Solution-Phase Bioconjugate Synthesis Using Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids

Jeffrey D. Kahl and Marc M. Greenberg*

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

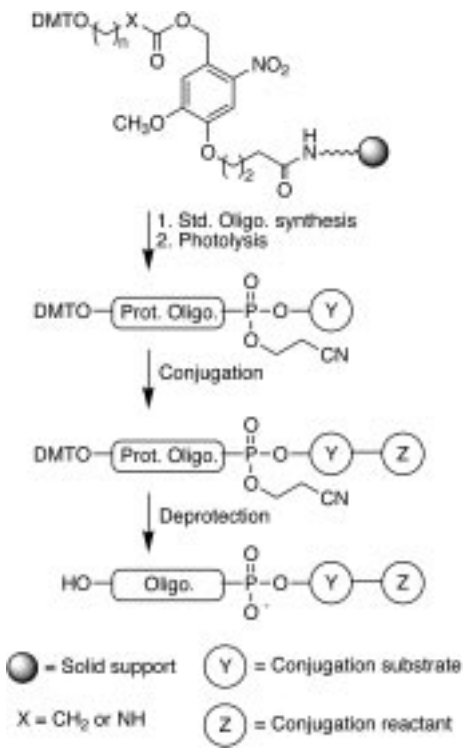
Received July 30, 1998

Protected oligonucleotides containing 3′-alkyl carboxylic acids are obtained from a photolabile solid-phase synthesis support (**1b**). The protected oligonucleotides are efficiently conjugated (>80%) with amines in solution to yield products of high purity under mild reaction conditions. This method is particularly well-suited for the synthesis of oligonucleotide−peptide conjugates containing a covalent linkage between the 3′ terminus of an oligonucleotide and the amino terminus of a peptide. High yields of nucleopeptides are obtained even when the peptide contains a hindered N-terminal amino acid.

Covalently modified oligonucleotides (bioconjugates) are useful for a variety of purposes. Bioconjugates often show increased cell permeability, add stabilization to complexes with other oligonucleotides, and demonstrate enhanced detectability relative to the respective unmodified biopolymer.[1−3] The two most common methods for the synthesis of oligonucleotide conjugates involve incorporation of the modification as an individual phosphoramidite (or preparation of a solid-phase support containing the modification) or postsynthetic modification of a specially functionalized, deprotected biopolymer.[4−7] These methods enable one to conjugate an oligonucleotide at either its 5′ or 3′ terminus, as well as at internal positions. Postsynthetic conjugation can be compromised by low yields, long reaction times, the need for large amounts of reagents, and formation of difficult-to-separate side products.[8−11] In addition, the phosphoramidite approach requires the individual synthesis of the monomeric component (or column) for each individual modification desired.[7] Moreover, phosphoramidites of larger molecules tend to couple more poorly than those of smaller organic molecules.[12]

We recently reported a general method for the synthesis of 3′-oligonucleotide conjugates, which have been considered the most difficult family of modified biopolymers to synthesize (Scheme 1).[3,13−15] This approach

