Comparison of Methods for Photochemical Phosphoramidite-Based DNA Synthesis

Michael C. Pirrung*,[1] and Jean-Claude Bradley

Department of Chemistry, P. M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27708-0346

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Toward the goal of preparing surface-bound arrays of short oligonucleotides using light-directed synthesis, we have studied novel photoremovable protecting groups for solid-phase DNA synthesis. We have protected the 5′-hydroxyls of all four nucleosides with our recently-described 3′,5′-dimethoxybenzoin carbonate (DMB-carbonate) group and converted the products to (cyanoethyl)-phosphoramidites. For comparison with another recently-described method for photochemical DNA synthesis, we have prepared the methylnitropiperonyl carbonate (MeNPOC) derivative of thymidine and its amidite. To permit chain attachment to supports, the thymidine DMB-carbonate succinoyl derivative was also prepared. High cycle yields (based on release of the photoproduct dimethoxyphenylbenzofuran) were obtained in initial photochemical DNA synthesis studies with DMB-carbonates. An increase in the deprotection half-life was seen with increasing oligomer length, but this effect was scale-dependent and should be minimal at the small scale at which most DNA arrays will be prepared. The oligonucleotides resulting from these syntheses contained some impurities. It was shown that 350 nm irradiation does not produce thymine dimers but does damage benzoylcytidine. This problem is solved through a change of protecting groups. However, other factors also affect the quality of the DNA prepared by photochemical synthesis, since direct comparisons between short oligomers of thymine prepared with DMTr, DMB-carbonate, and MeNPOC protecting groups show that photochemical deprotection results in diminished cycle yields compared to acid deprotection.

Introduction

Rapid and inexpensive methods of genetic analysis will be important in future methods for medical diagnosis and are crucial to achieving the goals of the human genome project (HGP).[2] In particular, current methods of DNA sequencing are too slow and too expensive to complete the 3.5×10^9 nucleotide sequence over the next decade. Consequently, the development of new sequencing technologies is important to the HGP. One of the novel methods proposed for DNA sequencing is sequencing-by-hybridization[3] (SBH, Figure 1), in which the presence of n-mer subsequences within an unknown target DNA is detected by hybridization to a complete library of n-mer oligonucleotides. The sequence of the target can then be reconstructed from the sequence overlaps of the individual positive hybridization probes.

A number of laboratories[4–7] are aiming to develop libraries of short DNA oligomers that can be used to detect complementary sequences in SBH. For example, a mock sequencing experiment has distinguished and sequenced 100–140 bp lengths of DNA using 156 10-mer and 11-mer probes.[8] While in theory the SBH method could be applied either with the probes in solution and the target(s) immobilized or the other way around (reverse blot), and in fact the initial experiments toward demonstrating this technology have used both, the advantages of light-directed synthesis[9] (Figure 2) in preparing large, high-density arrays of polymer sequences are necessary for and sufficient to enable the concept. Light-directed synthesis permits the preparation of thousands of different sequences at small locations on a surface. The pattern of illumination through a mask determines which regions of the surface are activated for chemical coupling. Light removes photolabile protecting groups from selected areas, and then the first of a set of

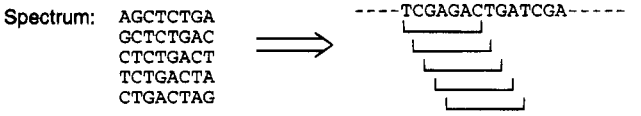

Figure 1.

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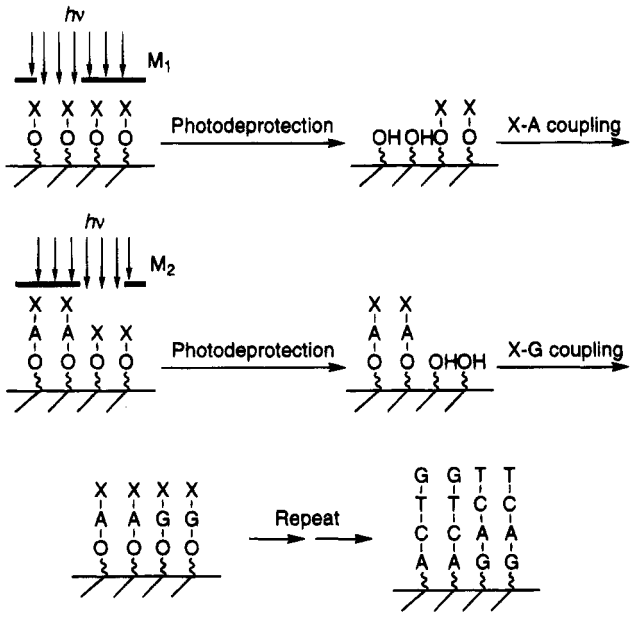

Figure 2.

building blocks (each bearing a photolabile protecting group) is exposed to the entire surface. It can react only with regions that were addressed by light in the preceding step. The substrate is illuminated through another mask for deprotection and coupling with a second building block. The steps of masking/irradiation and coupling are repeated to build up various oligomers. The pattern of masks and the sequence of reagents define the ultimate sequences synthesized and their locations on the surface. Each site is accessible for interaction with other molecules; such an array can be used to conduct binding assays with vast parallelism.

Because light-directed synthesis permits the chemistry of DNA preparation to be conducted in parallel at thousands of locations, the number of sequences prepared far exceeds the number of chemical reactions required. For the preparation of oligonucleotides of length l, the number of sequences is 4^l but the number of steps required is only $4l$. This means that a complete set of octamers (~65 000) can be prepared in only 32 steps and a complete set of dodecamers (1.67×10^7) in only 48 steps. If each location comprises 10 μm square, the complete array of dodecamers could be contained within an area ~4 cm square.

Like all solid-phase synthesis methods, light-directed synthesis takes advantage of the ability to separate reagents from the products of reactions by washing steps. It likewise requires that each deprotection and coupling reaction in each cycle proceed in as close as possible to quantitative yield in order to produce sequences of high fidelity with no deletions, since no intermediate purification steps can be performed. Because light-directed synthesis of different sequences is simultaneously conducted at many locations on the same surface, it also has the unique trait that the incorporation of "extra" monomer units can result from light falling in unintended regions. This latter limitation to the production of high-quality arrays of DNA can be addressed both through the physics of the masking process and through novel photochemistry. The former limitation can be addressed by optimizing the chemistry of both the deprotection and coupling steps; that topic provided the motivation for this work.

