Proofing of Photolithographic DNA Synthesis with 3′,5′-Dimethoxybenzoinyloxycarbonyl-Protected Deoxynucleoside Phosphoramidites

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We have evaluated in a microchip format the photochemical solid-phase phosphoramidite DNA synthesis method we previously developed. A set of nucleoside building blocks with "easy-off" base protecting groups was prepared bearing photolabile 5′-O-dimethoxybenzoincarbonate (DMBOC) groups. Photolysis rates and cycle yields for these DMBOC-protected nucleotides covalently attached to planar, derivatized glass surfaces were determined by fluorescence imaging-based methods earlier developed by McGall et al. and described in detail elsewhere. Data were obtained for both 280/310 and 365/400 nm irradiation in a range of solvents. Deprotection of the DMBOC occurs fastest in a nonpolar medium or without solvent. The coupling efficiency of these amidites in the synthesis of homopolymers was determined to be in the range 80−97%, with purines generally showing lower efficiency than pyrimidines. These DMBOC-protected monomers were used to prepare a 4 × 4 array of 16 decanucleotides of the sequence 5′-AAXTAXCTAC−chip, where X = A, C, G, or T. The array was hybridized with a target deoxyeicosanucleotide of the sequence fluorescein-5′-CTGAACG-**GTAGCATCTT**GAC. Surface fluorescence imaging demonstrated sequence-specific hybridization to this probe.

Introduction

The determination and utilization of DNA sequence information is crucial to modern biology. Avant garde methods for analysis of nucleic acid sequences include "chip" methodologies[1] in which DNA is attached at microscopic sites on a surface and permitted to hybridize to complementary sequences in an analyte DNA. The detection of such hybrid formation has been based on a variety of technologies, including fluorescence microscopy and primer extension reactions.[2] The preparation of microarrays can be accomplished by delivery of presynthesized oligonucleotides[3] or in situ synthesis.[4] The latter affords the opportunity for the principles of combinatorial chemistry to be applied so that the number of sequences synthesized far exceeds the number of chemical steps required. One combinatorial chemistry method that has been applied to the preparation of DNA on chip-like surfaces is light-directed synthesis.[5] We have developed a novel photoremovable protecting group, dimethoxybenzoincarbonate (DMBOC),[6] for light-directed synthesis and evaluated it for 5′-hydroxyl protection in phosphoramidite-based DNA synthesis.[7] We wished to evaluate this chemistry in a chip format using methods that have been developed earlier and described elsewhere.[8] A minor modification from earlier practice was required, involving rapidly removable base protecting groups (phenoxyacetyl (PAC) for A and G, isobutyryl (*i*-Bu) for C)[9] so that the final deprotection of the array could be carried out under mild conditions that would not cleave the oligonucleotides

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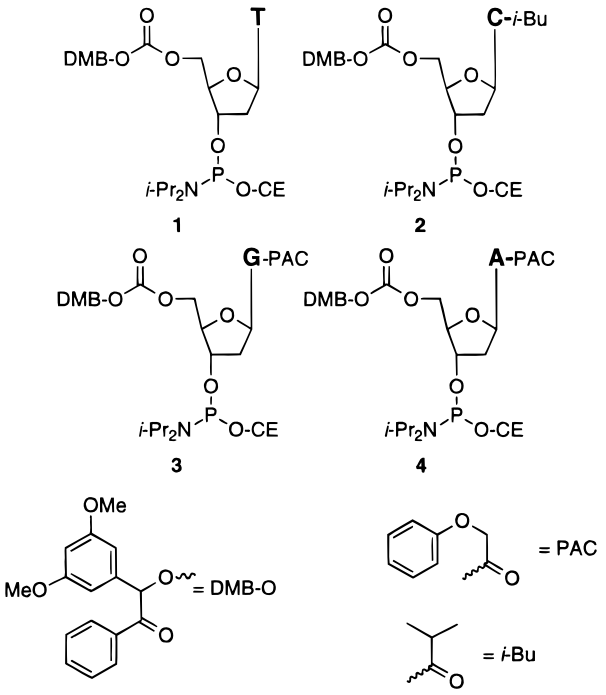
Chart 1



from the surface. We also earlier showed that benzoyl C was photoreactive,[7] so the benzoyl protecting group had to be avoided.

Results

Synthesis of three novel nucleoside phosphoramidite monomers **2**−**4** (Chart 1) needed for this study was accomplished along lines parallel to those used in our earlier work. That is, the azeotropically dried, base-protected A, G, and C nucleosides were treated with (dimethoxybenzoin)carbonylimidazolium triflate to selectively derivatize the 5′-hydroxyl. The 3′-hydroxyl was converted to the 2-cyanoethyl phosphoramidite using commercial reagents according to literature protocols.[10] These compounds were purified by repetitive precipitation from 6:1 hexanes/methylene chloride. Compound **1** was prepared in our earlier work.

Hydroxyalkylsilanated glass microscope slides[5a] were derivatized with a poly(ethylene glycol) linker terminated with a MeNPOC photoremovable nitrobenzyl group by coupling with MeNPOC−PEG−2-cyanoethyl phosphoramidite.[8] The resulting substrate (**5**) was clamped in a sealed flowcell through which a modified automated DNA synthesizer could deliver reagents.

