Cleavage of Oligonucleotides from Solid-Phase Supports Using *o*-Nitrobenzyl Photochemistry

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Oligonucleotides are cleaved from modified solid phase supports using the *o*-nitrobenzyl intramolecular photochemical redox reaction. Oligonucleotides can be cleaved before or after removal of protecting groups. Employment of phosphoramidites containing allyloxy protecting groups that are labile to Pd^0 enables one to deprotect oligonucleotides without the use of base. The yields of isolated oligonucleotides relative to yields obtained using conventional hydrolytic cleavage vary between 59% and 87%. Tritium labeling indicates that small amounts of thymidine·thymidine photodimers are formed during the photolysis time required to obtain good yields of oligonucleotides from the succinate linked photolabile support (**4**). Photodimer formation is minimized (<7%) during the prolonged irradiation required when cleaving oligonucleotides from **7** by employing bandpass filtering.

The use of modified oligonucleotides as diagnostic and mechanistic probes, as well as their potential applications for therapeutic purposes, continues to provide a strong driving force for the development of methods for their chemical synthesis.[1] Conjugation of synthetic oligonucleotides to various supports for binding studies, as well as to small organic molecules and other biopolymers, has spurred interest in the design of linkers that enable one to cleave protected oligonucleotides from their solid-phase supports without affecting the protecting groups associated with phosphorus and the nucleobase amines.[2] We have applied the *o*-nitrobenzyl intramolecular photoredox reaction to the development of such orthogonal linkers. This method enables one to cleave oligonucleotides from their solid-phase supports under neutral conditions prior to or after removal of the nucleobase and phosphodiester protecting groups.[3] The linkers are compatible with commercially available reagents and solid-phase synthesis protocols. The use of two such photolabile linkers, and characterization of oligonucleotides prepared using them, are described here (Figure 1).

Presently, the phosphite triester methodology is the most common strategy employed for the solid-phase synthesis of oligonucleotides. Commercial versions of this method utilize base-labile protecting groups for the exocyclic amines of the nucleobases and phosphodiesters, as well as a base-labile succinate linker between the solid-phase support and the 3′-terminus of the oligonucleotide (Figure 2). Typically, the protecting groups are removed, and the linkage between the oligonucleotide's 3′-terminus and solid-phase support is severed in one step using concentrated ammonium hydroxide for 8–16 h at 55 °C. Prolonged treatment with ammonium hydroxide is incompatible with many nonnative nucleosides. Consequently, protecting groups which can be removed in as little as 15 min using concentrated ammonium hydroxide are now available for unmasking the exocyclic amines of nucleosides.[4] Phosphodiesters can be revealed from the respective methyl phosphotriesters using anhydrous thiophenol and triethylamine in 1,4-dioxane.[5] More recently, the use of base to deprotect nucleobases and phosphodiesters has been eliminated completely by taking advantage of the ability to cleave *O*-allyloxy groups using Pd^0 chemistry at pH 5.5.[6]

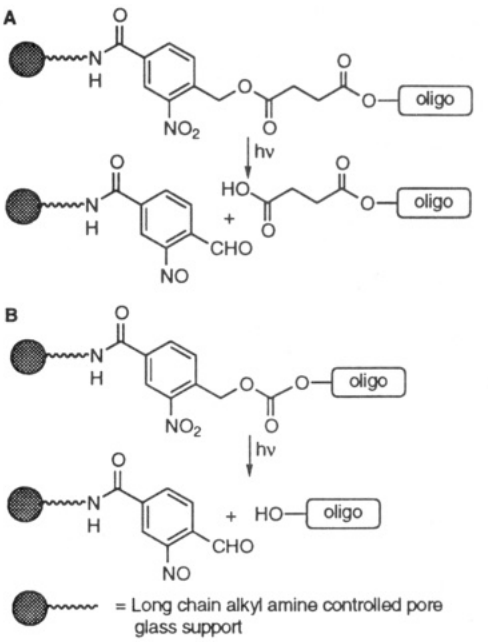

Figure 1. Photochemical cleavage of oligonucleotides from *o*-nitrobenzyl derivatized solid-phase supports.

Molecular constructs that enable one to cleave oligonucleotides from their solid-phase supports under milder conditions than required for commercially available succinate-linked resins (concentrated ammonium hydroxide

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(1) For recent reviews on oligonucleotide synthesis: (a) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123. (b) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 1925. (c) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223. (d) Caruthers, M. H. *Acc. Chem. Res.* **1991**, *24*, 278. (e) Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165. (f) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543.

(2) (a) Zon, G.; Geister, T. G. *Anti-Cancer Drug Design* **1991**, *6*, 539. (b) Maskos, U.; Southern, E. M. *Nucleic Acids Res.* **1992**, *20*, 1679. (c) Haralambidis, J.; Duncan, L.; Angus, K.; Tregear, G. W. *Nucleic Acids Res.* **1990**, *18*, 493.

(3) Greenberg, M. M. *Tetrahedron Lett.* **1993**, *34*, 251.

(4) (a) Schulhof, J. C.; Molko, D.; Teoule, R. *Tetrahedron Lett.* **1987**, *28*, 51. (b) Vu, H.; McCollum, C.; Jacobson, K.; Theisen, P.; Vinayak, R.; Spiess, E.; Andrus, A. *Tetrahedron Lett.* **1990**, *31*, 7269. (c) Sinha, N. D.; Davis, P.; Usman, N.; Pérez, J.; Hodge, R.; Kremsky, J.; Casale, R. *Biochimie* **1993**, *75*, 13.

(5) Gait, M. J. *Oligonucleotide Synthesis*; IRL Press: Oxford, 1984.

(6) Hayakawa, Y.; Wakabayashi, S.; Kato, H.; Noyori, R. *J. Am. Chem. Soc.* **1990**, *112*, 1691.

