Postsynthetic Conjugation of Protected Oligonucleotides Containing 3′-Alkylamines

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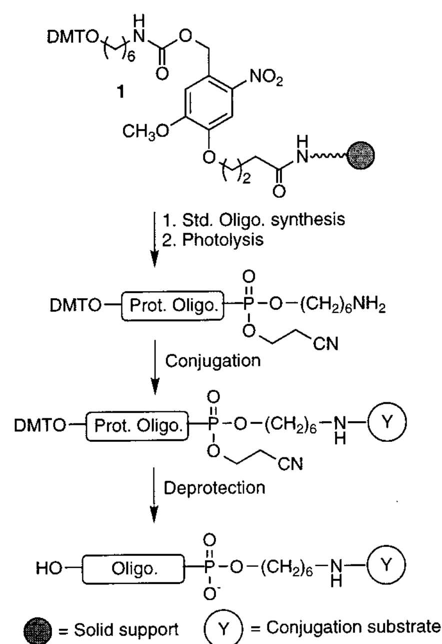
Abstract: The expansion of a highly efficient, convergent method for synthesizing 3′-oligonucleotide conjugates is described. 3′-Oligonucleotide conjugates containing amide or urea linkages between the oligonucleotide portion and the conjugated species were obtained by reacting protected oligonucleotides containing 3′-alkylamines in aprotic organic solvents with carboxylic acid and isocyanate substrates, respectively. The protected oligonucleotides are obtained via standard automated synthesis on a photolabile solid-phase synthesis support. Excellent yields (83−100%) of bioconjugates were obtained using carboxylic acids and aryl isocyanates with as few as 5 molar equivalents of conjugation reagents relative to protected oligonucleotide. More moderate yields were obtained using alkyl isocyanates as substrates (70−88%). In addition, this method has proven to be useful for synthesizing complementary oligonucleotide−peptide conjugates from a single oligonucleotide, in which the polarity of the peptide with respect to the oligonucleotide is determined by the bond-forming process employed.

We recently introduced a general method for the convergent synthesis of oligonucleotide conjugates.[1] This method utilizes fully protected oligonucleotides containing alkylamine substituents at their 3′-termini (Scheme 1). The use of protected oligonucleotides in conjugation reactions is analogous to the segmental synthesis of peptides and the "blockmer" approach to oligonucleotide synthesis, but is conceptually different than previously reported methods for the preparation of modified oligonucleotides.[2,3] Utilization of protected oligonucleotides eliminates any concern of side reactions involving other potential nucleophiles present throughout the biopolymer. A variety of oligonucleotide conjugates were obtained in greater than 80%, and in most cases >90%, isolated yield under very mild reaction conditions. We now report on the details and extension of this strategy.

Despite their importance in a variety of biological applications, in terms of reaction times, efficiency in use of reagents, yield, and purity of products, methods for the synthesis of oligonucleotide conjugates have lagged behind those for unmodified oligonucleotides.[4] 3′-Modification of oligonucleotides has in turn proven more difficult to efficiently achieve than at other sites within the biopolymers. Modified solid-phase supports have been developed containing FMOC-protected alkylamines, which can be used for preparing oligonucleotide conjugates at their 3′-termini.[5] The 3′-conjugates can be prepared by either derivatizing the solid support prior to oligonucleotide synthesis or postsynthetically using the fully deprotected biopolymer. The latter approach is attractive in that it is convergent, and a variety of oligonucleotide conjugates can be prepared from a single synthesis of an oligonucleotide. However, it is susceptible to the limitations incumbent upon all methods that involve solution-phase conjugation of deprotected biopolymers. These methods often require large excesses of reagents and long reaction times, produce low product yields, and can result in significant amounts of nonspecific covalent modification.[6] In contrast, utilization of specific phosphoramidites (which require independent synthesis) and modification of the solid-phase support prior to oligonucleotide synthesis are linear approaches, and often work best for synthesizing oligonucleotide conjugates of small molecules.[4a,7]

Scheme 1



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Solid-phase supports that enable postsynthetic modification of the 3′-termini of support bound oligonucleotides with small organic molecules have also been described.[8] This method takes advantage of nucleophilic attack of an amine on a thioester linkage between the oligonucleotide and solid-phase support. Yields of the conjugation reaction are believed to be good, but the process requires high concentrations of amine. Presumably, this is due to competing reactions within the biopolymer (e.g., elimination of β-cyanoethyl groups). Certainly, transamidation of fast deprotecting nucleobase protecting groups, if such groups are employed, would be a competitive process.[9] Thioesters have also proven to be very useful for ligating unprotected peptides to fully deprotected DNA in aqueous solution, as well as to other peptides.[10,11]

Conjugation of oligonucleotides with larger molecules, such as peptides, is of significant interest, because of the properties and potential applications of such molecules. Oligonucleotide−peptide conjugates impart a number of desirable properties upon antisense agents, including improved nuclease stability, membrane transport, and complex stability.[12,13] Oligonucleotide−peptide conjugates have also been employed as polymerase chain reaction primers.[14] Recently, oligonucleotide−peptide conjugates have been prepared linearly on a single support in moderate yields.[15] Alternatively, solution-phase methods are also available, but often suffer from the above-mentioned difficulties. One recent approach utilizing templates to increase the effective molarity of the biopolymer substrates results in very good yields of oligonucleotide−peptide conjugates, but still requires a large excess of peptidyl substrate, and lengthy reaction times.[10] Templates are also useful for effecting the ligation of oligonucleotides.[16]

The solution-phase conjugation of protected oligonucleotides described below enjoys the advantages of each of the above methods. It is convergent, makes efficient use of reagents, and produces high yields of pure products in short (2−4 h) reaction times. In addition, the method is general, and works well for a variety of types of molecules.