The rate of photolysis at two different wavelengths of these monomer units when bound to the glass surface was first determined by the protocol described in Scheme 1, wherein the black regions indicate the area of the surface irradiated in a given step.[8] Rear irradiation of this surface (contacted with dioxane) through a 0.8 × 12.8 mm horizontal striped photolithographic mask was used to generate (in quadruplicate) parallel regions of free hydroxyl groups. Successive coupling of phosphoramidites **1**−**4** to the hydroxylated regions was performed. This resulted in surface **6**. It was then irradiated through a single striped mask with a 0.4 × 12.8 mm vertical feature for increasing time intervals (0−200 s at 280/310 nm, 0−800 s at 365/400 nm), translating the mask by 0.4 mm between irradiation cycles. The resulting surface **7** was then coupled to a fluorescein phosphoramidite, the protecting groups were removed by brief base treatment, and the slide was subjected to quantitative fluorescence scanning using a confocal microscope. Examples of a typical image and kinetic plot are shown in Figures 1 and 2. The data show a first-order exponential increase

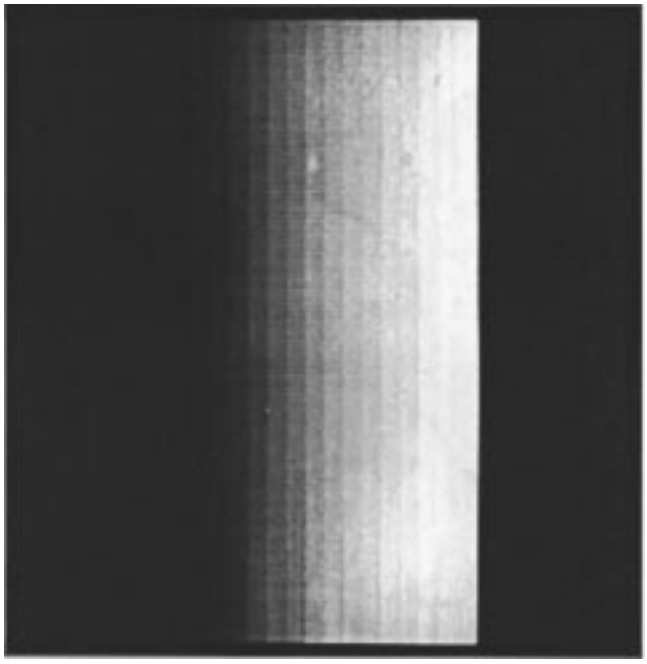
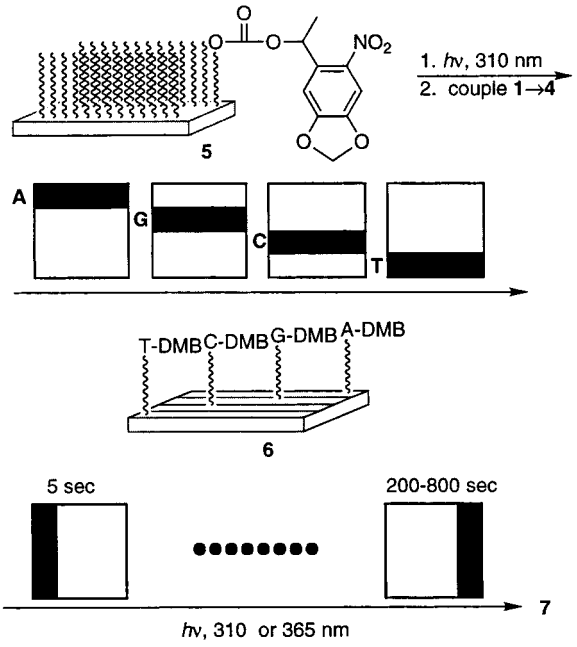


Figure 1. Surface fluorescence image of surface-bound DMBOC-protected isobutyryldeoxycytidine **7** after deprotection at 365 nm for up to 200 s followed by staining with fluorescein phosphoramidite.

Scheme 1



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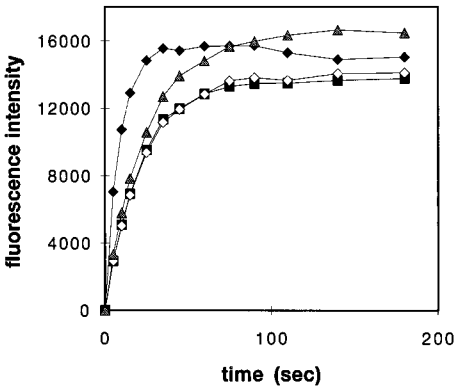

Figure 2. Plots of the data derived from Figure 1 of the photodeprotection at 365 nm of surface-bound DMBOC-protected (▲) deoxyadenosine, (■) deoxyguanosine, (◆) deoxycytidine, and (◇) thymidine in dioxane.

