Optimization and Mechanistic Analysis of Oligonucleotide Cleavage from Palladium-Labile Solid-Phase Synthesis Supports[1]

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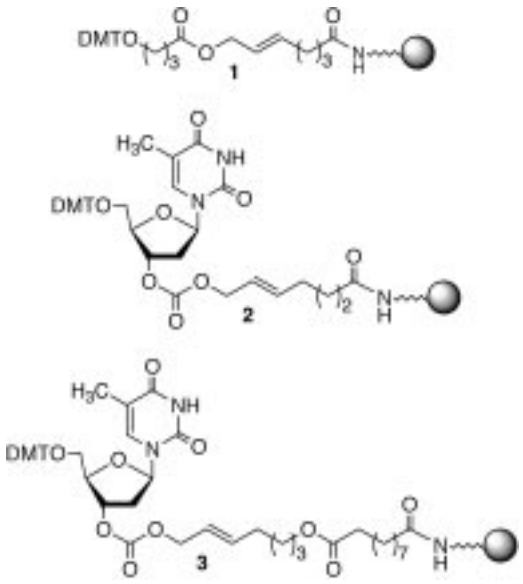
Pd(0)-labile solid-phase synthesis supports have been used to produce oligonucleotides containing 3′-alkyl carboxylic acid and 3′-hydroxy termini in quantitative yields. Optimization of the cleavage reaction conditions using tetrabutylammonium formate buffer resulted in quantitative yields of oligonucleotides using 4 molar equiv of $Pd_2(dba)_3 \cdot CHCl_3$ in 1 h at 55 °C. A proton source facilitates cleavage of the oligonucleotide from the supports. Trace amounts of water, acting as a nucleophile on the η^3-complex, presumably preventing back biting by the initially released oligonucleotide, are required to obtain reproducibly high yields of cleaved oligonucleotides during a 1 h reaction. The previously observed lability of β-cyanoethyl groups to the Pd(0) conditions has been examined using a mononucleotide substrate. Cleavage of the β-cyanoethyl group was shown to proceed to the exclusion of other alkyl groups. A mechanism involving initial insertion by Pd(0) into the carbon−oxygen bond of the β-cyanoethyl group is suggested to account for the cleavage of this group.

The Tsuji−Trost Pd(0) cleavage reaction has proven to be very useful in oligonucleotide synthesis. Noyori and Hayakawa were the first to utilize this reaction for deprotecting the phosphate diesters and exocyclic amines in oligonucleotides.[2] Their strategy has been used in conjunction with photolabile solid-phase supports to prepare oligonucleotides containing alkaline labile nucleotides at defined sites.[3] More recently, the Tsuji−Trost Pd(0) cleavage reaction has been employed to cleave oligonucleotides from their solid-phase supports (**1**).[4,5] The exocyclic amine, 5′-hydroxyl, and commercially available methyl phosphate protecting groups are unaffected by the Pd(0) cleavage reaction conditions.[4] Hence, the Pd(0)-labile supports can also be used to produce protected oligonucleotides in solution, which are useful for synthesizing oligonucleotide conjugates in high yield under mild conditions.[6] Supports **2** and **3** have expanded the array of functionalization obtainable from palladium-labile solid-phase synthesis supports to include optimal production of oligonucleotides containing 3′-hydroxy termini. The optimization of reaction conditions for oligonucleotide cleavage from these Pd(0)-labile supports, as well as relevant mechanistic observations, is described below.

Our original interest in Pd(0)-labile solid-phase synthesis supports was an outgrowth of work involving orthogonal photolabile solid-phase supports.[7] During the past decade, we and others have utilized photochemistry



and, in particular, the *o*-nitrobenzyl photoredox reaction in developing methods for solid-phase synthesis.[8−10] In our own research, the *o*-nitrobenzyl photoredox reaction has been used to synthesize solid-phase supports that release oligonucleotides (protected or unprotected) containing 3′-hydroxy, 3′-alkyl carboxylic acids, or 3′-alkylamines (**4**−**6**).[7] The supports are compatible with commercially available reagents and automated oligonucleotide

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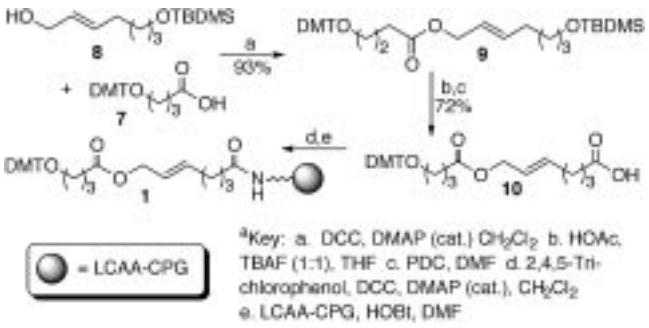
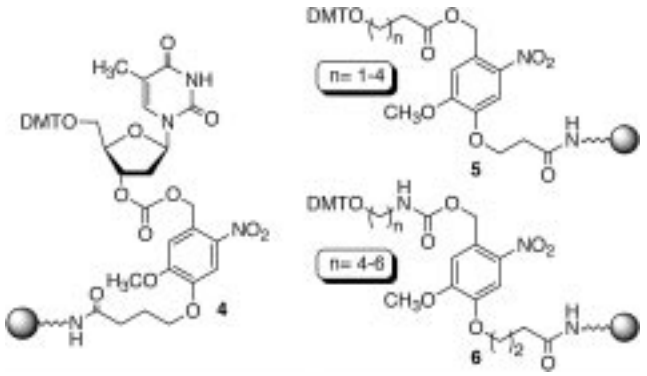
Scheme 1[a]



[a]Key: a. DCC, DMAP (cat.) CH_2Cl_2 b. HOAc, TBAF (1:1), THF c. PDC, DMF d. 2,4,5-Trichlorophenol, DCC, DMAP (cat.), CH_2Cl_2 e. LCAA-CPG, HOBt, DMF

synthesis protocols. Isolated yields as high as 98% of oligonucleotide containing photodamage below detectable limits are obtainable. However, decreases in oligonucleotide yields are observed when the length of the biopolymer is increased from 20 to 40 nucleotides.[7a] This decrease in yield prompted us to investigate the Tsuji–Trost reaction as a method for the cleavage of protected oligonucleotides from solid-phase supports, the yields of which we assumed would be independent of oligonucleotide length. Our preliminary experiments demonstrated that this was indeed the case.[4]

