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Photochemical Release of Protected Oligonucleotides Containing 3'-Glycolate Termini

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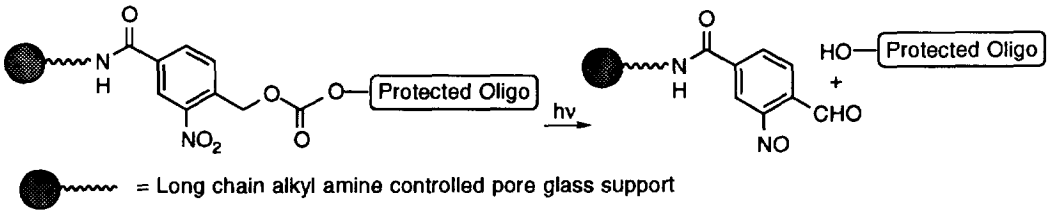
Abstract: Protected oligonucleotides containing 3'-glycolate termini that are suitable for further elaboration are released from their solid phase supports using the *o*-nitrobenzyl intramolecular photochemical redox reaction. The yields of isolated oligonucleotides of varying sequence, ranging between 12 and 20 nucleotides long are between 45% and 79%, under conditions that have been previously shown to form less than 2% thymine•thymine photodimers.

Oligonucleotides that are covalently bound to solid supports are useful as hybridization probes and affinity matrices. Furthermore, conjugation of oligonucleotides to other molecules in solution is useful for the development of oligonucleotide based therapeutics.[1] Synthesis of oligonucleotides with nucleophilic, or electrophilic functional groups at their 3'- or 5'-termini facilitates such derivatization. Conjugation of the oligonucleotide via a carboxylate, or phosphate moiety to a primary amine is an attractive strategy from the standpoint that robust amide, or phosphoramidate linkages are formed. However, execution of this synthetic strategy is hindered by a combination of side reactions involving the oligonucleotide, and/or inefficient conjugation of the oligonucleotide to the nucleophilic component.[2] Poor functionalization during carbodiimide mediated conjugation of deprotected oligonucleotides containing 5'-carboxylates with alkyl amine supports was attributed to competition by the nucleophilic components of the biopolymer with the support bound nucleophile. In a similar study, as much as 45% of the reactions between an oligonucleotide containing a 5'-primary amine and carboxyl containing support that were mediated by water soluble diimide, resulted in amide bond formation involving the exocyclic amine on deoxycytidine. Attempts to improve this methodology via prior activation of the support bound carboxylate resulted in inefficient functionalization due to competing hydrolysis of the N-hydroxysuccinimide esters under the aqueous conditions required to effect conjugation. These problems can be alleviated by cleaving oligonucleotides from solid phase supports that contain a functional group at either terminus, but retain all other protecting groups throughout the biopolymer. Such "orthogonal" solid phase supports would eliminate the possibility of side reactions at the exocyclic amines of nucleobases, and the requirement that the conjugation reactions be carried out under aqueous conditions.[3]

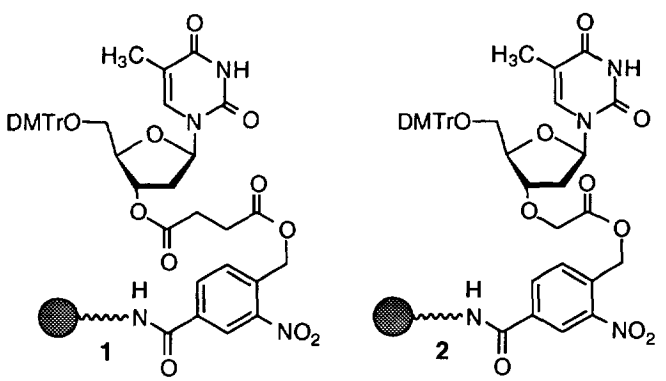
Recently, a versatile family of solid phase synthesis supports that are used to prepare oligonucleotides containing 3'-amines, or 3'-carboxylates were reported.[4] The disadvantage of these supports is that they are not orthogonal. The conditions used to effect cleavage partially deprotect the exocyclic amines and phosphodiesters as well. Disulfide based solid phase synthesis supports are orthogonal, but these are limited to carrying out