Table 1. Half-Lives for Photochemical Deprotection of DMB-carbonate-Protected Nucleosides on a Glass Surface in the Presence of Dioxane

	$t_{1/2}$, s	
DMB-carbonate nucleoside	310 nm	365 nm
dA	11	14
dG	13	17
dC	5.5	5.6
T	12	16

in fluorescence from which the half-lives were derived by curve fitting. Data on wavelength dependence were obtained with a mercury arc source filtered selectively in the "near-UV" (primarily Hg lines at 365 and 405 nm) and the "mid-UV" (lines at 280 and 310 nm). Summarized in Table 1 are the data from these experiments.

The effect of solvent on the rate of deprotection was examined for DMBOC−T and DMBOC-dC^{i-Bu}. Surface **8** (Scheme 2) was prepared by uniform irradiation of **5** and coupling with **1**. The kinetics of deprotection at 310 nm were determined using an experimental protocol similar to that described in Scheme 1, in the presence of dioxane, toluene, and methanol and without solvent. Data are given in Table 2.

The coupling efficiency of amidites **1**-**4** in the synthesis of homopolymers up to dodecamers was determined using the protocol described in Scheme 2.[8] Surface **8** and analogues bearing the other three bases were prepared by uniform irradiation of **5** and coupling with **1**−**4**. A photolithographic mask with a 6.4 × 12.8 mm vertical stripe was used in a first irradiation and coupling cycle. The irradiation was conducted for 8−10 half-lives based on the values earlier determined. Coupling of the phosphoramidite was performed, the mask was translated by 492 μm, and the cycle was repeated. Due to overlap of the areas of irradiation at each of the steps, this protocol leads to the synthesis of dodecanucleotides at the center of the slide, reflecting 12 coupling steps. The flanking regions reflect monotonically decreasing numbers of coupling cycles. After the full surface was coupled, half of the array was subject to uniform irradiation to free the 5′-hydroxyl groups, which were coupled with a fluorescein phosphoramidite. Fluorescence imaging with correction for background permitted the yield to be determined for each cycle on the basis of the relative fluorescence intensity of adjacent regions, whose oligonucleotides differ in length by one base. In Figure 3 is the fluorescence image for a sample $(dC)_n$ array. The

Scheme 2

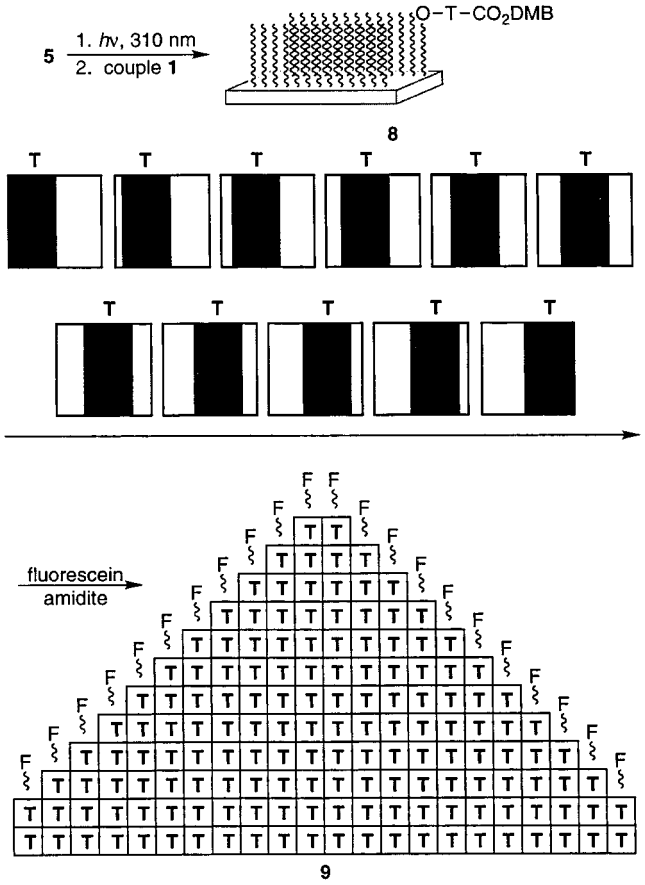

Table 2. Solvent Dependence of Half-Lives for Mid-UV Photochemical Deprotection of DMB-carbonate-Protected Nucleosides on a Glass Surface

	$t_{1/2}$	
solvent	T	dC
dioxane	12	5.5
toluene	9.3	6.5
methanol	12	8.4
acetonitrile	10	7.4
dry	5.8	5.6

surface fluorescence decreases somewhat toward the center of the pattern, as the yield of the full-length oligomer decreases with increasing length. Quantitative evaluation of the fluorescence intensity (I) data using eq 1 gives step yields.

$$\% \text{ yield (step } n) = 100 \times (I_n/I_{n-1}) \qquad (1)$$

Data for cycle yields and calculated aggregate yields for homopolymer synthesis from the experiment in Scheme 2 are tabulated in the Supporting Material. Representative data are shown in Figure 4. The ranges are as follows: T, 91 → 98%; C, 82 → 95%; G, 79 → 92%; A, 74 → 84%.

