$) to extend a primer following incorporation of **6** was examined by extending 5′-^{32}P-**20** in the presence of **19** only, isolating the extension product by denaturing polyacrylamide gel electrophoresis (PAGE), and rehybridizing with the complement to form 5′-^{32}P-**21**. Subsequent denaturing PAGE analysis of freshly isolated 5′-^{32}P-**21** incubated with Deep Vent (exo$^-$) and native dNTPs (1 mM) for 2 h showed no extension of the material containing **6** at its 3′-terminus (data not shown), indicating that the C-nucleotide is an absolute block for the polymerase under these conditions.

Since Deep Vent (exo$^-$) was developed for use in PCR, we explored the possibility that its acceptance of **19** would be enhanced under conditions in which such experiments are typically carried out. Consequently, a 287 bp PCR product was prepared from single stranded M13mp7 plasmid using 24 nt primers (one of which was labeled with ^{32}P at its 5′-terminus), as previously described.[40] A longer substrate than **20** also increased the statistical probability for incorporating a single molecule of **6**, which would be sufficient for producing an ICL. The 4 native nucleotide triphosphates (200 μM) and **19** (2 mM) were present in the reaction mixture. Control reactions contained only native dNTPs (200 μM). Following 25 PCR cycles, the reaction was phenol extracted and the full-length product purified by gel electrophoresis using DNA standards as markers. The presence of **6** was probed for by exposing the 5′-^{32}P-PCR products to ^{137}Cs (21 – 105 Gy) under anaerobic conditions. However, no ICL formation above that formed in the control that was produced only from native dNTPs was detected (data not shown). Finally, to further increase the statistical probability of incorporating **6**, linearized single-stranded M13mp7 plasmid (7,200 nt) was copied in the presence of native dNTPs (1 mM) and **19** (10 mM) using a 5′-^{32}P-primer and Sequenase as previously described.[41] However, γ-radiolysis up to 210 Gy also failed to produce any ICLs above background established by a control produced in the absence of **19** (data not shown).

Summary

Unlike the 5-halopyrimidines (BrdU and IdU) C-nucleotide aryl halides (**4**–**6**) produce interstrand cross-links when duplex DNA containing them is exposed to ionizing or UV-irradiation. γ-Radiolysis of DNA containing **6** in the presence of a hydroxyl radical quencher

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indicates that this species is not responsible for interstrand cross-linking. However, O_2 prevents cross-linking by γ-radiolysis but not UV-irradiation, suggesting that solvated electrons produced by the former react with the aryl halides to initiate product formation via σ-radicals. Selective cross-link formation under anaerobic conditions suggests that **4**–**6** could be useful as radiosensitizing agents in hypoxic cells, provided the nucleotide analogues could be incorporated enzymatically in DNA. The nucleotide triphosphate of **6** is incorporated preferentially opposite pyrimidines but the product formed is not extended further. These nucleotide analogues provide motivation for designing next generation molecules that could serve as radiosensitizing agents in cells. In addition, the utility of such nonnative nucleotide analogues in cells may be increased the evolution of DNA polymerases containing expanded substrate tolerance.[42,43]

Experimental

General Methods

Solvents used in reactions were purified by distillation before use. All reagents used in reactions were purchased from commercial sources and were used without further purification unless noted otherwise. All reactions were carried out under a positive pressure of argon atmosphere and monitored by TLC on Silica Gel G-25 UV_{254} (0.25 mm) unless stated otherwise. Spots were detected under UV light and/or by charring with a solution of ammonium molybdate or ceric ammonium sulfate in water and H_2SO_4. Column chromatography was performed on silica gel 60 (40–60 μm). The ratio between silica gel and crude product ranged from 100:1 to 20:1 (w/w).

Oligonucleotides were synthesized via standard automated DNA synthesis on an Applied Biosystems model 394 instrument. The coupling time for the phosphoramidites of modified nucleotides **1**5 min. The phosphoramidite for **4** and nucleoside analogue **4** were synthesized as previously described.[29] The UV spectrum of **4** was not reported previously (MeOH, λ_{max} = 246 nm, ε = 1700 $M^{-1}cm^{-1}$). Oligonucleotides were deprotected using 1:1 methylamine (40% in water) – concentrated NH_4OH at 65 °C for 75 min (oligonucleotides containing **4**–**6**), or concentrated NH_4OH at 25 °C for 9 h (oligonucleotides containing native nucleotides only). Oligonucleotides were purified by 20 % denaturing polyacrylamide gel electrophoresis (PAGE). All oligonucleotides containing modified nucleotides were characterized by MALDI-TOF MS. Oligonucleotides were 5′-^{32}P-labeled by polynucleotide T4 kinase (New England Biolabs) and γ-^{32}P-ATP (Perkin Elmer) using standard protocols.[44] Radiolabeled oligonucleotides were hybridized with 1.5 eq. of complementary oligonucleotides in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl at 90 °C for 5 min and cooled to room temperature. All anaerobic reactions were carried out in sealed Pyrex tubes, which were degassed and sealed using freeze-pump-thaw (three cycles, 3 min each) degassing techniques. Experiments involving radiolabeled oligonucleotides were analyzed following PAGE using a Storm 840 phosphorimager.