Scheme 1



conjugation reactions utilizing the thiol nucleophiles released from such resins.[5] Other functional groups can be unmasked at the 3'-terminus via alkaline treatment following reductive cleavage of the oligonucleotides from the solid phase synthesis supports. However, the alkaline treatment removes the nucleobase and phosphodiester protecting groups; eliminating potential applications of the disulfide resins as orthogonal synthesis supports. The preparation and utilization of a photolabile solid phase synthesis support that releases protected oligonucleotides containing a 3'-glycolate moiety (**2**) is reported herein.

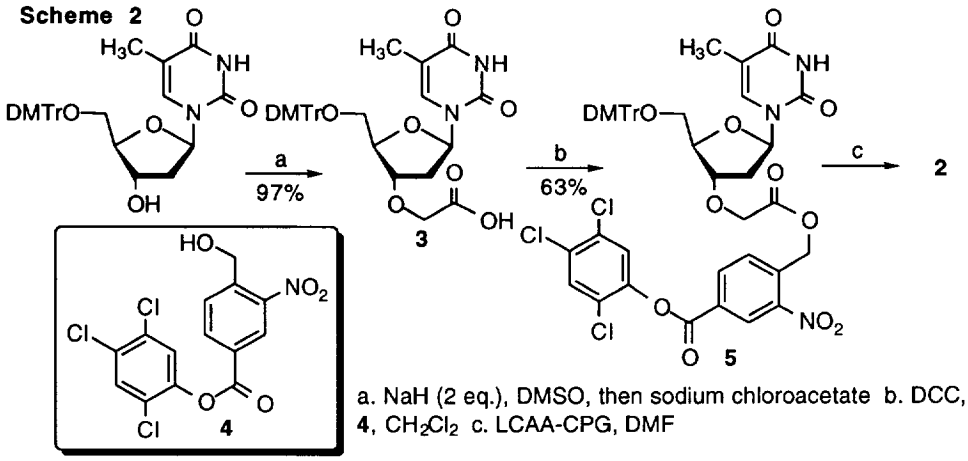
Previously, we described solid phase oligonucleotide synthesis supports that enable one to photochemically cleave the biopolymers from the long chain alkyl amine controlled pore glass (CPG) support at room temperature and neutral pH, without removing the nucleobase and phosphodiester protecting groups (Scheme 1).[6] The modified solid phase synthesis supports utilize the *o*-nitrobenzyl photoredox reaction.[7] Isolated yields of photochemically cleaved oligonucleotides using **1** were as high as 87% that obtained via conventional ammonolysis, under conditions that resulted in less than 2% photodimer formation (determined via tritium labeling). Resin **1** enables one to prepare oligonucleotides that contain 3'-carboxylates suitable for use in conjugation reactions. Alternatively, the succinato linkage can be cleaved under the mildly alkaline conditions encountered during gel electrophoresis to yield oligonucleotides containing 3'-hydroxy termini that are suitable for further enzymatic manipulation. This feature was recently exploited in the synthesis of an oligonucleotide containing 3'-termini suitable for further enzymatic manipulation, in which the alkaline labile, oxidative stress product, *5R*-5,6-dihydro-5-hydroxythymidine was incorporated site specifically in an oligonucleotide for the first



time.[8] With respect to the properties of bioconjugates synthesized from **1**, the presence of a relatively labile ester linkage between the 3'-nucleotide and the conjugated moiety is potentially useful for applications in which it is desired to release the biopolymer *in vivo*.[9] Construction of such bioconjugates can be accomplished using **1** in conjunction with Pd(0) labile phosphoramidites.[6,10] An orthogonal solid phase support that contains a less reactive linker between the 3'-terminal nucleoside and the moiety used for effecting conjugation is needed to form bioconjugates that will withstand the strongly alkaline conditions utilized to deprotect commercially available phosphoramidites (conc. NH_4OH, 55°C). Solid phase synthesis support **2** satisfies these requirements.