The amidites **1**−**4** were used in the preparation of a small oligonucleotide array for a hybridization study. Sixteen decanucleotides of the sequence 5′-AAXTAXC-TAC−chip were prepared, where the positions marked by X comprise all combinations of nucleotides. These oligonucleotides were prepared in the layout shown in Figure 5, using striped masks with 3.2 × 12.8 mm features, resulting in a 4 × 4 array of square hybridiza-

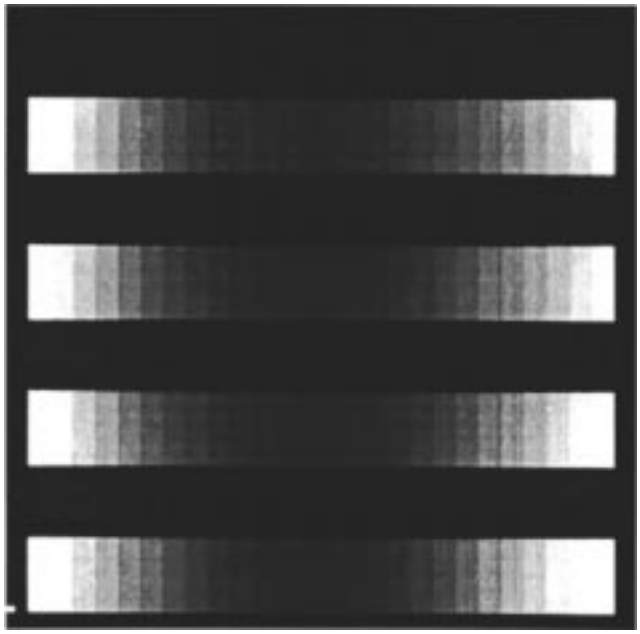

Figure 3. Fluorescence image of surface **9** after the synthesis of an array of $(dC)_n$ homooligomers followed by staining with fluorescein phosphoramidite.

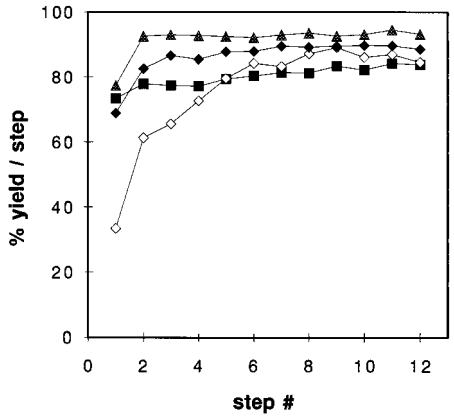

Figure 4. Plot of the calculated coupling efficiencies versus cycle number for $(dN)_n$ homooligomer synthesis: (■) deoxyadenosine, (◇) deoxyguanosine, (◆) deoxycytidine, and (▲) thymidine.

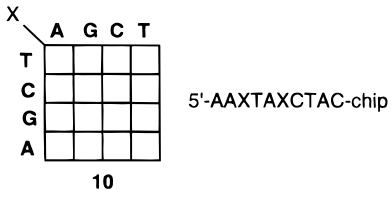

Figure 5. Format of a 16-element hybridization array **10** based on the decanucleotide sequence 5′-AAXTAXCTAC−chip.

tion areas of dimensions 3.2 × 3.2 mm. The array was hybridized at 23 °C with a target deoxyeicosanucleotide of the sequence fluorescein−5′-CTGAACG**GTAGCATCTT**GAC, where the emboldened residues are complementary to one of the sequences (the G, G hybridization area) of the surface **10**. The fluorescence image of the hybridized probe is shown in Figure 6. The average signal-to-background ratio of the hybridized region compared to the nonmatching cells is >6.

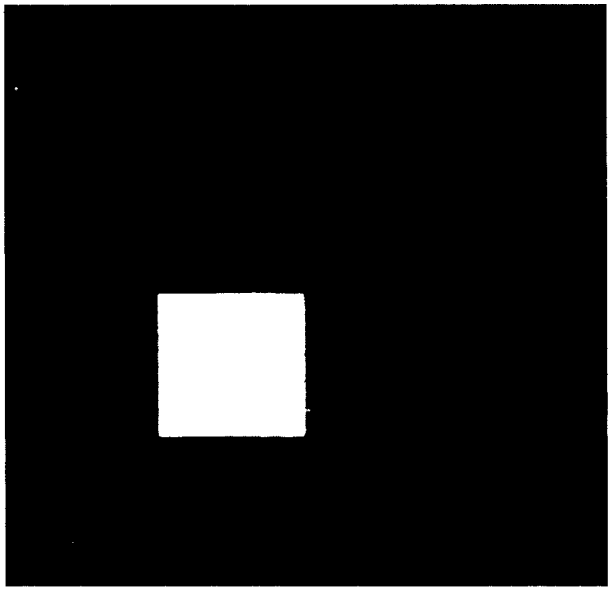

Figure 6. Fluorescence image of hybridization array surface **10** after staining at 23 °C with the probe fluorescein−5′-CTGAACG**GTAGCATCTT**GAC at 10 nM concentration. The region of complementarity with the target site in the array is emboldened.

