New Light-Sensitive Nucleosides for Caged DNA Strand Breaks

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Phototriggered bond cleavage has found wide application in chemistry as well as in biology. Nevertheless, there are only a few methods available for site-specific photochemical induction of DNA strand scission despite numerous potential applications. In this study we report the development of new photocleavable nucleotides based on the photochemistry of o*-nitrobenzyl esters. The light-sensitive moieties were generated through introduction of* o*-nitrophenyl groups at the 5′C position of the nucleoside sugar backbone. The newly synthesized, modified nucleosides were incorporated in oligonucleotides and are able to build stable DNA duplexes. In such a way modified oligonucleotides can be cleaved site-specifically upon irradiation with* >360 *nm light with high efficiency. Furthermore, we show that these modifications can be bypassed in DNA synthesis promoted by* Thermus aquaticus *DNA polymerase.*

KEYWORDS:

DNA cleavage · DNA damage · nucleosides · oligonucleotides · photochemistry

Introduction

Phototriggered bond cleavage has found wide application in protection-group and solid-phase chemistry as well as in biological studies in which caged, biologically active substances are released upon irradiation.[1] Nevertheless, there are only a few methods available for site-specific photochemical induction of DNA strand scission, despite numerous potential applications, for example, in studies of DNA topology and the dynamics of nucleic acid structure, DNA strand break/repair processes, or the activation and release of formally caged nucleic acid derived drugs such as ribozymes and antisense oligonucleotides.

Recently, we demonstrated that DNA containing 4′*C*-acylated nucleotides can be cleaved site-specifically upon irradiation with 320 nm light.[2] Unfortunately, application of longer wavelengths which would be more appropriate for biological applications failed to initiate efficient DNA strand cleavage. Another strategy to introduce strand breaks is based on the photochemistry of *o*-nitrobenzyl ethers and esters. Taylor and co-workers developed 1,2- and 1,3-diol-derived linkages to combine two oligonucleotide strands.[3] The linkage can be cleaved with longer wavelengths (>366 nm) due to the presence of *o*-nitrobenzyl ether and/or ester moieties. However, a non-nucleosidic linkage per se is unable to participate in selective enzyme or nucleic acid promoted transformation and recognition processes, which include the formation of distinct hydrogen-bonding patterns with the nucleobases or the sugar phosphate backbone of the DNA substrates. Thus, we aimed to develop new photocleavable DNA building blocks which combine a nucleic acid derived structure with the advantages of *o*-nitrobenzyl phosphate ester photochemistry. Herein we report for the first time the synthesis and chemical incorporation into DNA of such new, highly efficient, photocleavable building blocks. Furthermore, we show that the newly synthesized nucleotides are able to build stable DNA duplexes and participate in sequence-selective enzyme recognition processes.

Results and Discussion

Synthesis

Introduction of a 5′*C*-*o*-nitrophenyl substituent into the sugar backbone of DNA should generate light-sensitive DNA **1** due to the formation of an *o*-nitrobenzyl phosphate ester moiety in the DNA molecule (Scheme 1). Upon irradiation with 360 – 400 nm light **1** is expected to cleave site-specifically into phosphates **2** and **4** as outlined in Scheme 1.

First we envisaged the development of a synthetic pathway to form the 5′*C*-modified building blocks (Scheme 2). Treatment of aldehyde **6**[4] with *o*-nitrophenyl lithium (generated through

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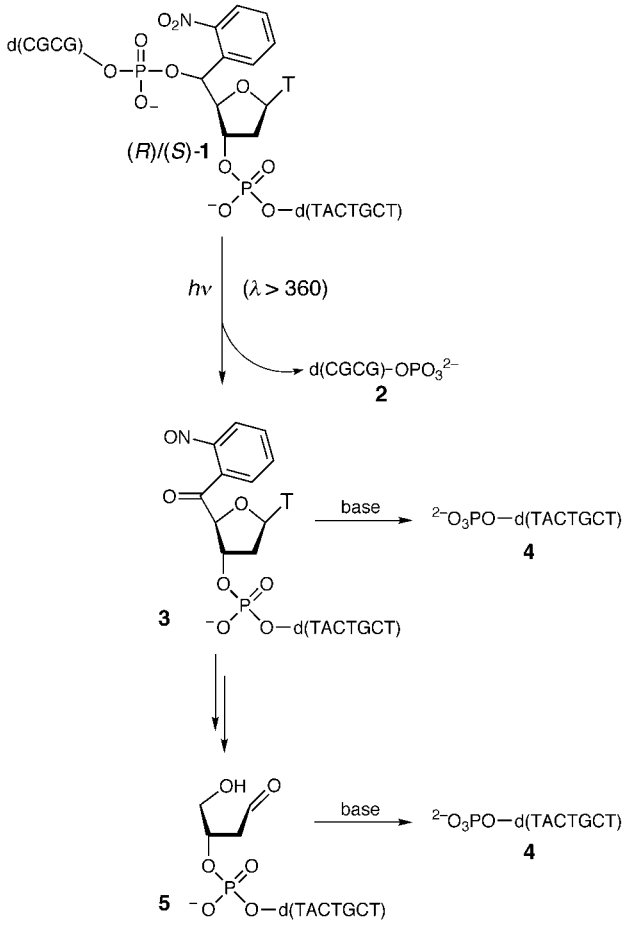
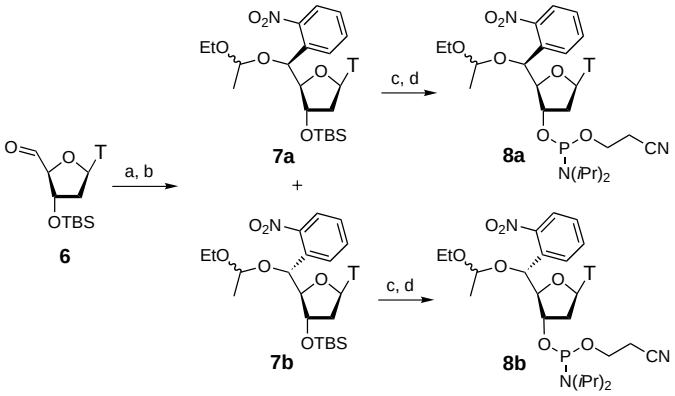


Scheme 1. *Proposed cleavage pathway of 5′C-o-nitrophenylated DNA. d = deoxy, A = adenosine, C = cytidine, G = guanosine, T = thymidine.*