Conventional solid-phase DNA synthesis has been essentially perfected (to the level of automation) through development of the phosphoramidite coupling method[10] using DMTr protection of the 5′-hydroxyl of nucleoside monomers. Adaptation of any solid-phase synthesis method to light-directed synthesis requires only the replacement of a conventional protecting group with a light-sensitive group. This group must therefore be removed in essentially quantitative yield by light of a wavelength that does not damage oligonucleotides. It also cannot give byproducts that damage DNA.

Recently, initial efforts in preparing DNA chips through light-directed synthesis and their use in mock sequencing experiments have been reported.[11] Using (cyanoethyl)-phosphoramidite chemistry and a light-sensitive methylnitropiperonyl carbonate (MeNPOC) replacement for the DMTr group, arrays of 256 octamers (four mixed nucleotide positions flanked by two CG clamps) have been prepared and used in hybridization experiments. However, no direct measures of the fidelity of the DNA synthesized in this work were reported. We have recently developed alternative photochemically-removable protecting groups for alcohols that can be used for the protection of nucleosides[12] and in principle used in light-directed DNA synthesis; we used one of these, 3′,5′-dimethoxybenzoin carbonate (DMB-O_2C), in solid-phase syntheses of simple trinucleotides.[13] In order to further investigate the quality of the DNA prepared by photochemical methods, we have performed solid-phase syntheses of several different short sequences, cleaved them from the support, and directly compared them with oligomers derived from conventional DMTr chemistry. Though the results were not equivalent, the problems are not unique to the DMB-carbonate, because the MeNPOC chemistry reported by Pease *et al.* gives similar results. These experiments are encouraging in that DNA can be effectively prepared with photochemical deprotection steps, but they suggest that a problem unique to photochemical DNA synthesis must be solved in order to prepare the highest-quality DNA arrays using light-directed synthesis.

Materials and Methods

General. Protected nucleosides, (cyanoethyl)(diisopropylamino)chlorophosphine, and long-chain alkylamine controlled-pore glass beads were obtained from Glen Research. Other reagents were from Aldrich. For ^{1}H NMR data, J values are given in Hz.

General Procedure for the Preparation of Nucleoside 3′,5′-Dimethoxybenzoin Carbonates. Methyl triflate (Aldrich, 2.71 mL, 24 mmol) was added dropwise to a solution of carbonyldiimidazole (1.96 g, 12 mmol) in 18.5 mL of nitromethane at 0 °C. The ice bath was removed, and the solution was stirred for 30 min at rt. This solution was transferred to a flask containing dimethoxybenzoin[14] (3.26 g, 12 mmol), freshly azeotroped from methylene chloride/benzene. The solution was stirred at rt for at least 1 h. The formation of the reagent (also contains 1 equiv of methylimidazolium triflate) may be verified by ^{1}H NMR by diluting a small aliquot (10 μL) of this solution into $CDCl_3$: δ 3.78 (s, 6H), 3.96 (s, 3H), 4.10 (s, 3H), 6.45 (s, 1H), 6.67 (s, 2H), 7.09 (s, 1H), 7.15 (s, 1H), 7.37 (s, 1H), 7.43 (t, J = 7.8, 2H), 7.49 (s, 1H), 7.57 (t, J

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= 6.6, 1H), 7.93 (d, J = 7.5, 2H), 9.54 (s, 1 H), 8.80 (s, 1H). No change in the ^{1}H NMR spectrum was observed after 1 month at rt under an argon atmosphere. One equiv of the 0.5 M solution prepared above was added dropwise over 10 min to an equal volume of a pyridine solution of 1 equiv of nucleoside twice azeotroped from pyridine. The resulting solution was stirred at rt for 1 h and evaporated under reduced pressure. Chromatography (1:1 hex/EtOAc to EtOAc/methanol mixtures) provided the title compounds.

Thymidine 5′-(3″,5″-dimethoxybenzoin)carbonate. Yield: 51%. ^{1}H NMR: δ 1.61 and 1.89 (2 × s, 3H), 2.1−2.5 (m, 2H), 3.6 (br s, 1H), 3.72 (s, 6H), 4.15 (m, 1H), 4.3−4.6 (m, 3H), 6.35 (m, 1H), 6.41 (s, 1H), 6.55 (s, 2H), 6.67 (s, 1H), 7.39 (t, J = 7.5, 2H), 7.47 (s, 1H), 7.52 (t, J = 7.5, 1H), 7.91 (d, J = 7.2, 2H), 9.51 (br s, 1/2H), 9.58 (br s, 1/2H). ^{13}C NMR: δ 12.01, 12.40, 40.14, 40.24, 55.37, 67.27, 67.61, 70.97, 71.29, 80.70, 80.89, 84.00, 84.08, 84.51, 84.86, 101.27, 101.33, 106.88, 106.99, 111.42, 111.48, 2 × 128.71, 133.82, 133.98, 134.22, 134.31, 135.63, 150.65, 154.22, 154.34, 161.22, 161.28, 163.97, 164.05, 192.81, 193.08. IR: 1754, 1696 cm^{-1}. MS (FAB, m/e): 541 (M$^+$ + 1), 509, 476, 381, 335. HRMS (FAB, M$^+$): for $C_{27}H_{28}O_{10}N_2$ calcd 540.1744, obsd 540.1747.

***N*-Benzoyldeoxyadenosine 5′-(3″,5″-Dimethoxybenzoin Carbonate).** Yield: 42%. ^{1}H NMR: δ 2.56 (m, 1H), 2.88 (m, 1H), 3.70 (s, 6H), 4.25 (m, 1H), 4.40 (m, 1H), 4.50 (m, 1H), 4.75 (m, 1H), 4.75 (m, 1H), 6.38 (s, 1H), 6.50 (m, 1H), 6.58 (s, 2H), 6.64 (s, 1H), 7.37 (t, J = 7.5, 2H), 7.4−7.6 (m, 4H), 7.89 (m, 2H), 8.00 (d, J = 6.0, 2H), 8.35 (s, 1H), 8.77 (s, 1H), 9.17 (br s, 1/2H), 9.23 (br s, 1/2H). ^{13}C NMR: δ 39.90, 39.96, 55.45, 67.41, 67.76, 71.48, 71.76, 80.67, 84.43, 84.63, 84.83, 101.40, 101.47, 106.79, 123.13, 127.95, 128.73, 128.82,, 133.00, 133.32, 133.85, 134.03, 134.38, 134.49, 142.05, 142.26, 149.14, 151.35, 151.48, 152.57, 154.21, 154.39, 161.21, 165.22, 193.20, 193.34. IR: 1753, 1703 cm^{-1}. MS (FAB, m/e): 654 (M$^+$ + 1), 613, 494, 460. HRMS (FAB, MH+): for $C_{34}H_{32}O_9N_5$ calcd 654.2200, obsd 654.2175.