Discussion and Conclusion

Similar (only ∼2-fold different) DMBOC deprotection half-lives were observed with 365 nm compared to 310 nm irradiation, though the intensity produced by the mercury source is much higher at the longer wavelength. This is not due simply to the lower absorptivity of the chromophore in this region, since it is almost negligible at $\lambda > 350$ nm. The quantum efficiency for removal of DMBOC groups is increased at longer wavelength.[11] Irradiation with wavelengths <340 nm should be avoided in any event for oligonucleotide synthesis, based on the potential photochemical damage to the DNA.[12] Solvent (including its complete omission) has only a modest influence on the deprotection rate of surface-bound DMBOC groups, with fastest rates observed without solvent. This is not to say that the surface was in contact with a vacuum during irradiation; it was rinsed with anhydrous acetonitrile and dried under a stream of argon, and indeed it is possible that some residual solvent adheres to the surface. The data do show that solvent is not a major influence with this protecting group. The significant variation in the deprotection half-lives for the four different nucleotides is somewhat surprising. It is interesting to compare the half-lives determined in this surface format with those earlier found[13] for DMB-phosphates (using a Rayonet reactor) in solution: T, 109 s; C, 105 s; A, 95 s; G, 81 s; 5′-Ac-T, 82 s; 5′-Ac-C, 71 s. There is not a consistent pattern within this latter data set, and trends between the sets are also not apparent. It was also earlier observed on larger scale[6,7] that the rate slows with greater extent of deprotection, but the concentration of photolabile groups in the chip format is sufficiently low that this is not observed.

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While 98% cycle yields have been obtained in a best case (T), none of the other heterocyclic bases is as efficient. Cytidine can also approach this efficiency, but cycle yields with purines can be lower. These coupling efficiencies agree with those determined in our earlier work in which the oligonucleotides were prepared on controlled-pore glass. The cause of the base dependence is unknown: we believe the base may influence the course of the product-forming photoreactions and thereby the final absolute yield of deprotected product (5′-OH). While these results demand improvement in order to maximize the fidelity of the in situ synthesized oligonucleotides and therefore of nucleic acid hybridization, it is interesting that the results of an actual hybridization experiment show little difficulty with discriminating a perfect hybrid from internal one-base mismatches despite the imperfect sequence fidelity that is a consequence of the less-than-quantitative cycle yields. The interest is increased by the fact that the purines, which are less efficient in the synthesis, are in the majority in the perfectly matched hybridization probe. An earlier experiment with a 16 × 16 array of octanucleotides gave comparable results in terms of the fidelity of hybridization.[5a] It is clear that light-directed in situ synthesis of short oligonucleotides will be an effective method to prepare hybridization surfaces ("DNA chips") for detection of complementary sequences. This work further highlights the issues of coupling efficiency in DNA array synthesis.

Experimental Section

General. The 2′-deoxynucleosides A, G, and T were obtained from Cruachem. Protected 2′-deoxycytidine was obtained from Sigma. DNA synthesis reagents were obtained from Applied Biosystems. Fluorescein phosphoramidite (Fluoreprime) and DMTr−thymidine−CE−amidite were obtained from Pharmacia. Other reagents were from Aldrich.

Preparation of a 0.50 M Solution of (3″,5″-Dimethoxybenzoin)carbonylimidazolium Triflate (DMIT) in Nitromethane. To a solution of 1.96 g (11.96 mmol) of carbonyldiimidazole (azeotroped twice from benzene) in 19.0 mL of nitromethane, cooled in an ice bath, was added 2.71 mL (23.95 mmol) of methyltrifluoromethanesulfonate dropwise over 10 min. The solution was stirred for 30 min at 25 °C and then transferred via cannula to a flask containing 3.26 g (11.97 mmol) of dimethoxybenzoin (azeotroped twice from benzene). The reaction mixture was allowed to stir for 3 h at 25 °C. Complete formation of the DMIT reagent was confirmed by ^{1}H NMR.

***N*-Isobutyryl-2′-deoxycytidine-5′-(3″,5″-dimethoxybenzoin)carbonate.** To a solution of *N*-isobutyryl-2′-deoxycytidine (0.83 g, 2.80 mmol, azeotroped twice from pyridine) was added 3.32 mL of 0.5 M DMIT followed by 2 mL of dry pyridine. The reaction mixture was stirred at 25 °C for 8 h and then concentrated in vacuo. The residual oil was taken up in dichloromethane and washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated. The resulting oil was purified by flash chromatography on a silica gel column with EtOH/CH_2Cl_2 (4:96 v/v) to give 0.83 g of the desired compound (50%). ^{1}H NMR ($CDCl_3$): δ 1.21 (6H, m), 2.10−2.23 (1H, m), 2.58−2.85 (2H, m), 3.76 (6H, m), 4.20−4.60 (4H, m), 6.35 (1H, m), 6.42 (1H, m), 6.57 (2H, m), 6.64 (1H, m), 7.38−7.60 (3H, m), 7.94 (2H, m), 8.08 (1H, d, J = 7.5 Hz), 8.17 (1H, d, J = 7.5 Hz), 8.5 (1H, br s). HRMS (FAB, MH^+) calcd for $C_{30}H_{34}N_3O_{10}$: 596.2244. Found: 596.2232. UV (CH_3CN): λ_{max} 245 (ϵ 10 350), 295 (ϵ 3740) nm (ϵ_{280} 3400, ϵ_{310} 3200, ϵ_{365} 30, ϵ_{405} 5).