Results and Discussion. The spectrum of scientific disciplines in which nucleic acids and their conjugates are utilized extends beyond organic chemistry into the realm of the biological sciences. In order to make photolabile solid phase supports palatable to as wide a range of nucleic acid scientists as possible, a photolabile support whose synthesis is simple was sought. Photolabile linker **2** was readily synthesized from 3'-*O*-carboxymethyl-5'-*O*-dimethoxytritylthymidine (**3**) and **4** (Scheme 2).[11] Carboxylic acid **3** was in turn prepared by reaction of the dianion of 5'-*O*-dimethoxytritylthymidine with sodium chloroacetate.[12] Coupling of components **3** and **4** was mediated by DCC. Reaction of excess **5** with long chain alkyl amine controlled pore glass support (CPG) in DMF produced **2** with a loading (as measured by trityl response) of 28 μmol/g.

Scheme 2



a. NaH (2 eq.), DMSO, then sodium chloroacetate b. DCC, **4**, CH_2Cl_2 c. LCAA-CPG, DMF

Previous experiments in our group revealed that oligonucleotides bound to their solid phase supports via an *o*-nitrobenzyl moiety are efficiently cleaved by irradiating with the band pass filtered (λ_{max}= 400 nm) output of a Hg-Xe arc lamp (800 W).[6a] Tritylated, or detritylated oligonucleotides can be photolytically cleaved from the solid phase synthesis support prior to, or after nucleobase and phosphodiester deprotection. Oligonucleotide **6** was isolated in 79% yield upon irradiation (3 h) of a detritylated dodecamer of thymidines synthesized on **2**, followed by treatment with concentrated ammonium hydroxide (55°C, 2 h) to remove the β-cyanoethyl protecting groups from the internucleotide linkages. Prior experiments on tritiated oligonucleotides indicate that these

Table 1. Isolated yields[a] of fully deprotected oligonucleotides obtained from **2**.

Sequence	Irradiation Time (h)	% Yield[a]
T_{12}-glycolate (**6**)	3	79
T_{20}-glycolate	3	62
5'-dTAC GCA ATG CTA GAT CTA AT-glycolate (**7**)	1	45
5'-dTAC GCA ATG CTA GAT CTA AT-glycolate (**7**)	3	57

[a]Isolated yields expressed relative to yield of oligonucleotide obtained without irradiation, but with identical ammonium hydroxide cleavage.

