N-(2-Cyanoethoxycarbonyloxy)succinimide: A New Reagent for Protection of Amino Groups in Oligonucleotides

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Introduction

During oligonucleotide synthesis, convenient amino group protection methodology is important not only for exocyclic amines but also useful for side-chain amino groups ("aminolinkers" or "aminotethers"). Conjugation of different ligands to improve the properties of oligonucleotides requires placement of protected amino groups with appropriate tethers in the building blocks and using them to synthesize oligonucleotides. The ligand of interest is attached postsynthetically either in the solution phase or in the solid phase. The amino group present in tethers can be conveniently deprotected and used for attaching various functionalities to modify the biological or chemical properties of oligonucleotides. The conjugated ligands can improve the uptake of antisense oligonucleotides by living cells. For example, attachment of polyamines and lipophilic molecules such as cholesterol improves cellular uptake of antisense oligonucleotides.[2] The ligands can serve as reporter groups (e.g., fluorescein or biotin), which are extensively used in DNA-based diagnostics and also used for following cellular trafficking of antisense oligonucleotides.[1] The attached moieties can act also as chemical nucleases to degrade the target pathogenic genes. Despite their widespread use, the conventional protecting groups used in oligonucleotide chemistry for aminotethers are either too labile during the monomer synthesis [e.g., trifluoroacetyl (CF_3CO-) and fluorenylmethoxycarbonyl (Fmoc)] or somewhat inert, requiring harsh conditions during oligonucleotide deprotection (e.g., phthalimido protecting group, which requires addition of methylamine to the standard ammonium hydroxide and heating at 55 °C for 12 h). The acid-labile monomethoxytrityl (MMTr−) group is another possibility, but it limits the use of the aminolinker to the 5′-end of the oligonucleotide. To overcome these problems, we and others have adopted the well-known allyloxycarbonyl (alloc) group[3,4] as a protecting group for aminolinkers. This group can be removed by the use of zerovalent palladium (Pd(0)) in either solution phase or solid phase.[4−7]

Because it is conveniently removed with ammonium hydroxide by β-elimination, the β-cyanoethyl ester group has been successfully used as a phosphate protecting group for decades.[8] If we could use the β-cyanoethoxycarbonyl group as a protecting group for amines (on nucleobases and/or on tethers) as well, both phosphate and amino group deprotection could be achieved in one step using the standard ammonium hydroxide treatment. If successful, one could envision using the β-cyanoethoxycarbonyl group for protection of the exocyclic amine of all nucleobases as well.

In the peptide literature, a related group, the cyano-*tert*-butoxycarbonyl group, has been used to protect the amino acid glycine. This group is cleaved by aqueous potassium carbonate or triethylamine by β-elimination at pH 10.[9] Recently, a related group, (2-cyano-1-phenyl)-ethoxycarbonyl (the CPEOC group), has been used as the base-labile protecting group for the 5′-OH in RNA synthesis.[10] The same research group suggests the protection of exocyclic amines of nucleobases using the chloroformate of β-cyanoethanol ($Cl(C{=}O)OCH_2CH_2CN$) and in situ activation of this reagent with *N*-methylimidazole in a patent application.[11] Surprisingly, this group and other analogues have not been widely used as a protecting group[6] for amino groups in nucleic acid synthesis, which may be related to the lack of a simple reagent to introduce this group.

Our desire to make a convenient reagent for cyanoethoxycarbonylation led us to prepare *N*-(2-cyanoethoxycarbonyloxy)succinimide (CEOC-*O*-succinimide), a stable, crystalline compound that can be synthesized from readily available commercial chemicals.

Results and Discussion

CEOC-*O*-succinimide **1** was generated by reacting 2-cyanoethanol with disuccinimidyl carbonate (DSC)[12−14] in acetonitrile in the presence of pyridine (Scheme 1). Pyridine was used as the base in place of triethylamine,[12] which is commonly used for the conversion of other

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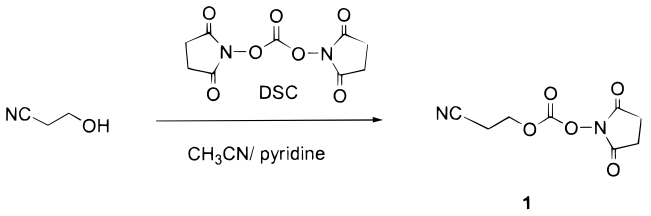
Scheme 1



alcohols to their carbonates to avoid any possible β-elimination during workup. The synthesis has been scaled up easily, and compound **1** has been prepared in multigram quantities several times. It is a stable compound that can be stored at room temperature for an extended period of time (more than a year) in a desiccator.