Scheme 2. *Synthesis of the photocleavable building blocks. a) Bromo-2-nitrobenzene, PhLi, THF, −105 °C, 5 h, 66%; b) ethylvinyl ether, PPTS, CH_2Cl_2, 18 h, **7a**: 72%, **7b**: 16%; c) nBu_4NF, THF, 2 h; d) 2-cyanoethyl-N,N-diisopropylphosphorochloroamidite, (i$Pr)_2EtN$, CH_2Cl_2, 2 h, **8a**: 77%, **8b**: 71% (two steps). TBS = tert-butyldimethyl silyl, THF = tetrahydrofuran, PPTS = pyridinium p-toluylsulfonate.*

metal–halide exchange) and subsequent transformation of the diastereomeric alcohols into the corresponding acetals yielded **7a** and **7b** in a ratio of 4.5:1 (5′*S*:5′*R*).[5, 6] The diastereomers **7a** and **7b** were separated easily by flash column chromatography, converted into **8a** and **8b**, and incorporated into oligonucleotides by standard automated DNA synthesis except that, for the modified nucleotides, the 5′-deprotection and coupling times were extended to 2 and 15 minutes, respectively. The integrity of modified oligonucleotides was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis.

Hybridization properties

Next, we investigated the capability of the 5′*C*-modified oligonucleotides to form stable duplexes. To assess the influence of the modifications on duplex stability, DNA melting curves of 12-mer oligonucleotide duplexes were measured. The duplexes were composed of oligonucleotides **1** (sequence shown in Scheme 1), which contained site-specifically one 5′*C-o*-nitrophenylated thymidine moiety, and the unmodified complementary strand **9**. We conducted thermal denaturating studies (UV-melting curves) and found that the modifications have only small destabilizing effects on the DNA double helix (Table 1).

Table 1. *T_m values of DNA duplexes composed of **9** with **1**, (R)-**1**, or (S)-**1**, respectively.*

DNA strand	T_m [°C]	ΔT_m [°C]
1	50.5	
(*R*)-**1**	46.4	−4.1
(*S*)-**1**	44.7	−5.8

In order to evaluate the influence of the 5′*C-o*-nitrophenyl substitutions on the conformation of the DNA duplexes, we next performed CD measurements. The data recorded are presented in Figure 1 and exhibit nearly congruent spectra for both modified and unmodified duplexes. Thus, the 5′*C*-modifications appear to have only a minor influence on the overall duplex structure. Nevertheless, local perturbation of the double helix caused by the modification can not be excluded. As determined

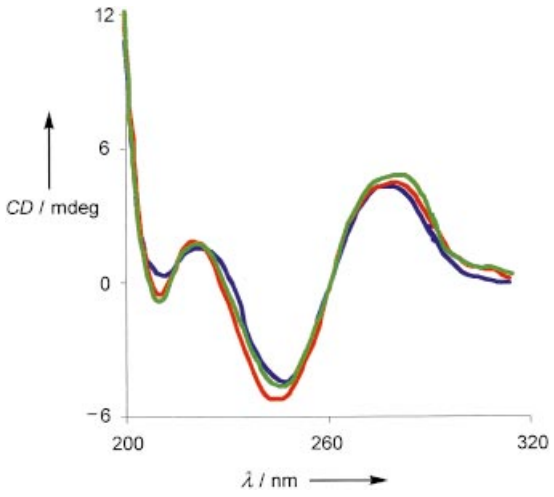


Figure 1. *CD spectra of DNA duplexes. Blue: unmodified DNA duplex; red: (5′R)-C-o-nitrophenylated oligonucleotide (R)-**1** and complementary strand **9**; green: (5′S)-C-o-nitrophenylated oligonucleotide (S)-**1** and complementary strand **9**.*

from the results depicted in Figure 1, both 5′*C*-modified DNA duplexes and their unmodified counterparts adopt the B-form DNA conformation.[7]

Photocleavage of 5′*C*-*o*-nitrophenylated oligonuclotides

To study the photocleavage properties of the modified oligonucleotides we focused first on single-stranded DNA. We investigated the photochemical properties of (*S*)-**1** which contained site-specifically one (*S*)-5′*C*-*o*-nitrophenyl substituent (Scheme 1). Irradiation of (*S*)-**1** with >360 nm light at neutral pH values and subsequent MS analysis revealed highly efficient and site-specific cleavage of the photolabile oligonucleotide after short irradiation times of only five minutes. (Figure 2 A).

It is noteworthy that no cleavage products could be detected when DNA modified with the previously described 4′*C*-acylated building blocks was irradiated with >360 nm light.[2] MALDI-TOF MS analysis gave preliminary insights into the cleavage pathway. Under neutral conditions the expected formation of 3′-phosphate **2** and 5′-phosphate **4** was accomplished together with the formation of two further species (Figure 2 A). The presumed reaction intermediate **3** could not be identified directly by MALDI-TOF MS, but we recorded a signal corresponding to the mass of **3** minus 46 mass units, which might be derived from **3** by liberation of NO_2. However, we were able to reduce **3** by addition of NaSH after irradiation and we identified the corresponding stable aniline derivative by MALDI-TOF MS. The mass of the other reaction product is in accord with that of **5**, which can be deduced from **3** through a reaction sequence involving photocleavage of the ketone and deglycosidation (Scheme 1). Both reaction intermediates should be base-labile, and indeed treatment of the reaction mixture with ammonia after photolysis led to the formation of **4** (Figure 2 B). Moreover, when photolysis of (*S*)-**1** was performed at pH 13 the formation of **2** and **4** was observed exclusively.

Next, we envisaged elucidating whether the new method is suitable to generate gapped DNA single-strand breaks through irradiation of site-specifically modified DNA duplexes. In order to assess the cleavage efficiency of the new building blocks we analyzed and quantified the reaction products with reverse-phase high-pressure liquid chromatography (RP-HPLC). Figure 3 shows an exemplary RP-HPLC trace of a DNA duplex composed of (*S*)-**1** and **9**. Irradiation for 5 minutes at >360 nm under aerobic conditions led to the consumption of (*S*)-**1** and formation of two products which were isolated and identified as **2** and **4** by MALDI-TOF MS. The data obtained in the quantitative investigations are summarized in Table 2; they show

Table 2. *Quantification of phototriggered cleavage of **1** with >360 nm light.*

DNA[a]	Irradiation conditions[b]	Yield of **2** [%]	Yield of **4** [%]
ss (*S*)-**1**	A	99	48
ss (*S*)-**1**	B	94	75
ds (*S*)-**1**	A	98	45
ds (*S*)-**1**	B	97	78
ss (*R*)-**1**	A	97	47
ss (*R*)-**1**	B	96	73
ds (*R*)-**1**	A	95	44
ds (*R*)-**1**	B	95	75

[a] ss = single-stranded **1**; ds = double-stranded complex containing **1** and the complementary strand **9**. [b] A: irradiation in 100 mM NaCl, 20 mM KH_2PO_4 (pH 7) at 20 °C; B: irradiation in 200 mM NaOH (pH 13) at 20 °C.