Scheme 1

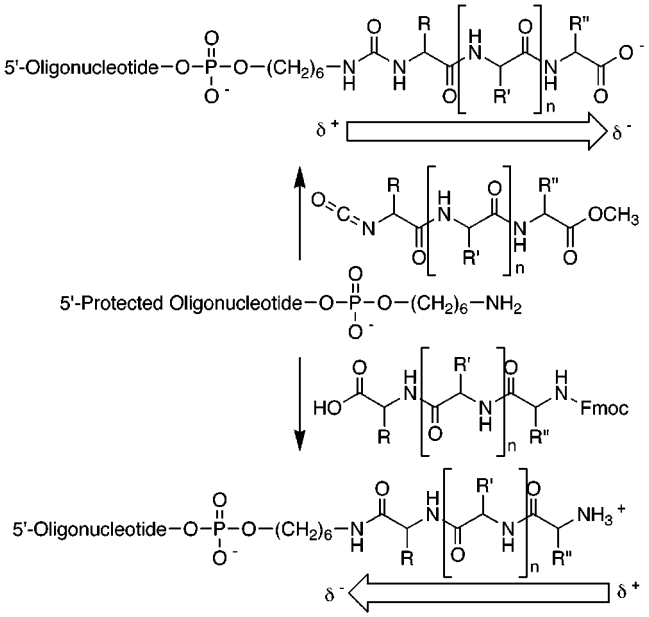


benefits from the convergence and convenience of postsynthetic oligonucleotide conjugation. However, by utilizing protected oligonucleotides containing 3′-alkylamine termini, the formation of undesired side products derived from reaction at the exocyclic amines of the biopolymer is eliminated.[8−10] The undamaged, protected oligonucleotides are obtained from photolabile solid-phase synthesis supports.[16] 3′-Amide and 3′-urea conjugates are typically obtained in greater than 80% isolated yield using a relatively small number of equivalents (5−20) of electrophilic reagents. Homogeneous products, as determined

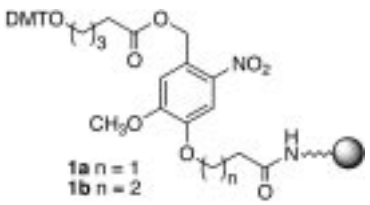
(1) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123.
(2) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 1925.
(3) Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165.
(4) Mullah, B.; Livak, K.; Andrus, A.; Kenney, P. *Nucleic Acids Res.* **1998**, *26*, 1026.
(5) Matteucci, M.; Lin, K.-Y.; Huang, T.; Wagner, R.; Sternbach, D. D.; Mehrotra, M.; Besterman, J. M. *J. Am. Chem. Soc.* **1997**, *119*, 6939.
(6) Nelson, P. S.; Kent, M.; Muthini, S. *Nucleic Acids Res.* **1992**, *20*, 6253.
(7) Nomura, Y.; Ueno, Y.; Matsuda, A. *Nucleic Acids Res.* **1997**, *25*, 6253.
(8) Ghosh, S. S.; Musso, G. F. *Nucleic Acids Res.* **1987**, *15*, 5353.
(9) Bischoff, R.; Coull, J. M.; Regnier, F. E. *Anal. Biochem.* **1987**, *164*, 336.
(10) Erout, M.-N.; Troesch, A.; Pichot, C.; Cros, P. *Bioconjugate Chem.* **1996**, *7*, 568.
(11) Manoharan, M.; Ramasamy, K. S.; Mohan, V.; Cook, P. D. *Tetrahedron Lett.* **1996**, *37*, 7675.
(12) (a) Jäschke, A.; Fürste, J. P.; Cech, D.; Erdmann, V. A. *Tetrahedron Lett.* **1993**, *34*, 301. (b) Kobertz, W. R.; Essigmann, J. M. *J. Am. Chem. Soc.* **1997**, *119*, 5960. (c) Hurley, D. J.; Tor, Y. *J. Am. Chem. Soc.* **1998**, *120*, 2194.
(13) McMinn, D. L.; Matray, T. J.; Greenberg, M. M. *J. Org. Chem.* **1997**, *62*, 7074.
(14) McMinn, D. L.; Greenberg, M. M. *J. Am. Chem. Soc.* **1998**, *120*, 3289.
(15) For another general method see the following: Hovinen, J.; Guzaev, A.; Azhayeva, E.; Azhayev, A.; Lönnberg, H. *J. Org. Chem.* **1995**, *60*, 2205.
(16) McMinn, D. L.; Greenberg, M. M. *Tetrahedron* **1996**, *52*, 3827.