In this paper, we demonstrate the use of this compound to protect pendant alkylamines in oligonucleotides. These alkylamines are present either in the 2′-position of RNA modifications or in nonnucleosidic tethers. In addition to helping functionalization of oligonucleotides by other compounds via amino group mediated conjugation chemistry, the nucleosidic aminolinkers can enhance affinity in binding to the target RNA (vide infra) and improve their biostability. Oligonucleotides containing short stretches of alkylamino tethers at their 3′-ends display extraordinary resistance against degradation by 3′-exonucleases.[15] The improvement in nuclease resistance is presumably due to the zwitterionic nature of the modified oligonucleotide. The amino group in the 2′-*O* substituent has a pK_a in the range of 9−10 and can therefore be expected to be protonated at physiological pH. The cationic group decreases susceptibility of oligonucleotides to nucleases.

The CEOC group was used to protect amino groups present in both in nucleosides and nonnucleosidic building blocks. Nucleosides[16,17] **4** and **7**, containing aminolinkers of different lengths (2′-*O*-$CH_2CH_2NH_2$ and 2′-*O*-$CH_2CH_2CH_2CH_2CH_2CH_2NH_2$), were reacted with the CEOC reagent to protect the amino groups. The synthesis of the 2′-*O*-(hexylamino) compound **7** was carried out according to a reported procedure.[16] Synthesis of compound **4** is shown in Scheme 2. This involves ring opening of 2,2′-anhydro-5-methyluridine by the borate ester generated from *N*-(2-hydroxyethyl)phthalimide and borane in THF.[17] The ring-opening reaction proceeded in 21% yield. Considering the cost of the inexpensive starting materials[18] and regioselective 2′-alkylation, this simple one-step process is convenient and economical, despite its poor yield. The nucleoside was protected at the 5′-end with dimethoxytrityl chloride and then treated with hydrazine to liberate the free amine. The amines **4** and **7** were protected by the CEOC reagent **1** to yield compounds **5** and **8**, which were converted to the corresponding β-cyanoethyl phosphoramidite compounds **6** and **9** and used under standard oligonucleotide automated synthesis conditions. Subsequently, standard deprotection conditions (concentrated ammonium hydroxide, 12 h, 55 °C) were used to deprotect the oligomers. The CEOC groups were removed without any side products (as detected by HPLC, NMR, and mass spectral analysis) in oligomers containing all four nucleobases. These bases contained standard protecting groups for exocyclic amines (benzoyl for deoxyadenosine and deoxycytidine; isobutyryl for deoxyguanosine). Oligomers synthesized using the CEOC strategy are shown in Table 1 along with their mass spectral and HPLC characteristics. The purified oligonucleotides with aminolinkers were used to conjugate other functionalities. They were also evaluated for their antisense properties in terms of oligonucleotide RNA target binding. The 2′-*O*-aminolinker-containing oligonucleotides were hybridized against RNA (Table 2). The modified oligonucleotides showed nearly a +1.0 °C increase in T_m (the midpoint for helix to coil transition) for each substitution compared to unmodified DNA.[19] This translates to nearly 1.8 °C increase per modification, when compared to 2′-deoxyphosphorothioates that are used as first-generation antisense oligonucleotide drugs.[20] This increase may be a result of overall reduction of negative charges in the oligomer−RNA duplex, as these amino groups are expected to be protonated. As noted previously, these zwitterionic oligonucleotides also improve the nuclease resistance of the antisense oligomers.[15]

A nonnucleosidic amino alcohol (6-aminohexanol) was also protected by CEOC (compound **10**) and converted to its phosphoramidite derivative (compound **11**; Scheme 4). This was coupled to the 5′-end of the oligonucleotides. The purified oligonucleotides with aminolinkers (both nucleosidic and nonnucleosidic) were used for conjugation chemistry (i.e., attachment of other functionalities such as fluorescein), in excellent yields starting with an isothiocyanate to give the product via a thiourea linker (Table 3).

Summary

A convenient crystalline reagent to protect aminolinkers (CEOC-*O*-succinimide) has been developed and used to protect nucleoside-based 2′-*O*-alkyl aminolinkers and nonnucleosidic aminolinkers. After oligonucleotide synthesis incorporating these aminolinkers, standard NH_4-OH treatment removes the CEOC group by β-elimination. The resultant oligonucleotides modified with 2′-*O*-alkylamines stabilize antisense oligomers toward RNA binding. Aminolinkers generated by this new method are also useful for conjugation chemistry.

In addition to the potential applications in the nucleic acid field, the CEOC reagent is of general use for amino groups in all classes of compounds. Our present efforts are directed toward this goal and will be reported in due course.