Results and Discussion

The oligonucleotides were synthesized on **1** using standard automated oligonucleotide synthesis cycles.[17] Solid-phase support **1** makes use of the facile *o*-nitrobenzyl photoredox reaction, which is finding increased application in solid-phase synthesis.[18] The protected oligonucleotides containing their 5′-*O*-dimethoxytrityl group were obtained via 2 h of photolysis at 365 nm using a transilluminator.[17] Stock solutions of the protected biopolymers used for conjugation were stored for long periods of time as aqueous acetonitrile solutions (1:1 by volume) at −80 °C. Solutions of protected oligonucleotides were quantified by acidolysis of an aliquot of solution (which was evaporated to dryness), and determination of the dimethoxytrityl cation released. Material for conjugation reactions was removed from these solutions and evaporated to dryness in the appropriate reaction vessel. Reagent solutions were added to the dried residue.

3′-Oligonucleotide Conjugates via Redox Condensation. The preliminary report concerning solution-phase conjugation of protected oligonucleotides contained examples utilizing eicosameric oligonucleotides only.[1] One might be concerned that longer synthetic biopolymers might react more slowly and/or give rise to a greater degree of side reactions due to a larger number of competing functional groups, such as the *N*7-position of purines. The heartiness of the nucleobase protecting groups made reactions at the exocyclic amines of nucleobases highly remote. A more likely reactive site whose concentration would increase with increasing biopolymer length is phosphate diesters that are revealed by the adventitious deprotection of phosphate triesters containing labile β-cyanoethyl substituents. The β-cyanoethyl phosphate protecting group is extremely labile to mild basic conditions. We have observed deprotection of this functional group in 5′-*O*-dimethoxytrityl nucleotides upon prolonged exposure to concentrated primary amines. The phosphate diesters revealed in this manner could be activated under the redox condensation conditions, and would form phosphoramidate side products upon reaction with amines. Admittedly, this process was not considered to be highly likely in the present system, as the protected oligonucleotides themselves are the only source of amines, and they are present in low concentration (~5 mM, including full length and deleted oligonucleotides). In addition, much higher concentrations of amines are typically used to form phosphoramidate conjugates under these conditions. Indeed, reaction of 30-mer **2** (~3 mM) with 10 equiv of pyrenebutyric acid (30 mM) and like amounts of activating agents produced **4** in 96 ± 12% yield.[19] Longer oligonucleotides, corresponding to dimerizations via the above process, were not observed. Yields of this magnitude, using such a slight excess of reagents and brief reaction times are unprecedented in oligonucleotide conjugation chemistry.

Having established that a longer oligonucleotide is conjugated in as high a yield as eicosamers, the efficiency of the redox condensation method was optimized with respect to the amount of reagents. In determining the minimum number of equivalents of reagents that can be employed, it is necessary to account for the total amount of alkylamine-containing oligonucleotides.

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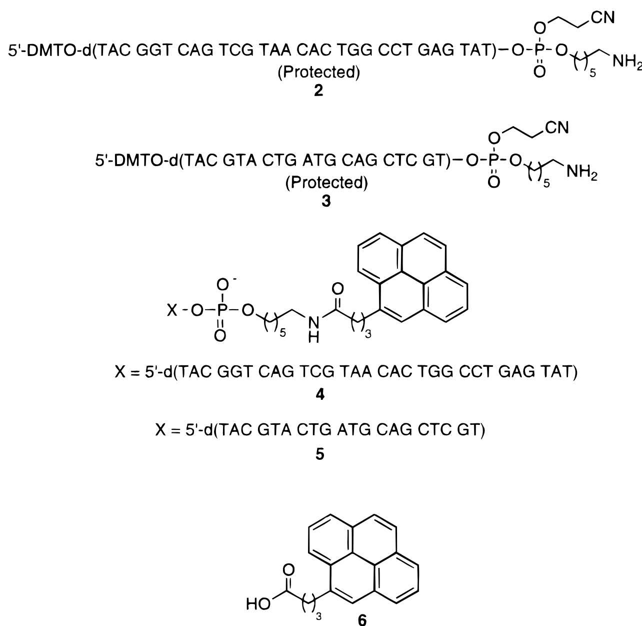
(19) Apparent yields of bioconjugates in excess of 100% are possible, because the yields are determined by comparing the OD_{260} of isolated bioconjugate versus the isolated OD_{260} of unreacted oligonucleotide substrate from the same synthesis. Since there are possible variations in the multiple handling steps, it is possible to isolate a greater amount of conjugated material than unreacted oligonucleotide.