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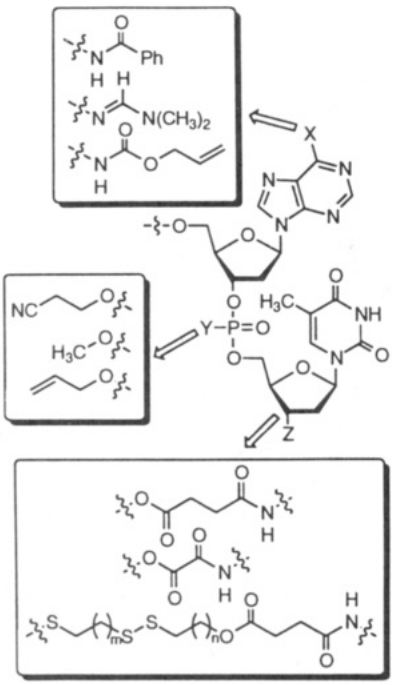


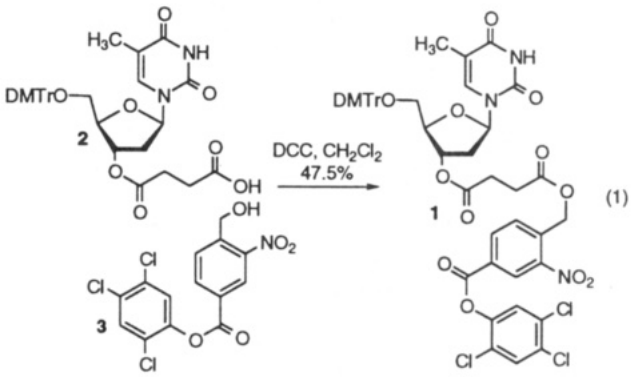
Figure 2. Examples of protecting groups and solid-phase support linkers utilized in the phosphite triester approach to solid-phase oligonucleotide synthesis.

for 1 h at 25 °C) have also been reported. Oligonucleotides containing 3′-hydroxy termini suitable for further enzymatic ligation have been generated under milder basic conditions by employing a more electrophilic carboxylate.[7] Alkaline cleavage has been eliminated in its entirety via the use of disulfides which are susceptible to reductive conditions.[8] In principle, when used in conjunction with palladium-labile allyloxy protecting groups, the disulfide linkers enable one to incorporate nucleosides that are unstable to concentrated ammonium hydroxide for even a brief period. However, the disulfide linkers suffer from the drawback that they require subsequent treatment with concentrated NH_4OH for the production of 3′-termini suitable for enzymatic ligation. Each of the photolabile linkers described below generates oligonucleotides containing 3′-hydroxyl termini under neutral or extremely mild basic conditions (such as gel electrophoresis buffer, pH 8.3).

Results and Discussion

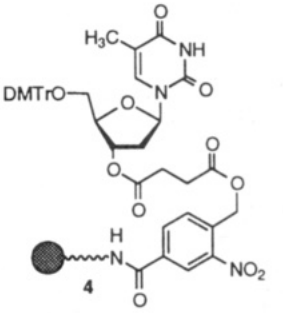
An ethereal linkage between the secondary hydroxyl of the 3′-terminal nucleoside and the photoreactive linker is the most obvious way of connecting the oligonucleotide to its photolabile solid-phase support so as to yield a free hydroxyl group upon photolysis. We reasoned that the succinate and carbonate constructs would also lead to 3′-hydroxy oligonucleotides upon photolysis (Figure 1). The carbonate linker would initially release a carbonic acid that decarboxylates spontaneously. The succinate linker should be stable under neutral conditions but undergoes saponification under slightly alkaline conditions. It was anticipated that the carbonate and succinate linkers would be more accessible synthetically than the respective ethereal linker, and for this reason would be attractive to a wide range of biological chemists.

We previously reported the loading of long-chain alkylamine-controlled pore glass support (CPG) by **1** and its utilization in oligonucleotide synthesis.[3] Activated ester **1** is easily prepared via DCC coupling of commercially available **2** and known **3** (eq 1).[9] Resin **4** was utilized in



(1)

the synthesis of oligonucleotides containing either *O*-methyl or commercially available β-cyanoethyl phosphoramidites using standard automated synthesis cycles. Ion-



exchange HPLC demonstrated the presence of an additional negative charge in the photochemically released oligonucleotide, which was removed upon subjection of the oligonucleotide to alkaline hydrolysis.[3] Conclusive evidence supporting the presence of the 3′-succinato moiety was obtained from electrospray mass spectrometry of a crude dodecamer of thymidine. The dodecamer examined by electrospray mass spectrometry was deprotected at phosphorous with diisopropylamine, photolytically cleaved, and then passed through a C_{18}-reversed-phase purification cartridge. The major peak at *m/z* 3688.3 corresponds to the expected molecular weight for fully protonated T_{12} containing a succinato moiety (Figure 3). The second most intense ion (*m/z* 3710.6) corresponds to substitution of a sodium atom for one of the hydrogens of a phosphodiester. The structure corresponding to the third largest ion detected (*m/z* 3742.6) is less certain but matches within one mass unit (experimental accuracy) material still containing a single β-cyanoethyl protecting group.

Yields of isolated oligonucleotides obtained using **4** were independent of the order in which the deprotection/cleavage steps were carried out (Table 1). They also were unaffected by slight changes in optical filters employed. One should note that the reported yields in Table 1 are expressed relative to the amount of material obtained via

(7) Alul, R. H.; Singman, C. N.; Zhang, G.; Letsinger, R. L. *Nucleic Acids Res.* **1991**, *19*, 1527.

(8) (a) Asseline, U.; Bonfils, E.; Kurfürst, R.; Chassignol, M.; Roig, V.; Thuong, N. T. *Tetrahedron* **1992**, *48*, 1233. (b) Gupta, K. C.; Sharma, P.; Sathyanarayana, S.; Kumar, P. *Tetrahedron Lett.* **1990**, *31*, 2471. (c) Zuckermann, R.; Corey, D.; Schultz, P. *Nucleic Acids Res.* **1987**, *15*, 5305.

(9) Lloyd-Williams, P.; Gairi, M.; Albericio, F.; Giralt, E. *Tetrahedron* **1991**, *47*, 9867.

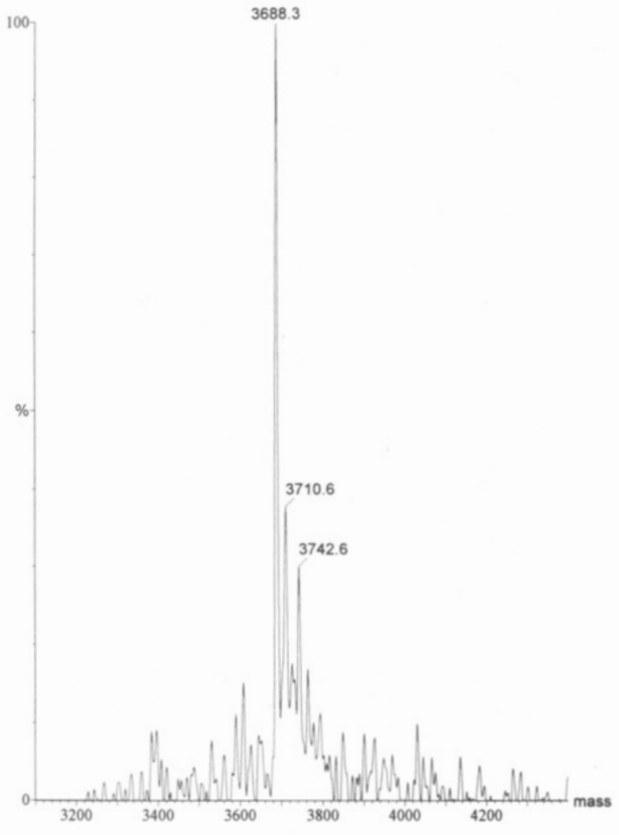


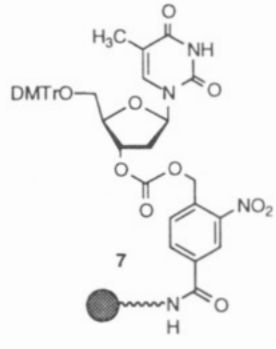
Figure 3. Electrospray mass spectrum of 3′-succianto T_{12}.

