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## The Efficiency of Light-Directed Synthesis of DNA Arrays on Glass Substrates

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**Abstract:** New methods based on photolithography and surface fluorescence were used to determine photodeprotection rates and stepwise yields for light-directed oligonucleotide synthesis using photolabile 5'-((( $\alpha$ -methyl-2-nitropiperonyl)-oxy)carbonyl)(MeNPOC)-2'-deoxynucleoside phosphoramidites on planar glass substrates. Under near-UV illumination (primarily 365 nm) from a mercury light source, the rate of photoremoval of the MeNPOC protecting group was found to be independent of both the nucleotide and length of the growing oligomer ( $t_{1/2} = 12$  s at 27.5 mW/cm<sup>2</sup>). A moderate dependence on solvent polarity was observed, with photolysis proceeding most rapidly in the presence of nonpolar solvents or in the absence of solvent (e.g.,  $t_{1/2} = 10$ –13 s at 27.5 mW/cm<sup>2</sup>). In solution, the photolysis rate was linearly dependent on light intensity over the range 5–50 mW/cm<sup>2</sup>. Average stepwise yields for the synthesis of dodecamer oligonucleotides were in the range of 92–94%, using monomers based on *N*<sup>6</sup>-(phenoxyacetyl)-2'-deoxyadenosine, *N*<sup>2</sup>-isobutyryl-2'-deoxyguanosine, *N*<sup>4</sup>-isobutyryl-2'-deoxycytidine, and thymidine. By comparison, an efficiency of 98%/step was obtained using a conventional 5'-dimethoxytrityl monomer with acid deprotection on the same support. The lower yields associated with the photochemical process appears to be due to incomplete recovery of free 5'-hydroxyl groups after photolysis on the support, although high yields of 5'-OH nucleosides ( $\geq 96\%$ ) are consistently observed when 5'-MeNPOC monomers are photolyzed in solution.

### Introduction

Arrays of immobilized oligonucleotide probes (DNA "chips") have emerged as powerful new tools for parallel hybridization-based analysis of DNA and RNA sequence.<sup>1</sup> Light-directed oligonucleotide synthesis provides an efficient and versatile method for microfabricating probe arrays with densities as high as 10<sup>6</sup> unique sequences/cm<sup>2</sup>.<sup>2</sup> In this approach, 5'-terminal protecting groups are selectively removed from growing oligonucleotide chains in predefined regions of a glass support by controlled exposure to light through photolithographic masks. This technique has been implemented using oligonucleotide building blocks with photolabile 5'-protecting groups<sup>2</sup> and, more recently, using conventional 5'-(4,4'-dimethoxytrityl) ("DMT")

building blocks in combination with polymeric semiconductor photoresist films as the photoimageable component.<sup>3</sup>

The development of chemistry and processes for DNA array

(1) (a) Lipshutz, R. J.; Morris, D.; Chee, M.; Hubbell, E.; Kozal, M. J.; Shah, N.; Shen, N.; Yang, R.; Fodor, S. P. A. *Biotechniques* **1995**, *19*, 442–447. (b) Cronin, M. T.; Fucini, R. V.; Kim, S. M.; Masino, R. S.; Wespi, R. M.; Miyada, C. G. *Hum. Mutat.* **1996**, *7*, 244–255. (c) Sapolsky, R. J.; Lipshutz, R. J. *Genomics* **1996**, *33*, 445–456. (d) Kozal, M. J.; Shah, N.; Shen, N.; Yang, R.; Fucini, R.; Merigan, T. C.; Richman, D. D.; Morris, M. S.; Hubbell, E.; Chee, M. S.; Gingeras, T. R. *Nat. Med.* **1996**, *2*, 753–759. (e) Yershov, G.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4913–4918. (f) Chee, M. S.; Huang, X.; Yang, R.; Hubbell, E.; Berno, A.; Stern, D.; Winkler, J.; Lockhart, D. J.; Morris, M. S.; Fodor, S. P. A. *Science* **1996**, *274*, 610–614. (g) Lockhart, D. J.; Dong, H.; Byrne, M. C.; Follettie, M. T.; Gallo, M. V.; Chee, M. S.; Mittmann, M.; Wang, C.; Kobayashi, M.; Horton, H.; Brown, E. L. *Nat. Biotechnol.* **1996**, *14*, 1675–1680. (h) Hacia, J. G.; Brody, L. C.; Chee, M. S.; Fodor, S. P. A.; Collins, F. S. *Nat. Genet.* **1996**, *14*, 441–47. (i) Shoemaker, D. D.; Lashkari, D. A.; Morris, D.; Mittmann, M.; Davis, R. W. *Nat. Genet.* **1996**, *14*, 450–456. (j) Southern, E. M. *Trends Genet.* **1996**, *12*, 110–115.

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fabrication requires access to routine procedures for measuring on-chip yields and efficiencies of the chemical and photochemical steps involved. Methods commonly employed in standard oligonucleotide synthesis, such as spectrophotometric monitoring of released trityl cation<sup>4</sup> and HPLC or CE analysis of products after cleavage from the support,<sup>5</sup> are not readily adaptable to DNA synthesis on planar, silanized glass supports. This is due to the fact that the surface coverage of the hydroxyalkylsilane synthesis sites ( $\sim 10\text{--}30\text{ pmol/cm}^2$ )<sup>6</sup> is too low for accurate determination of oligomers or protecting groups by absorbance spectrophotometry, either bound to the support or after subsequent release.

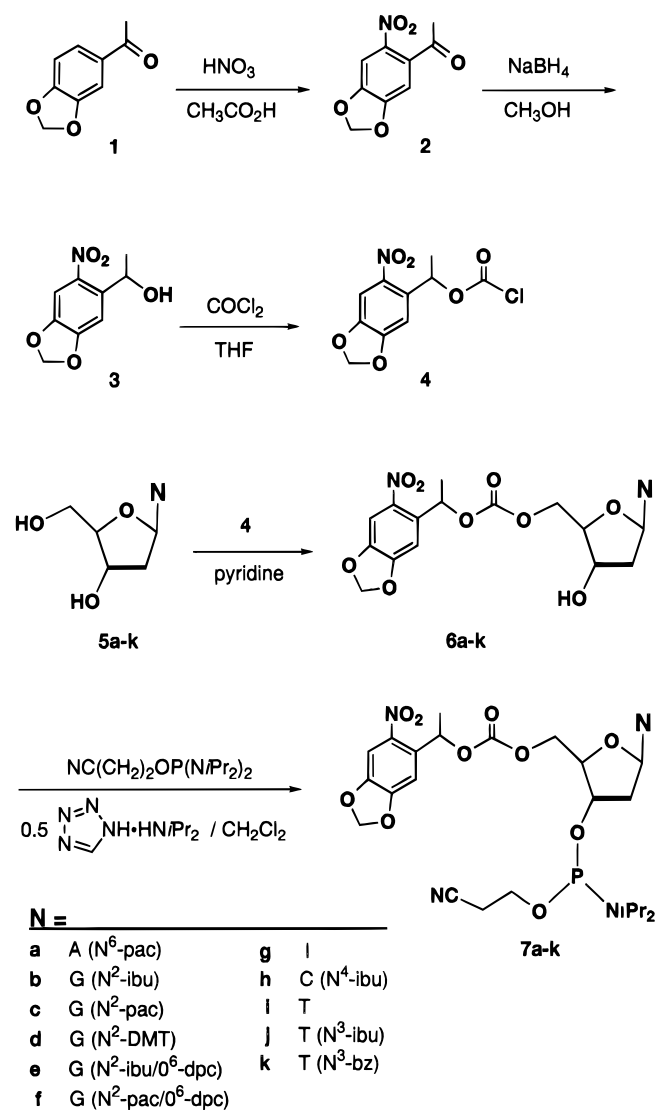
In this paper, we present a quantitative study of light-directed oligonucleotide synthesis on planar supports using procedures based on photolithography and surface fluorescence. These methods involve quantitative "labeling" of the unprotected 5'-hydroxyl groups associated with the bound nucleotides or oligonucleotides using a fluorescent phosphoramidite derivative and measuring surface fluorescence by confocal microscopy. Photochemical protection rates and stepwise yields for oligonucleotide synthesis can both be monitored directly on the support. Although the present study focuses on photolithographic synthesis using 5'-( $\alpha$ -methyl-2-nitropiperonyl)oxycarbonyl("MeNPOC")-2'-deoxynucleoside phosphoramidites,<sup>2b</sup> the techniques that are described here can be applied to the analysis of any reagent or process using *in situ* synthesis to fabricate arrays of biopolymers such as DNA, peptides, and analogs thereof.

## Results and Discussion

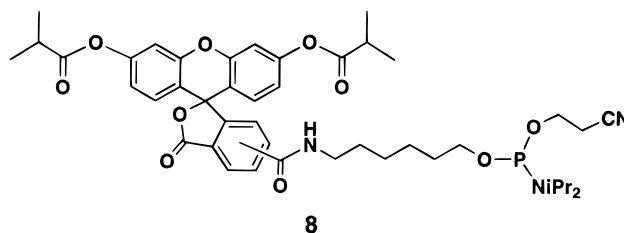
The 5'-MeNPOC-protected phosphoramidite building blocks **7a-k** were prepared according to the method outlined in Scheme 1.<sup>2b</sup> In order to develop reagents and processes for light-directed DNA array synthesis, one must be able to measure and monitor two basic parameters. One parameter is the rate of photolytic release of the 5'-protecting group, related to the reaction quantum efficiency, which is necessary to determine the minimum amount of light required to completely deprotect terminal residues on the support-bound oligomers during synthesis. The second parameter is the stepwise yield of monomer addition over the number of steps required to synthesize oligomers of the desired length.

**Surface Fluorescence Analysis.** In this work, the aforementioned parameters were obtained by measuring the relative surface coverage of unprotected 5'-terminal hydroxyl groups, in one case as a function of the time and intensity of light exposure and, in the other case, as a function of the number of complete cycles of combined monomer addition and photolysis. Photolithographic masking was used to confine light exposure and monomer coupling to specified regions of the substrate which could then be addressed separately for comparison. Surface hydroxyl groups were quantitatively reacted with a fluorescein phosphoramidite derivative (**8**) using the standard sequence of tetrazole-catalyzed coupling followed by capping

Scheme 1



(acetic anhydride/1-methylimidazole/2,6-lutidine/THF) and oxidation (I<sub>2</sub>/pyridine-H<sub>2</sub>O).<sup>7</sup> After removing the acyl protecting groups from the bound fluorescein, relative densities of hydroxyl groups in different regions of the support could then be determined from surface fluorescence intensities.