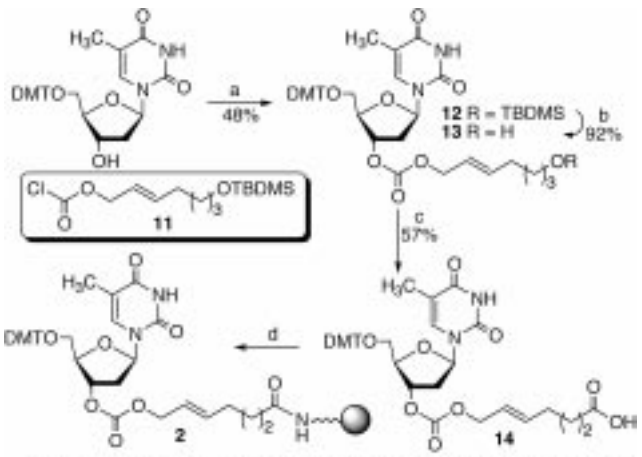


Results and Discussion

Synthesis of Pd(0)-Labile Solid-Phase Oligonucleotide Synthesis Supports. The general approach for the synthesis of **1** was presented previously (Scheme 1).[4] However, the experimental details are described in the Experimental Section of this paper. Two Pd(0)-labile supports (**2**, **3**) that release oligonucleotides containing 3′-hydroxy termini were designed on the basis of the successful utilization of **1**. When compared to **2** and previously described Pd(0)-labile (**1**) and photolabile (**4**–**6**) supports, solid-phase support **3** was designed to contain a longer tether between the long chain alkylamine controlled-pore glass support (LCAA-CPG) and the reactive center. This longer tether was introduced in order to examine whether increasing the distance between the support and the reactive functionality (thereby increasing the accessibility of reagents to the Alloc group) increased the efficiency of the cleavage reaction.

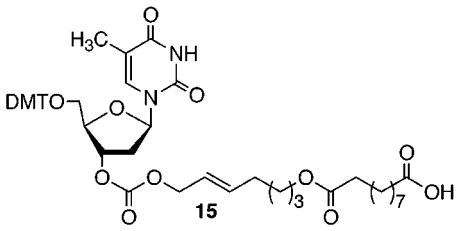
Solid-phase support **2** was prepared via coupling the chloroformate (**11**) of the previously reported alcohol (**8**) with the dianion of 5′-*O*-dimethoxytrityl thymidine (Scheme 2).[11] The major impurity of this reaction

Scheme 2[a]



[a]Key: a. NaH, THF then **11** b. AcOH, TBAF, THF c. PDC, DMF d. Pyridyl disulfide, PPh_3, DMAP, CH_3CN, 1,2-dichloroethane, LCAA-CPG

consisted of *N*-acylated product. Following desilylation of the coupling product (**12**), **13** was oxidized to the carboxylic acid (**14**), which was then loaded directly onto the LCAA-CPG.[12] Using the free carboxylic acid to load the LCAA-CPG marks a departure from previous syntheses of orthogonal solid-phase supports prepared in our group, which involved prior activation and isolation of the carboxylic acid as the respective trichlorophenyl ester.[4,7] The synthesis of **3** was accomplished using **13** as a branching point. Sebacic acid was coupled to **13**, and the resulting carboxylic acid (**15**) was loaded onto the LCAA-CPG.



The Effect of Reaction Buffer on the Efficiency of Pd(0)-Labile Solid-Phase Supports. Excellent yields of undamaged oligonucleotides were obtained from **1** using *n*-$BuNH_2/HCO_2H$ as reaction buffer. However, the reaction required 5 h to proceed to completion, and the workup of the biphasic Pd(0) reaction mixture was made difficult by residual reagents. Consequently, tetrabutylammonium formate (TBA) was investigated as an alternative buffer system.[13] TBA offered several potential advantages over *n*-$BuNH_2/HCO_2H$, including monophasic reaction conditions and a more facile workup. In addition, we anticipated that cleavage of the oligonucleotides from the solid-phase supports might proceed more quickly using the tetralkylammonium buffer, by eliminating the possibility for formation of weak σ-complexes between Pd(0) and the alkylamine in the buffer, which reduces the amount of Pd(0) available for reaction.

Indeed, quantitative yields of **16** were obtained from *O*-methyl phosphate protected polythymidylate in 1 h at 55 °C using 20 molar equiv of $Pd_2(dba)_3 \cdot CHCl_3$, 1,2-bis(diphenylphosphino)ethane (DIPHOS, 100 molar equiv), and 0.12 M TBA (Table 1). Control experiments carried

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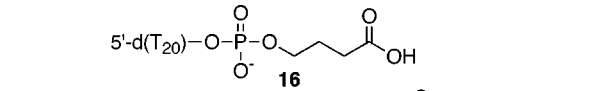
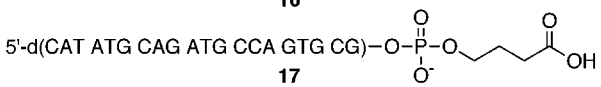
Table 1. Isolated Yields of Fully Deprotected Oligonucleotides Obtained via Pd(0)-Mediated Cleavage[a]

oligonucleotide	solid-phase support	reaction time (min)	isolated yield (%)[d,e]
16[b]	1	60	99 ± 5
16[c]	**1**	30	81 ± 3
17[b]	**1**	60	102 ± 6
18[c]	**2**	60	98 ± 16
18[c]	**3**	45	103 ± 3
18[c]	**3**	30	80 ± 9

[a] All reactions were carried out at 55 °C using 20 molar equiv of $Pd_2(dba)_3$·$CHCl_3$ and 100 molar equiv of DIPHOS relative to DNA. The concentration of TBA buffer was 0.12 M. [b] *O*-Methyl-protected phosphoramidites were used. [c] *O*-*β*-Cyanoethyl-protected phosphoramidites were used. [d] Yields reported with a standard deviation are the average of at least three separate reactions. [e] Isolated yields are determined via comparison of the isolated yield of oligonucleotide obtained via Pd(0) cleavage and subsequent NH_4OH treatment, versus that obtained via direct NH_4OH treatment of resin bound oligonucleotide from the same oligonucleotide synthesis.



16



17

5'-d(CAT ATG CAG ATG CGA GTG CT)

18

out in the absence of Pd(0) resulted in no detectable cleavage of the oligonucleotide. Isolated yields of oligonucleotides were independent of sequence (e.g. **17**). However, slightly lower yields were obtained upon shortening the reaction time 30 min. The reaction conditions that were successful for **16** and **17** from **1** gave rise to quantitative yields of **18** from **2** (Table 1). Unfortunately, the solid-phase support containing a longer tether (**3**) resulted in only marginally greater reactivity (Table 1). The integrity of the biopolymers, following aminolysis, which would result in cleavage at damaged sites (and result in less than the observed quantitative yields), were established by electrospray mass spectrometry, and/or enzymatic digestion, followed by reverse phase HPLC analysis of the nucleoside components.[14]

Optimization of Pd(0) Cleavage Conditions. During the course of these studies, it was determined that a slight excess of formic acid (≈0.5 equiv) relative to TBA was required in order to obtain quantitative yields of cleaved oligonucleotides. No DNA was cleaved from the resin in the absence of formic acid, suggesting that a proton source is required for a successful reaction. Additional acid results in degradation of the biopolymer, presumably due to cleavage of the glycosidic bonds. A proton source is available in the *n*-$BuNH_2$/HCO_2H buffer system used in previous experiments, but not when using TBA in the absence of additional formic acid.[2,4] We suggest that the proton facilitates the insertion of the Pd(0) into the carbon−oxygen bond by increasing the electrophilicity of the carboxyl group (Scheme 3). However, involvement of acid in a later step cannot be ruled out.