Synthesis of 5—A mixture of palladium acetate (60 mg, 0.27 mol) and triphenylarsine (159 mg, 0.52 mmol) in DMF (10 mL) was stirred under argon atmosphere at room temperature for 30 min. Then **8** (820 mg, 2.62 mmol), 1,4-anhydro-3,5-bis-*O*-(*t*-butyldimethylsilyl)-2-deoxy-*D*-erythro-pent-1-enitol (**4**, 810 mg, 2.35 mmol), and tributylamine (1.02 ml, 4.24 mmol) in DMF (10 mL) were added, and the resulting reaction mixture was stirred under argon at 70 °C for 15 h. The mixture was cooled to 0 °C, and 1 M tetrabutylammonium fluoride in THF (6 mL, 6 mmol) was added and stirred for 1.5 h. The reaction mixture was quenched with H_2O (30 mL) and extracted with EtOAc (50 mL × 2). The combined EtOAc layers were washed with saturated $NaHCO_3$ aq. (50 mL) and then dried over anhydrous $MgSO_4$. After filtration and evaporation to dryness under reduced

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pressure, the residue was purified by silica gel column chromatography (EtOAc–Hexanes, 1:2) to afford **9** (301 mg, 43%). Without further purification or characterization, **9** was dissolved in acetic acid (5 mL) and acetonitrile (5mL), the solution was cooled to 0 °C, and sodium triacetoxyborohydride (318 mg, 1.5 mmol) was added and stirred for 1 h. The reaction mixture was extracted with EtOAc (50 mL× 2) and saturated $NaHCO_3$ aq. (50 mL). The combined organic layers were dried over anhydrous $MgSO_4$. After filtration and evaporation, the residue was purified by silica gel column chromatography (CH_2Cl_2–CH_3OH, 20:1) to afford **5** (260 mg, 86%) as a white foam. ^{1}H-NMR (CD_3OD) δ 7.42-7.40 (m, 1H), 7.07-7.05 (m, 2H), 5.32 (dd, 1H, *J* = 10.0, 5.6 Hz), 4.27-4.24 (m, 1H), 3.92-3.89 (m, 1H), 3.80 (s, 3H), 3.66-3.63 (m, 2H), 2.33-2.27 (m, 1H), 1.76-1.69 (m, 1H); ^{13}C-NMR (CD_3OD) δ 158.3, 131.4, 128.4, 124.5, 122.2, 114.7, 88.6, 76.0, 74.3, 64.0, 56.2, 43.1. IR (NaCl plate) 3418, 3056, 2987, 1489, 1266, 1031 cm^{-1}. UV (MeOH) λ_{max} = 280 nm (ε = 2285 $M^{-1}cm^{-1}$). MALDI-TOF HRMS $C_{12}H_{15}O_4BrNa$ ($M + Na^+$) calcd. 325.0045, obsd. 325.0050.

Synthesis of 10a—Diol **5** (100 mg, 0.33 mmol) was azeotroped with pyridine (2 mL), after which a 2 mL solution of 4,4′-dimethoxytrityl chloride (168 mg, 0.50 mmol) in pyridine was added. The reaction mixture was stirred at room temperature for 6 h, at which time methanol (3 mL) was added to quench the reaction. The organic solution was removed in vacuo and the residue was purified by flash chromatography (EtOAc–Hexanes, 4:1 to 2:1) to afford compound the dimethoxytritylated C-nucleoside (133 mg, 67%) as a white foam. ^{1}H-NMR ($CDCl_3$) δ 7.52-7.49 (m, 2H), 7.42-7.25 (m, 8H), 7.10-7.07 (m, 1H), 7.00 (s, 1H), 6.89-6.85 (m, 4H), 5.39 (dd, 1H, *J* = 6.0, 9.6 Hz), 4.41-4.39 (m, 1H), 4.08-4.07 (m, 1H), 3.83 (s, 9H), 3.42 (dd, 1H, *J* = 4.8, 9.8 Hz), 3.30 (dd, 1H, *J* = 5.6, 9.8 Hz), 2.38-2.36 (m, 1H), 1.92-1.86 (m, 1H); ^{13}C-NMR ($CDCl_3$) δ 158.6, 156.8, 145.0, 136.2, 136.1, 130.24, 130.22, 130.19, 128.3, 128.0, 127.4, 126.9, 123.6, 121.3, 113.8, 113.3, 86.4, 85.7, 74.76, 74.72, 64.5, 55.7, 55.4, 42.1. IR (NaCl plate) 3055, 2938, 1509, 1463, 1285, 1033 cm^{-1}. MALDI-TOF HRMS $C_{33}H_{33}O_6BrNa$ ($M + Na^+$) calcd. 627.1353, obsd. 627.1357.