(2) (a) Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Stryer, L. T.; Lu, A.; Solas, D. *Science* **1991**, *251*, 767-773. (b) Pease, A. C.; Solas, D.; Sullivan, E. J.; Cronin, M. T.; Holmes, C. P.; Fodor, S. P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5022-5026.

(3) (a) McGall, G.; Labadie, J.; Brock, P.; Wallraff, G.; Nguyen, T.; Hinsberg, W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13555-13560. (b) Wallraff, G.; Labadie, J.; Brock, P.; Nguyen, T.; Huynh, T.; Hinsberg, W.; McGall, G. *Chemtech* **1997**, February, 22-32.

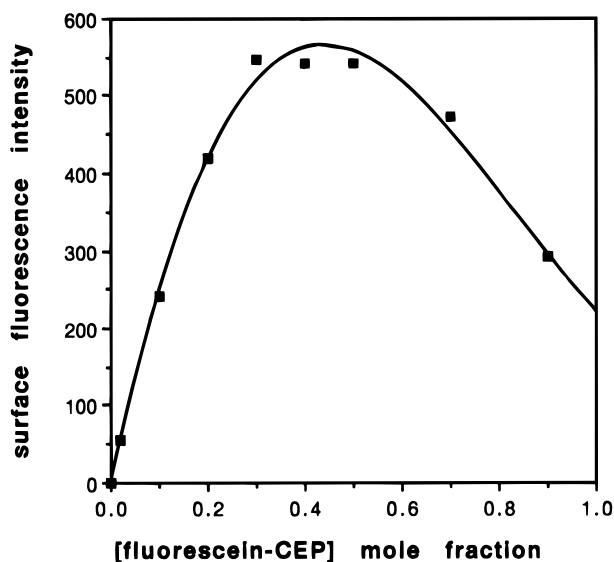
(4) Atkinson, T.; Smith, M. In *Oligonucleotide Synthesis. A practical Approach*; Gait, M. J., Ed.; IRL Press: Oxford, 1984; pp 35-81.

(5) (a) Warren, W. J.; Vella, G. In *Protocols for Oligonucleotides and Conjugates*; Agrawal, S., Ed.; Humana Press: Totowa, New Jersey, 1994; pp 233-264. (b) Andrus, A. *Ibid*; pp 277-300.

(6) McGall, G. H.; Barone, A. D. Unpublished results.

For the purpose of this study, it was not necessary to achieve an absolute measure of the amount of bound fluorescein in any given region of the substrate, although the photon-counting capability of the fluorescence microscope would, in principle, enable one to do so. Instead, differences in surface fluorescence were used to obtain *relative* values for surface density, providing a simple, internally consistent method for measuring chemical and photochemical efficiencies.

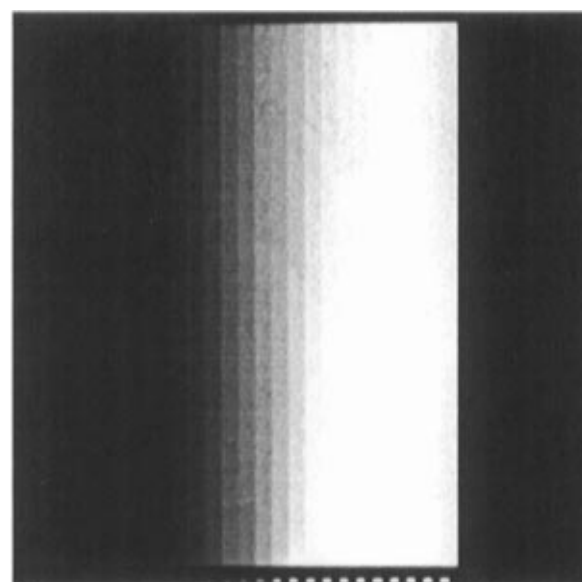
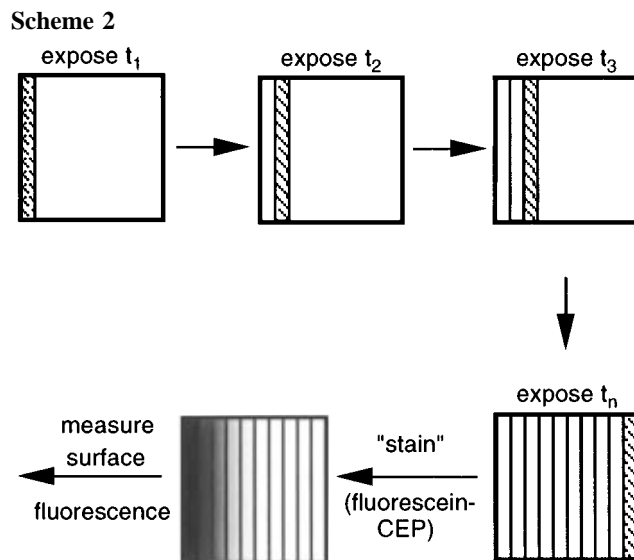
(7) Beaucage, S. L. In *Protocols for Oligonucleotides and Analogs*; Agrawal, S., Ed.; Humana Press: Totowa, New Jersey, 1993; pp 33-61.



**Figure 1.** Observed surface fluorescence intensity of hydroxyalkyl-silanated glass after coupling mixtures **8** and 5'-DMT-T-CEP in varying proportions. Total phosphoramidite concentration was 50 mM in all cases. Fluorescence data was obtained from images acquired using a scanning confocal fluorescence microscope with photon-counting electronics.

One potential source of interference with this kind of analysis is fluorescence quenching due to energy transfer interactions between adjacent fluorophores on the surface. The initial density of surface functional groups on the silanated glass substrates that were used in this work have been estimated to be in the range of 10–30 pmol/cm<sup>2</sup>.<sup>6</sup> Assuming that the initial silanation of the support gives a uniform distribution of molecules, the mean distance between sites will be  $\leq \sim 40$  Å. With such close proximity between fluorophores on the surface, significant quenching interactions would be expected,<sup>8</sup> and quantitative analysis would be complicated by the lack of direct proportionality between surface fluorescence intensity and fluorophore density. Quite strong fluorescence quenching was, in fact, observed when substrates were labeled with stoichiometric amounts of the fluorescent phosphoramidite **8**. However, by diluting the fluorescent reagent with an equally reactive, but nonfluorescent compound, quenching effects could be minimized. Figure 1 shows the dependence of surface fluorescence as a function of the mole fraction of **8**, when used with 5'-DMT-thymidine-3'-O-( $\beta$ -cyanoethyl)-N,N-diisopropylphosphoramidite (DMT-T-CEP) as the diluent reagent. Fluorescence intensity was seen to increase linearly with concentration of **8** up to a mole fraction of 0.2. The observed fluorescence reached a maximum and then dropped as the amount of fluorescein was increased further, consistent with increased quenching at higher surface fluorophore densities. These data established that quenching effects would be insignificant, provided that the diluent amidite was present in at least a 5-fold excess over the fluorescent amidite. In order to ensure that a reliably linear relationship was maintained between surface fluorescence and fluorophore density, a 10-fold excess of diluent amidite was used in all of the experiments described below.

**Surface Photolysis Rates.** Scheme 2 illustrates a typical strategy for determining the rates of photolytic deprotection for monomers coupled to the support. In all experiments, hydroxyalkylated glass substrates were first derivatized with a MeNPOC-protected hexaethyleneglycol (HEG)-based linker phosphoramidite, MeNPOC-[OCH<sub>2</sub>CH<sub>2</sub>]<sub>6</sub>O-CEP (**9**), to provide a starting

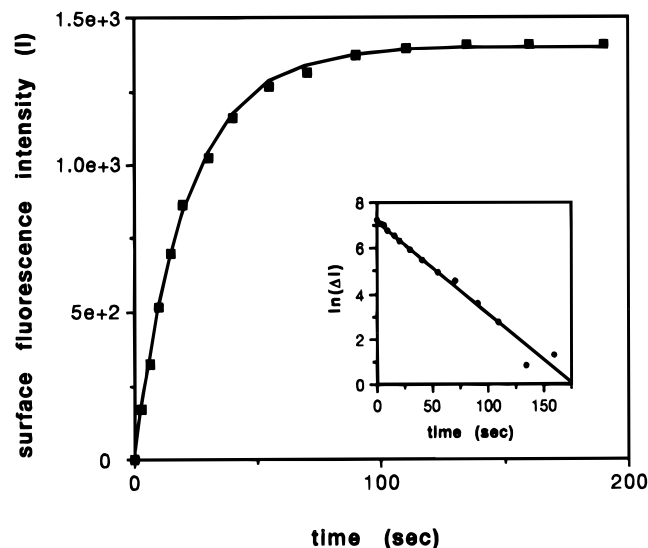


**Figure 2.** Surface fluorescence image of a substrate bearing a 5'-MeNPOC-protected nucleoside, exposed through verticle stripes in a photolithographic mask to near-UV light for increasing times, covering a range of 3–450 s, as outlined in Scheme 2. The image was acquired after “staining” the substrate with a 1:10 mixture of fluorescein- and 5'-DMT-thymidine phosphoramidites.

point for subsequent light-directed DNA synthesis. A photolithographic mask is positioned over the back of the substrate to allow a selected portion of the substrate, in this case an open verticle aperture  $0.8 \times 12.8$  mm, to be photolyzed by exposure to near-UV light from a constant-intensity mercury light source for a predetermined duration. The mask is then translated horizontally 0.8 mm, placing the aperture of the mask over an adjacent region for a subsequent, longer exposure. This process is continued, increasing the exposure time each time, to generate an array of stripes across the chip with a gradient of increasing exposure dose, and therefore increasing extent of deprotection. The pattern of surface deprotection is then “stained” by coupling the fluorescein phosphoramidite mixture.

In a final step, the isobutryl protecting groups are removed from the bound fluorescein by immersing the substrate in a base solution (1,2-diaminoethane in ethanol). The resulting surface fluorescence image, acquired with a scanning confocal microscope, is shown in Figure 2, and the fluorescence intensity change vs exposure time, extracted from the image, is plotted

(8) Forster, T. *Naturwissenschaften* **1946**, *6*, 166–175. (b) Fung, B. K. K.; Stryer, L. *Biochemistry* **1978**, *17*, 5241–5248.