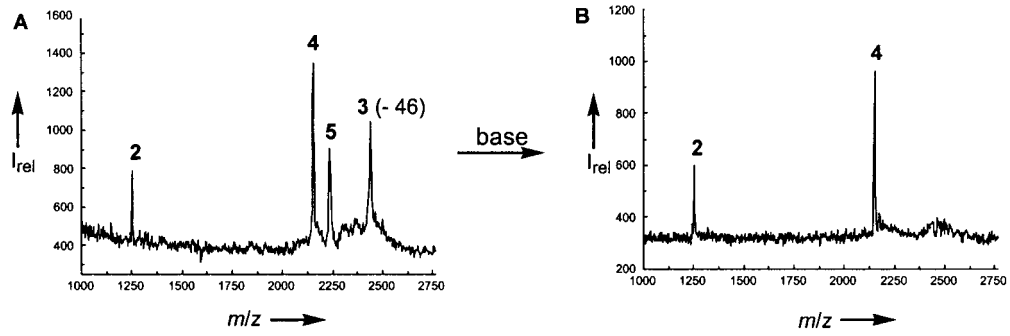


Figure 2. *MALDI-TOF MS analysis of the phototriggered cleavage of **1**. A) Spectra of a sample recorded after irradiation with >360 nm light for 5 min at pH 7. B) Same experiment as (A) but with prior treatment with ammonia for 4 hours. I_{rel} = relative intensity. **3**(− 46) = m/z of **3** minus 46 mass units.*

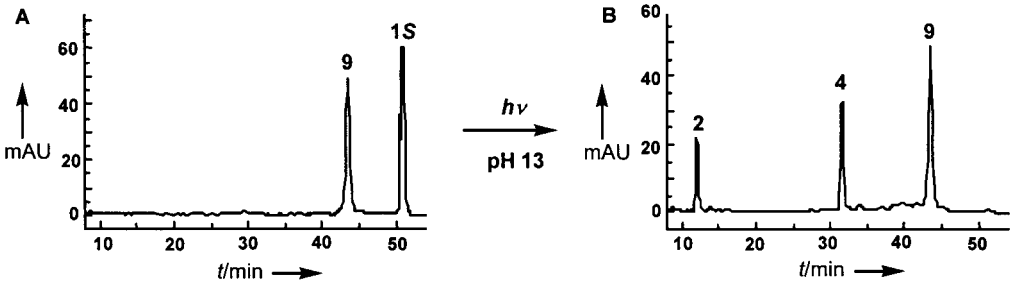


Figure 3. *RP-HPLC traces of a DNA duplex composed of **1** and the complementary strand **9** before (A) and after (B) photolysis at pH 13. AU = absorption units.*

that (*S*)-**1** is cleaved highly efficiently and **2** is formed in excellent yields both in single- and double-stranded DNA under the various conditions applied. The origin of the somewhat lower yields of **4** remains to be elucidated. Inefficient reactions following the strand scission might be a putative cause of this observation.

The stereochemistry at the 5′*C* position does not significantly influence the outcome of the photolysis (Table 2). Thus, a mixture of 5′*C* epimers could be used to achieve the same results as a single stereoisomer.

Enzymatic DNA synthesis past a 5′*C*-*o*-nitrophenyl moiety

In contrast to other non-nucleosidic photosensitive building blocks[3] the newly modified thymidines retain the nucleobase moiety and, thus, should be able to participate in enzymatic recognition processes. To prove this we investigated the ability of *Thermus aquaticus* (*Taq*) DNA polymerase to promote DNA synthesis past a template site containing the *o*-nitrophenyl modification. We designed a primer extension assay in such way that a site-specifically modified 33-mer template calls for nucleotide incorporation opposite the modified thymidine after elongating a 20-mer primer by four nucleotides (Figure 4).

20 nt primer
5′-^{32}P-GTG GTG CGA ATT CTG TGG AT
3′-CAC CAC GCT TAA GAC ACC TAG AGC T*GC ACG TAT
33 nt template

Figure 4. *DNA primer/template constructs used in primer extension studies. T* denotes thymidine, (5′S)-, or (5′R)-C-o-nitrophenylated thymidine.*

Our results are presented in Figure 5 and reveal that both the 5′*R* and 5′*S* modifications are bypassed by *Taq* DNA polymerase.[8] We observed predominately 34 nucleotide (nt) product formation since *Taq* DNA polymerase adds an additional nucleotide at the primer 3′ end, as reported previously.[9] Incorporation of a nucleotide opposite the modified nucleotide in the template strand appears not to be significantly hampered since only a minor accumulation of 24 nt DNA products is observed (Figure 5).

However, in the further elongation pathway significant pause sites are apparent beyond the modified thymidine in the template strand, which indicates unfavourable interactions of the modification with the DNA polymerase in the template–primer duplex. These observations are similar to previously published results where bypass syntheses with styrene oxide modified templates catalyzed by HIV-1 reverse transcriptase showed significant pause sites distal to the modifications.[10] Interestingly, DNA synthesis pausing is more pronounced in the case of the 5′*S* than the 5′*R* modification. X-ray analysis of a ternary complex composed of DNA template–primer, nucleoside triphosphate, and *Taq* DNA polymerase suggests that intensive contacts of the enzyme occur almost exclusively through the minor groove of the template–primer complex.[11] The 5′*S* modification points into the minor groove of double-stranded DNA while the 5′*R* modification is directed

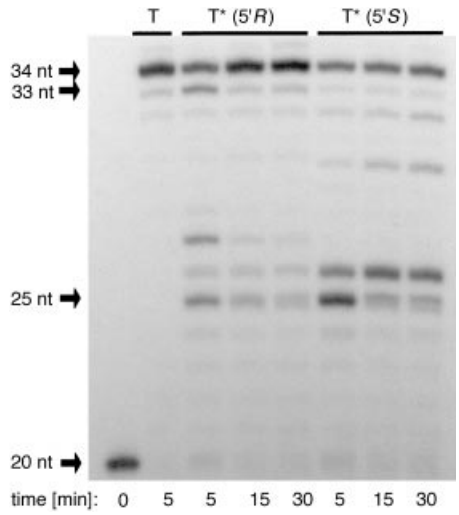


Figure 5. *Bypass DNA synthesis catalyzed by* Taq *DNA polymerase. An autoradiograph of a polyacrylamide gel (14%) containing 8 M urea is shown. The DNA templates used are indicated at the top of the figure. Every reaction comprised the same amount of the specific primer/template complex,* Taq *DNA polymerase, and all four dNTPs. The reactions were incubated at 72 °C for varied reaction times as indicated at the bottom of the figure. Experimental details and DNA substrate sequences employed are described in the Experimental Section.*

toward the major groove.[7] This might explain the difference in the ability of *Taq* DNA polymerase to bypass the stereoisomers of the 5′*C*-*o*-nitrophenyl modifications in the template strand.