To a solution of dimethoxytritylated C-nucleoside (80 mg, 0.13 mmol) and diisopropylethylamine (46 μl, 0.26 mmol) in dichloromethane (3 mL) was added 2-cyanoethyl *N*,*N*-diisopropylphosphoramidic chloride (39 μL, 0.17 mmol) at 0 °C. After warming to room temperature and stirring for 3 h, the reaction mixture was diluted with dichloromethane (20 mL) and washed with saturated aq. $NaHCO_3$ (20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in vacuo. The crude product was purified by silica gel column chromatography (EtOAc–Hexanes, 2:1) to afford **10a** (83 mg, 78%) as a white foam. ^{1}H-NMR ($CDCl_3$) δ 7.51-7.21 (m, 10H), 7.06-7.04 (m, 1H), 6.98-6.96 (m, 1H), 6.84-6.79 (m, 4H), 5.40-5.30 (m, 1H), 4.52-4.41 (m, 1H), 4.20 (s, 1H), 3.82-3.51 (m, 12H), 3.39-3.20 (m, 2H), 2.68-2.40 (m, 3H), 1.89-1.75 (m, 1H), 1.30-1.05 (m, 13H); ^{31}P NMR ($CDCl_3$) δ 148.3, 147.8. MALDI-TOF HRMS $C_{42}H_{50}N_2O_7BrNaP$ ($M + Na^+$) calcd. 827.2431, obsd. 827.2444.

Synthesis of 6—Diol **5** (100 mg, 0.33 mmol) was dissolved in pentanol (1 mL). To this solution was added sodium iodide (989 mg, 6.6 mmol) and *trans*-*N*,*N*′-dimethyl-1,2-cyclohexanediamine (**11**, 50 mg, 0.35 mmol). The flask was evacuated and backfilled with argon for 3 times. The reaction mixture was stirred at 130 °C for 3 h. The resulting suspension was cooled to room temperature and diluted with Et_2O (30 mL). After filtration, the organic layer was washed with saturated aq. $NaHCO_3$ (20 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4. After filtration and evaporation, the residue was purified by flash chromatography (CH_2Cl_2–MeOH, 20:1) to afford **6** (75 mg, 65%) as a yellow foam. ^{1}H-NMR (CD_3OD) δ 7.27-7.22 (m, 3H), 5.34-5.30 (m, 1H), 4.26-4.25 (m, 1H), 3.91-3.89 (m, 1H), 3.79 (s, 3H), 3.65-3.63 (m, 2H), 2.32-2.27 (m, 1H), 1.76-1.68 (m,

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1H); ^{13}C-NMR (CD_3OD) δ 158.2, 132.2, 130.9, 128.7, 120.6, 93.2, 88.6, 76.1, 74.4, 64.1, 56.2, 43.2. IR (NaCl plate) 3600, 3054, 2987, 1488, 1264, 1081 cm^{-1}. UV (MeOH) λ_{max} = 260 nm (ε = 1650 $M^{-1}cm^{-1}$). MALDI-TOF HRMS $C_{12}H_{16}O_4I$ ($M + H^+$) calcd. 351.0088, obsd. 351.0093.

Synthesis of 10b—Diol **6** (70 mg, 0.20 mmol) was azeotroped with pyridine (2 mL), after which 2 mL of a solution of 4,4′-dimethoxytrityl chloride (102 mg, 0.30 mmol) in pyridine was added. The reaction mixture was stirred at room temperature for 16 h and then quenched with methanol (3 mL). The organic solution was removed in vacuo and the residue was purified by flash chromatography (EtOAc–Hexanes, 5:1 to 2:1) to afford the dimethoxytritylated C-nucleotide (79 mg, 61%) as a white foam. ^{1}H-NMR ($CDCl_3$) δ 7.50-7.47 (m, 2H), 7.38-7.21 (m, 9H), 7.15 (s, 1H), 6.86-6.83 (m, 4H), 5.39-5.36 (m, 1H), 4.39-4.38 (m, 1H), 4.07-4.06 (m, 1H), 3.80 (s, 9H), 3.41-3.37 (m, 1H), 3.30-3.26 (m, 1H), 2.40-2.35 (m, 1H), 1.89-1.82 (m, 1H); ^{13}C-NMR ($CDCl_3$) δ 158.6, 156.7, 144.9, 136.1, 131.0, 130.2, 129.8, 128.3, 127.9, 127.6, 126.9, 119.4, 113.2, 92.5, 86.3, 85.7, 74.8, 74.6, 64.5, 55.6, 55.3, 42.1. IR (NaCl plate) 3054, 2989, 1588, 1422, 1264, 1178 cm^{-1}. MALDI-TOF HRMS $C_{33}H_{33}O_6INa$ ($M + Na^+$) calcd. 675.1214, obsd. 675.1214.

To a solution of the dimethoxytritylated C-nucleoside (69 mg, 0.11 mmol) and diisopropylethylamine (37 μL, 0.22 mmol) in dichloromethane (3 mL) was added 2-cyanoethyl *N*,*N*-diisopropylphosphoramidic chloride (31 μL, 0.14 mmol) at 0 °C. After stirring for 3 h at room temperature, the reaction mixture was diluted with dichloromethane (20 mL) and washed with saturated aq. $NaHCO_3$ (20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in vacuo. The crude product was purified by silica gel column chromatography (EtOAc–Hexanes, 2:1) to afford **10b** (63 mg, 70%) as a white foam. ^{1}H-NMR ($CDCl_3$) δ 7.51-7.14 (m, 12H), 6.84-6.80 (m, 4H), 5.36-5.34 (m, 1H), 4.50-4.46 (m, 1H), 4.20 (s, 1H), 3.84-3.54 (m, 12H), 3.30-3.23 (m, 2H), 2.65-2.40 (m, 3H), 1.84-1.79 (m, 1H), 1.28-1.05 (m, 13H); ^{31}P NMR 148.2, 147.7. MALDI-TOF HRMS $C_{42}H_{50}N_2O_7INaP$ ($M + Na^+$) ($CDCl_3$) δ calcd. 875.2293, obsd. 875.2294.