10.1021/jo9815148 CCC: $18.00
Published on Web 12/19/1998

Scheme 2



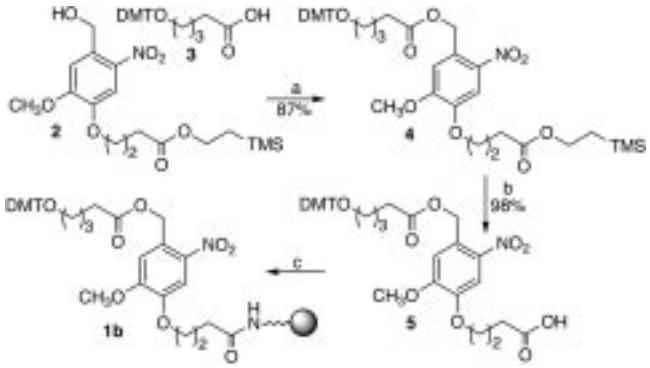
by a range of analytical methods, including HPLC, gel electrophoresis, electrospray mass spectrometry (ESMS), and enzymatic digestion, were obtained in 1−4 h reaction times. Despite these successful developments, there is room for improvement of and expansion of the scope of this synthetic method. For instance, 3′-oligonucleotide−peptide conjugates containing either polarity of the peptide dipole moment with respect to the nucleic acid domain are obtainable from protected oligonucleotides containing 3′-alkylamines (Scheme 2).[14] However, the synthesis of nucleopeptide conjugates consisting of a linkage between the oligonucleotide and the amino terminus of the peptide are limited to peptides containing unhindered and slightly hindered amino acids at their first and second positions, respectively.[14] We anticipated that utilizing protected oligonucleotides containing 3′-alkyl carboxylic acids as substrates in conjugation reactions would enable us to circumvent and overcome these limitations. The results of such studies are described below.



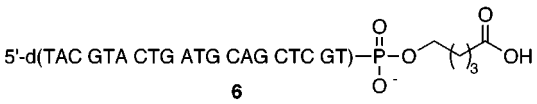
Results and Discussion

Chemical Synthesis of Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids for Use in Conjugation Reactions. Protected oligonucleotides containing 3′-alkyl carboxylic acids have been prepared on an orthogonal solid-phase support (**1a**) that releases the desired biopolymers upon photolysis at 365 nm.[17] This support, and others based upon the *o*-nitrobenzyl photoredox reaction, are fully compatible with standard automated synthesis cycles and commercially available reagents.[16−18] Protected oligonucleotides containing 3′-alkyl carboxylic acids employed in the current work were obtained from a slightly modified version (**1b**) of the previously described solid phase support **1a**. Preparation of **1b** is more direct than that for **1a** and eliminates a moderately yielding step that involves oxidation of a primary alcohol to a carboxylic acid. Support **1b** was prepared by coupling **2** and **3** (Scheme 3).[18,19] Following desilylation, the free carboxylic acid (**5**) was loaded directly onto the long-chain alkylamine-controlled pore-glass support. The slight structural difference between **1a** and **1b** was not expected to affect the yields of photocleaved oligonucleotides. Nonetheless, the yield of **6** obtained from **1b** was determined under photolysis conditions (2 h, 365 nm) identical to those that were previously employed for oligonucleotides prepared on **1a**.[16−18] These conditions have been shown to not damage the biopolymers. As expected, the isolated yields of **6** (80 ± 4%) were comparable to those obtained from the support that was more difficult to synthesize, **1a**.[17]

Scheme 3[a]



[a] Key: (a) DCC, DMAP (cat.), CH_2Cl_2, 25 °C; (b) TBAF, DMF, 25 °C; (c) $((NO_2)PyrS)_2$, PPh_3, DMAP, LCAA-CPG, dichloroethane, CH_2Cl_2 (1:1).