***N*-Isobutyroyldeoxyguanosine 5′-(3″,5″-Dimethoxybenzoin Carbonate).** Yield: 45%. ^{1}H NMR (acetone-d_6): δ 1.1−1.3 (m, 6H), 2.45 (m, 1H), 2.7−2.9 (m, 2H), 3.76 (s, 6H), 4.21 (m, 1H), 4.35−4.6 (m, 2H), 4.7−4.8 (m, 2H), (m, 2H), 6.35 (m, 1H), 6.48 (s, 1H), 6.75 (s, 2H), 6.92 (d, J = 10.0, 1H), 7.51 (m, 2H), 7.62 (m, 1H), 8.08 (m, 2H), 8.20 (m, 1H). ^{13}C NMR (acetone-d_6): δ 19.06, 36.33, 40.15, 40.20, 55.62, 68.35, 68.69, 72.08, 72.18, 80.76, 80.81, 84.56, 84.68, 85.20, 85.44, 101.54, 101.62, 107.46, 121.89, 129.37, 129.48, 134.44, 135.07, 135.67, 135.74, 137.74, 137.96, 148.90, 149.08, 154.98, 155.61, 162.03, 180.48, 193.77; IR: 1754, 1709, 1685 cm^{-1}. MS (FAB, m/e) 636 (M$^+$ + 1), 460. HRMS (FAB, MH$^+$): for $C_{31}H_{34}O_{10}N_5$ calcd 636.2306, obsd 636.2296.

***N*-Benzoyldeoxycytidine 5′-(3″,5″-Dimethoxybenzoin Carbonate).** Yield: 23% (using a nonoptimal aqueous workup). ^{1}H NMR: δ 2.15 (m, 1H), 2.71 (m, 1H), 3.68 (s, 6H), 4.2−4.6 (m, 4H), 6.3−6.4 (m, 2H), 6.55 (m, 2H), 6.64 (d, J = 7.0, 1H), 7.0−7.7 (m, 7H), 7.90 (m, 4H), 8.1−8.3 (m, 1H), 9.2 (br s, 1H). ^{13}C NMR δ 41.35, 55.22, 67.30, 67.36, 80.60, 84.61, 84.82, 87.15, 87.40, 96.80, 97.14, 101.30, 101.36, 106.46, 106.57, 127.56, 128.47, 128.56, 128.71, 132.86, 132.92, 133.62, 133.87, 134.22, 134.32, 144.19, 144.65, 154.17, 154.27, 154.95, 161.05, 162.29, 162.32, 171.05, 192.77, 192.88; IR: 1753, 1700, 1666 cm^{-1}. MS (FAB, m/e) 630 (M$^+$ + 1), 460. HRMS (FAB, MH$^+$): for $C_{33}H_{32}O_{10}N_3$ calcd 630.2088, obsd 630.2086.

Thymidine 5′-[1″-Methyl-6″-nitropiperonyl)oxycarbonate] (5). The protocol described above was used with the exception of the substitution of methylnitropiperonyl alcohol for dimethoxybenzoin. The title compound was obtained in 40% yield and showed properties consistent with its earlier synthesis in the work of Pease *et al.* ^{1}H NMR: δ 1.62 and 1.64 (s × 2, 3H), 1.86 (d, J = 4.8, 3H), 2.0−2.5 (m, 2H), 3.7 (br s, 1H), 4.14 (m, 1H), 4.3−4.5 (m, 3H), 6.10 (m, 2H), 6.28 (m, 2H), 6.98 (d, J = 1.5, 1H), 7.34 (d, J = 11.7, 1H), 7.45 (d, J = 2.7, 1H), 9.7 (br s, 1H).

Thymidine 5′-(3″,5″-Dimethoxybenzoin Carbonate) 3′-[*O*-(Cyanoethyl)phosphoramidite] (1). To 1 mmol of thymidine 5′-(dimethoxybenzoin carbonate) in dry methylene chloride (25 mL) was added diisopropylethylamine (12 mmol) followed by 2-(cyanoethoxy)(*N*,*N*-diisopropylamino)chlorophosphine (4 mmol). The reaction was stirred at rt for 30 min and quenched with 0.5 mL of methanol. The solution was quickly washed once with saturated aqueous sodium bicarbonate, dried over magnesium sulfate, and evaporated. The residue was chromatographed on triethylamine-treated silica gel (triethylamine was added to silica gel in methylene chloride solution and evaporated to dryness) using hexanes/ethyl acetate mixtures as eluent. The title compound was obtained in 80% yield. ^{1}H NMR: δ 1.1−1.3 (m, 12H), 1.62 and 1.94 (2 × s, 3H), 2.1−2.7 (m, 4H), 3.5−3.9 (m, 10H), 4.2−4.7 (m, 4H), 6.35 (m, 1H), 6.43 (s, 1H), 6.56 (m, 2H), 6.66 (s, 1H), 7.4−7.6 (m, 4H), 7.92 (d, J = 7.5, 2H), 8.15 (br s, 1H). ^{13}C NMR δ 11.98, 12.42, 20.19, 24.52, 24.61, 39.41, 39.50, 43.19, 43.36, 46.6, 55.39, 55.42, 55.51, 57.88, 57.96, 58.00, 58.12, 58.21, 58.38, 66.96, 67.28, 67.33, 67.40, 72.49, 72.71, 73.15, 73.37, 73.40, 80.68, 80.73, 80.89, 83.14, 83.22, 83.37, 83.45, 84.46, 84.54, 84.73, 101.25, 101.35, 106.92, 107.00, 111.42, 111.49, 117.56, 117.65, 128.72, 133.75, 134.00, 134.27, 134.38, 135.46, 150.25, 150.30, 150.36, 154.11, 154.16, 161.25, 161.30, 163.64, 163.70, 192.54, 192.58, 192.67, 192.74. ^{31}P NMR (acetone-d_6) δ 150.05, 150.19, 150.50, 150.63. ^{31}P NMR ($CDCl_3$): δ 149.37, 149.57, 149.75. IR: 1754, 1697 cm^{-1}. MS (FAB, m/e): 741 (M$^+$ + 1), 670, 523, 453. HRMS (FAB, MH$^+$): for $C_{36}H_{46}O_{11}N_4P$ calcd 741.2901, obsd 741.2896.