nonphotochemical cleavage of resin apportioned from the same synthesis. In expressing yields in this manner, one can compare the efficiency of the new method to that which is most commonly employed by practitioners of oligonucleotide synthesis. On average, yields dropped approximately 10% upon increasing oligonucleotide length from 12 to 20 nucleotides. Yet, there was no difference between an eicosamer of thymidines and an oligonucleotide of equal length containing all four naturally occurring nucleotides. We suggest that the decreased yields of photochemically cleaved biopolymers with increasing length are due to commensurate increases in ultraviolet absorption by the oligonucleotides, which tail into the region in which the *o*-nitrobenzyl moiety absorbs.

Ion-exchange HPLC analysis indicates that the 3′-succinato moiety readily undergoes saponification upon treatment with aqueous base or even denaturing polyacrylamide gel electrophoresis (PAGE, pH 8.3 buffer).[3] PAGE analyses of 5′-^{32}P-labeled 3′-succinato T_{12} and T_{12} deprotected using concentrated NH_4OH are consistent with the HPLC data (Figure 4). Only full-length dodecamer and single nucleotide deleted material are observable in each lane. Gel electrophoresis migratory behavior is commonly used to gain insight into the structural nature of end groups in oligonucleotides.[10] The photolytically cleaved oligonucleotide comigrates with the base deprotected material. If the succinato moiety survived electrophoresis, the additional negative charge would be expected to cause it to migrate faster than the comparable 3′-hydroxyl containing dodecamer.

The carbonate linker (**7**) yields the hydroxyl group directly. Activated ester **5** was synthesized in two steps from known materials (Scheme 1). Chloroformate **6** was prepared by reacting excess phosgene with the 2,4,5-trichlorophenyl ester of 4-(hydroxymethyl)-3-nitrobenzoic acid (**3**).[9] Crude chloroformate was characterized by FTIR and ^{1}H NMR. In order to obtain acceptable yields of **5**, it was necessary to deprotonate 5′-*O*-dimethoxytritylthymidine prior to coupling it to **6**. CPG was then loaded using a 20-fold excess of **5** in DMF and *N*-hydroxybenzotriazole. After washing and drying the resin, remaining free amines on the resin were capped with acetic anhydride and DMAP in pyridine.[5] Subsequent quantitative ninhydrin analysis indicated less than 3% of the original amines remained. Loadings ranged between 32 and 37.5 μmol of tritylated nucleoside per gram of resin, as determined by quantitation of dimethoxytrityl cation released into solution upon treatment of resin with a 0.1 N acetonitrile solution of *p*-toluenesulfonic acid.[11]

The efficiency of the photolabile carbonate resin was determined using a dodecamer of thymidine. The yield of isolated nucleotide was lower for the carbonate resin than for the analogous succinate resin (Table 1) using comparable photolysis conditions. Increasing the photolysis time to 9 h yielded 66% of the amount of isolated oligonucleotide as did hydrolysis of a portion of resin from the same synthesis with concentrated ammonium hydroxide. Extended photolysis (24 h) did not increase the yield of oligonucleotide to any measurable extent, nor was the yield increased by the addition of amines to the photolysis mixture to scavenge the resin-bound aldehyde. It is unclear why the carbonate resin (**7**) is less effective than **4** in releasing an identical oligonucleotide. While examples of *o*-nitrobenzyl carbonates are rare, there does not appear to be a general dependance of yields of photoreleased products on functional group type.[12]



Modest yields aside, when used in conjunction with palladium-labile allyloxy phosphoramidites the carbonate resin offers a strategy by which oligonucleotides containing enzymatically acceptable 3′-termini can be deprotected and purified under nonalkaline conditions. Dodecamers composed of allyloxy phosphoramidites were synthesized using slightly modified automated synthesis cycles than those prescribed in the literature. In our hands, recommended 70-s phosphoramidite coupling times resulted in average coupling yields of 94%, as judged by dimethoxytrityl cation response.[6] Doubling and tripling the reaction time increased the average coupling efficiency to 97% and 98%, respectively. Further increases in reaction time did

(10) (a) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. (b) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934. (c) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499.

(11) (a) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147. (b) Applied Biosystems Inc. User Bulletin No. 13, 1987.

(12) For a recent review see: Pillai, V. N. R. In *Organic Photochemistry*; Padwa, A., Ed.; Marcel Dekker: New York, 1987; Vol. 9.

Table 1. Isolated Yields of Oligonucleotides as a Function of Deprotection and Photochemical Cleavage Conditions

oligonucleotide sequence	photolabile linker	deprotection method	photolysis time (h), filter	% yield[a]
T_{12}	succinate	A; before *hν*	3; $CuSO_4$	87
T_{12}	succinate	B; before *hν*	3; $CuSO_4$	83
T_{20}	succinate	B; before *hν*	3; $CuSO_4$	70
T_{20}	succinate	B; after *hν*	3; $CuSO_4$	78
T_{20}	succinate	B; after *hν*	3; λ_{max} = 400 nm	74
T_{20}	succinate	B; after *hν*	6; λ_{max} = 400 nm	72
T_{20}	succinate	B; after *hν*	3; λ_{max} = 355 nm	74
T_{20}	succinate	B; after *hν*	6; λ_{max} = 355 nm	77
T_{12}	carbonate	B; before *hν*	1; $CuSO_4$	32
T_{12}	carbonate	B; before *hν*	3; $CuSO_4$	49
T_{12}	carbonate	B; before *hν*	9 $CuSO_4$	66
T_{12}	carbonate	B; before *hν*	24; $CuSO_4$	63
T_{12}	carbonate	B; after *hν*	6; λ_{max} = 400 nm	52
T_{12}	carbonate	B; after *hν*	9; λ_{max} = 400 nm	61
T_{12}	carbonate	C; before *hν*	9; $CuSO_4$	65
5′-dCATACTTAACTT	carbonate	C; before *hν*	9; $CuSO_4$	59
5′-dTACGCAATCCTAGATCTAAT	succinate	D; after *hν*	3; λ_{max} = 400 nm	76
5′-dTACGCAATCCTAGATCTAAT	succinate	D; after *hν*	6; λ_{max} = 400 nm	72

[a] Isolated yields expressed relative to yield of oligonucleotide obtained from identical deprotection procedure, but ammonium hydroxide cleavage. Deprotection method: (A) PhSH (1); Et_3N (2); 1,4-dioxane (2), 1 h; (B) neat diisopropylamine; 48 h; (C) ref 6; (D) concentrated NH_4OH, 12 h, 55 °C.