Cleavage of the protected oligonucleotides from the solid support releases the desired full length substrate (e.g., **3**), along with all deletions. Deletions are not accounted for in determining the concentration of oligonucleotide solutions for conjugation, because this is done via measuring the amount of dimethoxytrityl cation that is released, which detects only full length material (e.g., **3**). The maximum total amount of 3′-alkylamine-containing oligonucleotides was estimated on the basis of an average coupling yield of 97% during the automated oligonucleotide synthesis. For an eicosameric oligonucleotide, this results in ∼54% of full length material. Hence, approximately 2 equiv of carboxylic acid and activating agents would be a conservative estimate of the minimum amount of these reagents that one could use and still maintain a stoichiometric relationship between 3′-alkylamine oligonucleotides and a given electrophile.

Conjugation of **3** with 5 equiv of pyrenebutyric acid (and an equal number of equiv of PPh_3, $(PyS)_2$, and DMAP) gave rise to yields of **5** (99 ± 4%) within experimental error of those previously reported using 10 equiv.[1,20] Reaction of **3** with 2.5 equiv of the reagents employed above resulted in lower, but still good, isolated yields (60 ± 4%) of **5**. Increasing the number of equivalents of activating reagents to 10, and 3 equiv of **6**, improved the yield of **5** to 72 ± 3%. Increasing the reaction time from 2 to 24 h under these latter conditions did not raise the yield of **5** (70 ± 5%) to within the range obtained using 5−10 equiv of carboxylic acid. However, upon workup and detritylation with 80% aqueous acetic acid, some unconjugated starting material was acetylated, indicating that the activating reagents were still active, and that the decrease in yield is likely due to a kinetic problem associated with the lower concentration of carboxylic acid. The acetylated product was characterized by anion exchange HPLC and electrospray mass spectrometry. Nonetheless, these experiments reveal that very good yields of 3′-oligonucleotide conjugates can be obtained with as few as 3 equiv of reagents relative to full length oligonucleotide. Such a minimal sacrifice in conjugate yield as that described above could be tolerable when the carboxylic acid being conjugated is precious.

Conjugation Under Racemization Free Conditions. Short peptides have been shown to react under the redox condensation reaction conditions with a protected eicosameric oligonucleotide in as high of yields as small organic molecules. However, the activation of the peptide carboxy terminus for conjugation raises the possibility for epimerization. Epimerization of the carboxy terminus of peptidyl conjugants under redox condensation conditions was investigated by coupling (*R*)-methylbenzylamine to *N*-acetyl-L-histidine, which is comparable to the carboxy amino acid used in the original report on this method (eq 1). Examination of the coupling product by ^{1}H NMR revealed approximately 30% epimerization. Similar results were obtained upon coupling the *N*-FMOC protected histidine.

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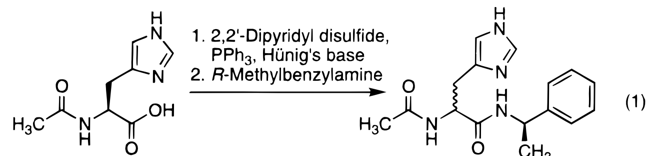
Table 1. Effects of Activating Agents on Isolated Yields of Oligonucleotide Conjugates from **3** and Pyrenebutyric Acid (**6**)

reaction conditions	isolated yield of **5** (%)[a]
PPh_3 (10 equiv), $(PyS)_2$ (10 equiv), DMAP (10 equiv), 55 °C	89 ± 1
PyBOP (10 equiv), $(iPr)_2EtN$ (10 equiv), 25 °C	95 ± 9
HBTU (10 equiv), HOBt (10 equiv), $(iPr)_2EtN$ (10 equiv), 25 °C	96 ± 2

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



Other methods were subsequently investigated for effecting amide bond formation, which would not induce epimerization during peptide coupling. It is well-known that HBTU/HOBt and PyBOP are effective peptide coupling agents that minimize racemization.[21] In addition, the (*R*)-methylbenzylamine adduct of *N*-acetyl-L-histidine prepared via HBTU/HOBt coupling was subjected to the aqueous ammonia conditions used to deprotect oligonucleotides. No base-induced epimerization was observed by ^{1}H NMR. The efficiency of these reagents under the concentration conditions employed during the oligonucleotide conjugation was investigated using **3** and pyrenebutyric acid (**6**), and found to be comparable, or superior, to the redox condensation process (Table 1).

Oligonucleotide−Isocyanate Conjugates. Recently, an isocyanate was successfully conjugated to a fully deprotected oligonucleotide in water by careful optimization of the reaction conditions.[22] However, under less carefully controlled conditions, the reaction of isothiocyanates and isocyanates yields significant amounts of side products that are attributable to nonspecific covalent modification of the oligonucleotide.[22,23] Such nonspecific reactivity is a nonissue when using protected oligonucleotides. Consequently, urea formation via reaction of isocyanates with 3′-alkylamine-containing oligonucleotides presents an alternative to the amide bond-forming approaches described above. In addition, the reaction of peptide isocyanates with protected 3′-alkylamine-containingoligonucleotides raises the possibility of preparing oligonucleotide−peptide conjugates with polarity opposite those obtained via amide bond formation from the same oligonucleotide (Scheme 2).