***N*-Benzoyldeoxycytidine 5′-(3″,5″-Dimethoxybenzoin Carbonate) 3′-[*O*-(Cyanoethyl)phosphoramidite] (2).** The above protocol was applied to *N*-benzoyldeoxycytidine 5′-(dimethoxybenzoin carbonate) to provide the title compound in 85% yield. ^{1}H NMR (acetone-d_6): δ 1.2−1.3 (m, 12H), 2.4 (m, 1H), 2.7−3.0 (m, 3H), 3.4−4.0 (m, 10H), 4.2 (br s, 1H), 4.3−4.8 (m, 4H), 6.32 (m, 1H), 6.47 (s, 1H), 6.75 (m, 2H), 6.95 (m, 1H), 7.5- 7.7 (m, 7H), 8.1−8.3 (m, 5H). ^{13}C NMR (acetone-d_6): δ 24.52, 24.62, 24.68, 40.63, 43.64, 43.80, 46.78, 55.52, 59.10, 59.17, 59.42, 67.87, 67.94, 68.00, 73.91, 74.13, 80.86, 84.33, 87.69, 87.93, 87.98, 97.23, 97.27, 97.48, 101.51, 101.56, 107.40, 107.47, 118.78, 118.84, 128.86, 128.99, 129.13, 129.43, 133.31, 134.41, 134.98, 135.64, 135.71, 144.83, 144.89, 145.14, 154.73, 154.81, 154.86, 161.97, 163.62, 163.26, 193.73, 193.78. ^{31}P NMR (acetone-d_6): δ 149.90, 150.01, 150.55, 150.60. IR: 1755, 1700, 1668 cm^{-1}. MS (FAB, m/e) 830 (M$^+$ + 1). HRMS (FAB, MH$^+$): for $C_{42}H_{49}O_{11}N_5P$ calcd 830.3166, obsd 830.3134.

***N*-Isobutyroyldeoxyguanosine 5′-(3″,5″-Dimethoxybenzoin Carbonate) 3′-[*O*-(Cyanoethyl)phosphoramidite (3).** The above protocol was applied to *N*-isobutyroyldeoxyguanosine 5′-(dimethoxybenzoin carbonate) to provide the title compound in 53% yield. ^{1}H NMR (acetone-d_6): δ 1.1−1.3 (m, 12H), 2.6−3.0 (m, 4H), 3.5−4.0 (m, 10H), 4.2−4.6 (m, 4H), 4.72 (br s, 1H), 6.35 (m, 1H), 6.47 (s, 1H), 6.76 (m, 2H), 6.92 (m, 1H), 7.45−7.7 (m, 3H), 8.1 (m, 3H). ^{13}C NMR (acetone-d_6): δ 18.96, 19.03, 24.54, 24.59, 24.64, 36.19, 39.13, 39.17, 39.28, 39.36, 43.58, 43.62, 43.74, 43.790, 46.63, 46.69, 46.87, 55.58, 59.13, 59.39, 67.91, 68.01, 74.37, 74.52, 74.75, 80.84, 84.69, 101.52, 101.60, 107.41, 118.89, 212.97, 128.85, 129.34, 129.42, 134.35, 134.41, 134.97, 135.01, 135.58, 137.83, 138.02, 148.85, 148.92, 149.10, 154.90, 154.95, 155.64, 161.97, 180.44, 180.50, 193.63, 193.70. ^{31}P NMR ($CDCl_3$): δ 148.79, 149.01, 149.28, 149.43. IR: 1749, 1702 cm^{-1}. MS (FAB, m/e) 836 (M$^+$ + 1). HRMS (FAB, MH$^+$): for $C_{40}H_{51}O_{11}N_7P$ calcd 836.3386, obsd 836.3392.

***N*-Benzoyldeoxyadenosine 5′-(3″,5″-Dimethoxybenzoin Carbonate) 3′-[*O*-(Cyanoethyl)phosphoramidite (4).** The above protocol was applied to *N*-benzoyldeoxyadenosine 5′-(dimethoxybenzoin carbonate) to provide the title compound in 63% yield. ^{1}H NMR: δ 1.2−1.3 (m, 12H), 2.6−3.0 (m, 4H), 3.6−3.9 (m, 10H), 4.4−4.6 (m, 3H), 4.82 (m, 1H), 6.40 (s, 1H), 6.53 (m, 1H), 6.60 (m, 2H), 6.64 (m, 1H), 7.4−7.7 (m, 6H), 7.92 (d, J = 7.5, 2H), 8.02 (d, J = 7.5, 2H), 8.35 (m, 1H), 8.81 (m, 1H), 9.02 (m, 1H). ^{13}C NMR: δ 24.44, 24.55, 24.65, 39.43, 43.21, 43.38, 46.6, 55.43, 58.07, 58.14, 58.33, 67.31, 67.54, 73.66, 73.87, 80.65, 80.74, 83.67, 84.13, 84.49, 84.81, 101.43, 101.50, 101.55, 106.71, 106.74, 117.54, 123.29, 127.80, 128.66, 128.75, 128.81, 132.70, 133.69, 134.14, 134.39, 134.55, 141.53, 141.79, 149.42, 149.47, 151.54, 152.64, 152.68, 154.10, 154.24, 161.17, 164.47, 164.51, 192.92. ^{31}P NMR ($CDCl_3$): δ 149.40, 149.51, 149.81, 149.86. IR: 1754, 1703 cm^{-1}. MS (FAB,

m/e): 854 ($M^+ + 1$), 636, 494, 453. HRMS (FAB, MH^+): for $C_{43}H_{49}O_{10}N_7P$ calcd 854.3230, obsd 854.3254.