**Figure 3.** Plot of fluorescence intensity vs exposure time, obtained from the image shown in Figure 2. Intensity values were taken as the average pixel intensity in counts per second, in each region of the substrate exposed for a given time. The data have been fitted to a first-order exponential (solid line). Inset shows linear plot of  $\ln(\Delta I = I_{\infty} - I)$  vs time.

**Table 1.** Photolysis Half-Lives for Base-Protected 5'-MeNPOC Nucleotides on Glass Substrates<sup>a</sup>

entry	monomer	linker	$t_{1/2}$ (s)
1	HEG		9
2	T	HEG	12
3	T(ibu)	HEG	11
3	dC(ibu)	HEG	12
5	dG(ibu)	HEG	12
4	dG(pac)	HEG	13
4	dG(DMT)	HEG	11
4	dG(pac/dpc)	HEG	12
6	dI	HEG	12
7	dA(pac)	HEG	12
8	dA(pac)	(dA) <sub>4</sub> (HEG)	10
9	dA(pac)	(dA) <sub>8</sub> (HEG)	12
10	dA(pac)	(dA) <sub>12</sub> (HEG)	12
11	dA(pac)	(dT) <sub>12</sub> (HEG)	13

<sup>a</sup> Photolysis carried out in the presence of dioxane under near-UV output from Hg source (365 nm/27.5 mW/cm<sup>2</sup>).

in Figure 3. The change in surface fluorescence with exposure time followed a first-order exponential increase from which the photolysis rate constant, or half-life ( $t_{1/2}$ ), was obtained by simple curve-fitting analysis.

To measure photocleavage rates for the other MeNPOC building blocks, portions of a MeNPOC-linker modified support were completely photolyzed (10 half-lives) to allow addition of the desired monomer, and the chip was then subjected to the same sequence of exposures, staining, imaging, and analysis described above. The photolysis data are summarized in Table 1. Rates for photolysis of MeNPOC monomers bound to the glass substrate were found to be very similar for all of the building blocks studied and in reasonably close agreement with values measured previously for several 5'-MeNPOC-deoxy-nucleosides in dioxane solution with monochromatic 365 nm light.<sup>2b</sup> In this work, exposures were not carried out with monochromatic light, but with collimated light from a commercially available high-pressure mercury arc lamp. The lamp was fitted with dichroic reflectors to selectively pass light in the near-UV region of the spectrum, which consists primarily of the Hg emission lines centered at 365, 405, and 436 nm. The 365 nm emission is almost exclusively responsible for photochemistry, as the absorbance of the MeNPOC chro-

**Table 2.** Dependence of Photolysis Half-Life on Solvent<sup>a</sup>

entry	solvent	$t_{1/2}$ (s)
1	(none)	8
2	toluene	10
3	dioxane	11
4	DCM	16
5	DMF	19
6	acetonitrile	20
7	methanol	30

<sup>a</sup> Data is for 5'-MeNPOC-dA(pac) in dioxane under near-UV output from Hg source (365 nm, 35 mW/cm<sup>2</sup>).

mophore ( $\lambda_{\max} = 345$  nm,  $\epsilon = 5 \times 10^3$  M<sup>-1</sup> cm<sup>-1</sup>) becomes negligible at wavelengths above 400 nm. This was confirmed by the observation of a 10-fold reduction in photolysis rates when filters were added to remove the 365 nm emission peak. No significant effect was observed upon eliminating the 400 nm or 436 nm emission lines. Photocleavage of the MeNPOC group is also induced by irradiation at lower wavelengths (data not shown). However, wavelengths below 340 nm were avoided due to the potential for photochemically-induced modification of the DNA.<sup>9</sup>

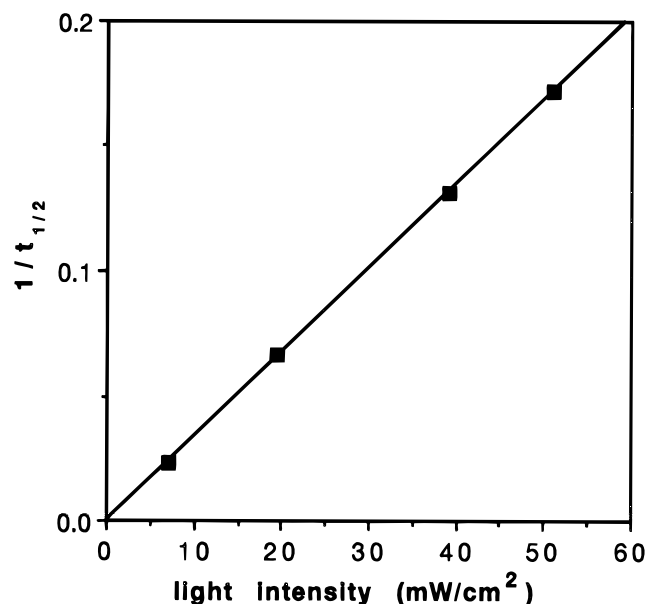
A brief examination of solvent effects (Table 2) revealed that photocleavage rates proceeded most rapidly under dry conditions, or when the substrate was maintained under a nonpolar solvent such as toluene or dioxane. A reasonably good correlation (not shown) between  $\ln(k_{\text{obsd}})$  and the Dimroth  $E_T$  solvent polarity scale<sup>10</sup> is suggested by the data. However, given the present understanding of the photochemistry of 2-nitrobenzyl compounds,<sup>11</sup> the mechanistic significance of the solvent effect is unclear. It is also worth noting that oligomer length does not significantly affect the photocleavage rate of the terminal nucleotide (Table 1, entries 7–11). For the automated multistep synthesis of arrays, this is a matter of some convenience as it allows a single photolysis time to be used for every cycle of monomer addition throughout the synthesis. Another practical consideration for synthesis of DNA probe arrays is the use of increasingly higher light intensities to reduce the photolysis time and therefore the overall duration of each synthesis cycle. Saturation of the excited state at high photon flux would present a potential obstacle or limitation to this approach. However, over the range of 5–50 mW/cm<sup>2</sup> at 365 nm (Figure 4), photocleavage half-lives obeyed an inverse-linear dependence on light intensity indicating the absence of any saturation of the excited state at these powers. At 50 mW/cm<sup>2</sup>, less than 1 min is required for complete (i.e., 10 half-lives) photolysis of the MeNPOC group. In principle, even higher intensity light, such as that provided by a nitrogen laser, could be used to achieve even shorter exposure times.

**Stepwise Synthesis Efficiency.** Scheme 3 illustrates how a similar methodology was used for the determination of stepwise synthesis efficiencies. In this experiment, a mask with a rectangular aperture was positioned over the substrate and exposed for sufficient time to allow complete photolysis (10 half-lives) of the linker. A MeNPOC-nucleoside phosphoramidite was coupled to the exposed region of the support, and after capping and oxidation, the mask was offset horizontally by  $1/n \times W$ , where  $W$  is the width of the open reticle and  $n$  is the oligomer length to be tested. The photolysis was repeated, and a second building block was added. This process was

(9) Cadet, J.; Vigny, P. In *Bioorganic Photochemistry*; Morrison, H., Ed.; Wiley: New York, 1990; Vol. 1, pp 1–272.

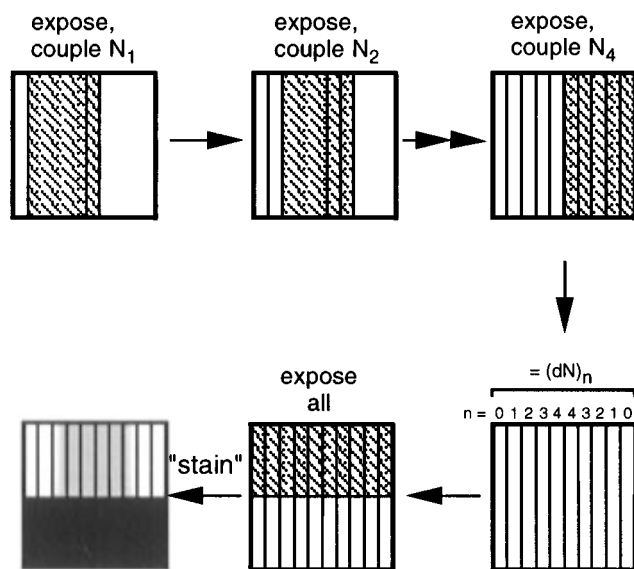
(10) Dimroth, K.; Reichardt, C.; Siepmann, T.; Bohlmann, F. *Ann. Chem.* **1984**, *661*, 1.

(11) For a recent review, see: Corrie, J. E. T.; Trentham, D. R. In *Bioorganic Photochemistry*; Morrison, H., Ed.; Wiley: New York, 1993; Vol. 2, pp 243–305.



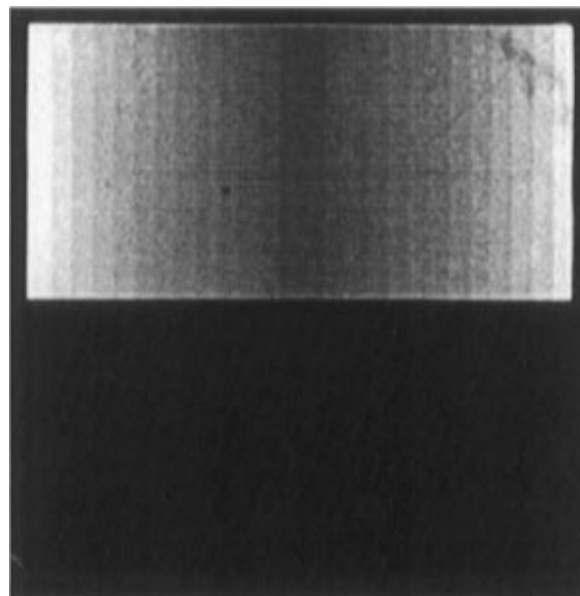
**Figure 4.** Dependence of the photolysis half-life on light intensity at 365 nm for compound **10** in dioxane at 200 nm.

### Scheme 3



repeated for a total of “*n*” cycles of photolysis and coupling to generate a duplicate set of stripes on the support comprising a complete set of oligomers of length 1 to *n*. Upon completion, half of the array was subjected to a final wide-field photolysis, as shown, to release photolabile groups from the 5′-termini of the finished oligomers, including a region of the previously unphotolyzed linker for comparison (“*n* = 0” control). Fluorescein-CEP was then added to label the free 5′-hydroxyl groups for quantitation as described above. The unphotolyzed half of the array was left to provide an internal control for background fluorescence due to nonspecific binding of the fluorescein in regions of the substrate where synthesis had occurred.