6

Optimization of Solution-Phase Conjugation of Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids. Solution-phase conjugation reactions of protected oligonucleotides are run under conditions where the biopolymer substrate is the limiting reagent. In previous reports, the electrophile which must be activated in situ was used in excess (5−10 equiv). We were concerned that having to activate the oligonucleotide substrate in situ might necessitate longer reaction times. However, this proved not to be the case for the conjugation of a polythymidylate (**7a**) under redox-condensation conditions (PPh_3, pyridyl disulfide, DMAP).[20] Reaction of **7a** with **8** (10 equiv) for 2 h at 55 °C yielded **9a** in comparable yield (91 ± 5%) to that previously reported using 3′-alkylamine-containing oligonucleotides (Scheme 4).[13]

Demonstration that the redox condensation conjugation conditions were compatible with protected hetero-oligonucleotides containing isobutyryl amide groups on

(17) Yoo, D. J.; Greenberg, M. M. *J. Org. Chem.* **1995**, *60*, 3358.

(18) Venkatesan, H.; Greenberg, M. M. *J. Org. Chem.* **1996**, *61*, 525.

(19) Greenberg, M. M.; Matray, T. J.; Kahl, J. D.; Yoo, D. J.; McMinn, D. L. *J. Org. Chem.* **1998**, *63*, 4062.

(20) Mukaiyama, T. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 94.

Scheme 4

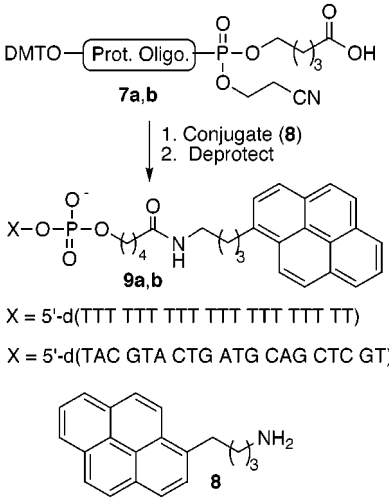


Table 1. Survey of Coupling Methods for the Conjugation of 7b with 8

coupling method	temp (°C)	time (h)	yield of **9b**[a] (%)
PPh_3, $(PyrS)_2$, DMAP (10 equiv each)	55	2	79 ± 10
PyBOP, $Et(iPr)_2N$ (10 equiv each)	55	2	95 ± 8
HBTU, HOBt, $Et(iPr)_2N$ (10 equiv each)	55	2	96 ± 7

[a] Isolated yields are average values of a minimum of two reactions ± the standard deviation from this value.

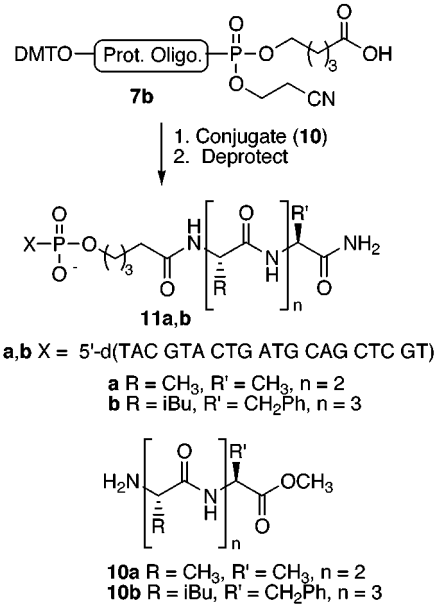
Table 2. Optimization of PyBOP Mediated Conjugation of 7b with 8

No. equivalents of reagents[a]	temp (°C)	time (h)	yield of **9b**[b] (%)
10	55	2	95 ± 8
10	25	2	102 ± 7
5	25	2	89 ± 4
5	25	0.5	93 ± 2
5	25	0.25	83 ± 6

[a] Reagents: PyBOP, **8**. [b] Isolated yields are average values of a minimum of two reactions ± the standard deviation from this value.

exocyclic amines and β-cyanoethyl groups on phosphates was achieved using **7b** and 4-(1-pyrene)butylamine (**8**) as reactants (Table 1). Once again, conjugation of a protected 3′-alkyl carboxylic acid containing oligonucleotide proceeded as efficiently (**9b**, 79 ± 10% isolated yield) as did reactions involving protected oligonucleotides containing 3′-alkylamines.[13,14] Subsequent exploration of alternative coupling methods revealed that both *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yloxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) were more effective activating agents than those used in the redox-condensation method (Table 1). Given the greater convenience of carrying out reactions mediated by PyBOP than by way of the redox condensation method, the effects of temperature, reaction time, and number of equivalents of activator and nucleophile on the coupling mediated by this reagent were investigated further (Table 2). Statistically, no detrimental effect on yield was observed upon reducing the reaction temperature to 25 °C and the number of equivalents of reagents (nucleophile and PyBOP) to five. In addition, the reaction time could be reduced to 15 min with little effect on the isolated yield of **9b**.