Thymidine 5′-[1″-Methyl-6″-nitropiperonyl)oxycarbonate] 3′-[*O*-(Cyanoethyl)phosphoramidite (6). Using the same protocol as described above, the title compound was obtained in 80% yield. ^{1}H NMR: δ 1.1–1.3 (m, 12H), 1.65 (s × 2, 3H), 1.90 (m, 3H), 2.1–2.5 (m, 2H), 2.62 (m, 2H), 3.4–3.9 (m, 4H), 4.1–4.6 (m, 4H), 6.11 (m, 2H), 6.30 (m, 2H), 7.00 (s, 1H), 7.3 (m, 1H), 7.49 (s, 1H), 8.5 (br s, 1H). ^{31}P ($CDCl_3$) 149.81, 149.84.

Thymidine 5′-(3″,5″-Dimethoxybenzoin Carbonate) 3′-Hemisuccinate (7). Thymidine 5′-(3″,5″-dimethoxybenzoin carbonate) (93 mg, 0.17 mmol), succinic anhydride (105 mg, 0.86 mmol), and DMAP (86 mg, 0.86 mmol) were stirred in 5 mL of methylene chloride for 4 h. The solution was washed with 0.5 N HCl, and the aqueous phase was extracted once with methylene chloride. The organic phases were combined, dried over magnesium sulfate, and evaporated. To the residue was added DMAP (23 mg), 5 mL of THF, and 3 drops of water to destroy excess succinic anhydride. After the mixture was stirred for 24 h at rt, the solvent was removed. The residue was taken up in methylene chloride and washed with 0.5 N HCl, and the aqueous phase was extracted once with methylene chloride. The organic phases were combined, dried over magnesium sulfate, and evaporated to yield 110 mg (96%) of the title compound. ^{1}H NMR: δ 1.56 and 1.93 (2 × s, 3H), 2.2–2.5 (m, 2H), 2.66 (m, 4H), 3.74 (s, 6H), 4.26 (m, 1H), 4.3–4.6 (m, 2H), 5.35 (m, 1H), 6.35 (m, 1H), 6.41 (s, 1H), 6.55 (s, 2H), 6.67 (s, 1H), 7.3–7.6 (m, 4H), 7.90 (d, J = 7.5, 2H), 10.01 (br s, 1/2H), 10.07 (br s, 1/2H). IR: 2600–2400 (br), 1750, 1701 cm^{-1}.

Preparation of Support Bearing T-DMB-O_2C. Long chain alkylamine-controlled porosity glass beads (LCAA-CPG, 87 μmol of amino groups/g) (500 mg, 43.5 μmol), **7** (110 mg, 167 μmol), HOBt (22.5 mg, 167 μmol), DCC (34.5, 167 mmol), DMAP (10.2 mg, 83.5 mmol), DMF (0.5 mL), and methylene chloride (7 mL) were combined and stirred for 18 h. The beads were washed with 10 mL each of DMF, methylene chloride, acetonitrile, and ether. Treatment with a capping solution consisting of 6 mL of pyridine, 20 mg of DMAP, and 0.4 mL of acetic anhydride for 10 min followed by washing with 10 mL portions of methylene chloride, acetonitrile, and ether followed by drying with argon yielded derivatized beads. The loading was assayed as 41.4 and 43 μmol/g on accurately weighed 5–10 mg samples irradiated for 30 min while suspended in acetonitrile, using the absorbance of dimethoxybenzofuran at 300 nm (ϵ = 27 500).

Protocols for DNA Synthesis. All phosphoramidite coupling on beads derivatized with photoremovable groups was carried out by a protocol adapted from Gait[15] and is summarized below. Capping and oxidizing solutions were prepared as described by Gait. DNA synthesis-grade tetrazole solution was purchased from Applied Biosystems. The synthesis was carried out in a capped 2 mL sintered glass funnel which was snugly fitted with the inside rim of a 24/40 rubber septum. Solvents and reagents were forced through the funnel by introducing argon through a needle inserted in the septum. The standard coupling protocol is as follows:

(1) Initial washing: A weighed sample of lcaa-cpg beads bearing the initial nucleoside was introduced into the funnel. Acetonitrile was washed down the sides of the funnel with a Pasteur pipette, and the septum was mounted. Argon pressure was introduced with a gentle flow until all the beads had settled. It is important never to attempt to mount or remove the septum while argon is flowing through the system since this could cause disruption of the beads.

(2) Capping: In the precapping step, 0.75 mL of DMAP solution and 0.25 mL of acetic anhydride/lutidine were mixed in a test tube and gently introduced over the beads with a Pasteur pipette. After 2 min, the reagent was forced out, and the beads were washed once with about 1.5 mL of acetonitrile, three times with methylene chloride, and three more times with acetonitrile, as described in step 1.

(3) Photodeprotection: Acetonitrile (1 mL) was introduced into the funnel, the septum was mounted, and the filtrate was allowed to slowly leak into a test tube while the funnel was irradiated near the center of a Rayonet photoreactor equipped with 16 350 nm lamps. After 7.5 min, the funnel was washed twice with acetonitrile and all of the washings combined to determine the total amount of benzofuran liberated by measuring the absorbance at 300 nm.[14] This process was repeated after 15 min and 45 min of total irradiation time to provide the total amount of benzofuran liberated as well as the half-life of photodeprotection. These time points were useful for a 200 nmol scale. For larger scale syntheses, the time points were appropriately increased.

(4) Coupling: The beads were washed with acetonitrile, and argon was forced through the funnel until the beads were dry. The phosphoramidite to be coupled was azeotroped from freshly-distilled benzene using a vacuum adapter fitted with a stopcock and a heat gun to provide gentle heating. Once the phosphoramidite had foamed, the evacuated flask was filled with argon. The flask was quickly mounted with a septum and flushed with argon briefly. A tetrazole solution (240 μL/30 mg of phosphoramidite) was added via a 500 μL syringe, and the resulting activated monomer was injected gently over the beads without argon flow. After 10 min the reagent was removed and the beads were washed three times with acetonitrile.

(5) Oxidation: About 1 mL of the stock iodine solution was introduced via Pasteur pipette and allowed to react for 1 min. The reagent was removed, and the beads were washed three times with acetonitrile.

(6) Chain extension: Steps 2–5 are repeated until the final coupling.