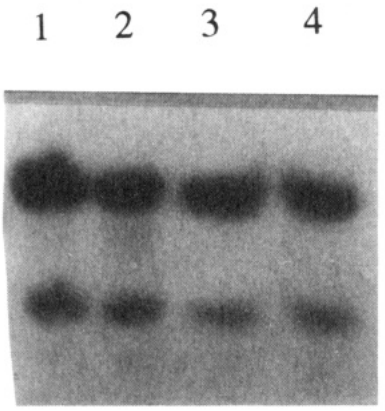
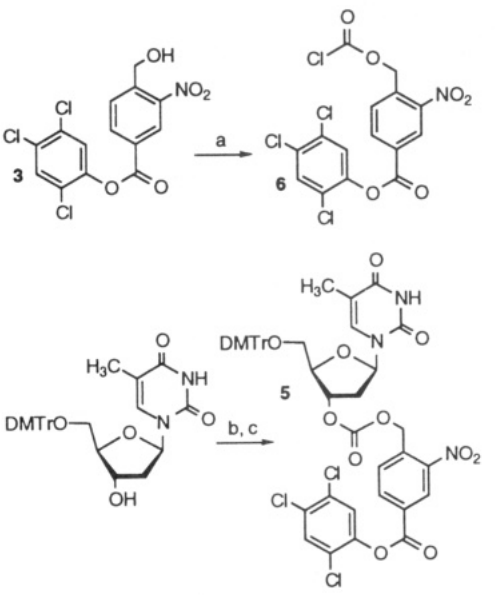


Figure 4. Denaturing gel electrophoresis (20%) of 5′-^{32}P labeled 3′-succianto T_{12}. Lanes 1 and 3: photochemically cleaved. Lanes 2 and 4: cleaved using concd NH_4OH. Lanes 3 and 4: subsequent treatment with 0.1 N NaOH.

not significantly increase the coupling yield. Consequently, oligonucleotides utilizing allyloxy phosphoramidites were synthesized using reaction cycles with a 210-s coupling time. In order to gauge the efficiency of the photocleavage of the dodecamer after treatment with Pd^0, resin from a synthesis utilizing allyloxy phosphoramidites was treated with $Pd(dba)_2$ and $P(Ph)_3$ as recommended in the literature.[6] Half of the resin was then subjected to $CuSO_4$-filtered photolysis for 9 h, while the remainder was cleaved using concentrated ammonium hydroxide for 2 h at 55 °C. As expected, the yield of photochemically cleaved material relative to that obtained via hydrolytic cleavage was close to that obtained for polythymidine using either β-cyanoethyl or allyloxy protecting groups (Table 1). The utility of photolabile resin **7** was further demonstrated in the preparation of an oligonucleotide containing thymidine, deoxyadenosine, and deoxycytidine nucleosides using allyloxy protecting groups. The isolated yield of the mixed sequence dodecamer (Table 1) was very near that of the comparable polythymidine.

The final issue which remains to be addressed is the integrity of the photochemically released oligonucleotides. The mixed sequence dodecamer (Table 1) was digested with snake venom phosphodiesterase. The resulting nucleotides were dephosphorylated with calf intestine

Scheme 1[a]



[a] Key: (a) 2 M phosgene in toluene, THF; (b) NaH, THF; (c) **6**, THF.

alkaline phosphatase.[13] HPLC analysis revealed the presence of several minor species in addition to deoxycytidine, thymidine, and deoxyadenosine (Figure 5). Digestion of a portion of the same oligonucleotide which was cleaved using ammonium hydroxide produced the same extraneous peaks, indicating that the additional peaks were not due to the photolysis conditions. Furthermore, the area ratios of the three nucleosides were within 5% of each other for the two different deprotection/cleavage schemes. The identification of the additional peaks in the chromatograms are unknown. We have independently verified that they are not allyloxy carbonyl containing nucleosides which were not deprotected during the palladium treatment and are not present in the enzyme or buffer solutions.

We were particularly concerned that the photolysis conditions would produce pyrimidine–pyrimidine pho-

(13) Eadie, J. S.; McBride, L. J.; Efcavitch, J. W.; Hoff, L. B.; Cathcart, R. *Anal. Biochem.* **1987**, *165*, 442.

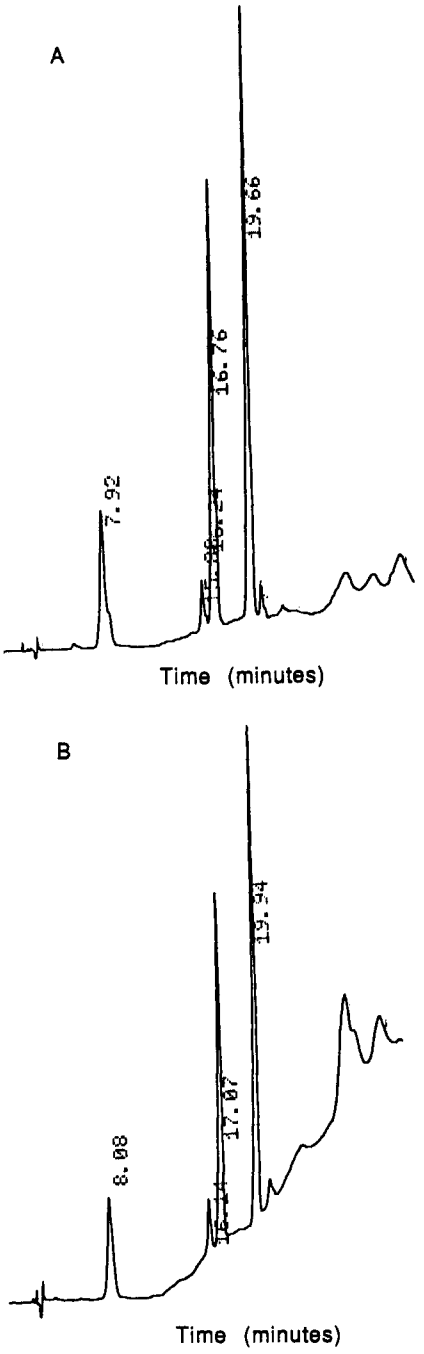


Figure 5. Base composition analysis of 5′-dCATACTTAACTT via enzymatic digestion. (A) Oligonucleotide cleaved using concentrated NH_4OH. (B) Oligonucleotide cleaved photochemically.