The final optimization of the Pd(0)-mediated cleavage conditions was carried out using **3**. The choice of support is arbitrary, as all three of the Pd(0)-labile supports

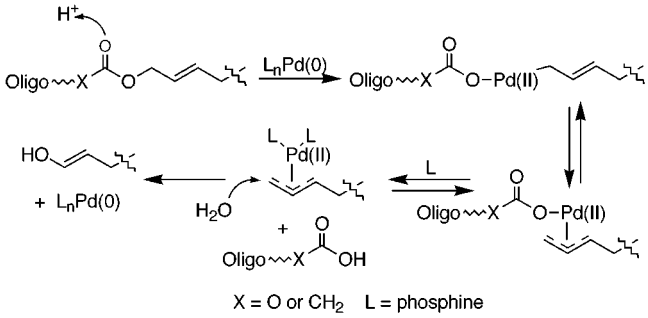
(14) See Supporting Information.

Table 2. Optimization of Isolated Yields of 18 Obtained from 3 via Pd(0)-Mediated Cleavage[a,b]

molar equiv of Pd(0)	[TBA] (M)	[H_2O] (M)	reaction time (min)	isolated yield (%)[c,d]
40	0.12	0.55	60	98 ± 2
40	0.06	0.55	60	94 ± 1
8	0.12	0.55	60	96 ± 7
8	0.06	0.55	60	94 ± 5
8	0.06	2.75	60	104 ± 4
4	0.12	0.55	60	77 ± 9
4	0.06	2.75	60	78 ± 3
4	0.12	0.55	120	102 ± 1
2	0.12	0.55	60	44 ± 5

[a] All reactions were carried out at 55 °C using 2.5 molar equiv of DIPHOS relative to Pd(0). [b] *O*-*β*-Cyanoethyl-protected phosphoramidites were used to prepare the oligonucleotides. [c] Yields reported with a standard deviation are the average of at least three separate reactions. [d] Isolated yields are determined via comparison of the isolated yield of oligonucleotide obtained via Pd(0) cleavage and subsequent NH_4OH treatment, versus that obtained via direct NH_4OH treatment of resin bound oligonucleotide from the same oligonucleotide synthesis.

Scheme 3



exhibited comparable reactivity. Although the cost of DNA on a molar basis is greater than that of $Pd_2(dba)_3$·$CHCl_3$, we sought to minimize the number of molar equivalents of Pd(0) employed in the cleavage reaction. While the molar ratio of DIPHOS to $Pd_2(dba)_3$·$CHCl_3$ was maintained at 5, we found that the Pd(0) could be reduced to 8 molar equiv relative to support-bound oligonucleotide without any sacrifice in isolated yield of **18** (Table 2). However, to obtain quantitative yields of **18** when using 2 molar equiv of $Pd_2(dba)_3$·$CHCl_3$, the reaction time had to be increased to 2 h. No adverse consequences in the yield of **18** were observed when the concentration of TBA was reduced to 60 mM. It is also relevant to note that high yields were unobtainable when using TBA as a buffer system under scrupulously dry conditions; whereas the addition of small amounts of water to the reaction mixture resulted in consistently high yields. We believe that when TBA is used as reaction buffer, water acts as a nucleophile in competition with the initially cleaved oligonucleotide ("biting back") to release the Pd(0) from the η^3-complex (Scheme 3).[15] Biting back by leaving groups, in this case the 3′-alkoxy oligoncleotide, during nucleophilic substitution of allylic substrates mediated by Pd(0) is not uncommon.[16]

Pd(0)-Mediated Cleavage of *β*-Cyanoethyl Phosphate Protecting Groups. ^{31}P NMR experiments on

(15) Upon initial cleavage of the oligonucleotide from **3**, it is expected that the biopolymer undegoes rapid decarboxylation to produce a 3′-terminal alkoxide. In the absence of a proton source, this oligonucleotide is believed to attack the η^3-complex, resulting in an oligonucleotide which is still bound to the solid-phase support.

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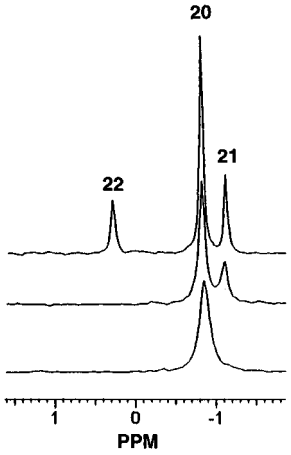
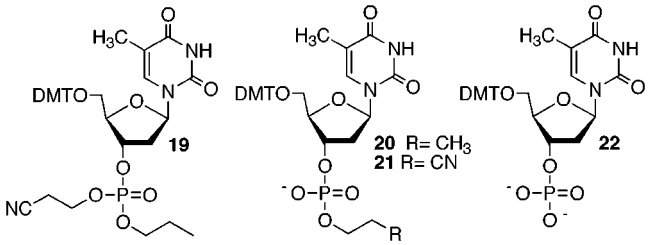


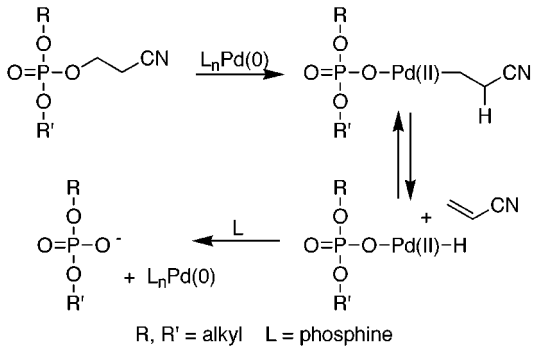
Figure 1. ^{31}P NMR of the reaction of **19** with Pd(0), DIPHOS, and TBA in THF: (bottom) reaction of **19**, (middle) reaction of **19**, spiked with **20** and **21**, (top) reaction of **19**, spiked with **20**−**22**.