Experimental Section

General Methods. All reagents and solvents were purchased from commercial sources unless otherwise noted. 2-Cyanoethanol, *N*,*N*′-disuccinimidyl carbonate (DSC), *N*-(2-hydroxy)phthalimide, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, and 6-aminohexanol were obtained from Aldrich Chemical

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Scheme 2

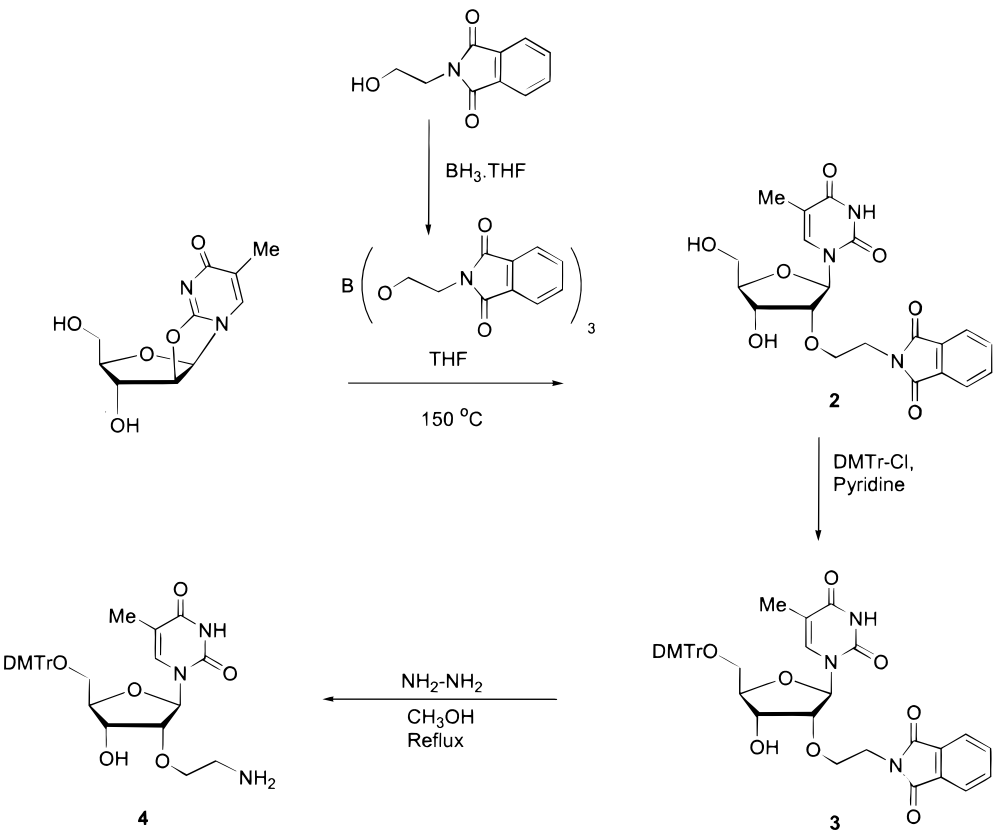


Table 1. Aminolinker-Containing Oligonucleotides Synthesized Using CEOC Strategy[a]

oligonucleotide no.	oligonucleotide sequence	modification 2′-O$(CH_2)_nNH_2$, *n*	mass calcd	mass found	HPLC t_R,[d] min
1	GAT*CT[e]	6	1895.00[b]	1895.57	20.05
2	T*CCAGGT*GT*CCGCAT*C	6	5599.00[b]	5597.24	24.01
3	CTCGTACT*T*T*T*CCGGTCC	6	5853.83[c]	5854.56	20.25
4	CTCGTACCT*TTCCGGTCC	6	5493.21[c]	5493.91	18.22
5	GAT*CT	2	1839.65[b]	1839.60	19.92
6	T*CCAGGT*GT*CCGGCAT*C	2	5368.00[b]	5370.40	23.88
7	L*-GCATCCCCCAGGCCACCAT	nonnucleosidic	6161.68	6160.72	17.86[f]

[a] The asterisked position indicates the site of modification. [b] DMT-on. [c] DMT-off. [d] HPLC conditions: Waters C-18 Delta-Pak reversed-phase column, 3.9 × 300 mm; solvent *A* = 100 mM triethylammonium acetate (TEAAc) pH 7; solvent B = CH_3CN; gradient of 8−18% B in 30 min; flow rate 1.5 mL/min, λ = 260 nm. [e] ^{31}P NMR (in D_2O, ppm) −0.05 (one), −0.38 (two), −0.46 (one). [f] Waters C-4, 3.9 × 300 mm, solvent *A* = 50 mm TEAAc, pH 7; solvent B = CH_3CN; gradient 5−60% B in 55 min; flow rate 1.5 mL/min, λ = 260 nm.