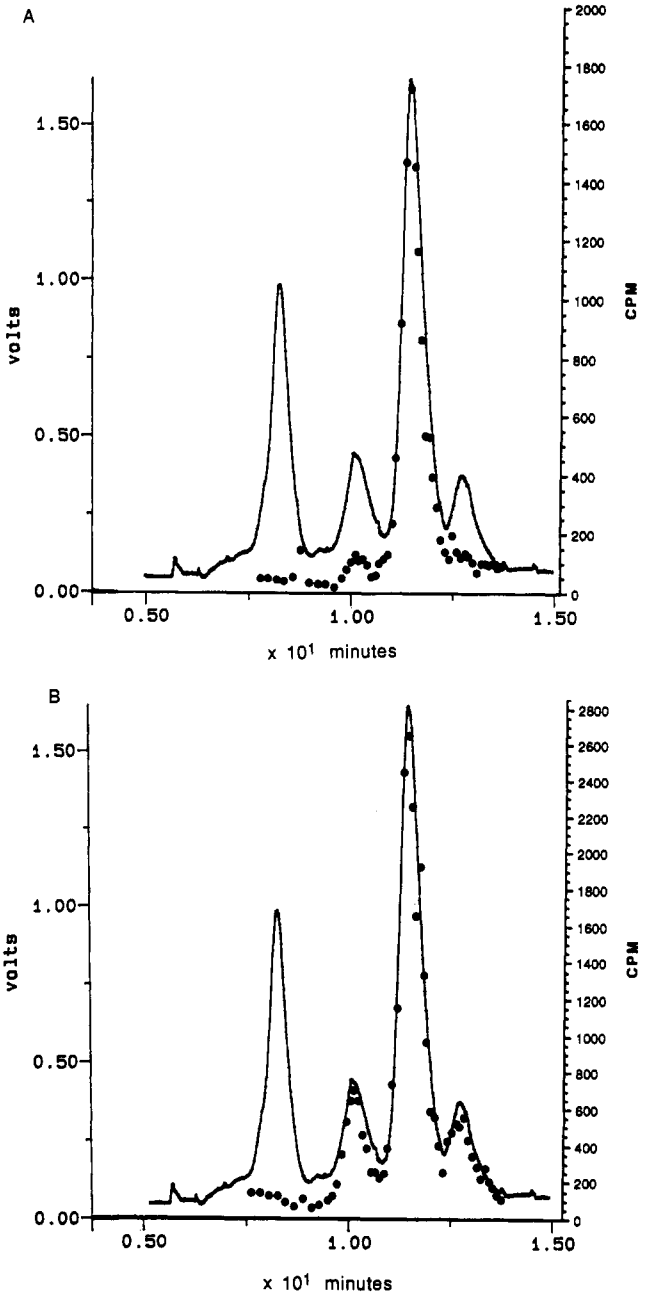


Figure 6. ^{3}H-Thymine–thymine dimer HPLC analysis. (A) 5′-T_3–[*methyl*-^{3}H]T–T_3–succinate photolyzed for 3 h. (B) 5′-T_3–[*methyl*-^{3}H]T–T_3–carbonate photolyzed for 9 h ($CuSO_4$ filter).

todimers (8–11). The cyclobutane adducts are a major lesion generated from nucleic acids via ultraviolet irradiation.[14] This possibility was investigated using two heptameric polythymidines in which the β-cyanoethyl groups were removed prior to photolysis using diisopropylamine. One oligonucleotide was prepared using the succinate linker, and the other utilized the carbonate moiety. Initially, oligonucleotides containing a single thymidine residue tritiated at its methyl group (specific activity: 3.95 Ci/mmol) incorporated at position four of the heptamer were subjected to 3- and 9-h $CuSO_4$-filtered photolyses for the succinate (4) and carbonate (7) linkers, respectively. After photolysis, the crude oligonucleotides were exhaustively hydrolyzed with formic acid (100 °C, 1 h), and the residue was analyzed by HPLC (Figure 6).[15] Formic acid hydrolysis releases the free nucleobases simplifying the HPLC analysis.

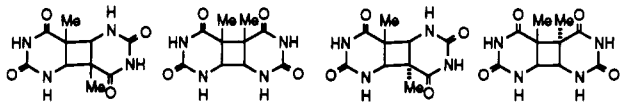
Conformational constraints of the oligonucleotide backbone prohibit formation of the *anti* photodimers (8, 10), leaving only two *syn* dimers (9, 11) as likely UV-induced damaged bases. Fortunately, both *syn* dimers are sepa-

(14) For a recent review see: Cadet, J.; Vigny, P. In *Bioorganic Photochemistry*; Morrison, H., Ed.; John Wiley: New York, 1990; Vol. 1.

(15) (a) Cadet, J.; Voituriez, L.; Hruska, F. E.; Kan, L.-S.; de Leeuw, F. A. A. M.; Altona, C. *Can. J. Chem.* **1985**, *63*, 2861. (b) Cadet, J.; Voituriez, L.; Hahn, B.-S.; Wang, S. Y. *J. Chromatogr.* **1980**, *195*, 139. (c) Jennings, B. H.; Pastra, S.-C.; Wellington, J. L. *Photochem. Photobiol.* **1970**, *11*, 215.

(16) Borer, P. N. In *Handbook of Biochem. and Mol. Biol.*; Fasman, G. D., Ed.; CRC Press: Boca Raton, 1975; p 589.

(17) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning A Laboratory Manual*; Cold Spring Harbor Laboratory: New York, 1982.



cis-anti (8) *cis-syn* (9) *trans-anti* (10) *trans-syn* (11)

rable from thymine by reversed-phase HPLC. The respective hydrolysates were resuspended in water and spiked with a mixture of nonradioactive thymine–thymine dimers and thymine.[15] Fractions were collected during the time in which the UV detector (230 nm) indicated that the nucleobase components eluted. A plot of radioactivity measured as a function of time indicated that a small amount of *cis-syn* photodimer (9) was formed during the 3 h in which the succinate resin was photolyzed. The amount of 9 was estimated to be <5% by both the cut and weigh method and the half-width peak height method. Superimposing the radiation profile and the UV chromatogram also suggests that a small amount of 11 is also formed during the photolysis of the succinate resin. Given this observation, it was not surprising that the longer photolysis time required to obtain moderate yields of oligonucleotide from the carbonate resin resulted in significantly larger amounts of photodimers. Nine hours of photolysis formed 14–17% of 9 relative to free thymine and approximately the same amount of 11. The amount of photodimers increased approximately linearly with irradiation time.