19 using catalytic $Pd_2(dba)_3{\cdot}CHCl_3$ and DIPHOS in TBA buffer confirmed the proposal that the β-cyanoethyl group was cleaved under these Pd(0) conditions (Figure 1).[4] Of the four possible products that can be formed from **19** (**20**−**22** and 5′-*O*-dimethoxytritylthymidine), only **20** is observed, indicating that cleavage of the β-cyanoethyl group occurs to the exclusion of the other two phosphate triester alkyl groups. β-Cyanoethyl cleavage occurs regardless of the buffer system used and requires the presence of palladium. On the basis of analogies to Pd(0) insertion into acyl halides, as well as halides, two possible mechanisms for the cleavage of the β-cyanoethyl group were considered. Although carbon−oxygen insertions into carboxylic acid esters do not occur, acid halides do react with Pd(0).[17,18] We considered the possibility that the lower pK_a of β-cyanoethanol compared to simple alcohols increased the reactivity of the phosphate triester such that its reactivity was closer to that of an acyl halide. Phosphorus−oxygen bond insertion, followed by hydrolysis, would yield the phosphate diester and β-cyanoethanol. In contrast, carbon−oxygen bond insertion (in analogy to reactions of alkyl halides[19]), followed by β-elimination, and subsequent reductive elimination would release the phosphate diester and acrylonitrile. Observation of acrylonitrile by GC/MS, but not β-cyanoethanol, leads us to propose that carbon−oxygen insertion is the pathway by which Pd(0) gives rise to deprotection of the β-cyanoethyl group in phosphate triesters (Scheme 4).

Summary. Oligonucleotides can be cleaved from solid-phase supports in quantitative yield in 1 h using a slight excess of the less costly Pd(0) reagent. When carried out in tetrabutylammonium formate buffer, the reaction needs a small amount of water and acid in order to obtain reproducible quantitative yields of product. In addition, the previously observed cleavage of β-cyanoethyl groups from phosphate triesters by Pd(0) is believed to occur via initial carbon−oxygen bond insertion, followed by elimination of acrylonitrile. This facile process suggests that the β-cyanoethyl group could be employed as an alternative to the Alloc protecting group.

Scheme 4



Experimental Section

General Methods. ^{1}H NMR spectra were obtained at 270 or 300 MHz. ^{13}C and ^{31}P NMR spectra were obtained at the respective frequencies using the same spectrometers; ^{31}P NMR spectra were referenced against external phosphoric acid. Reverse phase HPLC analysis utilized a Rainin Microsorb-MV C_{18} (5 μm) column. GC/MS was carried out on a DB1 fused silica capillary column. All reactions were carried out in oven-dried glassware, under a nitrogen atmosphere, unless otherwise stated. THF was distilled from Na^0/benzophenone ketyl. Pyridine, DMF, CH_3CN, 1,2-dichloroethane, acetic anhydride, and CH_2Cl_2 were distilled from CaH_2.

Oligonucleotides were synthesized using standard cycles, commercially available reagents, and the solid-phase supports described above. Commercially available DNA synthesis reagents were purchased from Glen Research (Sterling, VA). Deprotection of standard oligonucleotide (β-cyanoethyl or methyl protected) was carried out in concentrated NH_4OH at 55 °C for 12 h. All oligonucleotides were purified via 20% polyacrylamide denaturing gels [(20 × 40 × 0.1 cm), 5% cross-link, 45% urea (by weight)]. Oligonucleotides were visualized using 254 nm light. Bands were excised and eluted with a solution of NaCl (0.2 M) and EDTA (1 mM), filtered through Quick Sep filters desalted on C_{18} Sep-Pak cartridges. Oligonucleotides were quantitated by UV absorption at 260 nm. Molar extinction coefficients were calculated using the nearest neighbor method.[20]

Preparation of 7. 4,4′-Dimethoxytrityl chloride (5.0 g, 14.76 mmol) and 1,4-butanediol (6.41 g, 71.1 mmol) were stirred in pyridine (50 mL) at 0 °C overnight. After removal of the pyridine and excess alcohol in vacuo, the residue was taken up in diethyl ether (100 mL), washed with H_2O (25 mL) and brine (25 mL), and then dried over $MgSO_4$. Flash chromatography (hexanes:EtOAc 2:1 to 1:3) yielded 4.9 g (84%) of the dimethoxytritylated alcohol as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.41 (d, 2H, J = 8 Hz), 7.24 (m, 6H), 7.18 (m, 1H), 6.80 (d, 4H, J = 9 Hz), 3.76 (s, 6H), 3.61 (t, 2H, J = 6 Hz), 3.09 (t, 2H, J = 6 Hz), 2.99 (bd s, 1H), 1.66 (m, 4H); IR (film) 3357, 2934, 2868, 2835, 1607, 1508, 1463, 1445, 1301, 1249, 1176, 1034 cm^{-1}.

Pyridinium dichromate (14.2 g, 37.71 mmol) and the above alcohol (3.7 g, 9.43 mmol) were stirred in DMF (50 mL) for 18

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h at room temperature. The mixture was poured into H_2O (350 mL) and extracted with diethyl ether (5 × 100 mL). The combined organic layers were washed with brine (75 mL) and dried over Na_2SO_4. Flash chromatography (EtOAc:hexanes 1:2) yielded 1.62 g (42%) of **7** as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.45 (d, 2H, J = 9 Hz), 7.31 (m, 6H), 7.22 (m, 1H), 6.82 (d, 4H, J = 9 Hz), 3.78 (s, 6H), 3.14 (t, 2H, J = 6 Hz), 2.50 (t, 2H, J = 7.5 Hz), 1.94 (tt, 2H, J = 6, 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 179.8, 158.3, 145.1, 136.3, 130.0, 128.1, 127.7, 126.6, 113.0, 85.9, 62.1, 55.1, 31.2, 25.1; IR (film) 3600, 2932, 2835, 1707, 1608, 1509, 1445, 1300, 1250, 1176, 1075, 1035 cm^{-1}.

Preparation of 9. Silyl alcohol **8**[11] (530 mg, 2.17 mmol) and dimethoxytrityl carboxylic acid **7** (1.10 g, 2.71 mmol) were combined with DCC (582 mg, 2.82 mmol) and DMAP (26 mg, 0.22 mmol) in CH_2Cl_2 (17 mL) at 0 °C. The solution was filtered after allowing the reaction to stir and warm to room temperature over 3 h. The filter cake was washed with CH_2Cl_2. After removal of the solvents in vacuo, flash chromatography (Et_2O:hexanes 1:4) yielded 1.27 g (92.5%) of **9** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.42 (d, 2H, J = 9 Hz), 7.24 (m, 7H), 6.81 (d, 4H, J = 9 Hz), 5.73 (dt, 1H, J = 15, 7 Hz), 5.53 (dt, 1H, J = 15, 6 Hz), 4.48 (dd, 2H, J = 1, 6 Hz), 3.77 (s, 6H), 3.58 (dd, 2H, J = 3, 6 Hz), 3.09 (t, 2H, J = 6 Hz), 2.44 (t, 2H, J = 7.5 Hz), 2.04 (m, 2H), 1.91 (m, 2H), 1.47 (m, 4H), 0.89 (s, 9H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 173.3, 158.3, 145.2. 136.4, 136.2, 130.0, 128.1, 127.7, 126.6, 124.0, 113.0, 85.8, 65.1, 62.9, 62.3, 55.1, 32.3, 32.0, 31.5, 25.9, 25.4, 25.1, 18.3, −5.3; IR (film) 2930, 2857, 1735, 1608, 1509, 1463, 1445, 1301, 1251, 1175, 10907, 1037 cm^{-1}.