Synthesis 19—To a solution of **6** (53 mg, 0.15 mmol) and 1,8-bis-(dimethylamino)naphthalene (Proton Sponge®, 48 mg, 0.22 mmol) in trimethyl phosphate (2 mL) at 0 °C, was added phosphorous oxytrichloride (17 μL, 0.18 mmol). After the mixture was stirred at 0 °C for 3 h, a solution of tributylammonium pyrophosphate (178 mg, 0.32 mmol) in anhydrous DMF (1 mL) and tributylamine (220 μL, 0.92 mmol) was added dropwise. The reaction was stirred at room temperature for 10 min, followed by quenching with 1 M triethylammonium bicarbonate buffer (30 mL, pH 8.5). The quenched reaction was stirred for an additional 10 min. Lyophilization gave the crude product. The crude product was subjected to ion-exchange column (DEAE) and eluted using a 0 to 1 M TEAB gradient. Fractions was monitored by UV and checked by ESI-mass. Fractions was collected, lyophilized and purified by reverse-phase (C18) HPLC (0–50% CH_3CN in 0.1 M TEAB, pH 7.5) followed by lyophilization to afford the triphosphate as its triethylammonium salt (yield 3.5%) as a fluffy, white solid. The concentration of the triphosphate is determined by using the extinction coefficient at 260 nm (1650 $M^{-1}cm^{-1}$) for the nucleoside. ^{1}H NMR (D_2O) δ 7.44-7.35 (m, 2H), 7.28-7.25 (m, 1H), 5.41 (br s, 1H), 4.53 (br s, 1H), 4.23-4.08 (m, 3H), 3.75 (s, 3H), 2.30-2.23 (m, 1H), 2.06-1.93 (m, 1H); ^{31}P NMR (D_2O) δ −6.37 (br s), −11.11 (br s), −22.53 (br s); MS (ESI) m/z: 588.9 [M+3H], calc'd m/z: 588.9.

Kinetic study of incorporation of 19 by Deep Vent (exo$^-$) DNA polymerase

The primer-template duplex was obtained by hybridizing the 5′-^{32}P radiolabeled primer (1 μM) and the cold template (1.5 μM) in 20 mM Tris-HCl pH 8.8, 10 mM ammonium sulfate, 10 mM KCl, 2 mM $MgCl_2$, and 0.1% Triton X-100. The DNA was denatured at 90°C (5

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min) and slowly cooled to room temperature. A DNA duplex-enzyme cocktail (2×) stock solution (150 μL) was prepared by mixing Deep Vent (exo$^-$) DNA polymerase solution (10 μL, 2 nM) with the primer-template solution (30 μL, 200 nM), 100× BSA (3 μL), 10× thermopol buffer (30 μL), 1 mM DTT (3 μL) and water (74 μL). The extension reactions were carried out by adding 5 μL of the cocktail to the appropriate 2 × dNTP solutions (5 μL, 50–175 nM for dGTP, 40–90 μM for **19**), which were freshly prepared. After 6 min (**19** or dGTP) at 37 °C, the reactions were quenched with 95% formamide loading buffer (5 μL) containing 10 mM EDTA. The mixtures were heated at 90 °C for 2 min and cooled immediately in an ice-bath. Aliquots of the mixtures were subjected to 20% denaturing PAGE. Kinetic parameters were obtained by nonlinear regression analysis of velocity versus [dNTP]. The dNTP concentrations used were as follows: 50, 75, 100, 125, 150, 175 nM for dGTP; 40, 50, 60, 70, 80, 9 μM for **19**. Reaction conditions were chosen such that the maximum amount of extension was < 30%.

Full length Extension Reactions

A DNA primer-template enzyme solution (50 μL) was prepared by mixing 2 μL enzyme (500 nM) with the DNA solution (2 μL, 1 μM), 100 × BSA (5 μL), 1 mM DTT (1 μL), 10 × thermopol buffer (10 μL) and water (34 μL). The extension reactions were initiated by adding 10 μL of a premixed dNTP solution (200 μM dATP, 200 μM dCTP, 200 μM dTTP, 200 μM dTTP, or 200 μM **19**) to 10 μL DNA primer-template enzyme solution. Aliquots (8 μL) were taken and quenched with 95% formamide loading buffer (8 μL) containing 10 mM EDTA at 5, 15, 30, and 60 min. The mixture was heated to 90 °C for 2 min and chilled on ice. An aliquot of the mixture was loaded on to a 20% denaturing polyacrylamide gel.

Photoreactions

Photoreactions of the duplexes were carried out in Pyrex tubes in a Rayonet photoreactor fitted with 16 lamps having a maximum output at 300 nm. All photoreactions were carried out for 30 min in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl. After reaction, each sample (20 nM, 40 μL) was aliquoted into a 0.6-mL eppendorf tube and mixed with formamide loading buffer and subjected to 20% denaturing PAGE analysis. ICL yields were determined using the phosphor image by dividing the volume of the cross-link product by the summation of all of the DNA in the lane (cross-link product, intact DNA, cleaved DNA) and multiplying by 100.