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Scheme 2

Table 2. Isolated Yields of Oligonucleotide Conjugates Obtained from Reaction between **3** and Isocyanates

5'-DMTO-d(TAC GTA CTG ATG CAG CTC GT)–O–P–O–$(CH_2)_6$–NH_2 **3** (Protected)

1. R-N=C=O
2. Deprotection

5'-d(TAC GTA CTG ATG CAG CTC GT)–O–P–O–$(CH_2)_6$–NH–C(O)–NH–R **7a-e**

R	Isolated Yield (%)[a]
7a	99 ± 2
7b	99 ± 2
7c	99 ± 2
7d	98 ± 2
7e	88 ± 4

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

Reaction of **3** (~3 mM) with aryl isocyanates (30 mM) in 2 h at 55 °C in 1,2-dichloroethane–acetonitrile (1:1) produced quantitative yields of the respective conjugates (**7a**–**7d**, Table 2). The less reactive alkyl isocyanate was required in larger excess (20 equiv) to produce an 88 ± 4% yield of **7e**. Attempts at increasing the reaction rates between the protected oligonucleotides and alkyl isocyanate with cuprous chloride were unsuccessful.[24] Similarly, the coupling of **3** with peptide isocyanates also required a large excess of the electrophiles. The peptide coupling reactions faced additional structural limitations. The peptide isocyanates were required to contain a hindered amino acid at the position adjacent to the amino terminus in order to avoid hydantoin formation.[25] In addition, the peptides could not contain a hindered amino acid at the amino terminus (e.g., **8a**), as this significantly decreased the rate of the coupling reaction of the respective alkyl isocyanate. Coupling of **8a** with **3** proceeded in less than 20% yield, despite employing 20 equiv of the isocyanate for 4 h at 55 °C. In contrast, **9b** was obtained in 70 ± 4% yield upon reaction between **3** and **8b** under the same reaction conditions.

X = 5'-d(TAC GTA CTG ATG CAG CTC GT)–O–P–O–$(CH_2)_6$–NH–C(O)–NH–

Summary. The recently reported novel approach toward bioconjugate formation using protected oligonucleotides has been explored in greater detail. Excellent yields of bioconjugates are obtained under redox condensation conditions using as few as 5 equiv of reagents (relative to oligonucleotide substrate). In instances where the carboxylic acid is in short supply, very good yields are obtainable using as few as 3 equiv of these reactants. Epimerizable substrates can be conjugated to the protected oligonucleotides using commercially available peptide activating agents with no sacrifice in product yield. Oligonucleotide–peptide conjugates of either polarity of the peptide linkage are obtained from a single protected oligonucleotide containing a 3′-alkylamine by varying the nature of the peptidyl electrophile. Peptide isocyanates yield slightly lower yields of oligonucleotide–peptide conjugates than peptide carboxylic acids, and are limited with respect to the sequences of peptides that can be employed. It is anticipated that these limitations will be remedied by carrying out conjugations of protected 3′-alkyl carboxylic acid oligonucleotides with peptides containing free N-terminal amines.

Experimental Section

General Methods. All reactions were carried out in oven-dried glassware, under inert atmosphere, unless otherwise stated. All organic solvents were distilled over CaH_2. Oligonucleotides were synthesized using an Applied Biosystems Inc. 380B automated synthesizer. Standard ABI synthesis cycles were used. DNA synthesis reagents were purchased from Glen Research, with the exception of N^6-isobutyryldeoxycytosine phosphoramidite which was purchased from Pharmacia Biotech.[9] Photolytic cleavage of protected oligonucleotides was carried out using a UVP dual wavelength transilluminator containing 4 × 8 W lamps. Photolyses were performed at 365 nm as previously described.[17] Full length protected oligonucleotides were quantified by observing the 4,4′-dimethoxytrityl cation (UV absorbance at λ_{max} = 498 nm, $\epsilon = 7 \times 10^4$ M^{-1} cm^{-1}) resulting from the addition of 1000 μL of a 1 M solution of *p*-toluenesulfonic acid in CH_3CN to 20.0 μL of a ca. 1500 μL stock solution (in CH_3CN–H_2O; 1:1) of photolysate following evaporation of solvent. Retention times for oligonucleotides and respective conjugates were determined using anion-exchange HPLC purification (Vydac weak anion-exchange

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protein wax column; 0.75 × 5 cm; A, H_2O; B, 1 M NH_4Cl, H_2O; 0−100% B over 30 min; flow rate 2.0 mL/min). Gel purification of oligonucleotides was carried out on 20% polyacrylamide denaturing gels (5% cross-link, 45% urea by weight). Peptides were purchased from Bachem. Phosgene solution was purchased from Fluka.