Figure 5 shows the fluorescence image of a (dC)<sub>0–12–0</sub> array synthesized with 5′-MeNPOC phosphoramidites. Fluorescence decreases somewhat toward the center of the pattern as the yield of the full-length oligomer decreases with increasing length. After correcting for background fluorescence, the relative yield for each step in the synthesis was calculated from the ratio of the fluorescence intensities in adjacent stripes (eq 1).



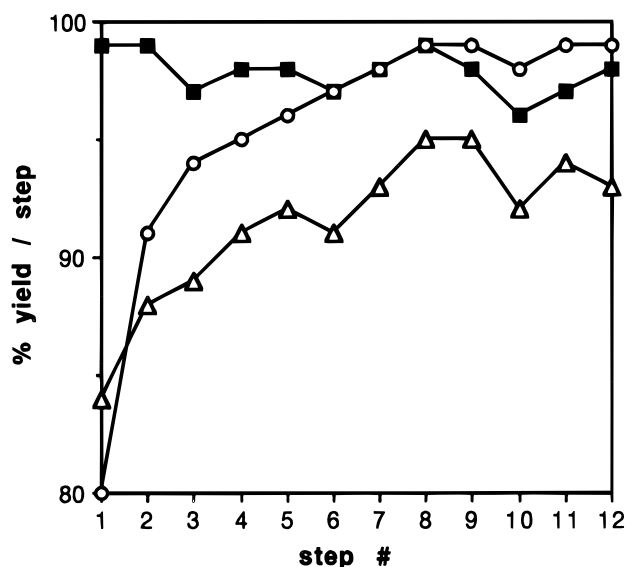
**Figure 5.** Surface fluorescence image of an array of poly(dC) oligomers ranging in length from 1–12, synthesized as outlined in Scheme 3. The image was acquired after completely photolysing the upper half of the array and “staining” with **8** as described in the text.

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

where  $I_n$  = fluorescence intensity for oligomer of length *n*. Representative data for the synthesis of (dC)<sub>12</sub> and T<sub>12</sub> using 5′-MeNPOC building blocks are plotted in Figure 6. This figure serves to illustrate a common feature observed with MeNPOC amidites, namely, an apparent increase in stepwise yields over the first ~6 steps of oligomer synthesis to maximum values in the range 92–98%. Also shown in Figure 6, for comparison, is the stepwise efficiency observed for the synthesis of T<sub>12</sub> using conventional 5′-DMT-protected phosphoramidite on the same substrates. The latter was determined in an experiment using a masking sequence similar to the one employed in the photolysis kinetics experiment (*vide supra*), wherein an open verticle aperture was translated horizontally across the substrate, completely deprotecting regions of “fresh” linker-modified substrate each time. In this case, after each exposure, a 5′-DMT-protected nucleoside phosphoramidite was coupled to the support and then detritylated by washing the support with acid (3% trichloroacetic acid in dichloromethane). The resulting (dN)<sub>0–12</sub> array of oligomers was stained and analyzed according to the protocol described above. As expected, synthesis with the DMT-monomers proceeded uniformly with very high efficiency (~98 ± 1%/step).

Table 3 summarizes the stepwise efficiency data for synthesis of a series of homopolymers with MeNPOC-protected monomers. Interestingly, the chemical protecting groups on the nucleobase can, in some cases, influence the observed efficiencies. For deoxyguanosine, substantially higher yields were observed when the exocyclic amine was protected with isobutyryl (iBu) compared to phenoxyacetyl (pac) or DMT. The addition of protecting groups (iBu, benzoyl) to the amide N<sup>3</sup>-position of thymidine had little discernable effect on the cycle yields, while supplemental protection of the O<sup>6</sup> (amide) of deoxyguanosine with *N,N*-diphenylcarbamoyl (dpc) reduced yields.

The accuracy of the values determined by the methods described above relies on the assumption that the fluorescence quantum efficiency of the terminal fluorescent label is independent of oligomer length. Two tests confirm that this is a



**Figure 6.** Observed stepwise synthesis efficiencies for  $(dC)_{12}$  (○) and  $T_{12}$  (△), using 5'-MeNPOC building blocks **7h.i**. Values were calculated from surface fluorescence as described in the text. Corresponding data for  $T_{12}$  using conventional 5'-DMT building blocks (■) is shown for comparison.

**Table 3.** Mean Stepwise Yields for the Synthesis of  $(dN)_{12}$  Homopolymers Using 5'-MeNPOC-Nucleoside Phosphoramidites

entry	nucleoside	mean stepwise yield (steps 6–12/1–12)
a	dA( $N^6$ -pac)	96/94
b	dG( $N^2$ -ibu)	93/91
c	dG( $N^2$ -pac)	88/85
d	dG( $N^2$ -DMT)	84/81
e	dG( $N^2$ -ibu/ $O^6$ -dpc)	88/87
f	dG( $N^2$ -pac/ $O^6$ -dpc)	86/83
g	dI	90/89
h	dC( $N^4$ -ibu)	98/94
i	T	96/92
j	T( $N^3$ -ibu)	91/88
k	T( $N^3$ -bz)	96/93

<sup>a</sup> Photodeprotection carried out in the presence of dioxane under near-UV output from Hg source (365 nm/27.5 mW/cm<sup>2</sup>).

reasonably good approximation. One is the stepwise yields determined using a DMT monomer with acid deprotection, which were uniformly high at every step, in accord with the known yields for these reagents on particulate supports. The second test was performed by “spiking” a MeNPOC phosphoramidite with known amounts of a chain-terminating phosphoramidite, such as 5'-*O*-methylthymidine-CEP, to compete for and eliminate active synthesis sites when the amidite is coupled to the substrate. This resulted in the expected decrease in the observed stepwise yield, proportional to the amount of terminator added (a 5% decrease yield was seen with a 95:5 mixture of MeNPOC/methyl-T-CEP).

The somewhat lower stepwise yields obtained with MeNPOC compounds, as compared to the standard DMT chemistry, appears to be related to the net yield of free 5'-hydroxyl groups after photolysis of the MeNPOC group. Inefficiency of the tetrazole-catalyzed coupling step itself is not responsible for the observed yields, since in all cases, yields were unaffected by increasing either the coupling time, reagent (i.e., tetrazole activator, amidite) concentration, or the total number of repetitions of “fresh” amidite addition for each coupling step. Stepwise yields were also unaffected by prolonged exposure times (up to 100 half-lives) or by variation of the solvent, light intensity, or wavelengths used (only the total time required for

complete ( $10t_{1/2}$ ) photolysis is affected, see above). Attempts to enhance yields by carrying out the photolysis step in the presence of mild acids, bases, oxidants, reductants, and other reagents during the photolysis step failed to identify conditions under which efficiencies were substantially improved over those obtained when exposures are performed under neat solvent or dry conditions. With respect to the latter approach, it should be noted that the range of possible conditions which one might contemplate using is somewhat restricted by the need to maintain compatibility with the surface oligonucleotide and linkage chemistry during the photolysis step.

Another possible explanation for the observed efficiencies was that the MeNPOC protecting group might be prone to either modification or removal during the activation/coupling, capping, or oxidation steps, such that competent, photolyzable synthesis sites were being capped or otherwise lost. However, tests which involved extended or repeated exposure to each of these reagents prior to the photolysis step, did not lead to any reduction in the observed cycle yields, establishing the overall stability of the MeNPOC group toward the reagents and conditions used in oligonucleotide synthesis.

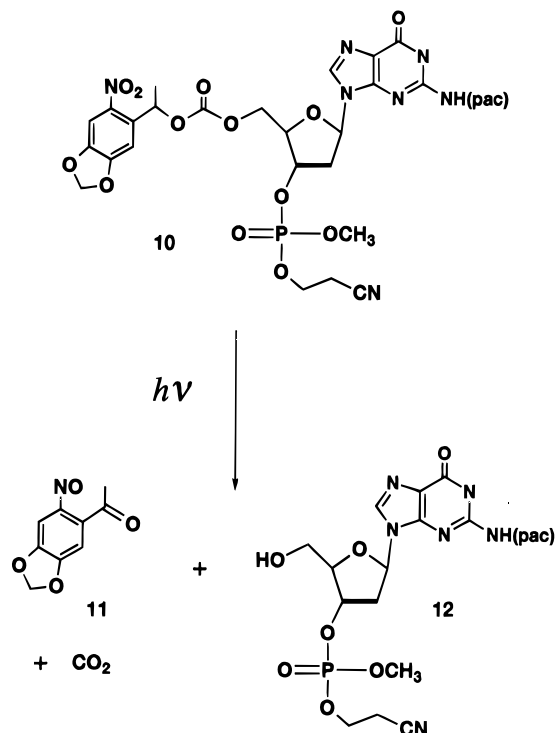
**Solution Phase Photochemistry.** Subquantitative yields for the photochemical cleavage may be due to an intrinsic partitioning of products from the excited state of the nitrobenzyl chromophore, such that, in addition to the desired products resulting from heterolytic cleavage of the benzylic C–O bond, one or more stable side products may be formed which remain attached to the 5'-oxygen, effectively blocking it from subsequent reaction. This hypothesis led us to study the photolysis of several model compounds in solution, where reactions could be performed on a scale that would allow a direct analysis of the products, to determine whether any unusual species were formed and to what extent.

Previously, base-protected 5'-MeNPOC (3'-OH) nucleosides were photolyzed in dioxane solution and analyzed by HPLC to determine photolysis rates, and only clean conversion to the free 5'-OH nucleoside was reported.<sup>2b</sup> This result was confirmed in the present study (data not shown). In addition, we also prepared the model nucleotide 5'-MeNPOC- $N^2$ -pac-2'-deoxyguanosine-3'-(2-cyanoethyl)methylphosphate (**10**, Scheme 4) which enabled us to study a closer structural analog to the terminal 5'-MeNPOC nucleotides involved in oligonucleotide synthesis. The pac-protected dG nucleotide was chosen in this case, since the surface-fluorescence analysis indicated the lowest stepwise yields with the corresponding phosphoramidite **7c** (av 85%/step, Table 3) and, hence, the greatest possibility of observing side products.