Scheme 5



Synthesis of Oligonucleotide−Peptide Conjugates Containing an Amide Linkage between the 3′-Terminus of the Oligonucleotide and the Amino Terminus of the Peptide. When compared to studies involving protected oligonucleotides containing 3′-alkylamines, the above results indicate that the efficiency of oligonucleotide-conjugate formation is unaffected by the polarity of the amide linkage between the two domains of the conjugate. Consequently, we sought to apply the PyBOP mediated coupling method to the synthesis of oligonucleotide−peptide conjugates in which the amino terminus of the peptide and the 3′-oligonucleotide terminus are covalently linked. As mentioned above, the prior method developed in our group for preparing oligonucleotide−peptide conjugates containing this topology of conjugants involved coupling a peptidyl isocyanate to a protected oligonucleotide containing a 3′-alkylamine (Scheme 2).[14] This method required longer reaction times and a larger number of equivalents of conjugant relative to oligonucleotide. Moreover, urea linkage formation was only effective with peptides containing unhindered amino acids at the amine terminus of the peptide and hindered amino acids at the adjacent position in the peptide.

In contrast, coupling **7b** to L-H_2N-Ala-Ala-Ala-CO_2Me (**10a**, 5 equiv) using PyBOP (5 equiv) for 15 min at room temperature provided **11a** in 89 ± 4% yield (Scheme 5). Furthermore, conjugation of the more hindered peptide (L-H_2N-$(Leu)_3$-Phe-CO_2Me, **10b**), which was an extremely reluctant partner in the isocyanate method (<20% yield), provided an 82 ± 4% yield of **11b** under the same conditions.[14] The yield of **11b** was increased to 95 ± 3% by extending the reaction time to 1 h.

Summary. The recently reported method for synthesizing 3′-oligonucleotide conjugates using protected biopolymers containing 3′-alkylamines has been expanded to include substrates containing 3′-alkyl carboxylic acids. The coupling efficiency of 3′-alkyl carboxylic acid containing protected oligonucleotides is equal to that of the analogous 3′-alkylamine-containing polymers. However, 3′-alkyl carboxylic acid containing oligonucleotides are superior for synthesizing oligonucleotide−peptide conjugates in which the amino terminus of the peptide is covalently linked to the oligonucleotide.

Experimental Section

Oligonucleotides were synthesized on an ABI 380B DNA synthesizer using standard 1 μmol cycles and β-cyanoethyl protected phosphoramidites. The exocyclic amines of deoxyadenosine, deoxycytidine, and deoxyguanosine were protected as their isobutyryl amides.[13,14,21] With the exception of solid-phase support **1b** and the phosphoramidite for deoxycytidine (Pharmacia), all DNA synthesis reagents were obtained from Glen Research, Inc. Oligonucleotides were cleaved from the solid support under N_2 using a transilluminator at 365 nm for 2 h. l-H_2N-Leu-Leu-Leu-Phe-CO_2Me and L-H_2N-Ala-Ala-Ala-CO_2Me were obtained from Bachem. *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yloxytri-pyrrolidinophosphonium hexafluorophosphate (PyBOP) were obtained from Aldrich. 4-(1-Pyrene)-butylamine was prepared as previously described.[14] Dimethylformamide (DMF), CH_2Cl_2, and CH_3CN were freshly distilled from CaH_2. Electrospray mass spectrometry was carried out using a Fisons VG-Quattro. Samples were precipitated twice from NH_4OAc prior to analysis. NMR spectra were collected at 300 MHz.