The extent of photodimer formation over the course of a 9-h photolysis is undesirable. Under the conditions used, the $CuSO_4$ solution filter should transmit less than 10% of the irradiation at 315 nm and should be opaque to wavelengths of light shorter than 310 nm. Considering that $CuSO_4$ solution filters are known to decay over time, the photocleavage process was investigated using more durable band pass filters.[18] Equally efficient photocleavage was achieved using band pass filters (Table 1). A vast improvement was observed with respect to the amount of photodimers formed. Irradiation of a tritiated heptathymidylate for 9 h using a band pass filter (λ_{max} = 400; 10% transmission at 330 nm) and subsequent hydrolysis showed less than 7% formation of 9 relative to liberated thymine (Figure 7). Extrapolation to shorter photolysis times required when using succinate resin 4 suggests that band pass filtering will reduce the amount of photodimers formed to less than 2%.

Summary

The intramolecular redox *o*-nitrobenzyl photochemical reaction enables one to photochemically cleave oligonucleotides from their solid-phase supports in good to excellent yield compared to standard alkaline hydrolysis. Eicosamers are sufficiently long to serve as genomic hybridization probes (>10^6 unique sites 20 base pairs long) and/or antisense therapeutics. Minor amounts of UV damage to the biopolymers are incurred upon prolonged irradiation required by carbonate resin 7. Other photochemical reactions should lessen the effects of this undesirable process by decreasing the photolysis time and/or shifting the irradiation wavelength to the red. When used in conjunction with phosphoramidites containing Pd^0 labile protecting groups, these linkers obviate the use of base during the deprotection of the oligonucleotide. The ability to carry out the cleavage reaction before or after deprotection of the nucleobases and phosphotriesters qualify these supports as orthogonal linkers. As such, the linkage of electrophilic and/or nucleophilic functionality between the 3′-terminal nucleosides and the photolabile moiety will facilitate the conjugation of such oligonucleotides in organic solvents.

(18) Murov, S. L. *Handbook of Photochemistry*; Marcel Dekker: New York, 1973.

Experimental Section

General Methods. ^1H NMR spectra were recorded at 300, 270, or 200 MHz. HPLC work was performed on a Waters 501 system with a Waters 490E programmable multiwavelength detector or a Waters 501 system with a Waters 440 fixed wavelength detector. Fractions were collected using an Isco Retriever II fraction collector. HPLC columns used were as follows: Waters μBondapak 10-μm C18 radial pak 8-mm i.d. (column A); Vydac weak anion-exchange oligonucleotide column 4.6 × 250 mm (column B); Nucleosil C18 5 μm 4.6 × 150 mm (column C). Photolyses were carried using a Oriel 1000-W high-pressure Hg/Xe lamp. $CuSO_4$ filters were 100 g/L, and the path length was 5 cm. Band pass filters were from Oriel (λ_{max} = 400 nm, #59820; (λ_{max} = 355 nm, #59810). Electrospray mass spectrometry was carried out on a VG Bio-Q. Radiation was counted using a Packard Tri-Carb 1500 liquid scintillation counter. [*methyl-*^3H]-Thymidine and γ-^{32}P ATP were purchased from Amersham.

All reactions were run under nitrogen atmosphere in oven-dried glassware, unless specified otherwise. Pyridine and diisopropylamine were freshly distilled from CaH_2. DMF was freshly distilled under aspirator pressure from CaH_2. Long-chain alkylamine-controlled pore glass support (CPG) and 5′-*O*-(4,4′-dimethoxytrityl)-3′-succinatothymidine were purchased from Sigma. Allyloxy phosphoramidites were prepared as described in the literature.[6] Snake venom phosphodiesterase and calf intestine alkaline phosphatase were obtained from Boehringer-Mannheim. T4 polynucleotide kinase was obtained from New England Biolabs.

Oligonucleotides were synthesized using an Applied Biosystem,s Inc., 380B automated synthesizer. Standard ABI synthesis cycles were used to prepare oligonucleotides containing β-cyanoethyl phosphoramidites and were altered to accommodate the coupling of allyloxy phosphoramidites. β-Cyanoethyl phosphorous protecting groups were removed prior to photolysis using anhydrous diisopropylamine or after photolysis with concentrated ammonium hydroxide.[7] Allyloxy protecting groups were removed as recommended.[6]

Polyacrylamide gel electrophoresis was carried out on 20% gels (19:1 *N,N′*-methylenebisacrylamide) containing 45% urea (w/v). Oligonucleotides were visualized using 254-nm light. Bands were cut out and eluted with 0.2 N NaCl, 1 mM EDTA, filtered through 0.22-μm Centrex filters, and desalted on C18 Sep-Pak cartridges. Oligonucleotides were quantitated by UV absorption at 260 nm. Molar extinction coefficients were calculated using the nearest neighbor method.[16] Oligonucleotides were labeled at their 5′-termini using T4 polynucleotide kinase and γ-^{32}P ATP as described.[17] Radiolabeled oligonucleotides were visualized via autoradiography using Kodak X-OMAT AR film.

Activated Photolabile Succinate (1). Dicyclohexylcarbodiimide (93 mg, 0.45 mmol) was added to a CH_2Cl_2 solution of 5′-*O*-(dimethoxytrityl)-3′-succinatothymidine (2, 268 mg, 0.415 mmol) and 3 (142 mg, 0.38 mmol). After 12 h, the solvent was removed in vacuo, and the residue was resuspended in Et_2O and filtered. The filter cake was washed twice with cold ether, and the organic layers were combined and concentrated. Column chromatography (1:4 EtOAc/CH_2Cl_2) yielded 180 mg (47.5%) of 1: mp 165–168 °C; ^1H NMR (270 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.80 (bd s, 1H), 8.42 (d, 1H, *J* = 6 Hz), 7.83 (d, 1H, *J* = 6 Hz), 7.60 (s, 1H), 7.57 (m, 2H), 7.40 (s, 1H), 7.38 (m, 1H), 7.27 (m, 7H), 6.82 (m, 4H), 6.44 (m, 1H), 5.65 (s, 2H), 5.50 (m, 1H), 4.13 (m, 1H), 3.78 (s, 6H), 3.45 (m, 2H), 2.72–2.84 (m, 4H), 2.42 (m, 2H), 1.38 (s, 3H); IR (KBr) 3325, 1737, 1690, 1623, cm^{-1}; ^{13}C NMR (67.9 MHz, $CDCl_3$) δ 172.2, 172.0, 164.1, 159.4, 151.0, 144.8, 138.7,