***N*-Phenoxyacetyl-2′-deoxyguanosine-5′-(3″,5″-dimethoxybenzoin)carbonate.** 2′-Deoxyguanosine (1.5 g, 5.61 mmol, azeotroped twice from pyridine) was dissolved in 30 mL of anhydrous pyridine and cooled in an ice bath. After the addition of 4.3 mL (33.9 mmol) of trimethylsilyl chloride, added dropwise over 20 min, the reaction mixture was allowed to stir for 1 h at 25 °C. In a separate round-bottom flask, 2.19 g (16.21 mmol) of 1-hydroxybenzotriazole was azeotroped from benzene and dissolved in 10 mL of dry CH_3CN. Phenoxyacetyl chloride (2.33 mL, 16 87 mmol) was slowly syringed into the solution of hydroxybenzotriazole and the reaction was allowed to stir for 5 min, when the mixture solidified. Pyridine and CH_3CN were added until the reaction mixture was homogeneous. The nucleoside was then transferred via cannula to the acylating solution and the reaction was allowed to stir overnight at 25 °C. The solution was cooled in an ice bath and quenched by the addition of 10 mL of H_2O followed by 5 mL of NH_4OH. The solution was stirred for 20 min and concentrated in vacuo. The residual oil was taken up in 100 mL of H_2O and washed with 50 mL of $CHCl_3$ (3×) and 50 mL EtOAc (1×), upon which a lavender solid began to precipitate out of the water. The solution was cooled in an ice bath and the solid collected and dried in vacuo to give 1.32 g of *N*-phenoxyacetyl-2′-deoxyguanosine (59%). ^{1}H NMR (DMSO): δ 2.24−2.33 (1H, m), 2.54−2.64 (1H, m), 3.50−3.60 (2H, m), 3.85 (1H, m), 4.39 (1H, m), 4.87 (2H, s), 4.98 (1H, m), 5.34 (1H, m), 6.23 (1H, t, J = 6.9 Hz), 6.99 (3H, m), 7.32 (2H, m), 8.26 (1H, s).

To a solution of *N*-phenoxyacetyl-2′-deoxyguanosine (0.93 g, 2.32 mmol, azeotroped twice from pyridine) was added 4.6 mL of 0.5 M DMIT followed by 4 mL of dry pyridine. The reaction mixture was stirred overnight at 25 °C and then concentrated in vacuo. The residual oil was taken up in dichloromethane and washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated. The resulting oil was purified by flash chromatography on a silica gel column using a step gradient of 2:98−5:95−10:90 EtOH/CH_2Cl_2 to give 0.91 g of the desired compound (56%). ^{1}H NMR ($CDCl_3$): δ 2.50 (1H, m), 2.60−2.80 (1H, m), 3.69 (6H, m), 4.25−4.80 (6H, m), 6.30 (1H, m), 6.36 (1H, m), 6.49 (2H, m), 6.54 (1H, m), 6.93 (2H, m), 7.05 (1H, m), 7.26−7.40 (4H, m), 7.46 (1H, m), 7.81 (2H, m), 7.95 (1H, m). UV (CH_3CN): λ_{max} 251 (ϵ 18 800), 276 (ϵ 13 000) nm (ϵ_{280} 14 200, ϵ_{310} 5800, ϵ_{365} 51, ϵ_{405} 16). Anal. Calcd for $C_{35}H_{33}N_5O_{11}$: C, 59.38; H, 4.84; N, 10.19; O, 25.59. Found: C, 59.51; H, 4.86; N, 10.08.

***N*-Phenoxyacetyl-2′-deoxyadenosine-5′-(3″,5″-dimethoxybenzoin)carbonate.** To a solution of 2.00 g (7.96 mmol) of 2′-deoxyadenosine (azeotroped twice from pyridine) in 80 mL of anhydrous pyridine, cooled in an ice bath, was added 5.10 mL (40.2 mmol) of trimethylsilyl chloride. The solution was allowed to stir for 30 min at 25 °C, followed by the addition of 2.20 mL (15.9 mmol) of phenoxyacetyl chloride. The reaction was stirred for 2 h at 25 °C and then quenched by the addition of 100 mL of saturated $NaHCO_3$. The pyridine was removed in vacuo, followed by the addition of 300 mL of CH_2Cl_2. The organic layers were combined and washed with saturated NaCl, dried over $MgSO_4$, and concentrated to give a yellow foam. The foam was purified by flash chromatography on silica gel using a step gradient of 5:95−10:90−15:85 EtOH/CH_2Cl_2 to give 1.38 g of *N*-phenoxyacetyl-2′-deoxyadenosine (46%). ^{1}H NMR (DMSO): δ 2.32−2.39 (1H, m), 2.74−2.83 (1H, m), 3.52−3.68 (2H, m), 3.91 (1H, m), 4.46 (1H, m), 5.04 (2H, s), 5.38 (1H, m), 6.47 (1H, t, J = 6.6 Hz), 6.95−7.00 (3H, m), 7.29−7.34 (2H, m), 8.69 (1H, s), 8.72 (1H, s).