***N*-Acetyl-(*S*)-histidinyl-(*R*)-methylbenzylamide.** To Fmoc-(*S*)-histidine(Fmoc)-OH (250 mg, 0.42 mmol) were added DMF (5 mL), Hünig's base (54 mg, 0.42 mmol), and HBTU (158 mg, 0.42 mmol), followed by HOBT (56.3, 0.42 mmol). To the dissolved reaction solution was added (*R*)-methylbenzylamine (51 mg, 0.42 mmol), and the reaction was stirred at room temperature for 1 h. To the reaction solution was added saturated NaCl (15 mL). The solution was extracted with EtOAc (3 × 10 mL). The combined organics were extracted successively with 10 mM HCl solution (10 mL) and saturated $NaHCO_3$ solution (10 mL). The organics were concentrated in vacuo. To the crude solid was added 20% piperidine in DMF (5 mL). The solution was stirred at room temperature for 1 h. Solvents were removed in vacuo. To the crude product was added H_2O (10 mL). The aqueous solution was extracted with Et_2O (3 × 3 mL). The aqueous layer was concentrated to give the crude deprotected intermediate. To the crude were added CH_2Cl_2 (5 mL) and acetic anhydride (233 mg, 2.28 mmol), and the acetylation mixture was stirred at room temperature for 1 h. Solvents were removed to provide the crude diacetylated product. The crude solid was dissolved in concentrated ammonia solution (1.5 mL, 28% NH_3). The solution was heated to 55 °C for 6 h with intermittent vortexing. Solvents were removed by evaporative centrifugation. Flash chromatography (0−20% MeOH in CH_2Cl_2) yielded 23 mg (18% over four steps) of *N*-acetyl-(*S*)-histidinyl-(*R*)-methylbenzylamide: mp 252 °C; ^{1}H NMR (CD_3OD) 7.55 (s, 1H), 7.21−7.10 (m, 5H), 6.79 (s, 1H), 4.86 (m, 1H), 4.54 (t, J = 7.2 Hz, 1H), 2.95 (dd, J = 6.9, 14.7 Hz, 1H), 2.85 (dd, J = 7.8, 14.7 Hz, 1H), 1.82 (s, 3H), 1.26 (d, J = 6.9 Hz, 3H); IR (thin film) 3269, 3062, 2975, 1650, 1547, 1447, 1374, 1107, 820, 761, 700, 667 cm^{-1}; HRMS FAB (M^+ + H) calcd 301.1664, found 301.1660.

***N*-Acetyl-(*R*)-histidinyl-(*R*)-methylbenzylamide.** The reaction of FMOC-*N*-FMOC-(*R*)-histidine (100 mg, 0.17 mmol) as described for *N*-acetyl-(*S*)-histidinyl-(*R*)-methylbenzylamide yielded 7.00 mg (14% over four steps) of *N*-acetyl-(*R*)-histidinyl-(*R*)-methylbenzylamide: mp 231 °C; ^{1}H NMR (CD_3OD) 7.53 (s, 1H), 7.28−7.11 (m, 5H), 6.68 (s, 1H), 4.93 (m, 1H), 4.59 (t, J = 7.2 Hz, 1H), 2.95 (dd, J = 6.9, 14.7 Hz, 1H), 2.82 (dd, J = 7.8, 14.7 Hz, 1H), 1.91 (s, 3H), 1.39 (d, J = 6.9 Hz, 3H); IR (thin film) 3291, 3100, 1647, 1542, 1456, 1375, 757, 669 cm^{-1}; HRMS FAB (M^+ + H) calcd 301.1664, found 301.1663.

Procedure for Mukaiyama Redox Coupling of *N*-Acetyl-(*S*)-histidine and (*R*)-methylbenzylamine. To *N*-acetyl-(*S*)-histidine (250 mg, 1.16 mmol) were added dipyridyl disulfide (282 mg, 1.28 mmol), triphenylphosphine (335 mg, 1.28 mmol), DMF (5 mL), and Hünig's base (161 mg, 1.28 mmol). To the dissolved solution was added (*R*)-methylbenzylamine (168 mg, 1.28 mmol). The reaction was allowed to stir at room temperature for 30 min. Solvents were removed in vacuo. The reaction mixture was dissolved in EtOAc (20 mL) and extracted with saturated $NaHCO_3$ (5 mL). Flash column chromatography (10% MeOH in CH_2Cl_2) yielded 97.9 mg (28%) of epimerized *N*-acetyl-(*R/S*)-histidinyl-(*R*)-methylbenzylamide.