Preparation of 10. An equimolar mixture of TBAF and HOAc (0.5 M; 24.4 mL) in THF was added to **9** (1.0 g, 1.58 mmol) in THF (10 mL) at 0 °C. After the mixture was allowed to warm to room temperature and stir overnight, the reaction was poured into saturated $NaHCO_3$ (25 mL) and extracted with Et_2O (2 × 75 mL). The combined organic layers were washed with brine (25 mL) and dried over $MgSO_4$. Flash chromatography (EtOAc:hexanes 1:2) yielded 817 mg (100%) of the desilylated product: ^{1}H NMR ($CDCl_3$) δ 7.40 (d, 2H, J = 9 Hz), 7.24 (m, 7H), 6.79 (d, 4H, J = 9 Hz), 5.72 (dt, 1H, J = 15, 6.5 Hz), 5.53 (dt, 1H, J = 15, 6 Hz), 4.48 (dd, 2H, J = 1, 6 Hz), 3.76 (s, 6H), 3.61 (t, 2H, J = 6 Hz), 3.08 (t, 2H, J = 6 Hz), 2.44 (t, 2H, J = 7.5 Hz), 2.06 (m, 2H), 1.91 (m, 2H), 1.51 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 173.4, 158.3, 145.1, 136.4, 135.8, 130.0, 128.1, 127.7, 126.6, 124.2, 112.9, 85.8, 65.0, 62.6, 62.3, 55.1, 32.1, 31.9, 31.5, 25.4, 25.0; IR (film) 3400, 2933, 1733, 1608, 1509, 1446, 1301, 1250, 1175, 1073, 1034 cm^{-1}.

PDC (2.03 g, 5.41 mmol) and the primary alcohol obtained above (800 mg, 1.55 mmol) were stirred at room temperature in DMF (12 mL) for 12 h. The solution was poured into H_2O (100 mL) and extracted with Et_2O (3 × 100 mL). The combined organics were washed with brine (50 mL) and dried over $MgSO_4$. Flash chromatography (EtOAc:hexanes 1:2) yielded 592 mg (72%) of **10**: ^{1}H NMR ($CDCl_3$) δ 7.40 (d, 2H, J = 9 Hz), 7.23 (m, 7H), 6.80 (d, 4H, J = 9 Hz), 5.69 (dt, 1H, J = 6.5, 15 Hz), 5.55 (dt, 1H, J = 6, 15 Hz), 4.49 (d, 2H, J = 6 Hz), 3.77 (s, 6H), 3.08 (t, 2H, J = 6 Hz), 2.44 (t, 2H, J = 7.5 Hz), 2.33 (t, 2H, J = 7.5 Hz), 2.08 (m, 2H), 1.91 (m, 2H), 1.71 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 179.1, 173.4, 158.3, 145.1, 136.4, 134.6, 130.0, 128.1, 127.7, 126.6, 125.1, 113.0, 85.8, 64.9, 62.3, 55.2, 33.2, 31.5, 31.4, 25.4, 23.7; IR (film) 3340, 2934, 2836, 1733, 1707, 1608, 1509, 1445, 1301, 1250, 1175, 1034 cm^{-1}.

Preparation of 1. 2,4,5-Trichlorophenol (260 mg, 1.32 mmol), DCC (272 mg, 1.32 mmol), **10** (560 mg, 1.05 mmol), and DMAP (13 mg, 0.11 mmol) were combined in CH_2Cl_2 (13 mL) at 0 °C. After warming to room temperature and stirring for 12 h, the reaction mixture was poured into H_2O (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. Flash chromatography (EtOAc:hexanes 1:8) yielded 624 mg (83%) of the requisite trichlorophenyl ester as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.52 (s, 1H), 7.42 (d, 2H, J = 9 Hz), 7.24 (m, 8H), 6.81 (d, 4H, J = 9 Hz), 5.72 (dt, 1H, J = 6, 15 Hz), 5.58 (dt, 1H, J = 6, 15 Hz), 4.50 (d, 2H, J = 6 Hz), 3.77 (s, 6H), 3.08 (t, 2H, J = 6 Hz), 2.59 (t, 2H, J = 7 Hz), 2.45 (t, 2H, J = 7.5 Hz), 2.16 (m, 2H), 1.87 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 173.3, 170.4, 158.3, 145.8, 145.1, 136.4, 134.2, 131.4, 131.0, 130.5, 129.9, 128.1, 127.7, 126.6, 126.1, 125.5, 125.3, 113.0, 85.8, 64.8, 62.3, 55.2, 33.0, 31.5, 31.3, 25.4, 23.8; IR (film) 2934, 2836, 1774, 1732, 1608, 1582, 1510, 1462, 1350, 1302, 1250, 1175, 1105, 1081, 1035 cm^{-1}; HRMS FAB (M^+) calcd 710.1605, found 710.1595.

A mixture of the trichlorophenyl ester (50 mg, 70 μmol), LCAA-CPG (100 mg, ≈5 μmol amine), and HOBt·hydrate (9.5 mg, 70 μmol) was shaken overnight in the dark at 25 °C using a vortexer. The resin was filtered, washed well with dry EtOAc, and dried under vacuum. Unreacted amine was capped by treatment with acetic anhydride (250 μL), pyridine (2 mL), and DMAP (25 mg) for 1 h. The resin was filtered, washed, and dried as described above. Free amine was measured on 1 mg of resin (**1**) via quantitative ninhydrin analysis.[21] Resin loading was measured by treatment with *p*-toluenesulfonic acid in CH_3CN and quantitation of the dimethoxytrityl cation by absorption spectroscopy (λ_{max} = 498 nm, $\epsilon = 7 \times 10^4$ M^{-1} cm^{-1}).