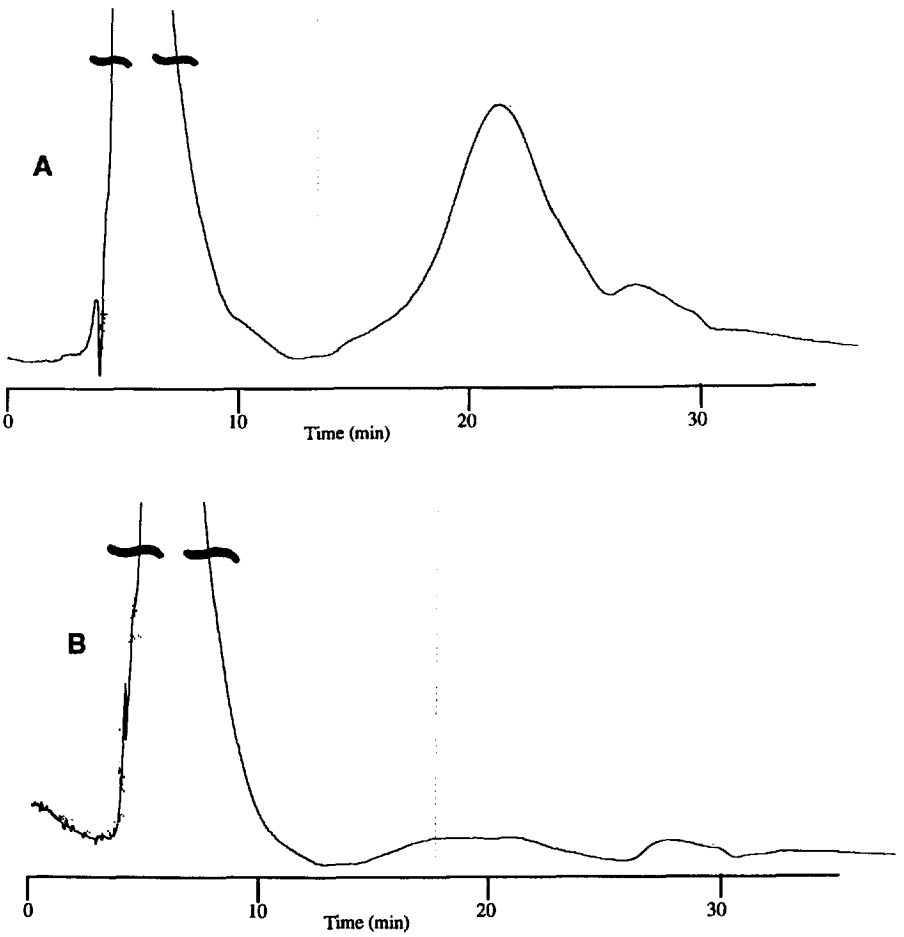


Figure 1. Normal phase HPLC analysis of tritylated, β-cyanoethyl protected, T_{20}-glycolate obtained from **2**. a) Oligonucleotide b) Blank. HPLC column C; Eluent A: CH_2Cl_2; Eluent B: MeOH; 20-100% B linearly over 20 min; 100% B for 10 min. Flow rate: 1 mL/min.

irradiation conditions produce less than 2% thymine•thymine photodimers.[6a] While longer oligonucleotides were cleaved in lower yield, there does not appear to be a strong dependance of yield on sequence (Table 1).

Oligonucleotides cleaved prior to removal of protecting groups were partially soluble in CH_3CN and very soluble in DMF. The protected oligonucleotides were isolable by HPLC using normal phase silica gel columns (Figure 1).[13] The broadness of these peaks prohibited separation of tritylated from detritylated material. The partially resolved peak eluting at approximately 27 minutes is attributed to extraction of plasticizers from the plastic tubes in which the protected oligonucleotide is isolated following photolysis (Figure 1b). Confirmation that the assigned peak was actually protected oligonucleotide was obtained by treating the concentrated eluent with concentrated NH_4OH, followed by detritylation. The oligonucleotide that was collected and deprotected in this way exhibited identical mobility on a 20% denaturing polyacrylamide gel as otherwise identical resin bound oligonucleotide which was not subjected to HPLC purification.

Evidence for the presence of a 3'-glycolate in oligonucleotides prepared using **2** was gleaned from ion exchange HPLC (Figure 2). A dodecamer of thymidines (**6**) was prepared on **2** using *O*-methyl phosphoramidites, which enabled deprotection of the phosphodiesters to be effected under mildly alkaline conditions (PhSH/Et_3N/dioxane) prior to photolysis.[14] Consistent with the presence of an additional negative charge contributed by the glycolate moiety, the crude photochemically cleaved oligonucleotide was retained approximately 2.5 min longer than an oligonucleotide of identical sequence prepared on conventional succinato