4-(1-Pyrenyl)butyl Mesylate. To 1-pyrenebutanol (400 mg, 1.5 mmol) in CH_2Cl_2 (1.5 mL) was added Hünig's base (340 mg, 2.6 mmol). The solution was cooled to 0 °C whereupon 1 mL of a 0.2 M solution of methanesulfonyl chloride in CH_2Cl_2 was added. The reaction was stirred at 0 °C for 3 h and then poured into ice water (4 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 2 mL), and combined organics were washed with brine (5 mL), dried over $MgSO_4$, and concentrated in vacuo to provide a quantitative yield (530 mg) of crude 4-(1-pyrenyl)butyl mesylate as a yellow solid: mp 58−59 °C; ^{1}H NMR ($CDCl_3$) δ 8.28−7.81 (m, 9H), 4.24 (t, J = 6.3 Hz, 2H), 3.37 (t, J = 6.9 Hz, 2H), 2.93 (s, 3H), 2.30−1.82 (m, 4H); IR (thin film) 3038, 2939, 2867, 1602, 1458, 1352, 1174, 972, 933, 846 cm^{-1}; HRMS FAB (M^+) calcd 352.1133, found 352.1135.

4-(1-Pyrenyl)butyl Azide. To 4-(1-pyrenyl)butyl mesylate (510 mg, 1.4 mmol) in DMF (9 mL) was added sodium azide (660 mg, 10 mmol). The reaction was stirred at room temperature for 24 h. Solvent was removed in vacuo, and the solid was dissolved in Et_2O−CH_2Cl_2 (3:1, 15 mL) and extracted with H_2O (10 mL). The aqueous layer was extracted with Et_2O−CH_2Cl_2 (3:1, 3 × 5 mL). The combined organics were washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL) and dried over $MgSO_4$. Following concentration of the organics in vacuo, flash chromatography (EtOAc−hexanes, 1:2) yielded 190 mg (79%) of 4-(1-pyrenyl)butyl azide as a white solid: mp 77−79 °C; ^{1}H NMR ($CDCl_3$) δ 8.28−7.86 (m, 9H), 3.42−3.32 (m, 4H), 2.02−1.91 (m, 2H), 1.84−1.72 (m, 2H); IR (thin film) 3048, 2938, 2862, 2092, 1278, 1245, 892, 840, 740 cm^{-1}; HRMS FAB (M^+ + H) calcd 299.1422, found 299.1425.

[4-(1-Pyrenyl)butyl]amine. To $LiAlH_4$ (120 mg, 3.0 mmol) in THF (3.0 mL) at 0 °C was added a solution of 4-(1-pyrenyl)butyl azide (370 mg, 1.24 mmol) in THF (3.1 mL). The reaction mixture was allowed to reach room temperature over 1.5 h, at which time 20% NaOH (5 mL) was added. The precipitate was filtered and successively washed with THF (3 × 10 mL). The yellow filtrate was acidified with 1 M HCl to pH 1. THF was removed in vacuo. The remaining solution was diluted with H_2O (100 mL) and extracted with CH_2Cl_2 (25 mL). The aqueous layer was brought to pH 11 with 20% NaOH and extracted with Et_2O (5 × 50 mL). The solvent was removed in vacuo, yielding 220 mg (67%) of [4-(1-pyrenyl)butyl]amine as a tan wax: ^{1}H NMR ($CDCl_3$) δ 8.30−7.86 (m, 9H), 3.38 (t, J = 7.6 Hz, 2H), 2.78 (t, J = 7.5 Hz, 2H), 1.91 (m, 2H), 1.64 (m, 4H); IR (thin film) 3334, 3039, 2927, 2856, 1586, 1458, 1314, 1182, 840, 753, 707 cm^{-1}; HRMS FAB (M^+ + H) calcd 274.1595, found 274.1590.

4-(1-Pyrenyl)butyl Isocyanate. To [4-(1-pyrenyl)butyl]amine (50 mg, 0.18 mmol) were added saturated $NaHCO_3$ (1.83 mL) and CH_2Cl_2 (1.83 mL). The biphasic mixture was cooled to 0 °C for 10 min under vigorous stirring. Stirring was stopped to afford the separation of solvent layers. To the CH_2Cl_2 layer was added a 1.93 M solution of phosgene in toluene (0.19 mL, 0.37 mmol). Stirring was resumed at 0 °C for 10 min. The layers were separated, and the aqueous layer was extracted with cold CH_2Cl_2 (3 × 5 mL). The organics were combined and removed in vacuo to yield 40 mg (73%) of crude 4-(1-pyrenyl)butyl isocyanate as a tan solid: ^{1}H NMR ($CDCl_3$) δ 8.28−7.79 (m, 9H), 3.42−3.10 (m, 4H), 2.02−1.66 (m, 4H); IR (thin film) 3038, 2925, 2857, 2263, 1690, 1617, 1508, 1458, 1181, 841 cm^{-1}; HRMS FAB (M^+ + H) calcd 299.1310, found 299.1307.

1-Pyrenyl Isocyanate. The reaction of 1-aminopyrene (100 mg, 0.46 mmol) with phosgene solution (0.477 mL, 0.920 mmol) as described for the preparation of 4-(1-pyrenyl)butyl isocyanate yielded 80 mg (71%) of crude 1-pyrenyl isocyanate as a yellow solid: ^{1}H NMR ($CDCl_3$) δ 8.19−7.79 (m, 8H), 7.69 (d, J = 8.1 Hz, 1H); IR (thin film) 3036, 2272, 1600, 1522, 1177, 1042, 839, 710 cm^{-1}; HRMS FAB (M^+ + H) calcd 243.0684, found 243.0683.