Preparation of 12. Phosgene (1.9 M in toluene, 3.1 mL) was added via syringe to **8** (250 mg, 1.0 mmol) in THF (2 mL). After the reaction mixture was stirred for 3 h, N_2 was bubbled through the solution for 1 h to remove excess phosgene. After removal of the solvent in vacuo, an aliquot of the crude product (**11**) was analyzed by IR and ^{1}H NMR ($CDCl_3$); IR showed one carbonyl stretch at 1778 cm^{-1}. ^{1}H NMR showed a shift of the allylic alcohol methylene protons from 4.07 to 4.69 ppm. The above analytical methods indicated that the reaction had gone to completion. The sodium alkoxide salt of 5′-*O*-(4,4′-dimethoxytrityl)thymidine [prepared by addition of sodium hydride (150 mg, 3.75 mmol) to 5′-*O*-(4,4′-dimethoxytrityl)thymidine dissolved in THF (6 mL) (820 mg, 1.5 mmol)] in THF (6 mL) was added, and the mixture was stirred under N_2 for 2 h at 25 °C. The reaction was diluted with EtOAc (40 mL) and poured into H_2O (100 mL). The aqueous layer was extracted with EtOAc (3 × 40 mL). The combined organic layers were washed with brine (2 × 100 mL) and dried over Na_2SO_4. Flash chromatography (EtOAc:hexanes 1:2) yielded **12** as a white foam (391 mg, 48%): ^{1}H NMR ($CDCl_3$) δ 8.01 (s, 1H), 7.58 (s, 1H), 7.37−7.21 (m, 9H), 6.82 (d, 4H, J = 9 Hz), 6.44−6.40 (m, 1H), 5.81 (dt, 1H, J = 6.6, 15 Hz), 5.55 (dt, 1H, J = 6.3, 15 Hz), 5.32 (d, 1H, J = 6 Hz), 4.55 (d, 2H, J = 6.6 Hz), 4.20 (s, 1H), 4.75 (s, 6H), 3.58 (t, 2H, J = 6 Hz), 3.55−3.40 (m, 2H), 2.57−2.37 (m, 2H), 2.10−2.03 (m, 2H), 1.58−1.38 (m, 4H), 0.86 (s, 9H), 0.01 (s, 6H); IR (film) 2930, 1744, 1696, 1607, 1508, 1458, 1253, 1176, 1103, 972 cm^{-1}. Anal. Calcd for $C_{45}H_{58}N_2O_{10}Si$: C, 66.31; H, 7.17; N, 3.49. Found: C, 66.55; H, 6.97; N, 3.42.

Preparation of 13. An equimolar solution of glacial acetic acid and TBAF in THF (0.5 M, 1.5 mL) was added to a solution of **12** (150 mg, 0.18 mmol) in THF (5 mL). After 24 h an additional 0.3 mL of the buffered TBAF solution was added. After 12 additional hours, the reaction was diluted with EtOAc (50 mL), washed with brine (100 mL), and dried over Na_2SO_4. Flash chromatography (EtOAc:hexanes 1:1) yielded **13** as a white foam (135 mg, 92%): ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 1H), 7.56 (s, 1H), 7.35−7.19 (m, 9H), 6.80 (d, 4H, J = 9 Hz), 6.41−6.36 (m, 1H), 5.79 (dt, 1H, J = 6, 14.5 Hz), 5.54 (dt, 1H, J = 7, 14 Hz), 4.53 (d, 2H, J = 7.5 Hz), 4.18 (s, 1H), 3.74 (s, 6H), 3.64−3.56 (m, 2H), 3.50−3.35 (m, 2H), 2.55−2.34 (m, 2H), 2.10−2.02 (m, 2H), 1.50−1.38 (m, 4H), 1.33 (s, 3H); IR (film) 3464, 3060, 2932, 1743, 1692, 1607, 1582, 1509, 1252, 1202, 1177, 1153, 1066, 973 cm^{-1}. Anal. Calcd for $C_{39}H_{44}N_2O_{10}$: C, 66.84; H, 6.33; N, 4.00. Found: C, 66.72; H, 6.32; N, 3.81.

Preparation of 14. PDC (210 mg, 0.56 mmol) was added to a solution of **13** (100 mg, 0.14 mmol) in DMF (1.3 mL). The reaction was allowed to stir for 15 h at 25 °C, after which the solution was poured into H_2O (50 mL) and extracted with Et_2O (3 × 30 mL). The combined organic layers were washed with saturated $NaHCO_3$ (50 mL) and then concentrated in vacuo. The residue was purified by flash chromatography (EtOAc:hexanes 3:2) to give **14** (58 mg, 57%) as a foam: ^{1}H NMR

(21) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.

($CDCl_3$) δ 9.19 (bd s, 1H), 7.57 (s, 1H), 7.35−7.18 (m, 9H), 6.82−6.77 (d, 4H, J = 9 Hz), 6.42−6.37 (m, 1H), 5.81−5.71 (m, 1H), 5.61−5.52 (m, 1H), 5.29 (d, 1H, J = 3.3 Hz), 4.59−4.46 (m, 2H), 4.19 (bd s, 1H), 3.74 (s, 6H), 3.51−3.36 (m, 2H), 2.56−2.29 (m, 4H), 2.14−2.05 (m, 2H), 1.76−1.66 (m, 2H), 1.33 (s, 3H); IR (film) 3172, 3035, 2930, 1736, 1702, 1552, 1509, 1462, 1252, 1202, 1070, 1033, 912, 703 cm^{-1}. Anal. Calcd for $C_{39}H_{42}N_2O_{11}$: C, 65.54; H, 5.92; N, 3.92. Found: C, 65.38; H, 6.10; N, 3.70.

Preparation of 15. DCC (30 mg, 0.14 mmol) was added to a solution of **13** (100 mg, 0.14 mmol), sebacic acid (113 mg, 0.56 mmol), and DMAP (9 mg, 0.07 mmol), in THF (1 mL). The solution was allowed to stir at room temperature for 6 h, at which time the reaction mixture was concentrated in vacuo, to give a white solid. The solid was resuspended in EtOAc (50 mL) and washed with H_2O (50 mL) and brine (3 × 50 mL). After drying over Na_2SO_4, flash chromatography (CH_2Cl_2:MeOH; 19:1) gave **15** (115 mg, 93%) as a white foam: ^{1}H NMR ($CDCl_3$) δ 9.12 (s, 1H), 7.57 (s, 1H), 7.35−7.17 (m, 9H), 6.79 (d, 4H, J = 9 Hz), 6.42−6.37 (m, 1H), 5.78 (dt, 1H, J = 6.6, 15 Hz), 5.55 (dt, 1H, J = 6.5, 15 Hz), 5.28 (d, 1H, J = 5.7 Hz), 4.60−4.46 (m, 2H), 4.18 (s, 1H), 4.01 (t, 2H, J = 6.3 Hz), 3.75 (s, 6H), 3.50−3.37 (m, 2H), 2.55−2.33 (m, 2H), 2.30 (t, 2H, J = 7.2 Hz), 2.24 (t, 2H, J = 7.5 Hz), 2.10−2.00 (m, 2H), 1.63−1.51 (m, 6H), 1.47−1.38 (m, 2H), 1.33 (s, 3H), 1.32−1.19 (m, 8H); IR (film) 3182, 3036, 2932, 1738, 1731, 1713, 1704, 1674, 1607, 1557, 1510, 1455, 1252, 1177, 1068, 1034, 973 cm^{-1}. Anal. Calcd for $C_{49}H_{61}N_2O_{11}$: C, 65.32; H, 6.82; N, 3.11. Found: C, 65.37; H, 6.84; N, 3.05.