As shown in Figure 7, HPLC analysis indicated that the photolysis of **10** in dioxane under near-UV illumination resulted in quite clean conversion to the free 5'-OH nucleoside **12** and nitrosoketone **11**, which were identified by comparison with the authentic materials. The HPLC also revealed the presence of several minor, unidentified byproducts, but these accounted for  $\leq 5\%$  of the total peak integral (excluding **11**), so this would not explain the 85–88% stepwise yield (Table 3) observed with **7c** on the glass support. Moreover, these byproducts appeared to be related to the subsequent photochemical breakdown of the primary photoproduct **11**, since the same peaks were observed in the HPLC when samples of authentic **11** were photolyzed independently under identical conditions.

The solution studies described thus far were performed at low concentrations ( $\sim 200$  mM) to avoid quenching the photocleavage rate by internal filtering. These concentrations would not specifically replicate the average close spacing ( $\sim 40$  Å) of MeNPOC groups linked to oligonucleotide chains on the surface

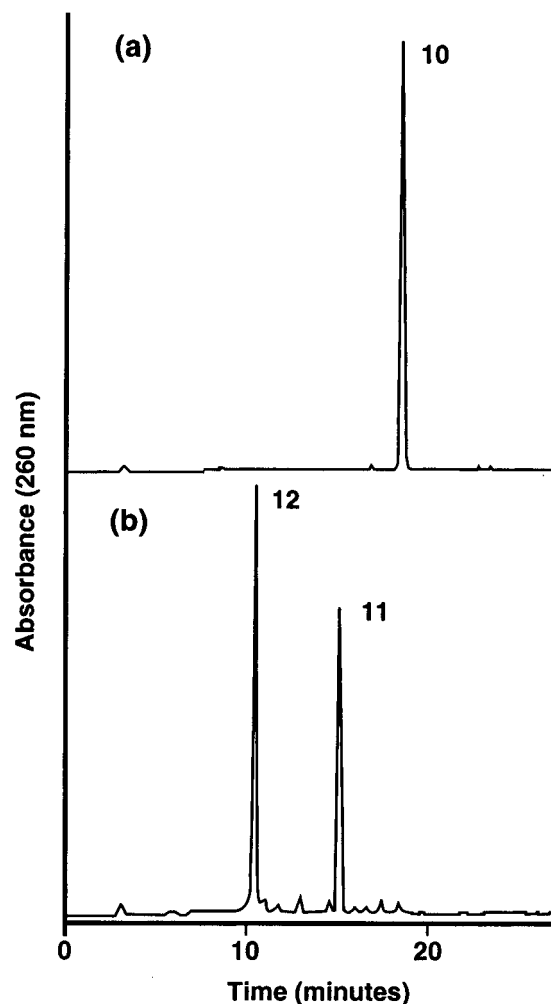
## Scheme 4



of the glass substrate. For this reason, the photolysis of 1,3-bis-MeNPOC-propanediol (**13**, Scheme 5) was examined in order to model the close spacing of chromophores on a support and to allow for the possibility that the nitrobenzyl excited states or reactive intermediates might undergo intermolecular interactions with neighboring molecules that would account for the observed photolysis efficiencies. However, as illustrated in Figure 8, compound **13** photolyzed rapidly and cleanly, generating 1,3-propanediol and nitrosoketone **11** almost exclusively (>96% yield). Further studies, in which **10** was photolyzed at high concentrations in solution (60 mM), failed to reveal any significant byproducts other than the nitrosoketone and its photochemical breakdown products.

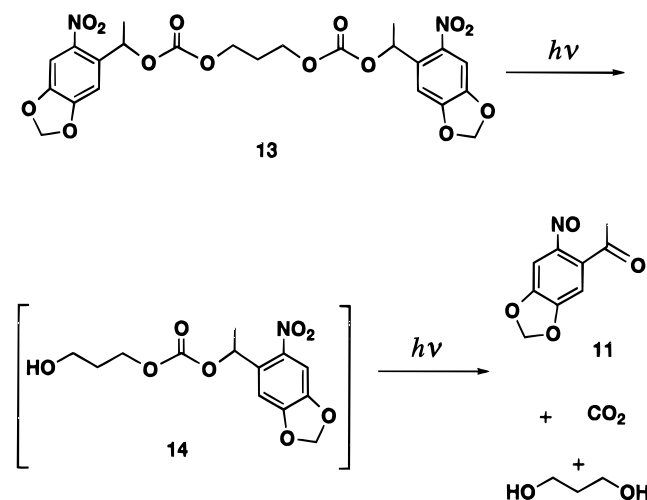
## Summary and Conclusions

With this work we have established a number of routine techniques, based on surface fluorescence, that can be generally applied to the analysis of light-directed oligonucleotide synthesis on planar glass supports. These methods were used to determine photodeprotection rates and stepwise efficiencies for oligonucleotide synthesis using a series of 5'-MeNPOC-nucleoside phosphoramidite building blocks. Using building blocks prepared from  $N^6$ -pac-dA,  $N^2$ -iBu-dG,  $N^4$ -iBu-dC, and T, DNA probe arrays can be synthesized with average stepwise yields in the range of 92–94%. These yields are somewhat lower than those achievable with conventional 5'-DMT phosphoramidites and chemical deprotection (98%/step), apparently due to incomplete recovery of free 5'-hydroxyl groups after photolysis. A working hypothesis is that photolysis on the support is accompanied by the occurrence of residual byproducts which remain attached to the 5'-hydroxyl termini and prevent further chain extension. Model experiments, however, indicate that photorelease of the MeNPOC protecting group proceeds with high yield ( $\geq 96\%$ ) in solution. In order to gain a better understanding of the mechanisms and factors influencing synthesis efficiency on glass substrates, further efforts are being undertaken to detect and characterize photolysis products



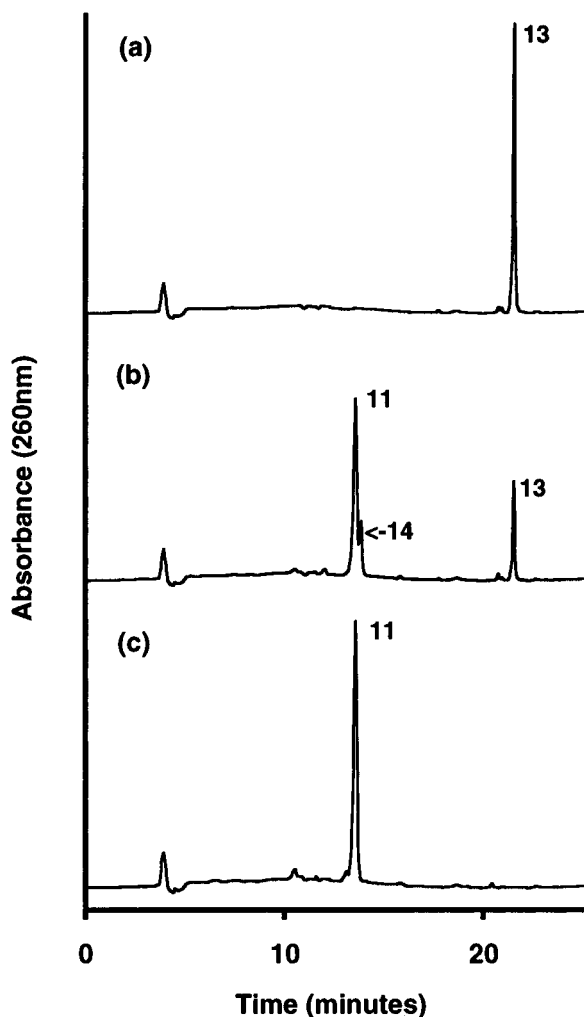
**Figure 7.** Analysis of the photolysis of compound **10** in dioxane by reverse-phase HPLC ( $[\mathbf{10}] = 200 \mu\text{M}$ ) (a) before photolysis and (b) after 300 s of exposure to near-UV light from a mercury arc source ( $10 \text{ mW/cm}^2$  at 365 nm). The products (**11** and **12**) were identified by comigration with authentic compounds.

## Scheme 5



directly on the support. Other photolabile protecting groups<sup>12,13</sup> are also being studied in order to identify potentially more efficient alternatives to the MeNPOC group.

(12) For recent reviews, see: ref 11. Pillai, V. N. R. In *Organic Photochemistry*; Padwa, A., Ed.; Marcel Dekker: New York, 1987; Vol. 9, pp 225–323.



**Figure 8.** Analysis of the photolysis of compound **13** (Scheme 5) in dioxane by reverse-phase HPLC ( $[13] = 200 \mu\text{M}$ ) (a) before photolysis, (b) after 30 s of exposure to near-UV light from a mercury arc source ( $10 \text{ mW}/\text{cm}^2$  at 365 nm), and (c) after 300 s of exposure. Intermediate **14** and products **11** and **12** were identified by comigration with the authentic compounds.

A detailed discussion of the impact of synthesis yields on the hybridization properties of photolithographically generated oligonucleotide probes is outside the scope of this report. However, as this question is relevant to the use of probe arrays for sequencing applications, a few general comments should be made. The observed photochemical synthesis yields imply that full-length probes will be accompanied on the support by significant quantities of incomplete or "truncated" sequences. Nonetheless, probe arrays prepared in this fashion exhibit excellent performance in a variety of applications in which sequence information can be "read" from hybridization data with single-base resolution.<sup>1b-i</sup> The thermodynamics of hybridization are such that conditions (e.g., temperature, ionic strength) can generally be manipulated to eliminate the hybridization of oligonucleotides which are substantially shorter than the full-length probe. Truncated sequences which are shorter by only a few nucleotides may still hybridize, but these comprise a relatively small fraction ( $\sim 10\%$ ) of the total amount of hybridizable probes on the surface, and consequently contribute little to the observed hybridization signal. Further improvement in the chemical efficiency of array synthesis remains an

important objective, however, as greater homogeneity of probe length will provide greater flexibility in the design and application of these arrays.

## Experimental Section

**General Methods and Materials.** HPLC analyses were performed on Beckman System Gold using absorbance detection at 260 nm and on a Beckman reverse-phase column ( $5 \mu\text{m}$  C18-silica, 4 mm I.D.  $\times$  250 mm *L*) eluted with a linear gradient of acetonitrile in 0.1 M aqueous triethylammonium acetate (TEAA)/pH 7.2 at a flow rate of 1 mL/min. UV-vis spectra were obtained on a Varian Cary 3E spectrophotometer.  $^1\text{H}$  and  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini-400 spectrometer. Mass spectra were recorded on the following instruments: electrospray ionization (ESI-MS), Hewlett Packard HP59987A; electron impact ionization (EI-MS), Hewlett Packard HP5989B; positive ion fast atom bombardment (FAB-MS), MicroMass ZABZ-EQ. Elemental analyses were performed by Quantitative Technologies, Inc., Whitehouse, NJ.