In conclusion, the present study provides a new nucleotide-based DNA building block, which is highly efficiently and site-specifically cleavable upon irradiation with >360 nm light in single- and double-stranded DNA. The ease of the synthesis and the duplex-formation properties, as well the ability of the building blocks to be bypassed by at least one DNA polymerase, make 5′*C*-*o*-nitrophenylated thymidines valuable tools for further chemical and biological applications.

Experimental Section

Synthesis: All temperatures quoted are uncorrected. All reagents are commercially available and were used without further purification. Solvents were purified and dried according to standard procedures. All reactions were conducted under rigorous exclusion of air and moisture. IR spectra were recorded on a Perkin Elmer 1600-FTIR spectrophotometer (wavelengths quoted in cm^{-1}). NMR spectra were recorded on a Varian Gemini 300 spectrophotometer with the solvent peak as the internal standard. Fast atom bombardment (FAB) MS were recorded on a VG 70–250, with a matrix of 3-nitrobenzyl alcohol (NBA) or NBA and KCl. Microanalysis was performed at the Mikroanalytisches Labor, University of Basel. Merck silica gel 60 was used for flash column chromatography and Merck precoated plates F_{254} were used for thin-layer chromatography.

3′-*O*-[(*tert*-Butyl)dimethylsilyl]-5′-*O*-[(1*R)-ethoxyethyl]-(5′*S*)-*C*-(2-nitrophenyl)-thymidine (7a) and 3′-*O*-[(*tert*-butyl)dimethylsilyl]-5′-*O*-[(1*R**)-ethoxyethyl]-(5′*R*)-*C*-(2-nitrophenyl)-thymidine (7b):** A precooled 1.3 M solution of phenyl lithium in pentane (41.5 mL, 53.9 mmol) was added to a solution of 1-bromo-2-nitrobenzene (10.95 g, 54.21 mmol) in THF (100 mL) at -105 °C, and the reaction mixture was stirred for 100 min. A solution of **6** (1.92 g, 5.42 mmol) in

THF (40 mL) cooled to −105 °C was added dropwise to the resulting dark solution, and stirring was continued at −105 °C for 5 h. The reaction mixture was then allowed to warm to room temperature and subsequently quenched with water and extracted into CH_2Cl_2. The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (pentane/ethyl acetate (3:1)) to yield 1.71 g (66%) of the addition products as a 4.6:1 diastereomeric mixture of pale yellow foam. IR (KBr, mixture of diastereomers): $\tilde{\nu}$ = 3355, 3200, 3066, 2930, 2856, 1694, 1527, 1364, 1471, 1277, 1253, 1196, 1107, 1063; MS (FAB, mixture of diastereomers): *m/z*: 516 [M^++K], 478 [M^++H]; elemental analysis (%, mixture of diastereomers): calcd for $C_{22}H_{31}N_3O_7Si$ (477.59): C 55.33, H 6.54, N 8.80; found: C 55.36, H 6.48, N 8.90.

Major diastereomer: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.13 (s, 6H, CH_3Si), 0.92 (s, 9H, $(CH_3)_3CSi$), 1.96 (d, ^3J(H,H) = 1.2 Hz, 3H, CH_3–C(5)), 2.16 (ddd, ^3J(H,H) = 2.4, 6.0, 13.1 Hz, 1H, H2′), 2.72 (ddd, ^3J(H,H) = 6.0, 8.5, 13.1 Hz, 1H, H2′), 4.29 (t, ^3J(H,H) = 2.2 Hz, 1H, H4′), 4.37 (d, ^3J(H,H) = 6.4 Hz, 1H, OH), 4.71 (td, ^3J(H,H) = 2.2, 6.0 Hz, 1H, H3′), 5.56 (d, ^3J(H,H) = 6.3 Hz, 1H, H5′), 5.90 (dd, ^3J(H,H) = 6.1, 8.3 Hz, 1H, H1′), 7.34 (d, ^4J(H,H) = 1.1 Hz, 1H, H6), 7.45 (t, ^3J(H,H) = 7.7 Hz, 1H, H3″), 7.66 (t, ^3J(H,H) = 8.2 Hz, 1H, H4″), 7.77 (d, ^3J(H,H) = 1.4 Hz, 1H, H5″), 8.02 (d, ^3J(H,H) = 1.2 Hz, 1H, H6″), 8.22 (s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = −4.3, 13.0, 18.5, 26.3, 40.1, 69.3, 74.3, 90.2, 90.4, 111.7, 125.0, 129.0, 130.2, 134.0, 137.8, 139.0, 148.1, 151.2, 164.5.

Minor diastereomer: ^{1}H NMR (300 MHz, $CDCl_3$): δ = −0.34 (s, 3H, CH_3Si), −0.17 (s, 3H, CH_3Si), 0.73 (s, 9H, $(CH_3)_3CSi$), 1.94 (d, ^3J(H,H) = 1.2 Hz, 3H, CH_3–C(5)), 2.08 (ddd, ^3J(H,H) = 1.5, 7.6, 13.7 Hz, 1H, H2′), 2.69 (m, 1H, H2′), 4.20 (dd, ^3J(H,H) = 1.5, 3.1 Hz, 1H, H4′), 4.33 (s, 1H, OH), 4.52 (td, ^3J(H,H) = 1.3, 5.7 Hz, 1H, H3′), 5.68 (s, 1H, H5′), 5.89 (dd, ^3J(H,H) = 5.8, 7.6 Hz, 1H, H1′), 7.29 (d, ^4J(H,H) = 1.2 Hz, 1H, H6), 7.45 (t, ^3J(H,H) = 7.7 Hz, 1H, H3″), 7.68 (t, ^3J(H,H) = 8.1 Hz, 1H, H4″), 7.79 (d, ^3J(H,H) = 1.4 Hz, 1H, H5″), 7.99 (d, ^3J(H,H) = 1.2 Hz, 1H, H6″), 8.31 (s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = −4.8, −4.4, 12.9, 18.2, 26.1, 40.5, 70.1, 71.6, 90.2, 90.1, 111.6, 125.49, 129.2, 130.2, 134.3, 136.2, 139.0, 148.1, 151.2, 164.5.