(7) Final coupling: The final couplings were carried out with a DMTr phosphoramidite to aid in the isolation process. After the oxidation step, the beads were washed twice with acetonitrile, three times with methylene chloride, and three times with acetonitrile and dried with a gentle argon flow.

(8) Solid support cleavage and base deprotection: Concentrated ammonia (1 mL) was added to the beads. A slight vacuum was applied via a needle inserted into the septum to prevent premature leakage of the ammonia solution. After 8–12 h, the ammonia solution was forced into a 1 dram vial via argon pressure, and the beads were washed three times with 0.5 mL of ammonia. The ammonia solution was sealed with a screw cap and parafilm and placed in a water bath kept at 55 °C for 8–12 h.

(9) Analysis of the DNA: the various methods of analysis, as referred to in specific experiments, follow:

(a) Quantitative HPLC: The 5′-terminally tritylated DNA was purified by HPLC on a reversed-phase column from an accurately measured volume of the ammonia solution. The eluent was normally 0.1 M triethylammonium acetate containing 24–32% acetonitrile. The peak was identified in comparison to an authentic tritylated oligo prepared by the standard DMTr protocol. The yield of the DMTr oligo was determined relative to the control (normal DMTr synthesis) by measuring the absorbance at 260 nm of the isolated product in buffer solution.

(b) Oligonucleotide purification cartridge (OPC): A sample of the ammonia solution can be subjected to a workup protocol involving a commercially available (Applied Biosystems) purification cartridge. The procedure has been specially designed to yield the *detritylated* pure oligonucleotide. Thus, the A_{260} values obtained can be used directly without a control to determine absolute yields. However, this procedure will only work well if there is no significant amount of tritylated impurities or decomposition products containing DMTr residues, since these compounds will be isolated along with the desired oligo.

(c) TT-Photodimer analysis: A sample of the ammonia solution was evaporated and treated with 3 N HCl at 100 °C for 20 min, as previously described.[16] Evaporation followed

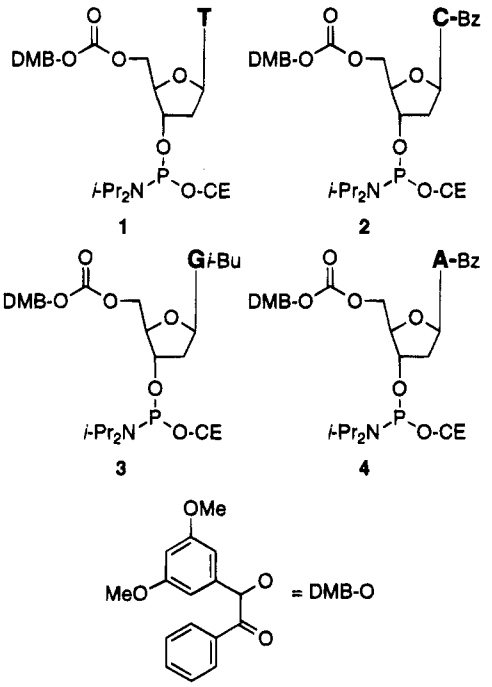
(15) Gait, M. J. *Oligonucleotide Synthesis*; IRL Press: Washington, DC, 1984; p 47.

(16) Cadet, J.; Voituriez, L.; Hruska, F. E.; Kan, L.-S.; De Leeuw, F. A. A. M.; Altona, C. *Can J. Chem.* **1985**, *63*, 2861.

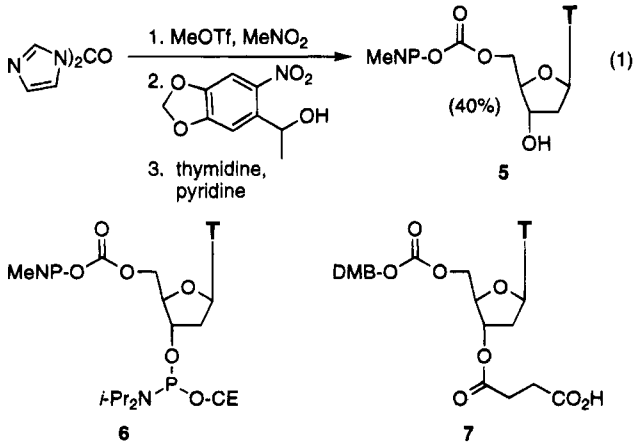
by redissolution in water and HPLC analysis was compared with an authentic sample of thymine–thymine photodimer.

Results

Monomer Synthesis. We previously reported[13] that 5′-DMB-O_2C-T was converted to its 3′-phosphoramidite derivative **1** by conventional means (with a nonaqueous workup). The other three phosphoramidites **2-4** were



obtained by a similar protocol; all were readily purified by twice precipitating them from 6:1 hexanes:methylene chloride or by chromatography on triethylamine-treated silica gel. The 5′-MeNPOC-protected nucleoside **5** was obtained in modest yield by treating methylnitropiperonyl alcohol with Rapoport's methylated carbonyldiimidazole reagent[17] and adding the intermediate carbonate imidazoliumide to thymidine in pyridine (eq 1). It was



converted into its 3′-phosphoramidite derivative **6** by a protocol similar to that used for **1**. Finally, the 3′-succinate of 5′-DMB-O_2C-T (**7**) was produced by conventional means.