All reagents and anhydrous solvents were obtained from Aldrich, except for the following: 20% w/v phosgene in toluene, from Fluka; thymidine, 2'-deoxyinosine, *N*<sup>2</sup>-(phenoxyacetyl)-2'-deoxyguanosine, *N*<sup>2</sup>-isobutryl-2'-deoxyguanosine, *N*<sup>4</sup>-isobutryl-2'-deoxycytidine, *N*<sup>6</sup>-(phenoxyacetyl)-2'-deoxyadenosine, and diisopropylammonium tetrazolide, from Chem-Impex (Wood Dale, IL); 2-(cyanoethyl)-*N,N,N',N'*-tetra-isopropylphosphorodiamidite, from ChemGenes (Waltham, MA); fluorescein phosphoramidite (Fluoreprime) and 5'-DMT-2'-deoxynucleoside 3'-phosphoramidites, from Pharmacia Biotech; DNA synthesis reagents and anhydrous acetonitrile from Glen Research (Sterling, VA); silica gel (60 Å pore size, 230–400 mesh for flash chromatography), E. Merck. *N*<sup>3</sup>-Benzoylthymidine was prepared as previously described.<sup>14</sup> *N*<sup>2</sup>-(4,4'-Dimethoxytrityl)-2'-deoxyguanosine (DMT-dG)<sup>15</sup> and *O*<sup>6</sup>-(*N,N*-diphenylcarbamoyl)-*N*<sup>2</sup>-(phenoxyacetyl)-2'-deoxyguanosine (dpc/pac-dG)<sup>16</sup> were prepared using modifications of reported methods which are available as Supporting Information. *O*<sup>6</sup>-(*N,N*-Diphenylcarbamoyl)-*N*<sup>2</sup>-isobutryl-2'-deoxyguanosine (dpc/iBu-dG) was prepared in the same way as dpc/pac-dG and converted directly to the 5'-MeNPOC derivative without isolation.

**Substrate Preparation.** Glass microscope slides (2 in  $\times$  3 in  $\times$  0.027 in, from Erie Scientific) were cleaned by soaking successively in NanoStrip (Cyantek, Fremont, CA) for 15 min, 10% aqueous NaOH/70 °C for 3 min, and then 1% aqueous HCl (1 min), rinsing thoroughly with deionized water between each step, and then spin drying for 5 min under a stream of nitrogen at 35 °C. The slides were then silanated for 15 min in a gently agitating 1% solution of *N*-(3-(triethoxysilyl)propyl)-4-hydroxybutyramide (PCR, Inc., Gainesville, FL) in 95:5 ethanol/water, rinsed thoroughly with 2-propanol, then deionized water, and finally spin-dried for 5 min at 90–110 °C.

**Photochemical Oligonucleotide Synthesis.** Phosphoramidites were used at a concentration of 50 mM in dry acetonitrile, and coupling reactions were performed on an Affymetrix Array Synthesizer, which consisted of a custom-built automated exposure tool and a 1 in diameter flowcell linked to a modified Applied Biosystems model 392 DNA synthesizer. Reagent delivery from the synthesizer was controlled using OligoNet software, with minor adjustments being made to the standard programmed coupling protocol to accommodate the particular volume and mixing requirements of the flowcell. In cycles adapted for photochemical synthesis, the detritylation step was replaced with a pause to allow the exposure tool to perform the automated mask positioning/alignment and timed light exposure. Exposures were made through a chrome-on-quartz mask in contact with the back of the substrate while the substrate remained clamped to the flowcell. Light was projected horizontally from a 500 W collimated light source (model 87330, Oriol Instruments, Stratford, CT) equipped with an Ushio model ush508sa super high pressure mercury lamp and dichroic reflectors to provide

(14) Sekine, M.; Fujii, M.; Nagai, H.; Hata, T. L. *J. Chem. Soc., Chem. Commun.* **1987**, 1119–1120.

(15) Hata, T. L.; Gokita, N.; Sakairi, N.; Yamaguchi, K.; Sekine, M.; Ishido, Y. *Bull. Soc. Chem. Jpn.* **1982**, 55, 2949–2955.

(16) Kamimura, T.; Tsuchiya, M.; Urakami, K.-I.; Koura, K.; Sekine, M.; Shinozaki, K.; Miura, K.-I.; Hata, T. L. *J. Am. Chem. Soc.* **1984**, 106, 4552–4457.

(13) (a) Pirrung, M. C.; Shuey, S. W. *J. Org. Chem.* **1994**, 59, 3890–3897. (b) Pirrung, M. C.; Bradley, J.-C. *J. Org. Chem.* **1995**, 60, 1116–1117. (c) Pirrung, M. C.; Bradley, J.-C. *J. Org. Chem.* **1995**, 60, 6270–6276.



output in the near-UV spectral range ( $\geq 340$  nm). Prior to the exposure step, the flowcell was usually filled with a solvent (see Table 2), delivered automatically by the DNA synthesizer from bottle no. 10.

Typically, the final step of a photolysis or synthesis efficiency experiment (see Results and Discussion) is fluorescence "staining", wherein fluorescein phosphoramidite **8** (5 mM, in a solution containing 50 mM DMT-T-CEP in acetonitrile) is coupled to the free hydroxyl groups on the substrate using the standard coupling protocol. Substrates were then removed from the flowcell and deprotected in a solution of 1,2-diaminoethane in ethanol (50%/vol) for 2 h at ambient temperature, rinsed with deionized water, and then dried under a stream of nitrogen.

The pattern and intensity of surface fluorescence was imaged with a specially constructed scanning laser confocal fluorescence microscope, which employed excitation with a 488 nm argon ion laser beam focused to a 2  $\mu$ m spot size at the substrate surface. Emitted light was collected through confocal optics with a 530( $\pm 15$ ) nm bandpass filter and detected with a photomultiplier equipped with photon-counting electronics. Output intensity values (photon counts/second) are proportional to the amount of surface-bound fluorescein, so that relative yields of free hydroxyl groups within different regions of the substrate could be determined by direct comparison of the observed surface fluorescence intensities. All intensity values were corrected for nonspecific background fluorescence, taken as the surface fluorescence within the nonilluminated regions of the substrate.

**Solution Photolysis Studies.** Compounds to be photolyzed in solution were dissolved in dioxane at a concentration of 200  $\mu$ M. Solutions were fully exposed, in a 1 mm path length quartz cuvette, to near-UV light from a 500 W collimated mercury light source (see above). Aliquots were removed at intervals for direct analysis by reversed phase HPLC (20–100% CH<sub>3</sub>CN over 25 min, A<sub>260</sub> detection). The primary photolysis products were identified by co-injecting authentic standards. Photolysis was followed by monitoring the disappearance of starting material, and half-times were calculated from ln[peak area] vs time plots ( $t_{1/2} = 0.693/\text{slope}$ ), using linear regression analysis.

**Methyl 3,4-(Methylenedioxy)-6-nitrophenyl Ketone (2).** A solution of 410 g (2.5 mol) 3,4-(methylenedioxy)acetophenone (**1**, Aldrich) in 1600 mL of glacial acetic acid was added dropwise over 45 min to 3400 mL of cold 70% HNO<sub>3</sub>. The solution was stirred continuously and maintained at 3–5 °C throughout the addition and for another 60 min afterward. The mixture was allowed to stir at ambient temperature for another 2 h and then slowly poured into 10 L of crushed ice. The resulting yellow solid was collected by filtration, washed with water, dried under vacuum, and recrystallized from THF/hexane to afford 345 g (66%) of **2**. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  7.55 (s, 1H), 6.75 (s, 1H), 6.18 (s, 2H), 2.49 (s, 3H). ESI-MS: 210 (M + H<sup>+</sup>), 232 (M + Na<sup>+</sup>). Anal. Calcd for C<sub>9</sub>H<sub>7</sub>NO<sub>5</sub>: C, 51.68; H, 3.37; N, 6.70. Found: C, 51.56; H, 3.21; N, 6.40. The product was contaminated with a small amount (3–5% by HPLC) of an inert byproduct, 1,2-(methylenedioxy)-4-nitrobenzene. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz):  $\delta$  7.93 (d,  $J = 10$  Hz, 1H), 7.80 (s, 1H), 7.17 (d,  $J = 10$  Hz, 1H), 6.30 (s, 2H).

**(*R,S*)-1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethanol (3).** Sodium borohydride (55 g, 1.45 mol) was added over 60 min to a cold, stirring suspension of 3,4-(methylenedioxy)-6-nitroacetophenone (690 g, 3.3 mol) in 3200 mL of methanol, maintaining a temperature of 5–15 °C with external cooling. Stirring was continued at ambient temperature until the reaction was complete (2–3 h), at which time the mixture was combined with 1600 mL of saturated aqueous ammonium chloride. The resulting suspension was extracted three times with CH<sub>2</sub>Cl<sub>2</sub>, and the combined extracts were washed with brine, dried with MgSO<sub>4</sub>, and evaporated to give 680 g (98%) of product after drying under vacuum. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 200 MHz):  $\delta$  7.46 (s, 1H), 7.27 (s, 1H), 6.11 (s, 2H), 5.46 (quartet,  $J = 6.5$  Hz, 1H), 1.54 (d,  $J = 6.5$  Hz, 3H). ESI-MS: 234 (M + Na<sup>+</sup>). Anal. Calcd for C<sub>9</sub>H<sub>9</sub>NO<sub>5</sub>: C, 51.19; H, 4.30; N, 6.63. Found: C, 51.24; H, 4.25; N, 6.59.

**(*R,S*)-1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethyl chloroformate (Menpoc-Cl) (4).** A solution of phosgene in toluene (1250 mL of 20% w/v, 2.4 mol) was added to a stirring suspension of (*R,S*)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethanol (211 g, 1.0 mol) in 500 mL of dry THF. Stirring was continued at ambient temperature under argon until the reaction was complete (36–48 h). Excess phosgene was

removed under low vacuum with an aqueous NaOH trap, before the remaining solvents were removed on a rotary evaporator. The residue was triturated with hexane to obtain a solid brown cake, which was then recrystallized from THF/hexane to afford 205 g of product as a light-brown powder which was at least 95% pure according to <sup>1</sup>H NMR. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 200 MHz):  $\delta$  7.52 (s, 1H), 7.05 (s, 1H), 6.47 (quartet,  $J = 6.5$  Hz, 1H), 6.16 (s, 2H), 1.78 (d,  $J = 6.5$  Hz, 3H). Anal. Calcd for C<sub>10</sub>H<sub>8</sub>NO<sub>6</sub>Cl: C, 43.90; H, 2.95; N, 5.12; Cl, 12.96. Found: C, 44.01; H, 2.98; N, 5.02; Cl, 12.96. The material was stored desiccated at –20 °C.