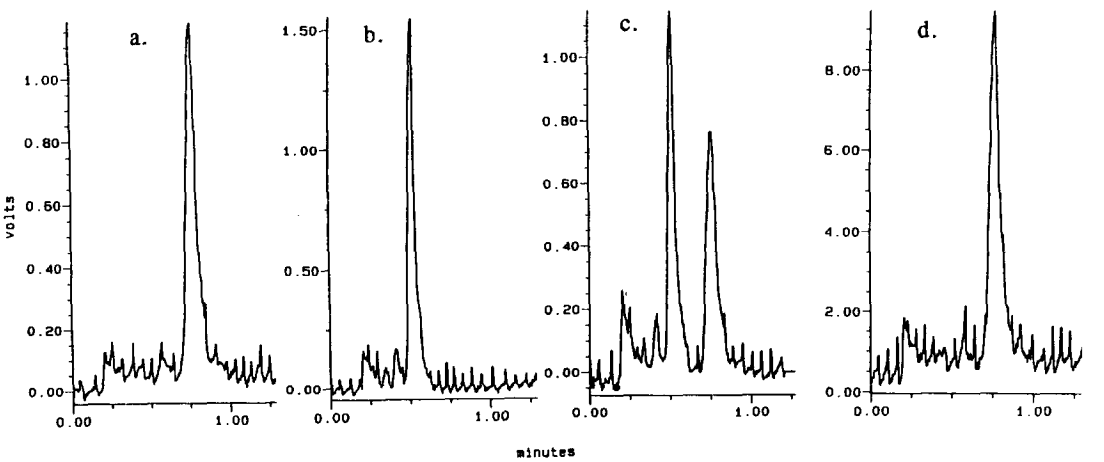


Figure 2. Ion exchange HPLC of 5'-T_{12}-glycolate obtained from **2**. a) 5'-T_{12}-glycolate (**6**) b) T_{12} standard c) Coinjection, **6** + T_{12} standard d) **6** after NaOH treatment. HPLC column B; Eluent A: 0.1 M $(NH_4)_2PO_4$ (pH 6.7), 20% CH_3CN (v:v). Eluent B: 0.3 M $(NH_4)_2PO_4$ (pH 6.7), 20% CH_3CN (v:v). 0-100% B linearly over 20 minutes. Flow rate: 1.5 mL/min.

type resin. Furthermore, subjection of **6** to 1 M NaOH at 55°C for 4 h had no effect on its chromatographic properties; indicating that the glycolate moiety is stable to the strongly alkaline conditions that one might employ in order to deprotect chemically synthesized oligonucleotides.

Further evidence that supports the contention that the 3'-glycolate moiety remains intact during the oligonucleotide synthesis, and subsequent deprotection was obtained by digesting **7** (Table 1) with Snake Venom Phosphodiesterase, followed by dephosphorylation of the resulting nucleotides with Calf Alkaline Phosphatase. Reverse phase HPLC analysis of the crude digest clearly indicated the presence of 3'-*O*-carboxymethylthymidine (**8**, Figure 3).