Table 2. T_m Data for Oligonucleotides[a] with 2′-*O*-(2-Aminoethyl) and 2′-*O*-(6-Aminohexyl) Modifications[b]

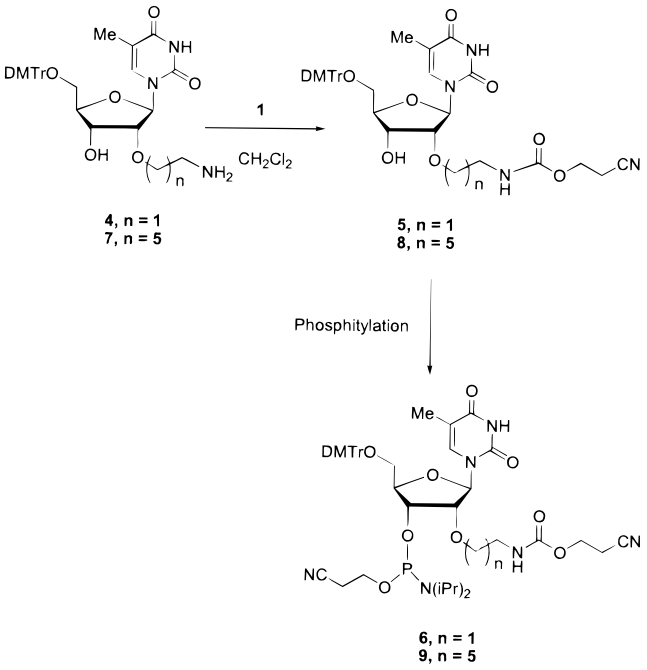
oligonucleotide no.	sequence	modification −*O*$(CH_2)_nNH_2$	T_m (°C) (against RNA target)	T_m (°C) (unmodified parent DNA)	ΔT_m/ modification (°C)
6	T*CCAGGT*GT*CCGGCAT*C	2	66.42	62.3	1.03
2	T*CCAGGT*GT*CCGCAT*C	6	65.54	62.3	0.81
3	CTCGTACT*T*T*T*CCGGTCC	6	66.2	61.8	1.1

[a] All nucleosides with asterisk contain 2′-*O*-(aminotether). [b] T_m conditions as described by Freier and Altmann.[19]

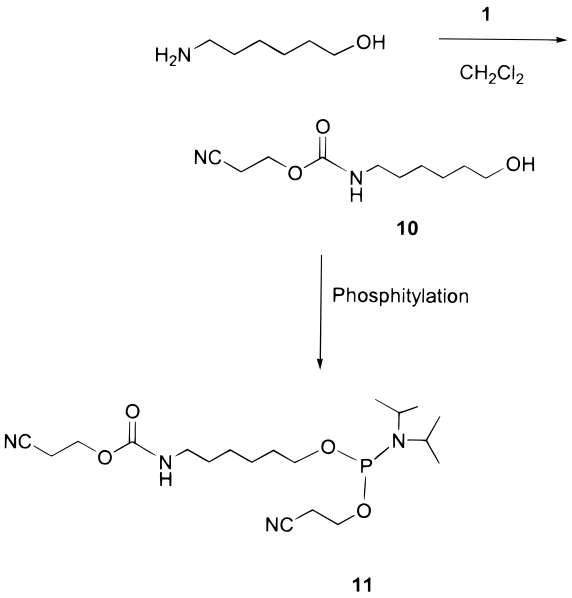
Co., Inc. (Milwaukee, WI). Reagents for the DNA synthesizer were purchased from PerSeptive Biosystems, Inc. (Framingham, MA). 2,2′-Anhydro-5-methyluridine was purchased from Ajinomoto (Tokyo, Japan). Flash chromatography was performed on silica gel (Baker, 40 μm). Thin-layer chromatography was performed on Kieselgel glass plates from E. Merck and visualized with UV light and *p*-anisaldehyde/sulfuric acid/acetic acid spray followed by charring.

***N*-(2-Cyanoethoxycarbonyloxy)succinimide (CEOC-*O*-succinimide) (1).** To a stirred solution of 2-cyanoethanol (7.23 g, 102 mmol) in 300 mL of anhydrous CH_3CN, under argon atmosphere, was added *N*,*N*′-disuccinimidyl carbonate (34.0 g, 133 mmol) followed by pyridine (11.3 mL, 140 mmol). The suspension became a clear solution after about 1 h. The solution was stirred for an additional 6 h and then concentrated in vacuo. It was redissolved in dichloromethane (200 mL) and extracted with saturated $NaHCO_3$ solution (3 × 50 mL) followed by saturated NaCl solution (3 × 50 mL). The organic layer was dried (anhydrous Na_2SO_4) and concentrated to give a white solid. Traces of pyridine were removed by coevaporation with dry acetonitrile. The white solid was dried overnight in vacuo and then triturated with ether (150 mL) to yield 20.23 g of a colorless amorphous powder of **1** (94%). This material is stable at room temperature in a desiccator for an extended period (1−2 years). For all derivatizations described in this paper, the material can be used as is. The proton and carbon NMR spectra revealed a homogeneous material even at this stage. The material was further purified by chromatography on silica gel using CH_2Cl_2/