Ethyl vinyl ether (2.80 mL, 27.0 mmol) was added to a solution of the alcohols (800 mg, 1.68 mmol) and pyridinium *p*-toluylsulfonate (50.0 mg, 0.20 mmol) in CH_2Cl_2 (20 mL), and the reaction mixture was stirred at 20 °C. After 6 h a second portion of ethyl vinyl ether (3.00 mL, 29.0 mmol) was added, and stirring was continued for an additional 12 h. The reaction mixture was then concentrated under reduced pressure and the resulting residue was purified by flash column chromatography (pentane/ethyl acetate (3:1)) to yield 154 mg (16%) of the 5′*R* stereoisomer **7b** and 707 mg (76%) of the 5′*S* stereoisomer **7a** as mixtures of diastereomers. Mixture of **7a** and **7b**: IR (KBr): $\tilde{\nu}$3191, 3073, 2955, 2931, 2895, 2857, 1696, 1528, 1472, 1349, 1277, 1253, 1196, 1134, 1106, 1056; MS (FAB, mixture of diastereomers): *m/z*: 588 [M^++K], 550 [M^++H]; elemental analysis (%, mixture of diastereomers): calcd for $C_{26}H_{39}N_3O_8Si$ (549.70): C 56.81, H 7.15, N 7.64; found: C 56.91, H 6.98, N 7.75.

7a (5′*S*) mixture of diastereomers: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.09, 0.10, 0.13, 0.14 (4s, 6H, CH_3Si), 0.91, 0.92 (2s, 9H, $(CH_3)_3CSi$), 1.19 (t, ^3J(H,H) = 7.0 Hz, 3H, C*H*$_3$-CH_2O), 1.35, 1.38 (2d, ^3J(H,H) = 5.2 Hz, 3H, C*H*$_3$-CHO_2), 2.02 (d, ^3J(H,H) = 1.2 Hz, 3H, CH_3–C(5)), 2.20 (m, 2H, H2′), 3.19, 3.45 (2m, 2H, CH_2O), 4.21 (t, ^3J(H,H) = 2.6 Hz, 1H, H4′), 4.26 (t, ^3J(H,H) = 1.8 Hz, 1H, H4′), 4.42 (q, ^3J(H,H) = 5.2 Hz, 1H, CHO_2), 4.44, 4.63 (2m, 1H, H3′), 4.75 (q, ^3J(H,H) = 5.2 Hz, 1H, CHO_2), 5.42 (d, ^3J(H,H) = 2.0 Hz, 1H, H5′), 5.63 (d, ^3J(H,H) = 1.7 Hz, 1H, H5′), 6.37, 6.44 (2t, ^3J(H,H) = 6.9, 1H, H1′), 7.48, 7.88, 7.92, 8.00 (4m, 4H, H_{ar}), 7.64, 7.66 (2d, ^4J(H,H) = 1.2 Hz, 1H, H6), 9.00 (s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = −5.0, −4.9, −4.8, −4.7, 12.6, 12.7, 14.7, 15.3, 17.9, 18.0, 20.4, 20.7, 25.6, 25.7, 40.9, 62.2, 62.3, 71.4, 72.8, 72.9, 74.1, 84.6, 84.8, 89.1, 89.4, 98.4, 101.8, 111.0, 124.2, 124.6, 128.5, 128.8, 129.8, 130.6, 133.0, 133.4, 134.3, 135.7, 135.7, 135.8, 147.6, 148.6, 150.3, 163.8

7b (5′*R*) mixture of diastereomers: ^{1}H NMR (300 MHz, $CDCl_3$): δ = −0.25, −0.15, −0.12, −0.04 (4s, 6H, CH_3Si), 0.75, 0.78 (2s, 9H, $(CH_3)_3CSi$), 0.87 (t, ^3J(H,H) = 7.1 Hz, 3H, C*H*$_3$–CH_2O), 1.35, 1.37 (2d, ^3J(H,H) = 1.0 Hz, 3H, C*H*$_3$–CHO_2), 1.95 (d, ^3J(H,H) = 1.1 Hz, 3H, CH_3–C(5)), 1.98 (d, ^3J(H,H) = 1.1 Hz, 3H, CH_3–C(5)), 2.05, 2.17 (2m, 2H, H2′), 3.24, 3.50 (2m, 2H, CH_2O), 4.07 (m, 1H, H4′), 4.42, 4.77 (2q, ^3J(H,H) = 5.2 Hz, 1H, CHO_2), 4.55 (dt, ^3J(H,H) = 1.8, 5.7 Hz, 1H, H3′), 5.45 (d, ^3J(H,H) = 5.2 Hz, 1H, H5′), 5.74 (d, ^3J(H,H) = 4.2 Hz, 1H, H5′), 6.32, 6.39 (2dd, ^3J(H,H) = 5.2, 9.4 Hz, 1H, H1′), 7.50, 7.74, 7.91, 8.00 (4m, 4H, H_{ar}), 7.29 (d, ^4J(H,H) = 1.2 Hz, 1H, H6), 8.43, 8.49 (2s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = −5.2, −5.0, −4.9, −4.7, 12.1, 12.4, 14.8, 15.3, 17.7, 20.4, 21.2, 25.6, 40.4, 40.9, 62.4, 64.1, 71.4, 72.6, 72.5, 74.0, 84.4, 85.0, 89.8, 89.9, 99.0, 101.7, 111.1, 124.6, 125.2, 128.5, 128.6, 129.1, 129.5, 133.3, 133.6, 134.0, 135.2, 135.3, 136.1, 148.3, 150.2, 163.5, 163.8.