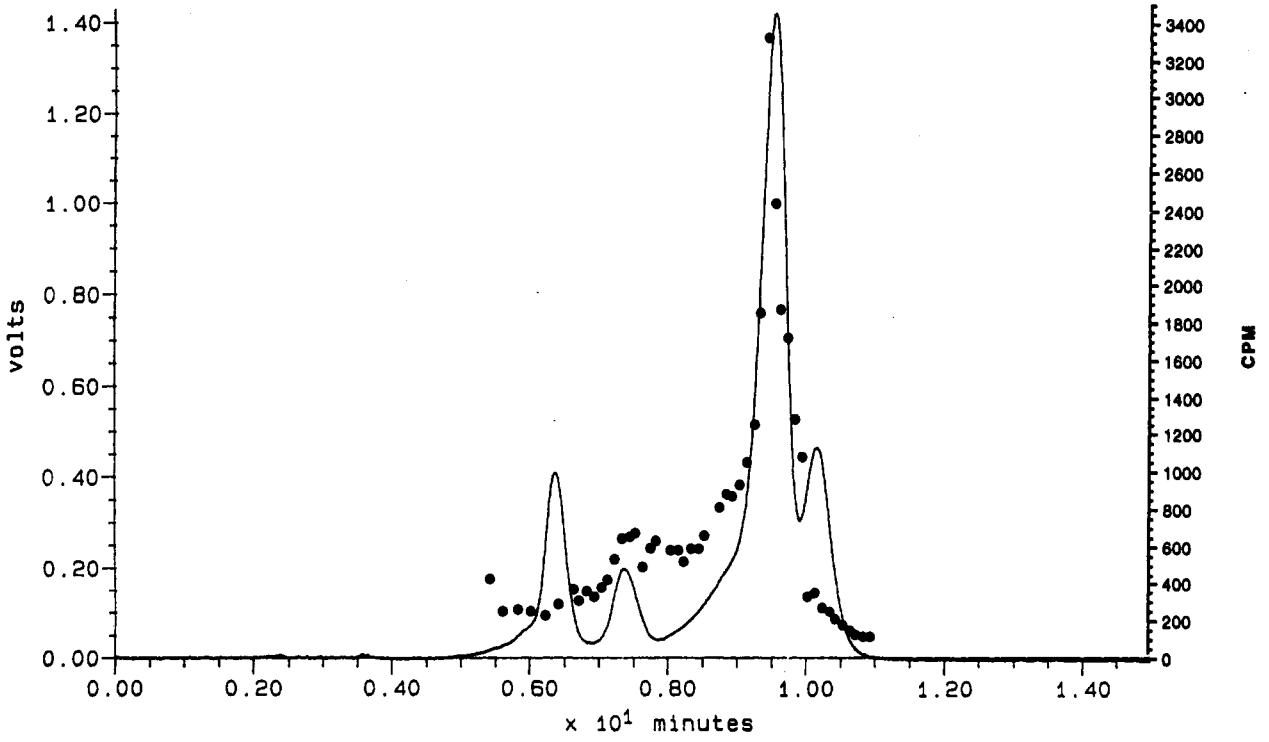


Figure 7. ^{3}H-Thymine–thymine dimer HPLC analysis of 5′-T$_3$–[*methyl*-^{3}H]T–T$_3$–carbonate photolyzed for 9 h (band pass filter; λ_{max} = 400 nm).

135.9, 135.8, 135.5, 131.9, 131.7, 130.7, 130.2, 130.1, 129.8, 128.8, 128.6, 128.4, 127.9, 127.7, 126.7, 125.8, 114.0, 113.8, 112.3, 87.9, 85.6, 85.0, 84.6, 64.3, 63.6, 55.9, 38.5, 29.6, 29.4, 12.2; HRMS FAB (M + Na) calcd 1026.1600, found 1026.1602.

Photolabile Succinate CPG Resin (4). A mixture of 1 (72 mg, 70 μmol), CPG (100 mg, ~3.5 μmol), and HOBt·hydrate (9.5 mg, 70 μmol) was shaken overnight in the dark at 25 °C using a vortexer. The resin was filtered, washed well with dry EtOAc, and dried under vacuum. Unreacted amine was capped by treatment with acetic anhydride (250 μL), pyridine (2 mL), and DMAP (25 mg) for 1 h. The resin was filtered, washed, and dried as described above. Free amine was measured on 1 mg of resin via quantitative ninhydrin analysis.[11a] Resin loading was measured by treatment with *p*-toluenesulfonic acid in CH_3CN and quantitation of the dimethoxytrityl cation by absorption spectroscopy (λ_{max} = 498 nm, $\epsilon = 7 \times 10^4$ M^{-1} cm^{-1}).[11b]

Activated Photolabile Carbonate (5). A solution of 3 (150 mg, 0.4 mmol) in THF (0.8 mL) was added to a phosgene/toluene solution (1.93 M, 0.83 mL, 1.6 mmol) at 25 °C. After the mixture was stirred for 2 h, nitrogen was bubbled through the reaction mixture for 1 h, and then the solvent was removed in vacuo. An aliquot of the crude product was analyzed by IR (solution cell, THF) and ^{1}H NMR (300 MHz, $CDCl_3$). IR showed two carbonyl stretches at 1760, 1784 cm^{-1}. ^{1}H NMR showed a shift of the benzylic protons from 5.14 to 5.75 ppm. The sodium alkoxide of dimethoxytritylthymidine (327 mg, 0.6 mmol) in THF (1 mL) prepared from NaH was added, and the reaction was stirred for 15 min at 25 °C. The reaction was quenched with $NaHCO_3$, diluted with EtOAc (100 mL), and washed with $NaHCO_3$ (50 mL) and brine (50 mL). The organic layer was separated, dried with Na_2SO_4, and concentrated to dryness under reduced pressure. The crude product was subjected to silica gel column chromatography (20 g silica gel) and eluted with EtOAc/hexanes (4:6) to afford 5 (175 mg, 47% yield): mp 138–142 °C; IR ($CDCl_3$) 3064, 2948, 2627, 1755, 1689, 1608, 1537, 1509, 1460, 1368, 1351, 1250, 1202, 1177, 1082, 1034, 960, 911, 829, 791, 734, 701, 672 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.93 (d, 1H, *J* = 1.7 Hz); 8.46 (dd, 1H, *J* = 1.70, 8.2 Hz), δ 8.33 (bs, 1H), 7.87 (d, 1H, *J* = 8.2 Hz), 7.61 (s, 1H), 7.59 (s, 1H), 7.44 (s, 1H), 7.22–7.40 (m, 9H), 6.81 (d, 4H, *J* = 8.9 Hz), 6.46 (q, 1H, *J* = 5.4, 9.1 Hz), 5.67 (s, 2H), 5.38 (d, 1H, *J* = 5.6 Hz), 4.23 (m, 1H); 3.77 (s, 6H), 3.42–3.55 (m, 2H), 2.45–2.60 (m, 2H), 1.38 (s, 3H); ^{13}C NMR (75.7 MHz, $CDCl_3$) δ 163.4, 161.2, 158.6, 153.7, 150.3, 146.9, 145.2, 143.9, 137.3, 135.0, 134.9, 134.8, 131.6, 131.1, 129.9, 129.1, 128.8, 127.9, 127.1, 126.8, 125.9, 125.0, 113.2, 111.6, 87.1, 84.1, 83.5, 79.5, 65.8, 63.5, 55.1, 37.7, 11.5; HRMS FAB (M$^+$) calcd 947.1472, found 947.1470.