γ-Radiolysis

γ-Radiolysis of the duplexes were carried out in Pyrex tubes in a J. L. Shepherd Mark I ^{137}Cs irradiator that has an output of 23 Gray/min. After reaction (315 Gy), each sample (20 nM, 40 μL) was aliquoted into a 0.6-mL Eppendorf tube and mixed with formamide loading buffer and subjected to 20% denaturing PAGE analysis. ICL yields were determined as described above.

Fe(II)-EDTA digestion of cross-linked DNA

Fe(II)-EDTA cleavage reactions of ICLs were carried out in 50 μM $(NH_4)_2Fe(SO_4)_2$, 100 μM EDTA, 1 mM sodium ascorbate, 5.0 mM H_2O_2, 100 mM NaCl and 10 mM potassium phosphate (pH 7.2), for 1 min at 25 °C (total volume of 20 μL each). The reactions were quenched with 100 mM thiourea (10 μL). Samples were lyophilized, resuspended in formamide loading buffer and subjected to 20% PAGE analysis.

Preparation of a 287 nt PCR fragment

A 287 nt PCR fragment was prepared from M13mp7 plasmid (10 fmol), which was amplified with primer 1 and primer 2 (250 pmol each), dNTP (0.5 mM each), Taq DNA

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polymerase (5 Units) in 100 μL of Taq DNA polymerase buffer (20 mM Tris, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8). PCR was performed using the following conditions: 94 °C, 30 sec for melting and 58 °C, 1 min for annealing and then 72 °C, 1 min for polymerase reaction. After repeating the cycle 60 times, the reaction solution was extracted by phenol and further purified by Microcon (MY-30) using a standard protocol. The concentration of the PCR fragment was determined by UV (ε_{260} = 20 $g^{-}{}_{1}$•cm^{-1}•L) and the quality of PCR fragment was determined by agrose gel (3%). Sequences of three primers and PCR fragment: 5′-CAC TGA ATC ATGGTC ATA GCT GTT-3′ (primer 1), and 5′-GGT GAA GGG CAA TCA GCT GTT-3′ (primer 2) used for primers. The sequence of the PCR fragment is 5′-GGT GAA GGG CAA TCA GCT GTT GCC CGT CTC ACT GGT GAA AAG AAA AAC CAC CCT GGC GCC CAA TAC GCA AAC CGC CTC TCC CCG CGC GTT GGC CGA TTC ATT AAT GCA GCT GGC ACG ACA GGT TTC CCG ACT GGA AAG CGG GCA GTG AGC GCA ACG CAA TTA ATG TGA GTT AGC TCA CTC ATT AGG CAC CCC AGG CTT TAC ACT TTA TGC TTC CGG CTC GTA TGT TGT GTG GAA TTG TGA GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT ATG ACC ATG ATT CAG TG-3′.

PCR experiments

PCR was performed in an overall volume of 50 μL containing 5 pM of the 287 nt template in thermopol buffer (20 mM Tris-HCl pH 8.8, 10 mM ammonium sulfate, 10 mM KCl, 2 mM $MgCl_2$, and 0.1% Triton X-100). The final mixtures contained dNTPs (200 μM of each dATP, dGTP, dCTP, and dTTP) in the presence or absence of **19** (2 mM), primers (25 pmol of each primer), 30 nM of Deep Vent (exo^{-}) DNA polymerase. PCR amplifications were performed employing the following program: initial denaturation at 95 °C for 2.5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 45 °C for 1 min and extension at 65°C for 5 min. The PCR solution was filtered with a Microcon YM-30 filter (Millipore) to remove the excess unincorporated dNTPs. The quality of PCR product was determined by 8% native PAGE analysis.

Preparation of the M13mp7 plasmid

GW5100 cells were grown overnight in 10 mL LB at 37 °C. The stock solution (2 mL) was diluted to 1 L in 2X-YT media and incubated at 37 °C for 2 h. To this solution was added 10 mL of M13mp7 and incubated for further 9 h. After that, the cells were store in ice for 10 min and centrifuged at 9500 rpm for 15 min at 4°C. The supernatant was decanted and precipitated at 4 °C overnight in NaCl (500 mM) and PEG (4%) buffer. The solution was pelleted at 9500 rpm for 15 min. The resulting pellet was resuspended in 10 mL TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0). The plasmid was purified by extraction with 3 ml phenol:isoamyl alcohol:chloroform for three times until the aqueous layer was clear. The aqueous layer was subjected to hydroxyapatite column (2 g, 18 ×1.2 cm) and eluted with 10 mL potassium phosphate (79 mM, pH 7). The sample was concentrated using a YM 100 Centricon filter (2 mL) and spun for 10 min at 1600 × g. Note: longer centrifuge time and use of the wrong size of Centricon leads to loss of most of the products. The concentration of the plasmid was determined by UV absorbance (ε_{260} = 7.152 ×10^7 L/mol*cm). One liter of GW5100 cell growth produced 2.7 nmol plasmid. The quality of plasmid was confirmed by 1% agarose gel. Plasmid was linearized by an *EcoR* I restriction cut. Restriction digestion of M13mp7 (100 pmol) with *EcoR* I (100 units) was carried out in 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol at 37 °C for 4 h. After incubation, the solution was heated to 90 °C for 5 min and immediately cooled in ice water to inactivate the enzyme. The linearized plasmid was stored at −20 °C until use. Complete digestion of M13mp7 was confirmed by comparison of the mobility in 1 % agarose gel electrophoresis between native plasmid and digested linear plasmid. The gel was stained with Syber-green. *EcoR* I cut plasmid migrated farther down the gel than native plasmid.