Preparation of 4. Benzyl alcohol **2** and dimethoxytrityl carboxylic acid **3** were prepared as previously described.[18,19] Benzyl alcohol **2** (0. 662 g, 1.6 mmol) was added to carboxylic acid **3** (0.703 g, 1.86 mmol), DCC (0.430 g, 2.1 mmol), and catalytic DMAP (21 mg) in CH_2Cl_2 (40 mL). The reaction was stirred for 1 h at room temperature, at which time it was filtered through a pad of Celite. The filtrate was poured into a separatory funnel containing saturated aqueous $NaHCO_3$ solution (15 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated under vacuum to yield a light yellow oil. Flash chromatography (silica gel, 1:2 ethyl acetate/hexanes) yielded 1.10 g (87%) of the coupled trimethylsilyl ester (**4**) as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.72 (s, 1H), 7.43 (d, J = 6.9 Hz, 2H), 7.20−7.34 (m, 8H), 6.98 (s, 1H), 6.83 (d, J = 9 Hz, 4H), 5.50 (s, 2H), 4.21 (d × d, J = 6.4, 6.1 Hz, 2H), 4.13 (t, J = 6.6 Hz, 2H), 3.89 (s, 3H), 3.79 (s, 6H), 3.09 (t, J = 6.3 Hz, 2H), 2.53 (t, J = 6.9 Hz, 2H), 2.43 (t, J = 6.9 Hz, 2H), 2.19 (p, J = 6.6 Hz, 2H), 1.67−1.81 (m, 4H), 1.01 (d × d, J = 6.6, 6.3 Hz, 2H), 0.06 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.9, 172.8, 158.3, 153.8, 147.4, 145.2, 139.8, 136.4, 129.9, 128.1, 127.6, 127.1, 126.5, 113.0, 110.5, 109.5, 85.7, 68.3, 63.0, 62.7, 62.6, 56.2, 55.1, 34.0, 30.6, 29.5, 24.2, 21.9, 17.3, −1.6; IR (film) 1737, 1731 cm^{-1}; HRMS FAB (M+) calcd for $C_{43}H_{53}O_{11}NSi$ 787.3388, found 787.3379.

Preparation of 5. To **4** (0.482 g, 0.612 mmol) in DMF (1.0 mL) was added 735 μL of a freshly prepared 1 M solution of tetrabutylamonnium fluoride.[22] The reaction was allowed to stir for 15 min, at which time it was poured into a separatory funnel containing ethyl acetate (30 mL) and saturated aqueous $NaHCO_3$ solution (20 mL). The layers were separated, and the aqueous layer was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine (15 mL), dried over $MgSO_4$, and concentrated under vacuum to afford a yellow oil. Flash chromatography (silica gel, 1:1 ethyl acetate/hexanes) yielded 0.414 g (98%) of **5** as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.28 (s, 1H), 7.42 (d, J = 6.0 Hz, 2 H), 7.19−7.35 (m, 7H), 6.97 (s, 1H), 6.82 (d, J = 9.0 Hz, 4H), 5.50 (s, 2H), 4.14 (t, J = 6.6 Hz, 2H), 3.89 (s, 3H), 3.79 (s, 6H), 3.08 (t, J = 6.3 Hz, 2H), 2.62 (t, J = 6.9 Hz, 2H), 2.42 (t, J = 7.8 Hz, 2H), 2.20 (p, J = 5.4 Hz, 2H), 1.69 (p, J = 5.3 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 178.3, 172.9, 158.3, 153.8, 147.3, 145.2, 139.8, 136.4, 129.9, 128.1, 127.6, 127.2, 126.6, 112.9, 110.6, 109.6, 85.7, 68.0, 63.0, 62.7, 56.2, 55.1, 34.0, 30.2, 29.5, 23.9, 22.0; IR (film) 1737, 1710 cm^{-1}; HRMS FAB (M+) m/z calcd for $C_{38}H_{41}O_{11}N$ 687.2680, found 687.2688.