Photolabile Carbonate CPG Resin (7). A mixture of 5 (80 mg, 85 μmol), long-chain alkylamine-controlled pore glass resin (130 mg, ~4.5 μmol free amine), and HOBt·hydrate (11.5 mg, 85 μmol) in DMF (2 mL) were treated as described above for the succinate resin.

General Procedure for Photolytic Cleavage of Oligonucleotides from Solid-Phase Supports. Resin was stirred in a Pyrex tube containing 3 mL of a 9:1 mixture of CH_3CN/H_2O. The mixture was sparged with N_2 for 30 min before photolysis. The volume above the solution was continuously purged with N_2 during photolysis. The output (800 W) of a high-pressure Hg/Xe lamp was filtered using an aqueous solution of $CuSO_4$.[18] The resin was filtered through a 0.45-μm filter upon completion of the photolysis. The tube and filter were washed with H_2O (3 × 1 mL). The filtrates were concentrated, combined, and subjected to the appropriate deprotection and/or purification method.

Anion-Exchange HPLC Characterization of Oligonucleotides. Column B was employed using a gradient. Eluent A: 0.1 M $(NH_4)_2PO_4$ (pH 6.7), 20% CH_3CN (v/v). Eluent B: 0.3 M $(NH_4)_2PO_4$ (pH 6.7), 20% CH_3CN (v/v). 0–30% B linearly over 20 min. Flow rate: 1.5 mL/min.

Enzymatic Digestion of 5′-dCAT ACT TAA CTT. Snake venom phosphodiesterase (30 μL; 0.1 unit/μL) in 0.1 M Tris–acetate buffer (pH 8.75) were added to an Eppendorf containing 0.1 OD of 5′dCAT ACT TAA CTT which had been concentrated to dryness. The tube was vigorously vortexed, spun briefly, and immersed in a 37 °C water bath. After 1.5 h, 1 μL of calf alkaline phosphatase (10 unit/μL) was added to the mixture. The solution was diluted to 250 μL after an additional hour at 37 °C, heated to 95 °C for 5 min, and immersed in an ice–water bath. The solution was centrifuged at 14 000 rpm for 10 min prior to HPLC analysis (column C). Gradient conditions: A, 0.1 M triethylammonium acetate (pH 7.0); B, CH_3CN. 0–30% B linearly over 30 min. Flow rate: 1.5 mL/min.

[*methyl*-^{3}*H*]-5′-*O*-(4,4′-Dimethoxytrityl)thymidine (12). [*methyl*-^{3}*H*]Thymidine (2.5 mCi; specific activity 82 Ci/mmol) and thymidine (121 mg, 0.5 mmol) were dried from pyridine (5 mL, 2 x). The mixture was resuspended in pyridine (5 mL) and allowed to react overnight at 0 °C with 4,4′-dimethoxytrityl chloride (186 mg, 0.55 mmol). The reaction was quenched with

CH_3OH (1 mL), diluted with EtOAc (50 mL), washed with saturated NH_4Cl (10 mL), and dried with Na_2SO_4. The product (235 mg, 86%) was purified by flash chromatography (EtOAc/hexanes/CH_3OH (3:6.5:0.5)). Spectra and melting point were identical to those for commercially available material. Specific activity: 3.95 Ci/mol.

[*methyl*-^{3}*H*]-5′-*O*-(4,4′-Dimethoxytrityl)thymidine-3′-(β-cyanoethyl-diisopropylphosphoramidite) (12) (40 mg, 0.073 mmol) was dissolved in CH_2Cl_2 (1 mL). To this was added bis(diisopropyl)ethylamine (2.0 equiv, 0.13 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (1.8 equiv, 0.15 mmol), and the reaction was stirred for 3 h at 0 °C. The reaction was quenched with CH_3OH, diluted with EtOAc, washed with saturated Na_2CO_3 and brine, and dried over Na_2SO_4. The product (28 mg, 52% yield) was purified by column chromatography (EtOAc/CH_2Cl_2 (1:9–3:7)). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (bs, 1H), 7.6 (d, 1H, J = 14 Hz), 7.21–7.40 (m, 9H), 6.81 (dd, 4H, J = 3.17, 8.86 Hz), 6.38 (dd, 1H, J = 6.06, 13.9 Hz), 4.62 (m, 1H), 4.23 (m, 1H), 3.76 (s, 6H), 3.40–3.70 (m, 4H), 2.02–2.70 (m, 6H), 1.40 (s, 3H), 0.9–1.38 (m, 12H); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 149.16 and 149.58; specific activity = 3.95 Ci/mol.

Tritium Quenching. A tritium quenching curve was constructed by using 10 standards with an activity of 262 154 CPM. Region A was counted from 0 to 3.5 keV. Region C was counted from 0 to 18.6 keV. The count ratio was determined by dividing region A by region C. The yield of quenching was determined by dividing region C by the activity of each standard. A graph of yield versus count ratio was constructed. The line was fourth order, and the correlation coefficient was 1.00. To correct for quenching, a sample is counted using the same regions and a count ratio is calculated. From this count ratio the yield can be determined from the quenching curve. The activity in region C is divided by the yield to obtain the corrected activity.

Thymine–Thymine Dimer Analysis. A heptamer of thymidines was synthesized on a 0.2 μmol scale using standard synthesis cycles in which the fourth thymidine from the 3′ end was tritiated. The oligonucleotide was deprotected at phosphorous using dry diisopropylamine for 24 h. Depending upon whether linker 4 or 7 was used, the oligonucleotide was cleaved from the resin by photolyzing for 3 or 9 h, respectively. The crude material was filtered and concentrated as described above. One fourth of the crude oligonucleotide was treated with formic acid for 1 h at 115 °C. After the formic acid was removed, the residue was resuspended in 100 μL of H_2O and coinjected with a mixture of the four diastereomers of thymine–thymine dimers and thymine on to the HPLC (column A). Isocratic eluent: 1% THF in H_2O. Flow rate: 1 mL/min. Fractions were collected every 6 s. These fractions were transferred to liquid scintillation vials, and 10 mL of liquid scintillation cocktail mixture was added. After being allowed to stand for 6 h, each fraction was counted for ^{3}H for 2 min and corrected for tritium quenching using channel ratioing.

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