Scheme 3



Scheme 4



EtOAc (50:50) to give a white crystalline compound (18.72 g, 87%): mp 105.5 °C. (R_f = 0.21 in the same solvent system); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.85 (t, J = 6.62 Hz, 2H), 2.86 (s, 4H) 4.45 (t, J = 5.96 Hz); ^{13}C NMR (80 MHz, DMSO-d_6) δ 17.33, 25.39, 65.86, 117.91, 150.91, 169.82; HRMS(FAB) calcd for $C_8H_9N_2O_5^+$ 213.0511, found 213.0509. Anal. Calcd for $C_8H_8N_2O_5$: C, 45.29; H, 3.80; N, 13.20. Found: C, 45.19; H, 3.45; N, 13.02.

Protection of Nucleosidic Aminolinkers. 2′-*O*-Phthalimidoethyl-5-methyluridine (2). *N*-(2-Hydroxyethyl)phthalimide (277 g, 1.45 mol) was slowly added to a solution of borane in tetrahydrofuran (1 M, 600 mL) with stirring. Hydrogen gas evolved as the solid dissolved. Once the rate of gas evolution subsided, the solution was placed in a 2 L stainless steel bomb. 2,2′-Anhydro-5-methyluridine (60 g, 0.25 mol) and sodium bicarbonate (120 mg) were added, and the bomb was sealed. After 30 min, the bomb was vented and placed in an oil bath and heated to 150 °C internal temperature for 24 h. The bomb was cooled to room temperature and opened. TLC (ethyl acetate−methanol 95/5) revealed the disappearance of starting material. The crude solution was concentrated, and the residue was purified by chromatography on silica gel starting with ethyl acetate to remove the excess phthalimide reagent followed by ethyl acetate−methanol 95/5 to elute **2 (**22.2 g, 20.6%): ^{1}H NMR (200 MHz, DMSO-d_6); δ 1.8 (s, 3H), 3.4−4.2 (m, 6H), 5.0−5.2 (m, 2H), 5.8 (d, J = 5.1 Hz, 1H), 7.65 (s, 1H), 7.8−8.0 (m, 4H), 11.2 (s, 1H).

2′-*O*-(2-Phthalimidoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine (3). 2′-*O*-Phthalimidoethyl-5-methyluridine (**2**, 22.2 g, 0.053 mol) was coevaporated with pyridine (2 × 75 mL) and then dissolved in 100 mL of pyridine. Dimethoxytrityl chloride (27 g, 0.080 mol) was added in one portion with stirring. TLC (ethyl acetate−hexanes 50:50) after 1 h indicated complete reaction. Methanol (10 mL) was added to quench the reaction. The mixture was concentrated and the residue was partitioned between ethyl acetate and saturated sodium bicarbonate solution (150 mL each). The organic layer was concentrated, and the residue was dissolved in a minimum amount of dichloromethane and applied to a silica gel column. The compound was eluted with ethyl acetate−hexanes−triethylamine (50:50:1 to 80:20:1) to give **3** (26.1 g, 68%) as a white foam: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.33 (s, 3H), 3.05 (d, J = 8.4 Hz, 1H), 3.49 (m, 2H), 3.8 (s, 6H), 3.9−4.1 (m, 4H), 4.18−4.26 (m, 1H), 4.47 (m, 1H), 5.88 (s, 1H), 6.84 (d, J = 8.78 Hz, 4H), 7.22−7.43 (m, 9H), 7.69−7.88 (m, 5H), 8.26 (s, 1H); HRMS(FAB) calcd for $C_{41}H_{39}N_3O_{10}Na^+$ 756.2533, found 756.2553.

2′-*O*-(2-Aminoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine (4). 2′-*O*-Phthalimidoethyl-5′-*O*-DMT-5-methyluridine (**3**, 21.1 g, 0.029 mol) was dissolved in methanol (500 mL). Anhydrous hydrazine (4.9 mL, 0.15 mol) was added, and the solution was heated to reflux. TLC after 3 h indicated a complete reaction. The residue was purified by chromatography on silica gel using methanol and then methanol−ammonium hydroxide (98:2) to give **4** (12.4 g, 70%). The material was completely soluble in methylene chloride, and any traces of silica from the leaching of the column were removed by filtration at this stage and reevaporation of the solution: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.39 (s, 3H), 2.98 (t, J = 3.48 Hz, 2H), 3.45 (d, J = 2.56 Hz, 1H), 3.53 (d, J = 1.96 Hz, 1H), 3.56−3.68 (m, 2H), 3.81 (s, 6H), 3.99 (m, 1H), 4.1 (t, J = 4.56 Hz, 1H), 4.17 (m, 1H), 4.45 (t, J = 5.06 Hz, 1H), 6.06 (d, J = 4.12 Hz, 1H), 6.86 (d, J = 8.9 Hz, 4H), 7.25−7.46 (m, 9H), 7.67 (s, 1H); ^{13}C (50 MHz, $CDCl_3$) δ 11.71, 40.55, 45.76, 55.03, 62.47, 69.15, 70.65, 82.64, 83.49, 86.62, 87.10, 110.98, 113.09, 126.91, 127.77, 127.97, 129.95, 135.35, 144.25, 151.27, 158.46, 164.97; HRMS (FAB) calcd for $C_{33}H_{37}O_8N_3Na^+$ 626.2478, found 626.2501.