5′-*O*-[(1*R)-Ethoxyethyl]-(5′*S*)-*C*-(2-nitrophenyl)-thymidine 3′-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (8a):** A 1 M solution of nBu$_4$NF (0.91 mL, 0.91 mmol) in THF was added to a solution of **7a** (100 mg, 0.18 mmol) in THF (15 mL) at 20 °C, and the reaction mixture was stirred for 2 h. The mixture was concentrated under reduced pressure and the resulting residue was purified by flash column chromatography (pentane/ethyl acetate (1:1)) to yield 68.0 mg (86%) of the alcohol as a pale yellow foam: (mixture of diastereomers): IR (KBr): $\tilde{\nu}$ = 3429, 3200, 3071, 2977, 2928, 1686, 1526, 1473, 1349, 1274, 1195, 1092, 1053; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85, 1.16 (2t, ^3J(H,H) = 7.0 Hz, 3H, C*H*$_3$-CH_2O), 1.34, 1.37 (2d, ^3J(H,H) = 5.3 Hz, 3H, C*H*$_3$–CHO_2), 1.98 (d, ^3J(H,H) = 1.2 Hz, 3H, CH_3–C(5)), 2.30 (m, 2H, H2′), 3.20, 3.49 (2m, 2H, CH_2O), 3.34 (d, ^3J(H,H) = 4.5 Hz, 1H, OH), 3.58 (d, ^3J(H,H) = 4.9 Hz, 1H, OH), 4.22 (dd, ^3J(H,H) = 2.4, 3.6 Hz, 1H, H4′), 4.28 (dd, ^3J(H,H) = 2.2, 3.0 Hz, 1H, H4′), 4.49, 4.82 (m, 1H, CHO_2), 4.54, 4.64 (2m, 1H, H3′), 5.53 (d, ^3J(H,H) = 2.2 Hz, 1H, H5′), 5.68 (d, ^3J(H,H) = 1.9 Hz, 1H, H5′), 6.42 (m, 1H, H1′), 7.46, 7.63, 7.81, 7.95 (4m, 5H, H6 and H_{ar}), 9.48, 9.50 (2s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.6, 14.7, 15.2, 20.4, 20.6, 38.3, 62.0, 62.1, 71.4, 72.0, 72.6, 72.8, 84.4, 84.5, 88.2, 98.7, 101.9, 111.1, 124.1, 124.6, 128.4, 128.8, 129.8, 130.6, 132.9, 133.3, 134.2, 135.4, 135.8, 147.6, 148.6, 150.5, 164.0; MS (FAB): *m/z*: 474 [M^++K], 436 [M^++H]; elemental analysis (%): calcd for $C_{20}H_{25}N_3O_8$ (435.44): C 55.17, H 5.79, N 9.65; found: C 55.33, H 5.69, N 9.90.

2-Cyanoethyl-*N*,*N*-diisopropyl-phosphorochloridamidite (104 μL, 0.47 mmol) was added to a solution of the alcohols (68.0 mg, 0.16 mmol) and *i*Pr$_2$EtN (0.15 mL, 0.87 mmol) in CH_2Cl_2 (5 mL) at 20 °C. After stirring for 2 h the reaction mixture was diluted with CH_2Cl_2 (20 mL) and extracted with saturated $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (pentane/ethyl acetate (3:1)) to yield 89 mg (90%) of **8a** as a 1:1 mixture of a pair of diastereomers. IR (KBr, mixture of diastereomers): $\tilde{\nu}$ = 3420, 3200, 3067, 2970, 2931, 2878, 1692, 1528, 1466, 1399, 1365, 1277, 1183, 1128, 1100, 1081, 1052; MS (FAB, mixture of diastereomers): *m/z*: 674 [M^+ + K], 636 [M^+ + H]; elemental analysis (%, mixture of diastereomers): calcd for $C_{29}H_{42}N_5O_9$ (635.66): C 55.17, H 5.79, N 9.65; found: C 55.33, H 5.69, N 9.90.

Pair of diastereomers A: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.82, 1.16 (2t, ^3J(H,H) = 7.0 Hz, 3H, C*H*$_3$–CH_2O), 1.20 (m, 12H, $(CH_3)_2CH$), 1.38, 1.39 (2d, ^3J(H,H) = 7.7 Hz, 3H, C*H*$_3$–CHO_2), 2.02, 2.03 (2d, ^3J(H,H) = 1.3 Hz, 3H, CH_3–C(5)), 2.35 (m, 2H, H2′), 2.71 (m, 2H, CH_2CN), 3.20, 3.40 (2m, 1H, CH_2O), 3.51, 3.60 (2m, 3H, CH_2O and C*H*$(CH_3)_2$), 3.89 (m, 2H, CH_2OP), 4.36, 4.42 (2m, 1H, H4′), 4.48, 4.84 (2m, 1H, CHO_2), 4.65, 4.78

(2m, 1H, H3′), 5.51 (d, ^3J(H,H) = 1.7 Hz, 1H, H5′), 5.66 (d, ^3J(H,H) = 1.8 Hz, 1H, H5′), 6.40 (m, 1H, H1′), 7.49, 7.62, 7.80, 7.91, 7.96 (5m, 4H, H_{ar}), 7.65, 7.67 (2d, ^4J(H,H) = 1.2 Hz, 1H, H6), 8.83 (2s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.6, 14.7, 15.3, 20.2, 20.3, 20.6, 20.7, 24.4, 24.5, 24.6, 39.5, 39.6, 43.2, 58.2, 58.4, 62.2, 71.4, 73.4, 74.7, 84.4, 84.7, 87.8, 88.0, 98.6, 102.2, 111.1, 117.7, 117.8, 124.2, 124.6, 128.4, 128.9, 129.8, 130.8, 132.9, 133.3, 134.2, 135.6, 135.7, 147.4, 148.6, 150.2, 150.3, 163.7; ^{31}P NMR (121 MHz, $CDCl_3$): δ = 149.1, 149.2.