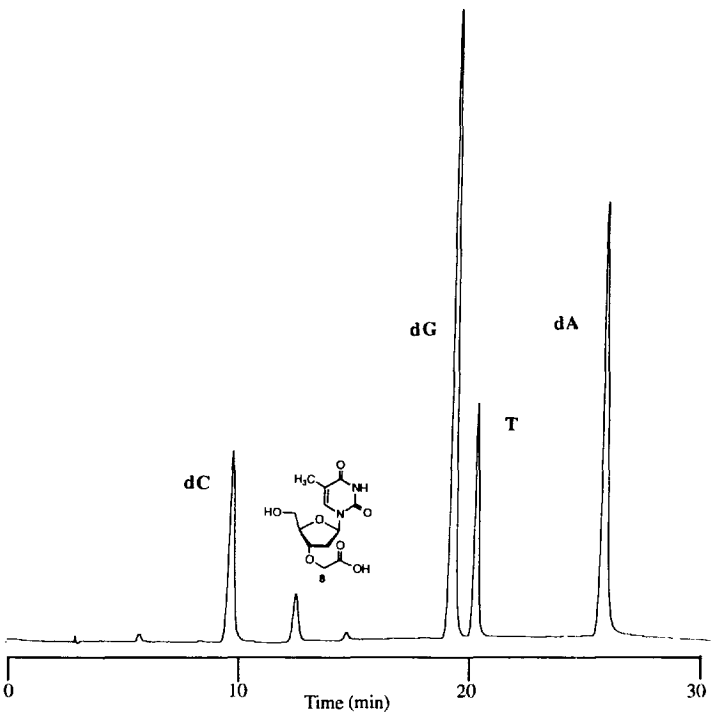


Figure 3. Reverse phase HPLC analysis of enzymatic digest of **7**. HPLC column A;. Gradient conditions: A, 0.01 M KH_2PO_4 (pH 6.8), 2.5% MeOH; B, 0.01 M KH_2PO_4 (pH 6.8), 20% MeOH; 0-100% B linearly over 15 min. Maintain 100% B for 20 min. Flow rate: 1.0 mL/min.

Conclusions. We have developed an orthogonal solid phase synthesis support with which one can directly prepare oligonucleotides containing 3'-terminal carboxylates in good yields. This approach is advantageous relative to previous methodology reported for releasing similarly substituted oligonucleotides, because the electrophilic moiety can be revealed prior to removal of the biopolymer's protecting groups. Furthermore, the linkage between the oligonucleotide and reactive moiety is stable to the strongly alkaline conditions employed for deprotecting commercially available phosphoramidites. These features suggest that **2**,

and similar solid phase synthesis supports will be very useful for preparing oligonucleotide conjugates. Efforts aimed at improving the purification (reverse phase chromatography) and yields of protected oligonucleotides are underway.

Experimental Section

General Methods. ^1H NMR spectra were recorded at 300, 270, or 200 MHz. HPLC work was performed on a Waters 501 system with a Waters 490E Programmable Multiwavelength Detector, or a Waters 501 system with a Waters 440 Fixed Wavelength Detector. HPLC columns used were: Column A, Rainin Microsorb MV C_{18} (4.6 x 250 mm); Column B, Vydac weak anion exchange oligonucleotide column (4.6 x 250 mm); Column C, Waters μBondapak Normal phase 10 μm radial pak 8 mm i.d.. Photolyses were carried using a Oriel 1000 W high pressure Hg/Xe lamp. The band pass filter was from Oriel (λ_{max}= 400 nm, #59820)

All reactions were run under nitrogen atmosphere in oven dried glassware, unless specified otherwise. Pyridine and diisopropylamine were freshly distilled from CaH_2. DMSO was freshly distilled under aspirator pressure from CaH_2. Long chain alkyl amine controlled pore glass support (CPG) was purchased from Sigma. Allyloxy phosphoramidites were prepared as described in the literature.[10] Snake Venom Phosphodiesterase and Calf Alkaline Phosphatase were obtained from Boehringer-Mannheim.

Oligonucleotides were synthesized using an Applied Biosystems Inc. 380B automated synthesizer. Standard ABI synthesis cycles were used. β-Cyanoethyl phosphorous protecting groups were removed prior to photolysis using anhydrous diisopropylamine, or after photolysis with concentrated ammonium hydroxide.[6a,13] Methyl phosphorous protecting groups were removed using PhSH/Et_3N/dioxane (1:2:2 by volume).

Polyacrylamide gel electrophoresis was carried out on 20% gels (19:1 N,N'-methylenebisacrylamide) containing 45% urea (w:v). Oligonucleotides were visualized using 254 nm light. Bands were cut out and eluted with 0.2 N NaCl, 1 mM EDTA; filtered through Quik Sep filters (Isolab) and 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.[15]