2′-*O*-[*N*-(2-Cyanoethoxycarbonyl)-2-aminoethyl]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine (5). To compound **1** (0.6 g, 2.8 mmol) in 10 mL of CH_2Cl_2 was added 0.5 mL of pyridine followed by 2′-*O*-(aminoethyl)-5′-*O*-DMT-5-methyluridine (**4**, 1.43 g, 2.35 mmol), and the mixture was stirred for 1 h. TLC (CH_2Cl_2/CH_3OH 9:1; R_f = 0.48) indicated the complete conversion of amine into the carbamate derivative. The mixture was diluted with CH_2Cl_2 (50 mL), washed successively with aqueous $NaHCO_3$ solution and saturated NaCl solution, and dried over $MgSO_4$. Chromatography over silica and elution with CH_2Cl_2/EtOAc gave the desired nucleoside **5** (1.2 g, 73%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.41 (s, 3H), 2.82 (t, J = 6 Hz, 2H), 3.18−3.3 (m, 4H), 3.56−3.64 (m, 2H), 3.94−4.08 (m, 2H), 4.11 (t, J = 6 Hz, 2H), 4.20−4.28 (m, 1H), 5.14 (d, J = 6.83 Hz, 1H), 5.82 (d, J = 4.06 Hz, 1H), 6.90 (d, J = 8.97 Hz, 4H), 7.24−7.42 (m, 9H), 7.48 (s, 1H), 11.38 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.70, 18.21, 40.77, 55.08, 59.07, 61.88, 68.87, 70.04, 82.59, 83.28, 86.63, 87.42, 111.03, 113.3, 117.3, 126.97, 127.87, 127.98, 129.95, 135.12, 136.21, 144.19, 150.77, 156.01, 158.50, 164,24; HRMS (FAB) calcd for $C_{37}H_{40}O_{10}N_4Cs^+$ 833.1799, found 833.1824.

2′-*O*-[*N*-(2-Cyanoethoxycarbonyl)-2-aminoethyl]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine 3′-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (6). Compound **5** (0.7 g, 1 mmol) was dissolved in dry CH_2Cl_2 (15 mL), and *N*,*N*-diisopropylammonium tetrazolide (0.085 g, 0.5 mmol) followed by 2-cyanoethyl-*N*,*N*,*N*′*N*′-tetraisopropyl phosphorodiamidite (0.420 mL, 1.1 mmol) was added slowly using a syringe under argon. The mixture was stirred at room temperature overnight, after which TLC (CH_2Cl_2/EtOAc 50:50) indicated almost complete reaction. An additional aliquot of the phosphitylation reagent (0.045 mL, 0.12 mmol) was added and the mixture stirred for an additional 2 h. TLC then indicated complete conversion of the starting material to the phosphoramidite (CH_2-

Table 3. Conjugation Chemistry of Oligonucleotides Derived from CEOC Strategy

oligonucleotide no.	sequence (5′-3′)	HPLC[a] t_R (min)	mass	
			calcd	found
4	CTCGTACCT*TTCCGGTCC	18.22	5493.21	5493.91
8	CTCGTACCT$_{FL}$TTCCGGTCC	22.08	5881.60	5880.89
7	L*-TGCATCCCCCAGGCCACCAT	17.96	6161.68	6160.72
9	L$_{FL}$-TGCATCCCCCAGGCCACCAT	23.74	6550.68	6550.01

[a] C-4 Waters, Delta-Pak C_4 column 3.9 × 300 mm, sSolvent A = 50 mM triethylammonium acetate pH 7; solvent B = acetonitrile, 5−60% B in 55 min; flow 1.5 mL/min, $\lambda = 260$ nm. L* = 2′-*O*-$(CH_2)_6NH_2$. L_{FL} = fluorescein-NHC(=S)NH$(CH_2)_6$O−. T* = 2′-O-(6-aminohexyl) ^{5Me}U. T_{FL} = 2′-O-(fluorescein-NHC(=S)NH$(CH_2)_6$−) ^{5Me}U.