Example of Loading of Free Carboxylic Acids onto LCAA-CPG Supports.[12] To a small glass vial containing **14** (5 mg, 7 μmol) was added DMAP (1 mg, 8.1 μmol) in CH_3CN (200 μmol), 2,2′-dipyridyl disulfide (2.3 mg, 8.1 μmol) in a 1:1 1,2-dichloroethane:CH_3CN (100 μL) solution, PPh_3 (2.1 mg, 8.1 μmol) in CH_3CN (100 μL), and LCAA-CPG (50 mg, 2.5 μmol of amines). The vial was shaken for 1 h, after which the resin was filtered and washed with EtOAc (10 mL), CH_3CN (10 mL), and Et_2O (10 mL). After air-drying, the resin was added to DMAP (9 mg, 0.073 mmol) and acetic anhydride (215 mg, 2.1 mmol) dissolved in dry pyridine (2 mL). After filtration and drying, it was determined (via trityl assay) that the resin was loaded to 44 μmol/g. Typical loadings ranged from 40 to 50 μmol/g.

Preparation of 19. To thymidine β-cyanoethyl phosphoramidite (100 mg, 0.13 mmol) stirring in CH_3CN (3 mL) were added a 0.1 M tetrazole solution (5 mL, 0.5 mmol) and *n*-propyl alcohol (0.5 mL, 8.3 mmol). The reaction was allowed to stir at room temperature for 30 min when TLC analysis (MeOH:CH_2Cl_2; 1:9) indicated that the starting amidite was completely consumed. To the reaction mixture was then added dropwise a 1.0 M solution of I_2 in THF:2,6-lutidine:H_2O (2:2:1), until an orange color persisted. The excess I_2 in solution was then treated with $Na_2S_2O_3$ solution (5 mol %), until the solution became clear. The contents of the flask were then poured into a separatory funnel containing CH_2Cl_2 (15 mL) and saturated $NaHCO_3$ (15 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 15 mL), and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo to afford crude product as a white foam. Flash chromatography (EtOAc:hexanes 3:1) afforded **19** as a white foam (76 mg, 76%): ^{1}H NMR (CD_3OD) δ 7.63 (s, 1H), 7.41 (d, 2H, J = 7.2 Hz), 7.27 (m, 7H), 6.87 (d, 4H, J = 8.4 Hz), 6.33 (t, 1H, J = 6.6 Hz), 5.17 (m, 1H), 4.27 (m, 1H), 4.21 (m, 2H), 4.03 (m, 2H), 3.77 (s, 6H), 3.46 (m, 2H), 2.82 (p, 2H, J = 5.7 Hz), 2.59 (m, 2H), 1.66 (m, 2H), 1.40 (s, 3H), 0.94 (m, 3H); ^{13}C NMR (CD_3OD) δ 166.3, 160.5, 152.4, 146.0, 137.5, 136.8, 131.6, 129.6, 129.2, 128.4, 118.6, 114.5, 112.2, 88.6, 86.1, 85.9, 80.4, 71.9, 64.5, 64.2, 55.9, 39.9, 24.8, 20.3, 12.2, 10.5; ^{31}P NMR (CD_3OD) δ −1.17, −1.46; IR (film) 3177, 3057, 2965, 2360, 1690, 1607, 1509, 1464, 1251, 1177, 1029, 828 cm^{-1}; HRMS (FAB) calcd 720.2686 (M + H), found 720.2664.

Preparation of 20. To **19** (65.9 mg, 0.09 mmol) stirring in MeOH (2 mL) was added 1.0 N NaOH solution (90 μL). The reaction was allowed to stir for 1 h while being monitored by TLC (MeOH:CH_2Cl_2 1:4) every 15 min. After 1 h, an additional portion of 1.0 N NaOH solution (10 μL) was added. TLC analysis after an additional 30 min indicated that starting material had been completely consumed. The reaction mixture was then poured into a separatory funnel containing CH_2Cl_2 (5 mL) and H_2O (10 mL). The layers were separated, and the H_2O layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were then washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Flash chromatography (MeOH:EtOAc 1:4) yielded 65.1 mg (99%) of the **20**: ^{1}H NMR (CD_3OD) δ 7.65 (s, 1H), 7.42 (d, 2H, J = 7.2 Hz), 7.30 (m, 7H), 6.86 (d, 4H, J = 8.7 Hz), 6.33 (dd, 1H, J = 8.1, 5.7 Hz), 4.99 (d, 1H, J = 6.3 Hz), 4.24 (m, 1H), 3.76 (s, 6H), 3.72 (m, 2H), 3.42 (m, 2H), 2.50 (m, 2H), 1.52 (m, 2H), 1.31 (s, 3H), 0.87 (t, 3H, J = 7.4 Hz); ^{13}C NMR (CD_3OD) δ 169.0, 160.5, 154.0, 146.2, 137.6, 137.0, 131.7, 129.7, 129.1, 128.3, 114.0, 112.1, 88.4, 86.9, 86.2, 77.4, 69.0, 68.4, 65.3, 55.9, 40.6, 26.7, 25.3, 12.5, 10.9; ^{31}P NMR (CD_3OD) δ −0.86; IR (film) 3410, 2962, 1690, 1607, 1508, 1464, 1250, 1176, 1074, 1033, 830 cm^{-1}; HRMS (FAB) calcd 666.2342 (M + H), found 666.2369.

Preparation of 21. To thymidine β-cyanoethyl phosphoramidite (100 mg, 0.13 mmol) stirring in CH_3CN (3 mL) was added 0.1 M tetrazole solution (1.5 mL, 0.15 mmol). The reaction was stirred at room temperature for 30 min when TLC analysis (MeOH:CH_2Cl_2 1:4) indicated that the starting amidite was completely consumed. To the reaction mixture was then added dropwise a 1.0 M solution of I_2 in THF:2,6-lutidine:H_2O (2:2:1), until an orange color persisted. The excess I_2 in solution was then consumed by dropwise addition of $Na_2S_2O_3$ solution (5 mol %) until the solution became clear. The contents of the flask were then poured into a separatory funnel containing CH_2Cl_2 (3 × 15 mL), and the combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Flash chromatography (MeOH:EtOAc 3:7) afforded 72.1 mg (79%) of **21** as a mixture of salts. The mixture of salts was passed through a cation exchange column (Dowex, Na^+ cation-exchange resin), affording predominantly the sodium salt of **21**: ^{1}H NMR (CD_3OD) δ 7.68 (s, 1H), 7.42 (d, 2H, J = 7.2 Hz), 7.25 (m, 7H), 6.86 (d, 4H, J = 8.4 Hz), 6.39 (dd, 1H, J = 7.8, 5.7 Hz), 5.05 (t, 1H, J = 6.3 Hz), 4.26 (m, 1H), 3.95 (m, 2H), 3.76 (s, 6H), 3.46 (m, 2H), 2.70 (t, 2H, J = 6.3 Hz), 2.55 (m, 2H), 1.32 (s, 3H); ^{13}C NMR (CD_3OD) δ 166.4, 160.5, 152.4, 146.1, 137.8, 136.9, 131.6, 129.7, 129.1, 128.3, 119.4, 114.4, 112.0, 88.4, 86.8, 86.1, 77.6, 65.2, 61.8, 55.9, 40.5, 20.5, 12.1; ^{31}P NMR (CD_3OD) δ −1.16; IR (film) 2956, 2835, 2359, 2341, 1692, 1607, 1508, 1467, 1250, 1176, 1075, 1032, 829 cm^{-1}; HRMS (FAB) calcd 677.2138 (M), found 677.2196.