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Polymerization of linearized plasmid by Sequenase

Linearized plasmid (30 pmol) was hybridized with 5′-^{32}P labeled primer (10 pmol, 5′-d(CAC TGA ATC ATG GTC ATA GCT GTT)) in 40 mM Tris·HCl (pH 7.5), 20 mM $MgCl_2$, and 50 mM NaCl at 90 °C for 5 min, followed by slow cooling to room temperature. Extension was carried out using Sequenase (26 units, Version 2.0 DNA polymerase) in the presence of **19** (10 mM) and native dNTPs (1 mM) at 37 °C for 30 min. After reaction, plasmid duplex was purified by Microcon filter (YM = 3) to remove excess reagents. Complete polymerization of linearized M13mp7 was confirmed by comparison of the mobility in 1 % alkaline agarose gel electrophoresis between enzymatic reaction product and 5′-^{32}P-labeled linear plasmid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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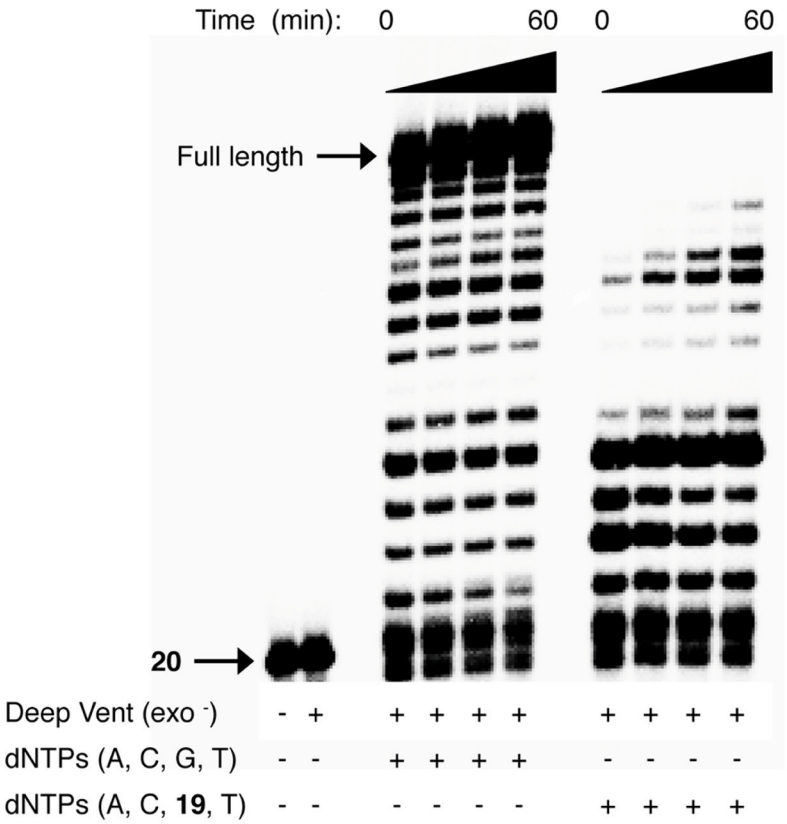
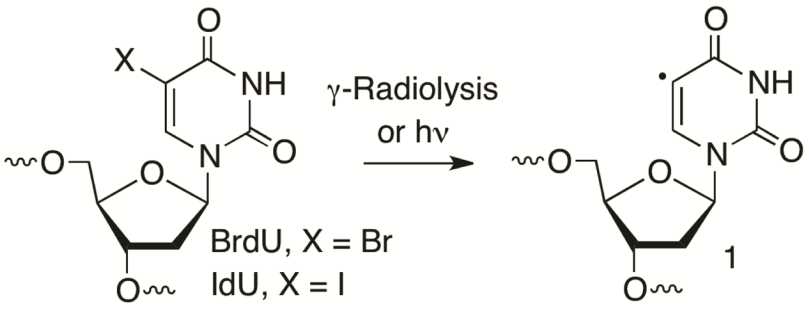


Figure 1.
Full-length extension of **20** in the absence or presence of **19**.



Scheme 1.