Luecylleucylleucylphenylalanine Methyl Ester Isocyanate (8a). The reaction of leucylleucylleucylphenylalanine methyl ester (50.0 mg, 0.09 mmol) with phosgene solution (93 μL, 0.18 mmol) as described for the preparation of 4-(1-pyrenyl)butyl isocyanate yielded 31.5 mg (64%) of crude **8a** as a white solid: ^{1}H NMR ($CDCl_3$) δ 7.30−7.08 (m, 6H), 6.92 (d, J = 7.8 Hz, 1H), 6.76 (d, J = 8.4 Hz, 1 H), 4.90−4.54 (m, 3H), 4.06 (t, J = 6 Hz, 1H), 3.68 (s, 3 H), 3.10 (d, J = 6 Hz, 2H), 1.84−1.48 (m, 9H), 0.98−0.87 (m, 18 H); IR (thin film) 3273, 3083, 2957, 2253, 1748, 1643, 1556, 1455, 1213, 700 cm^{-1}; HRMS FAB (M^+ + H) calcd 545.3339, found 545.3361.

Glycylprolylalanine Methyl Ester Isocyanate (8b). The reaction of glycylprolylalanine methyl ester (50 mg, 0.170 mmol) with phosgene solution (0.176 mL, 0.340 mmol) as described for the preparation of 4-(1-pyrenyl)butyl isocyanate yielded 15 mg (31%) of **8b** as a white solid: ^{1}H NMR ($CDCl_3$) δ 6.94 (d, J = 6.6 Hz, 1H), 4.59−4.45 (m, 2H), 3.97 (s, 2H), 3.65 (s, 3H), 3.53−3.30 (m, 2H), 2.36−1.94 (m, 4H), 1.42 (d, J = 7.2 Hz, 2H); IR (thin film) 3315, 2955, 2236, 1743, 1653, 1542, 1440, 1322, 1214, 1157, 1055, 907, 732 cm^{-1}; HRMS FAB (M^+ + H) calcd 284.1246, found 284.1257.

General Procedure and Representative Examples for the Reaction of Fully Protected Oligonucleotides with Isocyanates (7a). To a vacuum screw cap glass conical vial fitted with a spin vane was added a solution of **3** in CH_3CN−H_2O, 1:1 (119 μL, 4.2 μM **3**). The solution was frozen, and solvents were removed by evaporative centrifugation on a Savant Speed Vacced. The vessel and contents were further dried under high vacuum for 6 h. To the vessel was added a solution of

crude 1-pyrenyl isocyanate in acetonitrile−dichloroethane, 1:1 (15.2 μL, 33.3 mM). The reaction mixture was sealed and stirred at 55 °C for 2 h with intermittent vortexing every 30 min. The reaction contents were cooled to room temperature, the reaction vessel was centrifuged, and the reaction was quenched with MeOH (50 μL). The quenched reaction mixture was frozen with liquid nitrogen, and solvents were speed-vacced to dryness. To the crude material was added 28% NH_4OH (300 μL). The crude solution was heated to 55 °C for 6 h, frozen with liquid nitrogen, and speed-vacced to dryness. To the crude material was added 80% AcOH (150 μL). The crude solution was vortexed intermittently for 17 min. The solution was quenched with EtOH (150 μL), frozen with liquid nitrogen, and speed-vacced to dryness. The vessel was washed with CH_2Cl_2−H_2O, 1:1 (3 × 300 μL), and the eluent was collected in an eppendorf tube. The CH_2Cl_2 layer was extracted with H_2O (2 × 150 μL). Gel electrophoresis purification yielded 0.0242 μmol (quantitative yield when compared to an identical amount of starting material **3** deprotected and purified as above and quantified by UV spectral analysis: $\lambda_{max} = 260$ nm, $\epsilon = 188\,300$); retention time (anion-exchange HPLC) 13.94 min; ESMS calcd 6531.5, found 6531.0.

7b. The reaction of **3** with 1-naphthyl isocyanate performed and quantified as described for **7a** yielded **7b** (95%): retention time (anion-exchange HPLC) 14.03 min; ESMS calcd 6456.4, found 6457.0.

7c. The reaction of **3** with *o*-nitrophenyl isocyanate performed and quantified as described for **7a** resulted in a quantitative yield: retention time (anion-exchange HPLC) 13.70 min; ESMS calcd 6451.4, found 6451.0.

7d. The reaction of **3** with *m*-nitrophenyl isocyanate performed and quantified as described for **7a** resulted in a quantitative yield: retention time (anion-exchange HPLC) 14.32 min; ESMS calcd 6451.4, found 6452.0.

7e. The reaction of **3** with a stock solution of crude 4-(1-pyrenyl)-butyl isocyanate (15.2 μL, 66.0 mM) was performed and quantified as described for **7a**, with the exception of extending the reaction time to 4 h. Gel purification yielded **7e** (92%): retention time (anion-exchange HPLC) 13.76 min; ESMS calcd 6587.5, found 6587.0.