Cl_2/EtOAc 50:50; $R_f = 0.33$). The reaction mixture was diluted with 50 mL of CH_2Cl_2 and washed with saturated $NaHCO_3$ solution followed by saturated NaCl solution. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The crude foam was purified by chromatography on silica gel and eluted with 50:50 EtOAc/CH_2Cl_2 to give **6** (0.72 g, 81%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.02 (d, $J = 5.18$ Hz, 3H), 1.14−1.2 (m, 9H), 2.44 (t, $J = 6.14$, 2H), 2.654−2.78 (m, 4H), 3.21−3.47 (m, 4H), 3.51−3.71 (m, 4H), 3.75−3.95 (m, 4H), 3.81 (s, 6H), 4.1−4.16 (m, 2H), 4.28 (t, $J = 5.96$ Hz, 4H), 4.51−4.61 (m, 1H), 5.97−6.01 (m, 1H), 6.83−6.88 (m, 4H), 7.2−7.47 (m, 9H), 7.69 (s, 1H), 7.73(s,1H); ^{31}P NMR (80 MHz, $CDCl_3$) δ 149.5 and 150.5; MS (ES) *m*/*z* 923 $[M + Na]^+$.

2′-*O*-[*N*-(2-Cyanoethoxycarbonyl)-6-aminohexyl]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine (8). To a solution of **7** (3.3 g, 5 mmol)[13] in anhydrous CH_2Cl_2 (20 mL) was added anhydrous pyridine (1 mL) followed by compound **1** (1.2 g, 5.6 mmol). The reaction mixture was stirred for 2 h and tested by TLC (CH_2Cl_2/CH_3OH 9:1). The reaction was complete, and the reaction mixture was applied to silica gel equilibrated with CH_2Cl_2/CH_3OH 9:1 and eluted with the same solvent system (R_f = 0.51) to give **8** (3.13 g, 82%): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.28−1.6 (m, 11H), 2.6−2.78 (m, 3H), 3.21 (q, $J = 6.24$, 2H), 3.43 (d, $J = 8.4$ Hz, 1H), 3.57 (d, $J = 10.74$ Hz, 1H), 3.66−3.95 (m, 2H), 3.81 (s, 6H), 4.03−4.11 (m, 2H), 4.14−4.5 (m, 1H), 4.25−4.35 (m, 2H), 5.18 (br. s, 1H), 6.01 (d, $J = 2.94$ Hz, 1H), 6.85 (d, J = 8.8 Hz, 4H), 7.22−7.44 (m, 9H), 7.68 (s, 1H), 8.62 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 11.59, 18.2, 25.24, 26.03, 29.11, 29.37, 40.68, 55.05, 58.83, 62.10, 68.97, 70.66, 82.59, 83.38, 86.66, 87.1, 110.94, 113.09, 117.17, 126.94, 127.80, 127.97, 129.93, 135.09, 135.22, 144.14, 150.45, 155.44, 158.48, 164.14. HRMS(FAB) calcd for $C_{41}H_{48}N_4O_{10}Na^+$ 779.3268, found 779.3259.

2′-*O*-[*N*-(2-Cyanoethoxycarbonyl)-6-aminohexyl]-5′-*O*-(4,4′dimethoxytrityl)-5-methyluridine 3′-*O*-[(2-Cyanoethyl)*N*,*N*-diisopropyl) Phosphoramidite (9). Nucleoside **8** (1.51 g, 2 mmol) was dissolved in 30 mL of anhydrous CH_2Cl_2. To this solution was added *N*,*N*-diisopropylammonium tetrazolide (0.17 g, 1 mmol) followed by 2-cyanoethyl *N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite (0.99 mL, 2.6 mmol) under argon atmosphere. The reaction mixture was stirred for 16 h. TLC analysis (50:50 CH_2Cl_2/EtOAc) indicated completion of the reaction. The reaction mixture was then diluted with CH_2Cl_2 (100 mL), extracted with saturated $NaHCO_3$ solution (2 × 50 mL), washed with saturated NaCl solution (50 mL), and dried over $MgSO_4$. Evaporation to dryness yielded a white foam. This white foam was dissolved in CH_2Cl_2 and applied to a silica gel column equilibrated with CH_2Cl_2 containing 0.1% pyridine. Elution with 40:60 EtOAc/CH_2Cl_2 yielded **9** (1.3 g, 68%) as a foam: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.00 (d, $J = 6.74$ Hz, 3H), 1.11−1.4 (m, 12H), 1.46−1.78 (m, 8H), 2.62−2.77 (m, 4H), 3.13−3.22 (m, 2H), 3.4−3.72 (m, 4H), 3.79 (s, 6H), 3.84−3.98 (m, 2H), 4.14−4.5 (m, 5H), 4−4.12 (m, 2H), 5.98−6.09 (m, 1H), 6.78−6.9 (m, 4H), 7.21−7.48 (m, 9H), 7.59 (s, 1H), 7.71 (s, 1H); ^{31}P NMR (80 MHz, CDCl3) δ 150.5, 151 ppm; MS (ES) *m*/*z* 979 $(M + Na]^+$.