3'-*O*-Carboxymethyl-5'-*O*-dimethoxytritylthymidine (3). 5'-*O*-Dimethoxytritylthymidine (545 mg, 1 mmol) in DMSO (3 mL) was added via syringe to NaH (46 mg, 2mmol) in DMSO (1 mL) at 25°C. After stirring for 1 h, sodium chloroacetate (116 mg, 1 mmol) in DMSO (3 mL) was added via syringe. The mixture was stirred for 10 h, quenched with H_2O (20 mL), and extracted with EtOAc (2 x 30 mL). The aqueous layer was adjusted to pH 6 via the dropwise addition of 10% aqueous acetic acid, and extracted again with EtOAc (3 x 30 mL), and dried over Na_2SO_4. Carboxylic acid **3** (585 mg, 97%) was obtained as an oil, and was used without further purification. ^1H NMR ($CDCl_3$) δ 9.43 (bd s, 1H), 7.52 (d, 1H, J= 6 Hz), 7.40-7.14 (m, 9H), 6.82 (d, 4H, J= 6 Hz), 6.37 (m, 1H0, 4.29 (m, 1H0, 4.18 (m, 1H), 4.04 (d, 2H, J= 3 Hz), 3.77 (s, 6H), 3.50-3.36 (m, 2H), 2.43 (m, 1H), 2.23 (m, 1H), 1.40 (s, 3H). IR (film) 3300, 2921, 2829, 1702, 1665, 1647, 1500, 1463, 1247, 1174, 1100, 1022 cm^{-1}.

Photolabile Trichlorophenyl Ester (5). Dicyclohexylcarbodiimide (120 mg, 0.59 mmol) was added to a CH_2Cl_2 (4 mL) solution of **3** (353 mg, 0.59 mmol). After 5 min, **4** (146 mg, 0.39 mmol) and DMAP (7.2 mg, 0.06 mmol) were added. The mixture was stirred for 12 hours, at which time the solvent was removed in vacuo, the residue was resuspended in Et_2O (10 mL) and filtered. The filter cake was washed with cold ether

(10 mL). Column chromatography (EtOAc:CH_2Cl_2; 1:4) yielded 355 mg (63%) of **5**. mp: 124-126°C. ^{1}H NMR ($CDCl_3$) δ 8.91 (bd s, 1H), 8.41 (d, 1H, J= 7 Hz), 7.82 (d, 1H, J= 7 Hz), 7.61 (s, 1H), 7.56 (s, 1H), 7.41 (s, 1H), 7.36-7.22 (m, 10 H), 6.82 (d, 4H, J= 6Hz), 6.36 (dd, 1H, J= 3, 7 Hz), 5.63 (d, 2H, J= 1.5 Hz), 4.34 (m, 1H), 4.22 (d, 2H, J= 1.5 Hz), 4.14 (m, 1H), 3.78 (s, 6H), 3.52-3.34 (m, 2H), 2.54 (m, 1H), 2.25 (m, 1H), 1.48 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 169.8, 164.4, 159.4, 151.5, 148.2, 146.1, 145.0, 138.1, 136.1, 136.0, 135.7, 131.9, 130.7, 130.2, 130.0, 129.8, 128.7, 128.5, 127.9, 127.7, 127.6, 126.7, 125.9, 114.0, 113.8, 112.0, 87.7, 85.4, 84.7, 81.7, 67.1, 64.3, 63.7, 56.0, 38.4, 12.5. IR (film) 3300, 2920, 2893, 1688, 1629, 1534, 1509, 1458, 1249, 1099 cm^{-1}. HRMS FAB (M^+ + 1) calcd 960.1705, found 960.1675.

Photolabile Thymidine glycolate CPG resin (2). A mixture of **5** (88 mg, 91.5 μmole) and CPG (120 mg, ~4.2 μmole) in DMF (2 mL) 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 hour. The resin was filtered, washed and dried as described above. Resin loading was measured by treatment with *p*-toluenesulfonic acid in CH_3CN, followed by quantitation of the dimethoxytrityl cation by absorption spectroscopy (λ_{max} = 498 nm, ε = 7 x 10^4 M^{-1}cm^{-1}).[16]