9b. The reaction of **3** with a stock solution of crude **8b** (15.2 μL, 66.0 mM) was performed and quantified as described for **7a**, with the exception of extending the reaction time to 4 h. Gel purification yielded **9b** (73%): retention time (anion-exchange HPLC) 12.03 min; ESMS calcd 6555.5, found 6556.0.

General Procedure and Representative Example for *o*-Benzotriazol-1-yl-*N, N, N′, N′*-tetramethyluronium Hexafluorophosphate (HBTU) Mediated Amide Formation Using Fully Protected Oligonucleotide (3). To pyrenebutyric acid (9.5 mg, 33 μmol) were added DMF (1 mL), diisopropylethylamine (4.2 mg, 33 μmol), and 1-hydroxybenzotriazole (4.5 mg, 33 μmol), followed by HBTU (12.5 mg, 33 μmol). The solution was stirred at room temperature for 5 min. To **3** (0.05 μmol) in a vacuum screw cap glass conical vial fitted with a spin vane was added 15.2 μL of the above reaction mixture. The reaction was stirred at room temperature for 2 h with intermittent vortexing every 30 min. Starting oligonucleotide preparation, reaction workup conditions, and deprotections were carried out as for **7a**. Anion-exchange HPLC purification (retention time 13.50 min) yielded **5** (97% when compared to an identical amount of starting material **3** deprotected and purified as above and quantified by UV spectral analysis: $\lambda_{max} = 260$ nm, $\epsilon = 188\,300$). Mass spectral characterization and anion-exchange HPLC retention times are in agreement with those reported for **5**.[1]

General Procedure and Representative Example of (Benzotriazol-1-yloxy)tripyrrolidinophosphonium Hexafluorophosphate (PyBOP) Mediated Amide Formation Using Fully Protected Oligonucleotides (3). To pyrenebutyric acid (9.5 mg, 33 μmol) were added DMF (1 mL) and diisopropylethylamine (12.7 mg, 99.0 μmol), followed by PyBOP (12.5 mg, 33 μmol). The solution was stirred at room temperature for 5 min. To **3** (0.05 μmol) in a vacuum screw cap glass conical vial fitted with a spin vane was added 15.2 μL of the above mixture. The reaction was stirred at room temperature for 2 h with intermittent vortexing every 30 min. Starting oligonucleotide preparation, reaction workup conditions, and deprotections were carried out as for **7a.** Anion-exchange HPLC purification yielded **5** (86% when compared to an identical amount of starting material **3** deprotected and purified as above and quantified by UV spectral analysis: $\lambda_{max} = 260$ nm, $\epsilon = 188\,300$). Mass spectral characterization and anion-exchange HPLC retention times are in agreement with those reported for **5**.[1]

Preparation of 4. To a mixture of 4-(1-pyrenyl)butyric acid (28.5 mg, 99 μmol), *N,N*-(dimethylamino)pyridine (12.1 mg, 99 μmol), dipyridyl disulfide (21.8 mg, 99 μmol), and triphenylphosphine (26.0 mg, 99 μmol) was added acetonitrile−dichloroethane, 1:1 (3 mL). The mixture was stirred at room temperature for 5 min. To **2** (0.05 μmol) in a vacuum screw cap glass conical vial fitted with a spin vane was added 15.2 μL of the above reaction mixture. The reaction was stirred at 55 °C for 2 h with intermittent vortexing every 30 min. Starting oligonucleotide preparation, reaction workup conditions, and deprotections were carried out as for **7a**. Gel electrophoresis purification yielded **4** (84% when compared to an identical amount of starting material **2** deprotected and purified as above and quantified by UV spectral analysis: $\lambda_{max} = 260$ nm, $\epsilon = 188\,300$) retention time (anion-exchange HPLC) 15.93 min; ESMS calcd 9671.5, found 9672.0.

Preparation of 5. To a mixture of 1-pyrenebutyric acid (14.2 mg, 49 μmol), *N,N*-(dimethylamino)pyridine (6.0 mg, 49 μmol), dipyridyl disulfide (10.9 mg, 49 μmol), and triphenylphosphine (13.0 mg, 49 μmol) was added acetonitrile−dichloroethane, 1:1 (3 mL). The mixture was stirred at room temperature for 5 min. To **3** (0.05 μmol) in a vacuum screw cap glass conical vial fitted with a spin vane was added 15.2 μL of the above reaction mixture. The reaction was stirred at 55 °C for 2 h with intermittent vortexing every 30 min. Starting oligonucleotide preparation, reaction workup conditions, and deprotections were carried out as for **7a**. Gel electrophoresis purification yielded **5** (89% when compared to an identical amount of starting material **3** deprotected and purified as above and quantified by UV spectral analysis: $\lambda_{max} = 260$ nm, $\epsilon = 188\,300$): ESMS calcd 6557.5, found 6557.1.

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Supporting Information Available: Electrospray mass spectra of **4**, **7a−e**, and **8b** and enzymatic digest of **5** and synthesis/characterization of the modified pyrenebutyric acid obtained upon digestion (7 pages, print/DF). See any current masthead page for ordering information and Web access instructions.

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