**Synthesis of 5'-MeNPOC-2'-deoxynucleosides (6a–k).** All but one (**6j**) of the 5'-*O*-MeNPOC-2'-deoxynucleosides were prepared from the base-protected nucleosides using the following general procedure: The base-protected nucleoside (90 mmol) was dried by coevaporating three times with 250 mL of anhydrous pyridine, dissolved or suspended in 300 mL of anhydrous pyridine under argon, and then cooled to –15 °C with an ice/methanol bath. A solution of 27.5 g (100 mmol) MeNPOC-Cl in 100 mL of dry CH<sub>2</sub>Cl<sub>2</sub> was then added dropwise with stirring. After 30 min, the ice bath was removed, and the solution allowed to stir overnight at room temperature. After evaporating the solvents, the crude material was taken up in EtOAc and extracted with water and brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to obtain a yellow foam. The crude products were generally purified by flash chromatography on silica gel (0–6% MeOH gradient in CH<sub>2</sub>Cl<sub>2</sub> or 1:1 CH<sub>2</sub>Cl<sub>2</sub>/EtOAc), except **6g**, which was recrystallized from DCM. Products (mixture of diastereomers) were obtained in 65–85% yield, with purity  $\geq 96\%$  as determined by reverse-phase HPLC (15–100% CH<sub>3</sub>CN over 15 min).

**5'-*O*-[(*R,S*)-1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethoxy]carbonyl-N<sup>3</sup>-isobutryl-2'-thymidine (6j).** Chlorotrimethylsilane (2.48 g, 2.9 mL, 23 mmol) was added to a solution of MeNPOC-T (**6i**, 5.5g, 11.5 mmol) in 50 mL of dry DCM containing 2.5 g (3.27 mL, 25 mmol) of triethylamine (TEA). Stirring was continued at room temperature until HPLC analysis indicated complete silylation (~4 h) and then 2 mL each of triethylamine and pyridine were added, followed by 1.5 g (1.5 mL, 14.3 mmol) of isobutryl chloride. HPLC analysis indicated complete acylation after stirring overnight, at which time the mixture was evaporated and redissolved in 100 mL of THF. The resulting solution was filtered to remove TEA·HCl, then 50 mL of water and 25 mL of HOAc were added, and stirring was continued for another 30 min to desilylate the nucleoside. EtOAc and saturated aqueous NaHCO<sub>3</sub> were then added (100 mL of each), and the organic phase was separated and washed with brine (100 mL), dried with Na<sub>2</sub>SO<sub>4</sub>, and evaporated to dryness. The crude product was purified by flash chromatography (DCM/EtAc (9:1 to 7:3 step gradient) to obtain 3.5g of **6j** (55%).

**Synthesis of 5'-MeNPOC-2'-deoxynucleoside 2-Cyanoethyl 3'-*N,N*-Diisopropylphosphoramidites (7a–k).** All 5'-MeNPOC-deoxynucleosides were phosphorylated using the procedure of Barone et al.<sup>17</sup> and purified by flash chromatography (silica gel, DCM/EtOAc containing 1% triethylamine). Yields were in the range of 60–90% with purity  $\geq 96\%$ , as determined by reverse-phase HPLC (40–100% CH<sub>3</sub>CN over 15 min).

**18-*O*-[(*R,S*)-1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethoxy]carbonyl]-3,6,9,12,15,18-hexaoxaoctadec-1-yl *O*'-2-Cyanoethyl-*N,N*-Diisopropylphosphoramidite (MeNPOC-HEG-CEP, 9).** Hexaethylenglycol (Aldrich, 200 g, 710 mmol) was coevaporated twice with 400 mL of dry pyridine and then dissolved in 500 mL of dry pyridine and DCM (4:6). MeNPOC-Cl (**4**) (64.5 g, 236 mmol) in 500 mL of CH<sub>2</sub>Cl<sub>2</sub> was then added dropwise with stirring over 2 h. After another hour of stirring, the mixture was transferred to a separatory funnel and washed three times with 150 mL of water and once with 150 mL of saturated brine. The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to give ~125 g of crude material as an oil, which, according to HPLC analysis, consists of an 8:2 mixture of mono- and bisacylated products. The crude material was purified by flash chromatography (0–5% MeOH in DCM/EtOAc) to give 75 g of 18-[(*R,S*)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethoxy]carbonyl]-3,6,9,12,15,18-hexaoxaoctadecan-1-ol ( $\geq 98\%$  purity by RP-HPLC). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400

(17) Barone, A. D.; Tang, A.-Y.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 4051–4061.

MHz):  $\delta$  7.50 (s, 1H, H<sub>Ar-MeNPOC</sub>), 7.09 (s, 1H, H<sub>Ar-MeNPOC</sub>), 6.27 (quartet,  $J = 6.5$  Hz, 1H, H<sub>ArCH-MeNPOC</sub>), 6.12 (br s, 2H, H<sub>OCH<sub>2</sub>O-MeNPOC</sub>), 4.80 (br s, 1H, H<sub>OHI</sub>), 4.31–4.19 (m, 2H, H<sub>C17</sub>), 3.73–3.59 (m, 22H, H<sub>C1-16</sub>), 1.64 (d,  $J = 8$  Hz, 3H, H<sub>CH<sub>3</sub>-MeNPOC</sub>). ESI-MS: 520 (M + H<sup>+</sup>), 537 (M + H<sub>2</sub>O<sup>+</sup>), 542 (M + Na<sup>+</sup>).

MeNPOC-HEG-OH (80 g, 154 mmol) was coevaporated three times with 300 mL of dry toluene, and combined with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (55.7 g, 185 mmol) and diisopropylammonium tetrazolidine (6.4 g, 77 mmol) in 600 mL of dry DCM under argon at room temperature. The solution was stirred overnight and then transferred to a separatory funnel and washed twice with saturated aqueous NaHCO<sub>3</sub> and once with saturated brine (400 mL each). After drying with Na<sub>2</sub>SO<sub>4</sub>, the organic phase was evaporated and the crude product was purified by flash chromatography (0–50% EtOAc in DCM/0.5% triethylamine) to give 87 g (78.5%) of **9** (purity 98% pure by HPLC; 100% by <sup>31</sup>P NMR). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  7.50 (s, 1H, H<sub>Ar-MeNPOC</sub>), 7.09 (s, 1H, H<sub>Ar-MeNPOC</sub>), 6.27 (quartet,  $J = 6.5$  Hz, 1H, H<sub>ArCH-MeNPOC</sub>), 6.12 (br s, 2H, H<sub>OCH<sub>2</sub>O-MeNPOC</sub>), 4.30–4.18 (m, 2H, H<sub>C17</sub>), 3.90–3.80 (m, 3H, H<sub>C1, CE-a</sub>), 3.76–3.58 (m, 23H, H<sub>C2-16, CH-*ipr*, CE-a</sub>), 2.65 (t,  $J = 8$  Hz, H<sub>CE-b</sub>), 1.65 (d,  $J = 8$  Hz, 3H, H<sub>CH<sub>3</sub>-MeNPOC</sub>), 1.21–1.16 (m, 12H, H<sub>CH<sub>3</sub>-*ipr*</sub>). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz):  $\delta$  +148.7. ESI-MS: 720 (M + H<sup>+</sup>), 742 (M + Na<sup>+</sup>), 821 (M + Et<sub>3</sub>NH<sup>+</sup>). Anal. Calcd for C<sub>31</sub>H<sub>51</sub>N<sub>3</sub>O<sub>14</sub>P: C, 51.66; H, 7.13; N, 5.83; P, 4.30. Found: C, 51.70; H, 6.82; N, 5.73; P, 4.04.

**5'-O-(*R,S*)-(1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethoxy)carbo-nyl)-N<sup>2</sup>-(phenoxyacetyl)-2'-deoxyguanosine 3'-O-(2-Cyanoethyl)-methyl Phosphate (10).** Anhydrous methanol (1.73 mL, 39 mmol) was added to a solution of MeNPOC-(pac)dG-CEP (**7c**, 3.26 g, 3.9 mmol) in 10 mL of dry CH<sub>3</sub>CN under argon, and a solution of tetrazole (0.45 M in CH<sub>3</sub>CN, 10 mL, 4.5 mmol) was added slowly with stirring. After 15 min, 50 mL of EtOAc was added, and the solution was washed with saturated aqueous NaHCO<sub>3</sub> and brine (50 mL each). The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated, and the crude product purified by flash chromatography (DCM/MeOH, 97:3) to obtain 1.7 g (57%) of 5'-MeNPOC-*N*<sup>2</sup>-(phenoxyacetyl)-2'-deoxyguanosine 3'-O-(2-cyanoethyl)methyl phosphite (mixture of diastereomers). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  9.50 (br s, 1H, H<sub>N2</sub>), 7.86, 7.80 (2s, 1H, H<sub>C8</sub>), 7.40, 7.36 (2s, 1H, H<sub>Ar-MeNPOC</sub>), 7.34–7.28 (m, 2H, H<sub>Ar-PAC</sub>), 7.05 (t, 1H, H<sub>Ar-PAC</sub>), 7.00–6.94 (m, 2H, H<sub>Ar-PAC</sub>), 6.99, 6.86 (2s, 1H, H<sub>Ar-MeNPOC</sub>), 6.32–6.26 (m, 1H, H<sub>C17</sub>), 6.24–5.98 (m, 3H, H<sub>MeNPOC</sub>), 5.10–4.95 (br d, 1H, H<sub>C3'</sub>), 4.72–4.62 (m, 2H, H<sub>pac</sub>), 4.43–4.32 (m, 3H, H<sub>C5',4'</sub>), 4.08–4.02 (m, 2H, H<sub>CE</sub>), 3.60 (d, 3H, H<sub>CH<sub>3</sub>O-P</sub>), 2.93–2.82 (m, 1H, H<sub>2'a</sub>), 2.70 (t, 2H, H<sub>CE</sub>), 2.65–2.47 (m, 1H, H<sub>C2'b</sub>), 1.62–1.56 (m, 3H, H<sub>CH<sub>3</sub>-MeNPOC</sub>). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz):  $\delta$  +140.6.