To a solution of *N*-phenoxyacetyl-2′-deoxyadenosine (1.20 g, 3.11 mmol, azeotroped twice from pyridine) was added 6.23 mL of 0.5 M DMIT followed by 10 mL of dry pyridine. The reaction mixture was stirred overnight at 25 °C and then concentrated in vacuo. The residual oil was taken up in dichloromethane and washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated. The resulting oil was purified by flash chromatography on a silica gel column using a step gradient of 2:98−6:94 EtOH/CH_2Cl_2 to give 0.58 g of the desired compound (30%). ^{1}H NMR ($CDCl_3$): δ 2.55−2.66 (1H, m), 2.86−2.95 (1H, m), 3.68−3.75 (6H, m), 4.27−4.56 (3H, m), 4.73−4.89 (3H, m), 6.41 (1H, m), 6.56 (1H, m), 6.59 (2H, m), 6.64 (1H, s), 7.05 (3H, m), 7.30−7.60 (5H, m), 7.91 (2H, m), 8.32 (1H, m), 8.77 (1H, s), 9.40

(1H, br s). HRMS (FAB, MH^+) calcd for $C_{35}H_{34}N_5O_{10}$: 684.2305. Found: 684.2308. UV (CH_3CN) λ_{max} 271 (ϵ 28300), 256 (ϵ 27500) nm (ϵ_{280} 21200, ϵ_{310} 1900, ϵ_{365} 68, ϵ_{405} 19).

UV Spectra. To enable the relative photoefficiencies of the different compounds under different experimental conditions to be calculated, detailed UV data are provided for the foregoing compounds and the known thymidine-5′-(3″,5″-dimethoxybenzoin)carbonate): UV (CH_3CN) λ_{max} 250 (ϵ 21 400) nm (ϵ_{280} 8900, ϵ_{310} 1140, ϵ_{365} 22).

***N*-Isobutyryl-2′-deoxycytidine-5′-(3″,5″-dimethoxybenzoin)carbonate-3′-cyanoethyl(diisopropyl)phosphoramidite (2).** To a solution of *N*-*i*-Bu-5′-(DMBOC)-2′-deoxycytidine (0.70 g, 1.18 mmol, azeotroped twice from benzene) in 20 mL of anhydrous CH_2Cl_2, cooled in an ice bath, was added 410 μL (2.35 mmol) of diisopropylethylamine and 315 μL (1.41 mmol) of (2-cyanoethoxy)(diisopropylamino)chlorophosphine. The reaction was allowed to stir overnight at 25 °C. The reaction mixture was taken up in EtOAc and washed with 10% Na_2CO_3 and saturated NaCl, dried over Na_2SO_4, and concentrated in vacuo. Purification of the phosphoramidite was accomplished by repetitive trituration of the residual oil from 6:1 hexane/CH_2Cl_2 to give 0.65 g of the desired compound as a white foam (70%). ^{1}H NMR ($CDCl_3$): δ 1.14−1.29 (20H, m), 2.20 (1H, m), 2.60−2.80 (4H, m), 3.59 (2H, m), 3.74 (6H, s), 4.25−4.75 (4H, m), 6.32 (1H, m), 6.42 (1H, m), 6.57 (2H, m), 6.65 (1H, m), 7.42 (2H, m), 7.53 (1H, m), 7.93 (2H, m), 8.04 (1H, m), 8.15 (1H, m), 8.39 (1H, br s). ^{31}P NMR ($CDCl_3$): δ 149.94, 149.57, 149.38, 150.09. HRMS (FAB, MH^+) calcd for $C_{39}H_{51}N_5O_{11}P$: 796.3322. Found: 796.3295.

***N*-Phenoxyacetyl-2′-deoxyguanosine-5′-(3″,5″-dimethoxybenzoin)carbonate-3′-cyanoethyl(diisopropyl)phosphoramidite (3).** To a solution of *N*-phenoxyacetyl-5′-(DMBOC)-2′-deoxyguanosine (0.75 g, 1.07 mmol, azeotroped twice from benzene) in 20 mL of anhydrous CH_2Cl_2, cooled in an ice bath, was added 374 μL (2.14 mmol) of diisopropylethylamine and 287 μL (1.29 mmol) of (2-cyanoethoxy)(diisopropylamino)-chlorophosphine. The reaction was allowed to stir overnight at 25 °C. The reaction mixture was taken up in EtOAc and washed with 10% Na_2CO_3 and saturated NaCl, dried over Na_2SO_4, and concentrated in vacuo. Purification of the phosphoramidite was accomplished by repetitive trituration of the residual oil from 6:1 hexane/CH_2Cl_2 to give 0.64 g of the desired compound as a white foam (66%). ^{1}H NMR ($CDCl_3$): δ 1.15−1.30 (14H, m), 2.55 (1H, m), 2.65 (2H, m), 2.90 (1H, m), 3.66 (2H, m), 3.74 (6H, m), 4.30−4.80 (6H, m), 6.28 (1H, m), 6.40 (1H, m), 6.53 (2H, s), 6.59 (1H, m), 6.95−7.12 (3H, m), 7.30−7.55 (5H, m), 7.80−7.98 (3H, m). ^{31}P NMR ($CDCl_3$): δ 149.71, 149.61, 149.57. HRMS (FAB, MH^+) calcd for $C_{44}H_{51}N_7O_{12}P$: 900.3333. Found: 900.3312.