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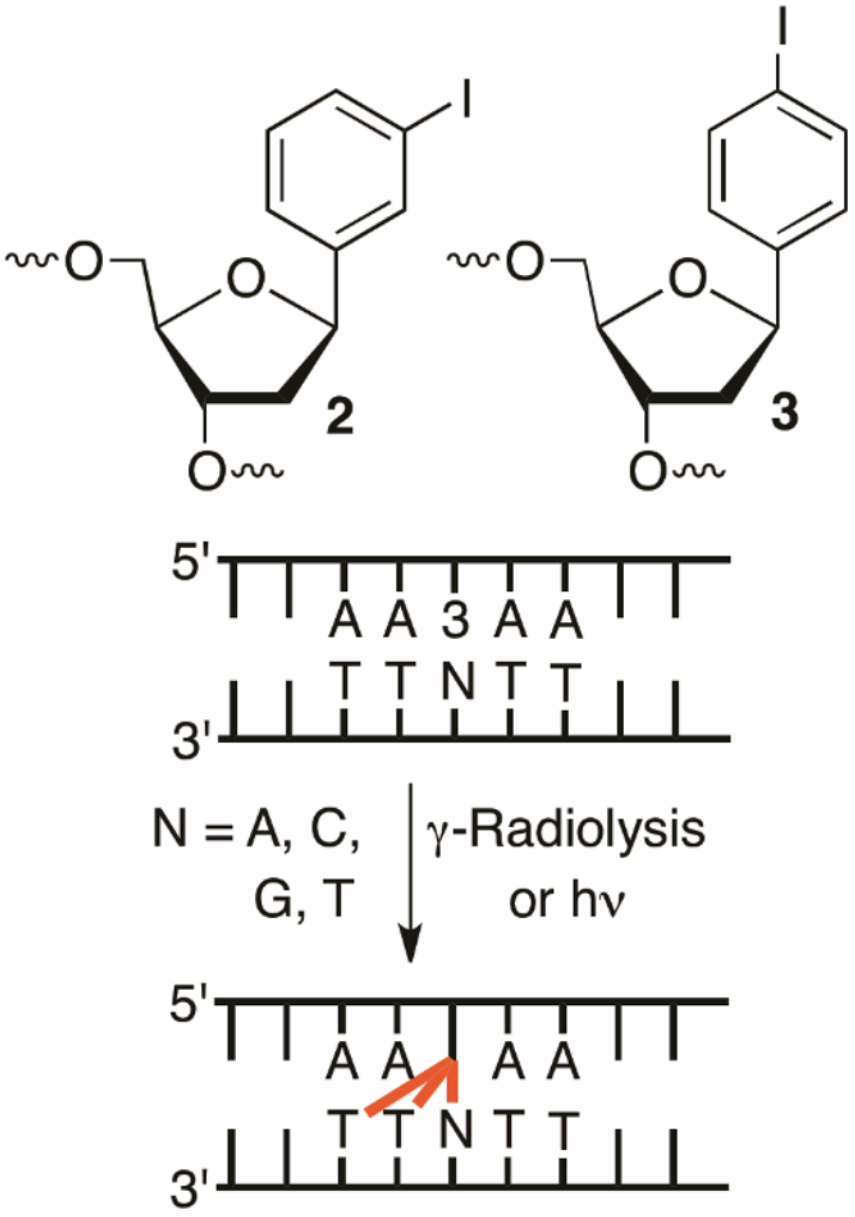

Scheme 2.

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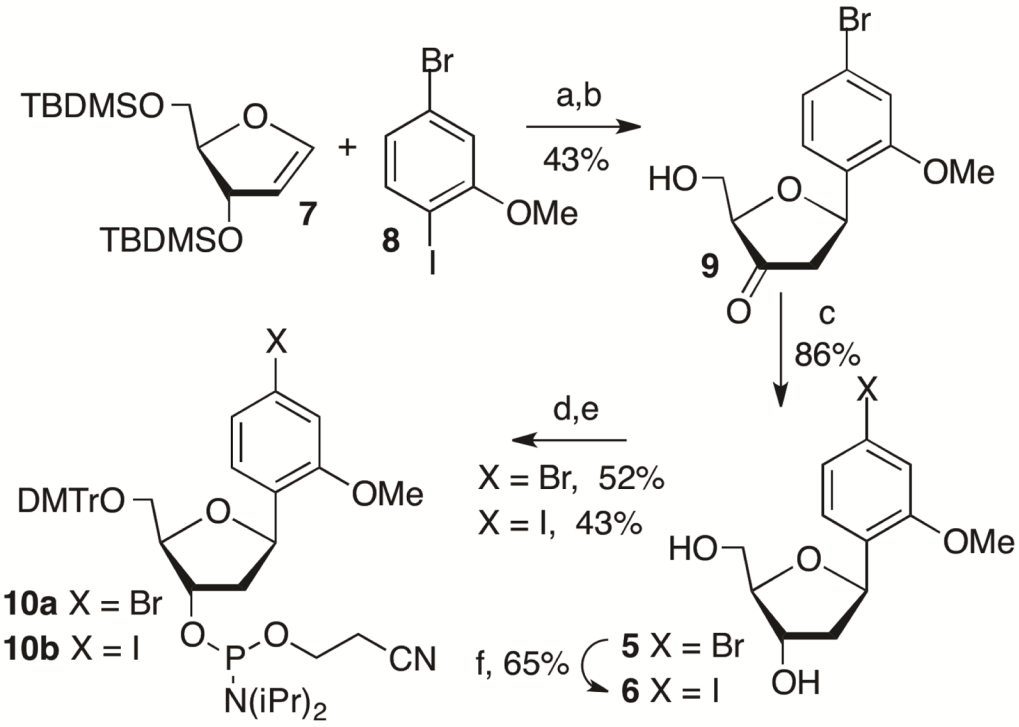

[a]Key: a. Ph_3As, $Pd(OAc)_2$, DMF b. Bu_4NF, THF c. $NaB(OAc)_3H$, AcOH, CH_3CN d. DMTrCl, pyridine e. 2-Cyanoethyl-*N,N*'-diisopropyl phosphoramidic chloride, diisopropylethylamine CH_2Cl_2 f. **11**, CuI, NaI, pentan-1-ol.