The nucleoside 3'-phosphite triester (1.5g, 1.95 mmol) was combined with 5 mL of 10% aqueous H<sub>2</sub>O<sub>2</sub> in 5 mL of THF. After 5 min, 25 mL of EtOAc was added, and the solution was washed with saturated aqueous NaHCO<sub>3</sub> and brine (25 mL each). The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated, and the crude product purified by flash chromatography (DCM/MeOH, 97:3) to obtain 1.2 g (77%) of **10** (mixture of diastereomers). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  9.77, 9.51 (2d, 1H, H<sub>N2</sub>), 7.85, 7.79 (2s, 1H, H<sub>C8</sub>), 7.40, 7.36 (2s, 1H, H<sub>Ar-MeNPOC</sub>), 7.35–7.29 (m, 2H, H<sub>Ar-pac</sub>), 7.06 (t, 1H, H<sub>Ar-pac</sub>), 7.02–6.96 (m, 2H, H<sub>Ar-pac</sub>), 6.98, 6.85 (2s, 1H, H<sub>Ar-MeNPOC</sub>), 6.32–6.26 (m, 1H, H<sub>C17</sub>), 6.24–5.98 (m, 3H, H<sub>MeNPOC</sub>), 5.33–5.16 (br d, 1H, H<sub>C3'</sub>), 4.74–4.58 (m, 2H, H<sub>pac</sub>), 4.46–4.26 (mm, 5H, H<sub>C5',4'</sub>, H<sub>CE</sub>), 3.86 (d, 3H, H<sub>CH<sub>3</sub>O-P</sub>), 3.07–2.93 (m, 1H, H<sub>C2'a</sub>), 2.82 (t, 2H, H<sub>CE</sub>), 2.80–2.62 (m, 1H, H<sub>C2'b</sub>), 1.62–1.56 (m, 3H, H<sub>Me-MeNPOC</sub>). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz):  $\delta$  +0.15. Anal. Calcd for C<sub>33</sub>H<sub>53</sub>N<sub>7</sub>O<sub>15</sub>P: C, 48.92; H, 4.11; N, 12.48; P, 3.94. Found: C, 48.54; H, 4.00; N, 12.06; P, 3.89. FAB-MS(HR):  $m/z$  calcd for C<sub>33</sub>H<sub>53</sub>N<sub>7</sub>O<sub>15</sub>P (M + H<sup>+</sup>) 786.17723, obsd 786.17741.

***N*<sup>2</sup>-(Phenoxyacetyl)-2'-deoxyguanosine 3'-O-(2-Cyanoethyl)methyl Phosphate (12).** The same method described above for the preparation of **10** was used to prepare 5'-DMT-*N*<sup>2</sup>-pac-2'-deoxyguanosine 3'-O-(2-cyanoethyl)methyl phosphite from the commercially available phosphoramidite. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  9.50 (s, 1H, H<sub>N2</sub>), 7.85 (2s, 1H, H<sub>8</sub>), 7.42–6.78 (m, 18H, H<sub>Ar-DMT,pac</sub>), 6.30 (t, 1H, H<sub>17</sub>), 5.34–5.22 (br m, 1H, H<sub>3'</sub>), 4.69 (s, 2H, H<sub>pac</sub>), 4.29–4.25 (m, 1H, H<sub>4'</sub>), 4.25–4.15 (m, 2H, H<sub>CE</sub>), 3.82 (d, 3H, H<sub>MeO-P</sub>), 3.77 (s, 6H, H<sub>MeO-DMT</sub>), 3.42–3.32 (m, 2H, H<sub>5'</sub>), 2.88–2.65 (m, 4H, H<sub>2'</sub>, H<sub>CE</sub>). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz):  $\delta$  +0.90.

A portion (180 mg, 220  $\mu$ mol) of the DMT nucleoside phosphate

triester was detritylated by dissolving it in 2.5 mL of DCM/MeOH (6:1) containing 6% trichloroacetic acid. After 5 min, 10 mL of EtOAc was added, and the solution was washed with saturated aqueous NaHCO<sub>3</sub> and brine (5 mL each). The organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated, and the crude product purified by flash chromatography (DCM/MeOH, 9:1) to obtain 12 mg (79%) of **12**. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  9.72 (br s, 1H, H<sub>N2</sub>), 8.02 (2s, 1H, H<sub>C8</sub>), 7.32 (t, 2H, H<sub>Ar-pac</sub>), 7.05 (t, 1H, H<sub>Ar-pac</sub>), 6.99 (d, 2H, H<sub>Ar-pac</sub>), 6.39 (t, 1H, H<sub>C17</sub>), 5.31 (br s, 1H, H<sub>C3'</sub>), 4.62 (s, 2H, H<sub>pac</sub>), 4.38 (br s, 1H, H<sub>C4'</sub>), 4.34–4.27 (m, 2H, H<sub>CE</sub>), 3.98–3.93 (m, 1H, H<sub>C5'a</sub>), 3.84 (d, 3H, H<sub>CH<sub>3</sub>O-P</sub>), 3.84–3.76 (m, 1H, H<sub>C5'b</sub>), 3.03–2.94 (br m, 1H, H<sub>C2'a</sub>), 2.81 (br m, 1H, H<sub>CE</sub>), 2.72–2.64 (m, 1H, H<sub>C2'b</sub>). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz):  $\delta$  +0.93. Anal. Calcd for C<sub>22</sub>H<sub>25</sub>N<sub>6</sub>O<sub>9</sub>P: C, 48.18; H, 4.59; N, 15.32; P, 5.65. Found: C, 48.65; H, 4.58; N, 14.73; P, 5.40. FAB-MS(HR):  $m/z$  calcd for C<sub>22</sub>H<sub>26</sub>N<sub>6</sub>O<sub>9</sub>P (M + H<sup>+</sup>) 549.14989, obsd 549.14916.

**1,3-bis-[(*R,S*)-((1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethoxy)-carbonyloxy]propane (13).** A solution of 2.5 g (9.15 mmol) in 3 mL of dioxane was added slowly with stirring to a cold solution of 1,3-propanediol in 5 mL of dry pyridine under argon. After stirring overnight at room temperature, 50 mL of EtOAc was added, the solution was filtered to remove pyr-HCl, and the solvents were evaporated. The crude products were purified by flash chromatography (hexane/EtOAc, 3:2) to obtain 1.2 g of **13** (mixture of diastereomers). UV-vis (dioxane): 245 nm ( $\lambda_{\max}$ , 2.5  $\times 10^4$ ), 294 nm (sh, 6.5  $\times 10^3$ ), 344 nm ( $\lambda_{\max}$ , 1.03  $\times 10^4$ ). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz):  $\delta$  7.49 (2s, 2H), 7.06 (s, 2H), 6.27–6.21 (m, 2H), 6.12 (s, 4H), 4.22–4.11 (m, 4H), 1.99 (quintet, 2H), 1.63 (d, 6H). EI-MS: 573 (M<sup>+</sup>). Anal. Calcd for C<sub>13</sub>H<sub>15</sub>N<sub>3</sub>O<sub>8</sub>: C, 49.84; H, 4.83; N, 4.87. Found: C, 49.55; H, 4.82; N, 4.55.

**3-[(*R,S*)-((1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethoxy)carbo-nyloxy]propan-1-ol (14)** was prepared by the method described for **9**. UV-vis (dioxane): 244 nm ( $\lambda_{\max}$ , 1.23  $\times 10^4$ ), 294 nm (sh, 3.4  $\times 10^3$ ), 345 nm ( $\lambda_{\max}$ , 5.2  $\times 10^3$ ). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz):  $\delta$  7.50 (s, 1H), 7.07 (s, 2H), 6.26 (quartet, 1H), 6.12 (s, 2H), 4.31–4.19 (octet, 2H), 3.70 (br m, 2H), 1.89 (quintet, 2H), 1.64 (d, 3H). ESI-MS: 314 (M + H<sup>+</sup>), 336 (M + Na<sup>+</sup>). Anal. Calcd for C<sub>23</sub>H<sub>22</sub>N<sub>2</sub>O<sub>14</sub>: C, 50.19; H, 4.03; N, 5.09. Found: C, 50.18; H, 3.95; N, 4.99.

**Methyl 3,4-(Methylenedioxy)-6-nitrosophenyl Ketone (11).** A solution of 1.2 g  $\alpha$ -methyl-3,4-(methylenedioxy)-6-nitrobenzyl alcohol (**3**) in 40 mL of acetonitrile was circulated through a 75  $\times$  75 mm (1 mm path length) quartz flowcell under continuous exposure to near-UV irradiation (10 mW/cm<sup>2</sup> at 365 nm) from a 500 W collimated mercury arc light source (model 87330, Oriel Instruments, Stratford, CT) for 4 h. The partially photolyzed mixture was evaporated to dryness and purified by flash chromatography (DCM). The product eluted as a green band (TLC,  $R_f = 0.35$ /DCM), which was reduced *in vacuo* to induce crystallization of the product as green-yellow crystals (250 mg), purity 97% by HPLC (5–80% CH<sub>3</sub>CN over 25 min). UV-vis (dioxane): 263 nm ( $\lambda_{\max}$ , 1.4  $\times 10^4$ ), 328 nm (sh, 4.3  $\times 10^3$ ), 382 nm ( $\lambda_{\max}$ , 9.5  $\times 10^3$ ). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz):  $\delta$  7.49 (s, 1H), 6.35 (s, 2H), 6.82 (s, 1H), 2.79 (s, 3H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz):  $\delta$  210.8, 161.0, 154.7, 150.4, 143.7, 106.9, 104.2, 89.0, 33.2. EI-MS: 193 (M<sup>+</sup>). Anal. Calcd for C<sub>9</sub>H<sub>7</sub>NO<sub>4</sub>: C, 55.96; H, 3.65; N, 7.25. Found: C, 55.73; H, 3.75; N, 7.07.

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**Supporting Information Available:** Synthetic procedures and analytical data (NMR, MS, elemental) are available for *N*<sup>2</sup>-(4,4'-dimethoxytrityl)-2'-deoxyguanosine, *O*<sup>6</sup>-(*N,N*-diphenylcarbamoyl)-*N*<sup>2</sup>-(phenoxyacetyl)-2'-deoxyguanosine, all 5'-MeNPOC-2'-deoxynucleosides (**6a–k**), and phosphoramidites (**7a–k**) (12 pages). See any current masthead page for ordering and Internet access instructions.