***N*-Phenoxyacetyl-2′-deoxyadenosine-5′-(3″,5″-dimethoxybenzoin)carbonate-3′-cyanoethyl(diisopropyl)phosphoramidite (4).** To a solution of *N*-phenoxyacetyl-5′-(DMBOC)-2′-deoxyadenosine (0.54 g, 0.79 mmol, azeotroped twice from benzene) in 12 mL of anhydrous CH_2Cl_2, cooled in an ice bath, was added 276 μL (1.58 mmol) of diisopropylethylamine and 212 μL (0.949 mmol) of (2-cyanoethoxy)(diisopropylamino)-chlorophosphine. The reaction was allowed to stir overnight at 25 °C. The reaction mixture was taken up in EtOAc and washed with 10% Na_2CO_3, saturated NaCl, dried over Na_2SO_4 and concentrated in vacuo. Purification of the phosphoramidite was accomplished by repetitive trituration of the residual oil from 6:1 hexane/CH_2Cl_2 to give 0.50 g of the desired compound as a white foam (71%). ^{1}H NMR ($CDCl_3$): δ 1.19−1.29 (14H, m), 2.60−3.0 (4H, m), 3.63 (2H, m), 3.75 (6H, m), 4.35−4.60 (3H, m), 4.83 (1H, m), 4.88 (2H, s), 6.42 (1H, m), 6.52 (1H, m), 6.60 (2H, m), 6.65 (1H, m), 7.30−7.60 (5H, m), 7.93 (2H, m), 8.33 (1H, m), 8.79 (1H, m), 9.39 (1H, m). ^{31}P NMR ($CDCl_3$): δ 149.92, 149.89, 149.59, 149.47. HRMS (FAB, MH^+) calcd for $C_{44}H_{51}N_7O_{11}P$: 884.3384. Found: 884.3366.

DNA Synthesis. Glass substrates were prepared for oligonucleotide synthesis by derivatization with *N*,*N*-bis(hydroxyethyl)(aminopropyl)triethoxysilane as described previously[8] and then by adding to the surface a protected linker, MeNPOC−hexaethylene glycol−(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite. A 50 mM solution of each nucleoside phosphoramidite monomer was prepared in anhydrous CH_3CN. All phosphoramidite coupling reactions were performed on a custom-built automatic exposure tool and flowcell interfaced to a modified Applied Biosystems 392 DNA synthesizer for reagent delivery. The desired synthesis order containing coupling and photolysis cycle data was programmed into a computer interfaced with both the synthesizer and lithographic equipment. The synthesis program consisted of the base sequence to be coupled to the surface, the mask position during each coupling step, and the irradiation time. Minor adjustments were made to the standard instrument coupling program to accommodate the particular volume and mixing requirements of the flowcell, to eliminate the detritylation step, and to pause for automated mask positioning and exposure. Exposures were made through a chrome-on-quartz mask in contact with the back of the substrate. Light was from a 500 W collimated mercury arc light source (model 87330, Oriel Instruments, Stratford, CT) with interchangeable dichroic reflectors providing output in either the mid-UV (280 nm/0.55 mW/cm^2, 310 nm/5.0 mW/cm^2) or near-UV (365 nm/44.5 mW/cm^2, 405 nm/98 mW/cm^2) range. The glass substrate was held in a flow cell under argon allowing DNA coupling reagents to be delivered to the surface under anhydrous conditions. The output of the UV light source was measured through the photolithographic mask and glass substrate to be 5.0 mW/cm^2 at 310 nm and 44.5 mW/cm^2 at 365 nm. After the synthesis was complete, all substrates were deprotected in a 50% solution of 1,2-diaminoethane or ethanolamine in ethanol for 1 h at RT to remove all base, phosphate, and fluorescein protecting groups, then rinsed with deionized water, and dried under a stream of nitrogen. Deprotected surface hydroxyl groups were visualized and quantitated by fluorescence staining, wherein the chip is treated with a solution of 5 mM fluorescein CE-amidite/50 mM DMTr-thymidine-CE-amidite in acetonitrile, with tetrazole, and subjected to conventional oxidation.

Imaging. The pattern and intensity of the surface fluorescence was imaged with a specially constructed scanning laser fluorescence microscope, with 488 nm argon ion laser excitation. Emitted light was collected through confocal optics with a 530 ± 15 nm band-pass filter and detected with a photomultiplier tube equipped for photon counting. Relative quantitation of surface-bound fluorescein molecules in various regions of the substrate was taken directly from output intensity values in photon counts/s. All values were corrected for nonspecific background fluorescence, measured as the surface fluorescence in nonilluminated regions of the substrate.

Hybridization. Array hybridization was carried out in a flowcell fixed to the stage of the microscope with the labeled oligonucleotide at 10 nm concentration in 6× SSPE buffer (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.5) for 60 min. After removal of the oligonucleotide solution, the array was washed briefly with 6× SSPE, and the image was obtained.

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Supporting Information Available: Table of coupling yields (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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