Pair of diastereomers B: δ = 0.83, 1.16 (2t, ^3J(H,H) = 7.0 Hz, 3H, CH_3–CH_2O), 1.21 (m, 12H, $(CH_3)_2CH$), 1.37, 1.38 (2m, 3H, CH_3–CHO_2), 2.02 (s, 3H, CH_3–C(5)), 2.28, 2.47 (2m, 2H, H2′), 2.66 (m, 2H, CH_2CN), 3.21, 3.42 (2m, 1H, CH_2O), 3.49, 3.63 (2m, 3H, CH_2O and $CH(CH_3)_2$), 3.75, 3.88 (2m, 2H, CH_2OP), 4.36, 4.40 (2m, 1H, H4′), 4.46, 4.80 (2m, 1H, CHO_2), 4.65, 4.75 (2m, 1H, H3′), 5.47, 5.65 (2d, ^3J(H,H) = 1.9 Hz, 1H, H5′), 6.40 (m, 1H, H1′), 7.49, 7.62, 7.82, 7.88, 7.99 (5m, 4H, H_{ar}), 7.65, 7.66 (2s, 1H, H6), 8.73 (2s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.6, 14.7, 15.3, 20.3, 20.4, 20.5, 20.6, 24.5, 24.6, 39.5, 43.2, 43.3, 57.6, 58.0, 62.0, 62.1, 71.3, 73.1, 74.5, 75.2, 84.4, 84.5, 87.4, 87.7, 98.4, 102.2, 111.1, 117.6, 128.4, 128.8, 129.9, 130.7, 132.9, 133.3, 134.1, 135.8, 147.5, 148.6, 150.3, 163.6; ^{31}P NMR (121 MHz, $CDCl_3$): δ = 148.6, 148.7.

5′-*O*-[(1*R)-Ethoxyethyl]-(5′*R*)-*C*-(2-nitrophenyl)-thymidine 3′-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (8b):** A 1 M solution of nBu$_4$NF (2.20 mL, 2.20 mmol) in THF was added to a solution of **7b** (120 mg, 0.22 mmol) in THF (15 mL) at 20 °C and stirred for 2 h. The reaction mixture was concentrated under reduced pressure and the resulting residue was purified by flash column chromatography (pentane/ethyl acetate (1:1)) to yield 85.0 mg (89%) of the alcohol as a pale yellow foam: (mixture of diastereomers): IR (KBr): $\tilde{\nu}$ = 3424, 3201, 3072, 2976, 2930, 1688, 1527, 1474, 1348, 1197, 1106, 1092, 1054; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.92, 1.18 (2t, ^3J(H,H) = 7.0 Hz, 3H, CH_3-CH_2O), 1.33, 1.48 (2d, ^3J(H,H) = 5.0 Hz, 3H, CH_3–CHO_2), 1.94, 1.97 (d, ^3J(H,H) = 1.1 Hz, 3H, CH_3–C(5)), 2.17, 2.34 (2m, 2H, H2′), 2.84, 2.97 (2s, 1H, OH), 3.27, 3.54 (2m, 2H, CH_2O), 4.02 (m, 1H, H4′), 4.48, 4.81 (2m, 1H, CHO_2), 4.59 (m, 1H, H3′), 5.54 (d, ^3J(H,H) = 6.1 Hz, 1H, H5′), 5.77 (d, ^3J(H,H) = 5.2 Hz, 1H, H5′), 6.29 (m, 1H, H1′), 7.17 (d, ^4J(H,H) = 1.2 Hz, 1H, H6), 7.47, 7.68, 7.77, 7.94 (4m, 4H, H_{ar}), 9.05 (2s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.2, 12.4, 14.8, 15.3, 20.4, 21.0, 39.8, 40.1, 62.5, 63.5, (71.4, 71.5, 72.8, 73.0, 83.9, 84.3, 88.3, 88.4, 99.3, 101.3, 111.3, 111.4, 124.5, 124.9, 128.5, 128.7, 129.0, 129.3, 133.3, 133.6, 133.8, 135.1, 135.2, 135.8, 148.4, 150.3, 163.6, 163.9; MS (FAB): m/z: 474 [M^++K], 436 [M^++H]; elemental analysis (%): calcd for $C_{20}H_{25}N_3O_8$ (435.44): C 55.17, H 5.79, N 9.65; found: C 55.33, H 5.69, N 9.90.

2-Cyanoethyl *N*,*N*-diisopropylphosphorochloridamidite (123 μL, 0.55 mmol) was added to a solution of the alcohols (80.0 mg, 0.18 mmol) and iPr$_2$EtN (0.19 mL, 1.10 mmol) in CH_2Cl_2 (5 mL) at 20 °C. After stirring for 2 h the reaction mixture was diluted with CH_2Cl_2 (20 mL) and extracted with saturated $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (pentane/ethyl acetate (3:1)) to yield 94 mg (80%) of **8b** as a 1:1 mixture of a pair of diastereomers. IR (KBr, mixture of diastereomers): $\tilde{\nu}$ = 3420, 3200, 3056, 2971, 2933, 2878, 1691, 1528, 1466, 1365, 1278, 1251, 1183, 1128, 1083, 1052, 972; MS (FAB, mixture of diastereomers): m/z: 674 [M^++K], 636 [M^++H]; elemental analysis (%, mixture of diastereomers): calcd for $C_{29}H_{42}N_5O_9$ (635.66): C 55.17, H 5.79, N 9.65; found: C 55.33, H 5.69, N 9.90.

Pair of diastereomers A: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.89, 1.11, 1.22 (3m, 15H, CH_3–CH_2O and $(CH_3)_2CH$), 1.37 (m, 3H, CH_3–CHO_2), 1.94 (d, ^3J(H,H) = 1.3 Hz, 3H, CH_3–C(5)), 1.96 (d, ^3J(H,H) = 1.1 Hz, 3H, CH_3–C(5)), 2.16, 2.39 (2m, 2H, H2′), 2.63 (m, 2H, CH_2CN), 3.25, 3.51, 3.90 (3m, 6H, CH_2O, $CH(CH_3)_2$ and CH_2OP), 4.19, 4.26 (2m, 1H, H4′), 4.45, 4.79 (2m, 1H, CHO_2), 4.75 (m, 1H, H3′), 5.53 (d, ^3J(H,H) = 5.0 Hz, 1H, H5′), 5.78 (d, ^3J(H,H) = 3.7 Hz, 1H, H5′), 6.33 (dd, ^3J(H,H) = 5.4, 8.6 Hz, 1H, H1′), 6.37 (dd, ^3J(H,H) = 5.3, 9.1 Hz, 1H, H1′), 7.26 (s, 1H, H6), 7.49, 7.74, 7.98 (3m, 4H, H_{ar}), 8.22 (s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.1, 12.3, 14.9, 15.3, 20.1, 20.5, 20.2, 21.5, 24.3, 24.4, 24.5, 39.1, 43.3, 58.0, 58.1, 62.4, 63.9, 71.4, 73.5, 72.5, 84.0, 84.8, 88.1, 88.5, 99.1, 101.7, 111.1, 111.3, 117.5, 124.5, 125.1, 128.6, 128.9, 129.1, 129.9, 133.3, 133.7, 135.0, 135.2, 135.7, 136.0, 148.2, 150.1, 150.2, 163.4, 163.7; ^{31}P NMR (121 MHz, $CDCl_3$): δ = 148.9, 149.2.