Solid-Phase Synthesis. Attachment of the succinoylated DMB-O_2C nucleoside **7** to a lcaa-cpg support was accomplished using DMAP, DCC, and HOBt in a 14:1 methylene chloride/DMF solvent mixture.[18] Loadings of 35–42 μmol/g were obtained, as determined by a benzofuran (BF) assay after irradiation. As described previously,[13] 5′-DMB-O_2C-T yields T in near-quantitative yield when irradiated in acetonitrile solution with the 350 nm phosphor lamps of a Rayonet photochemical reactor. Reaction in acetonitrile is 2-fold slower than irradiation in benzene in solution,[13] but acetonitrile is much more compatible with the other reagents used in phosphoramidite-based DNA synthesis cycles and so was chosen for these studies. 5′-DMB-O_2C-A-Bz and 5′-DMB-O_2C-G-*i*-Bu are likewise deprotected in very high yield by irradiation in acetonitrile. The isolated yield of dimethoxyphenylbenzofuran, the byproduct of the deprotection process, is also high (86–88%). In work to be detailed elsewhere, we have found that the amount of the benzofuran released is only 50–60% of theoretical when this reaction is performed on the solid phase. Consequently, the actual loading could be as much as 100% higher than measured. It was therefore not possible to accurately determine the loading onto supports of the DMB-O_2C-protected nucleosides using BF release, though it can be used to follow the deprotection half-time or the relative cycle yield. Initial attempts to prepare mixed sequence decanucleotides (terminated with a DMTr-nucleoside phosphoramidite to facilitate oligonucleotide purification, HPLC analysis, and yield determination) on solid phase were encouraging in that very high coupling yields (based on BF release) were obtained throughout each synthesis. The inaccuracy of BF release in determining loading discussed above does not diminish its value in measuring cycle yields so long as there is no systematic variation in the yield as the synthesis progresses, and the following experiments showed that there is not. BF release did show an increase in the half-time for deprotection as the synthesis proceeds, and this phenomenon was found to be scale-dependent. An attempted synthesis of 5′-GCTAAAGTTT on a 820 nmol scale (average step yield, 93.1%) showed an increase of half-time from 8 to 20 min from the first to the last coupling. An attempted synthesis of 5′-ACATGGTTTT on a 400 nmol scale (average step yield, 97.0%) showed an increase from 4 min to 15 min over the synthesis. However, the attempted synthesis of 5′-CAGTTTTTTT on a 170 nmol scale (average step yield, 96.2%) showed an essentially constant half-time of 3–4 min. Because the scale of the synthesis in the preparation of DNA arrays is much smaller than used here (pmol/mm^2 of surface area), this behavior should not present a problem. However, analysis of the oligonucleotides produced in these three syntheses showed a multitude of products. An obvious concern was the role of photochemistry in producing these failures. To address this problem, three parallel syntheses of the decanucleotide 5′-ACTTGGTTTA were conducted, one using conventional DMTr chemistry with acid deprotection, one using DMB-O_2C chemistry with photochemical deprotection, and one using conventional DMTr chemistry involving irradiation of the support at 350 nm in acetonitrile after acid removal of each DMTr group. HPLC analysis of the DMTr-oligonucleotide cleaved from the irradiated support (average step yield, 96.2%) showed

(17) Saha, A. K.; Shultz, P.; Rapoport, H. *J. Am. Chem. Soc.* **1989**, *111*, 4856.

(18) Montserrat, F. X.; Grandas, A.; Pedroso, E. *Nucleosides Nucleotides* **1993**, 967.

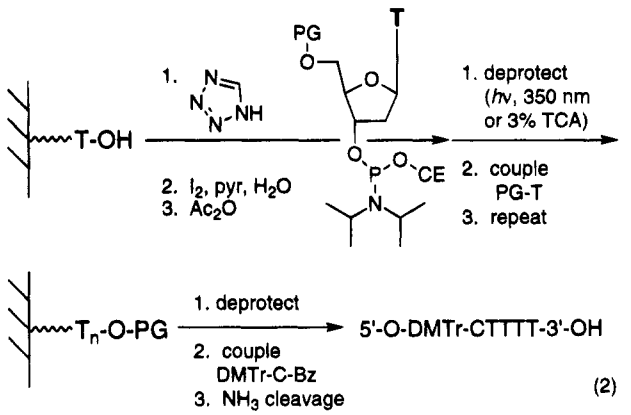
the same family of peaks as in the DMB-O_2C syntheses. The product of the DMTr synthesis that was not irradiated showed a single peak (average step yield, 100.6%, 33.9% isolated after OPC purification) for the expected oligomer. Since this result suggested that the photochemical step was damaging the DNA, the basis of the damage was investigated.

Since the target sequence had several adjacent T residues, it was reasonable to suggest the formation of T–T dimers had occurred, as was recently found by Greenberg in the photochemical release of DNA from solid supports.[19] Although our wavelength of irradiation is longer than is generally required to form thymine dimers, it was possible that some of the broad emission of the phosphor lamps overlapped slightly with the absorption band of thymine and promoted the photocycloaddition reaction. However, a conventional DMTr synthesis of the trinucleotide 5′-ATT on a 470 nmol scale that included an irradiation for 12 h after the initial coupling produced no T–T dimer. A single, strong HPLC peak was seen at a retention time identical to dark-synthesized control, and no dimer was detectable by a modification of Greenberg's method (acidic hydrolysis and HPLC analysis with comparison to an authentic sample). As we have described,[13] neither is there evidence for the formation of T–T dimer in the DMB-O_2C-based syntheses of the same sequence. The photochemical damage of the growing DNA chain must be attributed to other reactions.

The absorption spectra of the protected nucleosides show that benzoyl-C has the longest wavelength absorption, so an experiment similar to that used to address thymine dimerization was performed to determine the stability of C during photochemical DNA synthesis. The sequence 5′-ACT was prepared in two parallel 480 nmol syntheses using conventional DMTr chemistry, with one including an irradiation for 12 h after the coupling of the C. HPLC analysis of the DMTr-trinucleotide cleaved from the irradiated support showed little of the strong, single peak produced from the dark-synthesized control. At least one problem, C, had been identified. To confirm this finding, the photochemistry of benzoyldeoxycytidine was examined. It disappears, as shown by monitoring of its UV spectrum, after just a few minutes of 350 nm irradiation in acetonitrile. We therefore attempted syntheses of decanucleotide and octanucleotide sequences that omit C. However, a family of peaks was again seen in the region of the HPLC profile at which the authentic DMTr-oligonucleotide elutes. Two conclusions are drawn from these experiments, that a novel protecting group must be used for C that minimizes its long-wavelength absorption and that other factors also contribute to the diminished quality of DNA prepared photochemically. Under conditions identical to those used to show the photolability of benzoyldeoxycytidine, however, both acetyldeoxycytidine and [(allyloxy)carbonyl]deoxycytidine[20] are completely stable. It is interesting that in the DMB-O_2C-based syntheses of decanucleotides described above, the apparent cycle yields and the deprotection half-lives for couplings of C were comparable to those for the other nucleosides. These data show that imputing the purity of a photochemically-synthesized sequence based on cycle yields[11] is unreliable.

Another possibility for damage during photochemical DNA synthesis was a reaction between the dimethoxyphenylbenzofuran byproduct of deprotection and the oligonucleotide. This was investigated by irradiation in the presence of (dimethoxyphenyl)benzofuran after each deprotection during a conventional DMTr-based synthesis of the sequence CTTT. No loss in yield or quality was noted.