Preparation of Photolabile Solid-Phase Support (1b). Carboxylic acid **5** (10 mg, 14.5 μmol), triphenyl phosphine (3.8 mg, 14.5 μmol), 2,2′-dinitropyridine disulfide (4.5 mg, 14.5 μmol), and DMAP (1.8 mg, 14.5 μmol) were combined in an oven-dried 1-dram vial with long-chain alkylamine-controlled pore-glass support (200 mg, ~7 μmol amine) in 1:1 dichloroethane/CH_3CN (1.2 mL) for 2 h at 25 °C. The resin was filtered, washed with copious amounts of ethyl acetate, dried under aspirator vacuum, and capped (acetic anhydride, pyridine). Dimethoxytrityl analysis of **1b** indicated a loading of 34.1 μmol/g.

General Procedure for Redox-Condensation-Mediated Conjugation of Protected Oligonucleotides. A 66 mM solution of the coupling reagents (14.5 mg of pyridine disulfide, 17.3 mg of triphenyl phosphine, and 8.0 mg of DMAP dissolved to make a 1.0-mL DMF solution) was prepared in an oven-dried vial. In a second oven-dried vial a 66 mM solution of 4-(1-pyrene)butylamine was prepared in DMF. The protected oligonucleotide, which was stored as a solution (1:1 CH_3CN/H_2O) at −80 °C, was prepared for reaction by transferring the desired amount of material to a clean reaction flask, and then it underwent evaporative centrifugation.[14] To the dried DNA (50 nmol) was added 8 μL of the solution of coupling reagents. The reaction was stirred at room temperature for 5 min, at which time 8 μL of the solution of the pyrene amine was added. The reaction vessel was sealed, and the reaction was stirred at 55 °C for 2 h, cooled to room temperature, and then quenched with H_2O (100 μL). The reaction mixture was evaporated to dryness and treated with concentrated aqueous ammonia (300 μL) for 6 h at 55 °C. Following evaporation, the DNA was treated with 80% acetic acid (150 μL) for 20 min. The reaction was quenched with ethanol (150 μL) and concentrated in vacuo. Denaturing gel purification of the conjugated oligonucleotide was carried out on 20% polyacrylamide. The product band was excised, crushed, and eluted with NaCl (0.2 M) and EDTA (1 mM) overnight. Following desalting (C_{18} cartridge), isolated yields were determined by comparing the amount of oligonucleotide obtained (OD_{260}) from the conjugation reaction to the amount of unconjugated material recovered from the identical deprotection, purification, and isolation conditions.

General Procedure for HBTU and PyBOP Mediated Conjugation of Protected Oligonucleotides. The appropriate solutions of reagents and DNA were prepared as described above, with the exception that PyBOP or HBTU and associated ancillary reagents (e.g., HOBt) were substituted for pyridine disulfide, triphenyl phosphine, and DMAP. After the appropriate reaction time (25 °C), the conjugation reactions were quenched, treated with concentrated aqueous ammonia, treated with acetic acid, and purified as described above.

Acknowledgment. We are grateful for the financial support of this research we received from the National Science Foundation (CHE-9732843) and the Colorado Advanced Technology Institute Center for RNA Chemistry. M.M.G. thanks the Alfred P. Sloan Foundation for a fellowship. We thank Dustin L. McMinn for helpful discussions. Mass spectra were obtained on instruments supported by the National Institutes of Health shared instrumentation grant GM49631.

Supporting Information Available: Electrospray mass spectra of **9a**, **9b**, **11a**, and **11b** (4 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.

JO9815148

(21) McMinn, D. L.; Greenberg, M. M. *Tetrahedron Lett.* **1997**, *39*, 1103.

(22) Majetich, G.; Casares, A.; Chapman, D.; Behnke, M. *J. Org. Chem.* **1986**, *51*, 1745.