Preparation of 22. To **21** (67 mg, 0.1 mmol) stirring in MeOH (2 mL) at 55 °C was added 1.0 N NaOH (0.1 mL). The reaction was stirred for 1 h, with monitoring by TLC (MeOH:CH_2Cl_2 1:1) every 15 min. After 1 h, an additional portion of 1.0 N NaOH solution (10 μL) was added. After an additional 2 h, TLC indicated that the starting material had been completely consumed. The reaction mixture was concentrated in vacuo to afford 64 mg (106%) of a white film as the desired product in >95% purity according to ^{1}H NMR: ^{1}H NMR (CD_3OD) δ 7.65 (s, 1H), 7.42 (d, 2H, J = 7.2 Hz), 7.25 (m, 7H), 6.84 (d, 4H, J = 8.4 Hz), 6.54 (dd, 1H, J = 9.0, 5.4 Hz), 4.97 (t, 1H, J = 6.3 Hz), 4.33 (m, 1H), 3.72 (s, 6H), 3.51 (dd, 1H, J = 10.2, 3.0 Hz), 3.41 (dd, 1H, J = 10.2, 2.4 Hz), 2.66 (m, 1H), 2.39 (m, 1H), 1.23 (s, 3H); ^{31}P NMR (CD_3OD) δ 0.24; IR (film) 3365, 2935, 1692, 1659, 1606, 1510, 1463, 1439, 1300, 1252, 1118, 984 cm^{-1}; ESMS (M) calcd 622.6, found 623.0.

General Procedure for Cleavage of Oligonucleotides From Pd(0)-Labile Resins. Initial preparation for this reaction entailed the preparation of several reagent solutions. In an oven-dried scintillation vial was placed THF (15 mL) which was sparged with argon for 30 min. In an oven-dried 1 dram vial equipped with a septum was weighed out $Pd_2(dba)_3$·$CHCl_3$ and DIPHOS in a 1:2 mass ratio. The reagent mixture was diluted with the appropriate amount of sparged THF. In an oven-dried scintillation vial, tetrabutylammonium formate (TBA) was weighed out under an argon blanket and then diluted with the appropriate amount of sparged THF. In an oven-dried reaction vessel purged with argon was placed ≈1.0 mg of resin containing protected oligonucleotide. The vessel

was purged a second time with argon, followed by the addition of the Pd(0) solution (100 μL) and the TBA solution (100 μL). The reaction vessel was then capped and placed in a heating block at 55 °C for the appropriate reaction time. The mixture was vortexed every 15 min. After the prescribed reaction time, the vessel was cooled to room temperature, and the solution was removed via pipet. The resin was washed with THF (1 mL), H_2O (1 mL), and THF (1 mL) again. The washings were collected into a single sample vial. The washings were then concentrated in vacuo and resuspended in CH_3CN (0.5 mL), followed by filtration through a 0.2 μm filter, which was washed with (3 × 0.7 mL) CH_3CN and CH_3CN:H_2O (1:1, 3 × 0.7 mL). The filtrate was collected into a sample vial and concentrated in vacuo. The residue was taken up into CH_3CN:H_2O (1:1, 3 × 0.3 mL) and transferred to an Eppendorf tube. The solution was concentrated in vacuo, treated with aqueous ammonia, analyzed by denaturing gel electrophoresis, isolated, and quantitated as described in the General Methods section.

Preparation of Oligonucleotides for ESMS. To the gel-purified oligonucleotide (6−10 nmol) were added H_2O (150 μL) and NH_4OAc (50 μL, 5 M, pH 5.2). The solution was vortexed and allowed to stand at 25 °C for 5 min. Cold ethanol (0.6 mL) was added and the mixture was inverted several times. After freezing at −78 °C for 15 min, the solution was centrifuged for 15 min. The supernatants were removed and saved. The procedure was repeated a second time. Before being submitted for ESMS analysis, the oligonucleotide was requantitated by UV absorption at 260 nm as described in the General Methods section.

Snake Venom Phosphodiesterase (SVPD) Digestion of DNA. SVPD digests were carried out at 37 °C in a solution (50 μL) containing oligonucleotide (5−10 nmol), SVPD (0.006 units), and reaction buffer [5 μL; $MgCl_2$ (15 mM), Tris acetate (0.1 M, pH 8.8)]. The reaction was initiated by addition of enzyme and was allowed to react for 1 h at 37 °C, after which calf intestinal alkaline phosphatase (10 units) was added. Digestion was allowed to proceed for an additional 12 h at 37 °C. Upon completion of the digestion, the enzymatic components were removed by precipitation prior to analysis by reverse phase HPLC. Precipitation was achieved by addition of NaOAc (5 μL, 3 M, pH 5), followed by addition of EtOH (200 μL). After freezing (12 min), the supernatants were reprecipitated by addition of NaOAc (50 μL, 0.3 M, pH 5), followed by addition of EtOH (200 μL). After freezing and centrifugation as described above, the supernatants were removed and dried in vacuo. Following resuspension of the nucleosides in water, analysis was carried out using reverse phase HPLC. Reverse phase HPLC analysis of enzyme digests of oligonucleotides utilized a Rainin Microsorb-MV C_{18} 5 μm column. Gradient conditions: eluent A, 10 mM KH_2PO_4 (pH 7.0), 2.5% MeOH (v:v); eluent B, 10 mM KH_2PO_4 (pH 7.0), 20% MeOH (v:v); 0−100% B linearly over 15 min; maintain 100% B for 20 min; flow rate 1.0 mL/min.

GC/MS Analysis of Reaction of 19 with Pd(0). Phosphate triester **19** was reacted with Pd(0) as described above for oligonucleotides, with the exception that Pd(0) was used in 20 mol %. β-Cyanoethanol and acrylonitrile were analyzed for by GC/MS. Conditions were as follows: injector, 250 °C; detector, 270 °C; column oven, $T_i = 35$ °C, $t_i = 4$ min, $R = 50$ °C/min, $T_f = 200$ °C.

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Supporting Information Available: Electrospray mass spectrum and HPLC chromatogram of enzyme digest of **18** (2 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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