3'-*O*-Carboxymethylthymidine (8). 3'-*O*-Carboxymethyl-5'-*O*-dimethoxytritylthymidine (**3**, 200 mg, 0.33 mmol) was stirred in 80% acetic acid (10 mL) for 12 h. The solvents were removed in vacuo, and the residue azeotroped with EtOH (2 x 20 mL). The residue was taken up in H_2O (10 mL) and extracted with diethyl ether (3 x 25 mL). The aqueous layer was lyophilized to dryness, and then relyophilized from D_2O (2 mL) to yield 94 mg (95%) of **7** as a colorless oil. ^{1}H NMR (MeOH-d_4) δ 7.74 (s, 1H), 6.24 (dd, 1H, J= 4,7 Hz), 4.63 (m, 1H), 4.47 (m, 1H), 3.78 (s, 2H), 2.44 (m, 1H), 2.22 (m, 1H), 1.86 (s, 3H). IR (film) 3400, 3008, 2921, 1699, 1665, 1469, 1436, 1409, 1273, 1098, 1017 cm^{-1}. HRMS FAB (M^+ + 1) calcd 301.1036, found 301.1047

General Procedure for Photolytic Cleavage of Oligonucleotides from Solid Phase Supports. Resin was stirred in a Pyrex tube containing 3 mL of a 9:1 mixture of CH_3CN:H_2O. The mixture was sparged with N_2 for 20 minutes before photolysis. The volume above the solution was continuously purged with N_2 during photolysis. The output (800 W) of a high pressure Hg/Xe lamp was filtered using a band pass filter (λ_{max}= 400 nm). Following photolysis, the resin was filtered through a 0.45 μ filter. The photolysis tube and filter were washed with CH_3CN (3 x 1 mL), followed by H_2O (3 x 1 mL). The filtrates were concentrated, combined and subjected to the appropriate deprotection and/or purification method.

Normal Phase HPLC Characterization of Phosphodiester Protected Oligonucleotides. Column C was employed using a gradient. Eluent A: CH_2Cl_2; Eluent B: MeOH; 20-100% B linearly over 20 min; 100% B for 10 min. Flow rate: 1 mL/min.

Anion Exchange HPLC Characterization of Fully Deprotected Oligonucleotides. Column B was employed using a gradient. Eluent A: 0.1 M $(NH_4)_2PO_4$ (pH 6.7), 20% CH_3CN (v:v). Eluent B: 0.3 M $(NH_4)_2PO_4$ (pH 6.7), 20% CH_3CN (v:v). 0-100% B linearly over 20 minutes. Flow rate: 1.5 mL/min.

Enzymatic Digestion of 5'-dTAC GCA ATG CTA GAT CTA AT-glycolate. Snake Venom Phosphodiesterase (30 μL; 0.1 unit/μL) in 0.1 M Tris-acetate buffer (pH 8.75) were added to an Eppendorf containing 0.3 OD of **7** which had been concentrated to dryness. The tube was vigorously vortexed, spun briefly and immersed in a 37°C water bath. After 6 h, 1 μL of Calf Alkaline Phosphatase (10 unit/μL) was added to the

mixture. After an additional 1 h at 37°C, the sample was precipitated via the addition of 20% NaOAc (5 μL) and EtOH (120 μL), and freezing (-78°C). The solution was centrifuged at 14,000 rpm for 10 minutes. After which, the supernatants were carefully removed so as to not perturb the salt pellet at the bottom of the tube. The supernatants were then reprecipitated via the addition of EtOH (350 μL). The supernatants were separated from the salt pellet as described above, and removed in vacuo (Savant Speed Vac). The residue was resuspended in H_2O (50 μL). Samples were passed through 0.45 μ filters prior to HPLC analysis (Column A). Gradient conditions: A, 0.01 M KH_2PO_4 (pH 6.8), 2.5% MeOH; B, 0.01 M KH_2PO_4 (pH 6.8), 20% MeOH; 0-100% B linearly over 15 min. Maintain 100% B for 20 min. Flow rate: 1.0 mL/min.

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