Pair of diastereomers B: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85 – 1.31 (m, 15H, CH_3–CH_2O and $(CH_3)_2CH$), 1.36 (m, 3H, CH_3–CHO_2), 1.96 (d, ^3J(H,H) = 1.2 Hz, 3H, CH_3–C(5)), 1.98 (d, ^3J(H,H) = 1.1 Hz, 3H, CH_3–C(5)), 2.15, 2.46 (2m, 2H, H2′), 2.61 (m, 2H, CH_2CN), 3.26, 3.48 – 3.90 (2m, 6H, CH_2O, $CH(CH_3)_2$ and CH_2OP), 4.16, 4.27 (2m, 1H, H4′), 4.41, 4.78 (2m, 1H, CHO_2), 4.73 (m, 1H, H3′), 5.52 (d, ^3J(H,H) = 4.7 Hz, 1H, H5′), 5.75 (d, ^3J(H,H) = 3.3 Hz, 1H, H5′), 6.32 (dd, ^3J(H,H) = 5.3, 9.3 Hz, 1H, H1′), 6.40 (dd, ^3J(H,H) = 5.2, 9.3 Hz, 1H, H1′), 7.27 (m, 1H, H6), 7.47, 7.69, 7.80, 7.98 (4m, 4H, H_{ar}), 8.48 (s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 12.1, 12.4 (Cp, CH_3–C(5)), 14.9, 15.3 (Cp, CH_3–CH_2O), 20.3, 20.4, 21.2, 21.5, 24.1, 24.2, 24.3, 24.4, 24.5, 24.6, 39.2, 39.7, 43.1, 43.2, 57.7, 57.8, 62.4, 64.0, 72.5, 73.7, 73.0, 84.0, 84.8, 88.3, 88.4, 99.0, 101.7, 111.2, 111.3, 117.7, 124.6, 125.2, 128.6, 128.7, 129.1, 129.6, 133.4, 133.8, 135.1, 135.2, 136.1, 148.0, 150.2, 163.4; ^{31}P NMR (121 MHz, $CDCl_3$): δ = 125, 148.1, 148.6.

Oligonucleotide synthesis: The synthesis of oligonucleotides was carried out on an Expedite 8909 (Perseptive Biosystems) DNA synthesizer on a 1 μmol scale. A standard method for 2-cyanoethyl-phosphoramidites was used, with the exception that, for the modified nucleotides, the 5′-deprotection period was extended to 2 min and the coupling step to 15 min, respectively. We determined the yields of the incorporation steps by monitoring the trityl concentrations after deprotection. The unmodified nucleotides were incorporated in at least 95%. These yields decreased to 75 – 80% for the modified thymidines **8a**, **b**. Purity and integrity of all oligonucleotides were controlled by anion-exchange HPLC, RP-HPLC, and MALDI-TOF MS.

General procedure for irradiation and subsequent analysis: modified oligonucleotides (1 – 2 nmol) with or without the complementary strand were irradiated in the irradiation buffer (A: 100 mM NaCl, 20 mM KH_2PO_4 (pH 7); B: 200 mM NaOH (pH 13)) at 20 °C with an Osram high-pressure mercury arc lamp (500 W, 360 nm cut-off filter) for 5 min. For MALDI-TOF analysis (Vestec Benchtop II, laser wavelength 337 nm, acceleration voltage 25 kV, negative ion mode, matrix: 2,4-dihydroxyacetophenone), the buffered solutions were desalted by the use of membrane filters (Millipore) prior to analysis. For reduction of **3**, NaSH (2 mg) was added after irradiation and the mixture was incubated at 20 °C for 1 h, followed by subsequent desalting and analysis as described above. For quantification of the irradiation products, the irradiation mixtures were analyzed directly without further work up by analytical RP-HPLC (Waters Alliance) with UV detection at 260 nm. To neutralize samples irradiated at pH 13, KH_2PO_4 (100 mg) was added prior to HPLC analysis. The peak areas were divided by the calculated extinction coefficients of the corresponding oligonucleotides and quantified with external calibration. For identification, the samples separated by RP-HPLC were collected and lyophilized as recently described.[12] The masses of the samples were determined by MALDI-TOF MS and were, in each case, in agreement with the proposed structure.

DNA melting curves: Modified oligonucleotides (0.5 nmol) with the complementary strand (0.5 nmol) were dissolved in buffer A (1 mL; as above). The reaction mixture was heated to 85 °C for 2 min and slowly cooled to room temperature prior to use. Melting curves were recorded on a Lambda 2 device (Perkin Elmer) equipped with PTP-6

temperature control according to standard procedures. T_m values are derived from three heating and cooling cycles.

CD spectra: Modified oligonucleotides (2 nmol) with the complementary strand (2 nmol) were dissolved in buffer A (1 mL; as above). The reaction mixture was heated to 85 °C for 2 min and slowly cooled to room temperature prior to use. CD spectra were recorded on Jasco 720 spectrophotometer. An average of 15 spectra were recorded in each experiment.

Primer extension reactions: Primer stands were labeled at the 5′-end by the use of T4 polynucleotide kinase (New England Biolabs) followed by purification with Quick spin columns (Roche Diagnostics). Labeled 20 nt primer (5′-GTG GTG CGA ATT CTG TGG AT-3′; 1 pmol) was annealed to its complementary site on the 33 nt template (5′-TAT GCA CGT* CGA GAT CCA CAG AAT TCG CAC CAC-3′, T* = thymidine, 5′*S*-, or 5′*R*-*C*-*o*-nitrophenylated thymidine; 3 pmol) by heating a mixture of both DNA strands in the reaction buffer (tris(hydroxymethyl)aminomethane – HCl (pH 7.5; 20 mM); $MgCl_2$ (10 mM), 1,4-dithiothreitol (0.1 mM)) to 90 °C and then cooling it to 25 °C within one 1 h. Each primer extension reaction (100 μL) contained dATP, dGTP, dCTP, and TTP (500 μM). The reactions were initiated by addition of *Taq* DNA polymerase (Promega, 5 U) and incubated at 72 °C. At several time points aliquots (10 μL) were removed and subsequently quenched by addition of 80% formamide solution containing 20 mM ethylenediaminetetraacetate (EDTA) and heating to 90 °C. The aliquots were analyzed with polyacrylamide gel electrophoresis (14%) containing 8 M urea, transferred to filter paper, dried under vacuum, and visualized by autoradiography.

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