In order to systematically investigate the cause(s) of the diminished quality of photochemically-synthesized DNA, the targets were simplified to oligomers of T terminated by DMTr-C. Solid-phase syntheses were performed on 150–200 nmol scales with 3′-[*O*-(cyanoethyl)phosphoramidite] nucleosides bearing three 5′-protecting groups: DMTr, DMB-O_2C (**1**), and MeNPOC (**6**). To make the comparisons as close as possible, commercially-available DMTr-T-derivatized lcaa-cpg glass beads were used to initiate each synthesis. Tri- (5′-CTT), tetra- (5′-CTTT), and pentanucleotides (5′-CTTTT) were prepared, with the final coupling being performed with DMTr-C-Bz (eq 2). This permitted direct comparison



(2)

between oligomers prepared using different protocols using a simple analysis of the 5′-DMTr-oligomer by quantitative reversed-phase HPLC and/or yield determination by DMTr release. It also eliminated concerns with nitrogen protecting groups, particularly the undesired photochemistry of C-Bz.

The DMTr-based synthesis of 5′-DMTr-CTTTT (the control) proceeded in an average step yield of 97.8% as determined by trityl release from the penultimate coupling. The DMB-O_2C-based synthesis of the same sequence was performed in duplicate. The step yields of the first coupling in each, because they were determined by BF release, appeared abnormally low (57.7 and 59.3%), but subsequent step yields were >90%. These data provide further confirmation of our inference that the release of BF from irradiated supports is not quantitative and suggest that yields for all three cycles were likely >90%. However, quantitative HPLC analysis of the oligonucleotides cleaved from the support showed only ~25% of the amount of pentamer as was obtained in the DMTr synthesis.

A shorter oligomer, 5′-DMTr-CTTT, was prepared with all three protecting groups. The DMTr-based synthesis (the control) proceeded in an average step yield of 97.9% based on DMTr release from the cleaved oligomer. A similar synthesis that included irradiation after the acid removal of the DMTr group in each cycle gave an average step yield of 97.2% based on DMTr release and a preparative yield of 104.2% of the control. This result suggests that the difficulty in the photochemical synthesis of the 5′-ACTTGGTTTA sequence was indeed the photochemistry of C-Bz. The DMB-O_2C-based synthesis

(19) Greenberg, M. M.; Gilmore, J. L. *J. Org. Chem.* **1994**, *59*, 746.
(20) Pirrung, M. C.; Fallon, L.; Lever, D. C.; Shuey, S. W. *J. Org. Chem.*, in review.

of 5'-DMTr-CTTT gave step yields of 68% (low, as predicted) and 93%, an average step yield of 96.2% based on the final DMTr release, and a quantitative HPLC yield of 64% of the control. MeNPOC-based synthesis does not permit the determination of individual step yields; it did give an average step yield of 92.7% for the preparation of the target sequence based on DMTr release, and the quantitative HPLC yield was 60% of the control.

An even shorter sequence, 5'-DMTr-CTT, was also prepared that uses only one coupling cycle involving novel protecting groups. The DMTr-based synthesis (the control) proceeded in an average step yield of 92.7% based on DMTr release from the cleaved oligomer. The DMB-O_2C-based synthesis of the same sequence gave a step yield of 59% (low, as expected), an average step yield of 92.0% based on DMTr release, and a quantitative HPLC yield of 76.5% of the control. The MeNPOC-based synthesis gave an average step yield of 89.1% based on DMTr release and a quantitative HPLC yield of 77.6% of the control.

Discussion

This study has demonstrated that photochemical deprotection steps in solid-phase DNA synthesis can result in oligonucleotides of diminished quality. This loss does not derive from T–T dimer formation. The photochemical degradation of benzoylcytidine was identified as one problem, but it could be solved by changing protecting groups. Other side reactions must also contribute to the lower quality of the DNA. Possibilities include a photochemical or thermal reaction between the side products of the deprotection reactions (carbon dioxide and dimethoxyphenylbenzofuran for DMB-carbonate, carbon dioxide, and a nitrosoacetophenone for MeNPOC) and the growing DNA strand or direct photochemical damage of other bases or functionalities. It seems unreasonable that CO_2 could damage oligonucleotides under most circumstances. Sheehan reported that the (dimethoxyphenyl)benzofuran byproduct of the deprotection of dimethoxybenzoin esters is subject to photochemical [2 + 2] dimerization, which suggests it could also participate in photochemical reactions with heterocyclic bases.[21] The participation of dimethoxyphenylbenzofuran in a photochemical reaction with the growing DNA strand is disfavored on the basis of the negative result in an irradiation conducted in its presence, but this experiment does not exclude the possibility that BF is reactive when released on the lcaa-cpg beads in locally-high concentrations as deprotection occurs. The nitrosocarbonyl byproducts of nitrobenzyl photochemically-removable protecting groups are widely recognized to participate in both photochemical and thermal reactions with a variety of functional groups,[22] so it is reasonable to ascribe the lower quality of DNA prepared with these groups to this property. However, the quite comparable yields in syntheses with the two different photochemically-removable groups suggest that the problem is intrinsic to photochemical deprotection, though the controls involving irradiation in the DMTr-based syntheses argue against this idea.

These data raise concern about the quality of the DNA that has been prepared in array synthesis experiments. If the yield for two couplings observed here (~66%) is representative of similar reactions on a flat surface, the amount of the intended DNA sequences within each synthesis area in the experiments reported by Pease *et al.* (involving four photochemical couplings) would be <50%. When this yield is extrapolated to the octanucleotides necessary for reasonable DNA reading length in SBH, the amount of the desired sequences would be <20%, and this value reflects only errors that can be ascribed to chemistry. Errors arising from imperfection in the masking process would further diminish the specificity of the sequences synthesized in each area. This dilution of intended sequences by "junk" DNA will obviously raise the signal-to-noise ratio in subsequent hybridization experiments. The initial good successes with SBH in the reverse blot format despite this problem suggest that improvement in the quality of the DNA synthesized photochemically on solid phase will significantly improve the reliability of this method. Our future efforts will be directed toward this goal.

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Supporting Information Available: NMR spectra for the compounds characterized by HRMS (8 pages). 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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