Protection of a Nonnucleosidic Aminolinker. *N*-(2-Cyanoethoxycarbonyl)-6-aminohexanol (10). 6-Aminohexanol (0.5 g, 4.23 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL). Compound **1** (1.08 g, 5.09 mmol) was added, and the mixture was stirred for 2 h. The reaction was followed by TLC (5% MeOH in CH_2Cl_2). Solvent was removed *in vacuo*, and the residue was placed on a flash column and eluted with 5% MeOH in CH_2Cl_2 to get **10** as a white powder (0.883 g, 96% yield): R_f = 0.28, 5% MeOH in CH_2Cl_2; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.6−1.39 (m, 8H), 2.73 (t, $J = 6.12$ Hz, 2H,), 3.68 (t, $J = 3.71$ Hz, 3H), 4.3 (t, $J = 6.12$ Hz, 2H,), 4.9 (br, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 18.49, 25.30, 26.35, 29.73, 32.47, 40.92, 59.01, 62.49, 117.34, 155.64; HRMS (FAB) calcd for $C_{10}H_{19}N_2O_3^+$ 215.1396, found 215.1395.

***N*-(2-Cyanoethoxycarbonyl)-6-aminohexyl *O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (11).** Compound **10** (0.72 g, 3.36 mmol) was mixed with *N*,*N*-diisopropylammonium tetrazolide (0.29 g, 1.68 mmol). The mixture was then dried over P_2O_5 *in vacuo* overnight at 40 °C. The reaction flask was flushed with argon. Anhydrous acetonitrile (17 mL) was added, followed by dropwise addition of 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.52 mL, 5.04 mmol). The reaction mixture was stirred at room temperature for 4 h under argon. Solvent was removed in vacuo. The residue was placed on a flash column and eluted with ethyl acetate/hexane 1:1 to get **11** as an oil (0.62 g, 44% yield): R_f (0.05, EtOAc/hexane 50:50); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.22 (s, 6H), 1.22 (s, 6H), 1.48−1.7 (m, 8H), 2.62−2.75 (m, 4H), 3.21 (q, $J = 6.46$ Hz, 2H), 3.53−3.95 (m, 6H), 4.29 (t, J = 6.24 Hz, 2H); ^{31}P NMR (80 MHz, $CDCl_3$) δ 147.78; MS (FAB) *m*/*z* 437 $[M + Na]^+$.

Oligonucleotide Synthesis. Compound **6**, **8**, or **11** was dissolved in anhydrous acetonitrile to give 0.1 M solution and loaded onto an Expedite Nucleic Acid Synthesis system (Millipore 8909) to synthesize the oligonucleotides. The coupling efficiencies were more than 98%. For the coupling of the modified amidites (**6**, **8**, or **11**), coupling time was extended to 10 min, and this step was carried out twice. All other steps in the protocol supplied by Millipore were used as such. The oligomers were cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions (12 h) using concentrated aqueous NH_4OH (30%) at 55 °C. 5′-*O*-DMT-containing oligomers were then purified by reversed-phase high-performance liquid chromatography [(C-4 column, Waters, 7.8 × 300 mm, A = 50 mM triethylammonium acetate, pH = 7, B=acetonitrile, 5−60% of B in 55 min, flow 2.5 mL/min), $\lambda = 260$ nm]. Detritylation of the purified full-length oligonucleotide with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column, gave modified oligonucleotides. Oligonucleotides were analyzed by HPLC, capillary gel electrophoresis and mass spectrometry.

Oligonucleotide Conjugation Chemistry. Oligonucleotides **4** and **7** (Table 1) containing the tethered amino functionality were used to conjugate fluorescein to the oligonucleotide. Purified oligonucleotides (15 OD/mL) in water were evaporated, and the residue was dissolved in 100 μL of 1 M $NaHCO_3$/Na_2CO_3 buffer (pH 9.2). A solution of fluorescein isothiocyanate (100 μL, 1 M solution in DMF) was added to the solution of oligonucleotides and kept at room temperature for 24 h. Unreacted fluorescein isothiocyanate was removed by passing the reaction mixture through a column of Sephadex G-25 and eluting with water. Conjugated oligonucleotides were then purified by reversed-phase HPLC and characterized by electrospray mass spectrometry, analytical HPLC, and CGE (Table 3).

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Supporting Information Available: ^{1}H and ^{13}C NMR and high-resolution mass spectra of compounds **1**, **4**, **5**, **8**, and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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