Scheme 3[a]

Key: a.Ph_3 As, $Pd(OAc)_2$, DMF b. Bu_4NF, THF c. $NaB(OAc)_3H$, AcOH, CH_3CN d. DMTrCl, pyridine e. 2-Cyanoethyl-*N,N*prime;-diisopropyl phosphoramidic chloride, diisopropylethylamine CH_2Cl_2 f. **11**, Cul, Nal, pentan-1-ol.

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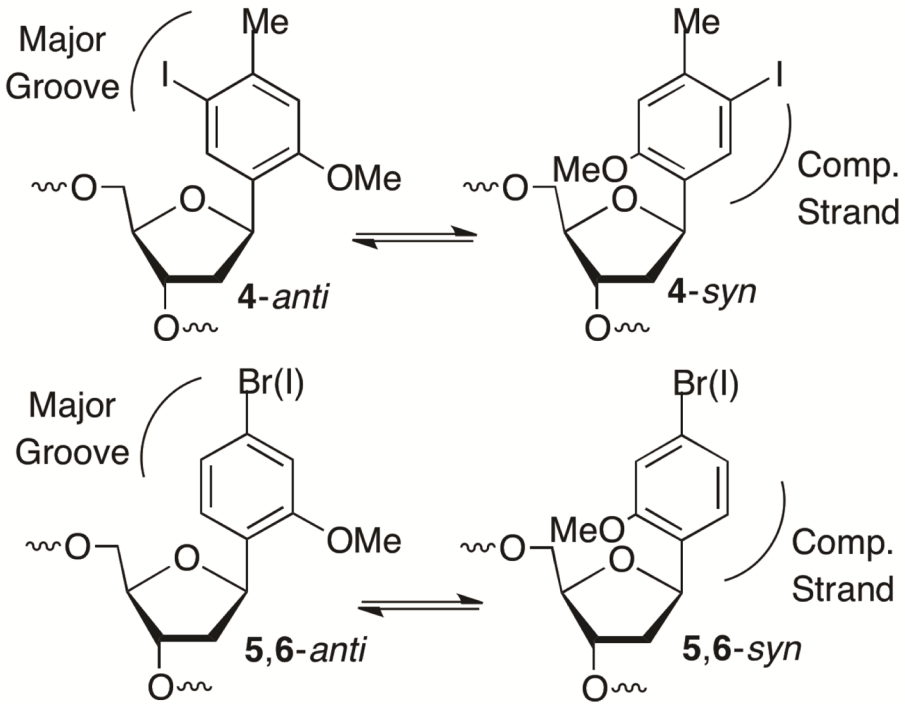

Scheme 4.

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Table 1

Interstrand cross-link yields following UV-irradiation of 5′-^{32}P-**16a–d** - 5′-^{32}P-**18a–d**.[a]

5′-^{32}P-d(CGA GTA CTG C A A X AA CGT GTA CAG C) 3′-d(GCT CAT GAC GT$_{15}$T$_{14}$ Y$_{13}$TT GCA CAT GTC G) ICL Yield (%)				
X	**Y = A**	**Y = C**	**Y = G**	**Y = T**
4	7.7 ± 2.3	11.0 ± 1.6	10.4 ± 1.5	12.9 ± 2.6
5	17.7 ± 3.1	27.2 ± 4.1	65.1 ± 12.4	25.0 ± 6.1
6	24.5 ± 4.5	34.8 ± 3.4	24.4 ± 1.2	27.4 ± 2.2

[a]Yields are the average ± std. dev. of 3 samples.

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Table 2

Interstrand cross-link yields following ^{137}Cs-irradiation of 5′-^{32}P-**16a**–**d** - 5′-^{32}P-**18a**–**d**.[a]

5′-^{32}P-d(CGA GTA CTG C A A X AA CGT GTA CAG C) 3′-d(GCT CAT GAC GT$_{15}$T$_{14}$ Y$_{13}$TT GCA CAT GTC G) ICL Yield (%)				
X	**Y = A**	**Y = C**	**Y = G**	**Y = T**
4	15.1 ± 1.0	13.3 ± 0.5	11.1 ± 0.8	13.2 ± 0.8
5	9.6 ± 1.2	9.3 ± 0.5	6.5 ± 0.6	9.6 ± 1.3
6	15.2 ± 2.0	17.5 ± 1.0	9.4 ± 1.5	13.0 ± 1.1

[a]Yields are the average ± std. dev. of 3 samples.

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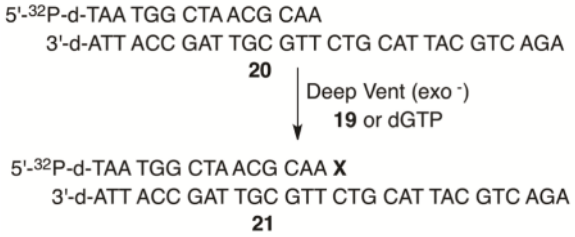
Table 3

Steady-state incorporation kinetics of nucleotides opposite dC in **20**.[a]



X	dNTP	V_{max} (%/min)	$K_{m(app)}$ (nM)	V_{max}/K_m (%/(min·nM))
G	dGTP	12.4 ± 0.5	94.9 ± 8.0	130.5 ± 12.2
6	**19**	6.9 ± 0.3	(63.0 ± 64) × 10^3	0.1 ± 0.01

[a] Kinetic constants are the average ± std. dev. of 3 experiments, each containing 3 replicates.