Efficient Solution Phase Synthesis of Oligonucleotide Conjugates Using Protected Biopolymers Containing 3′-Terminal Alkyl Amines

Dustin L. McMinn, Tracy J. Matray, and Marc M. Greenberg*

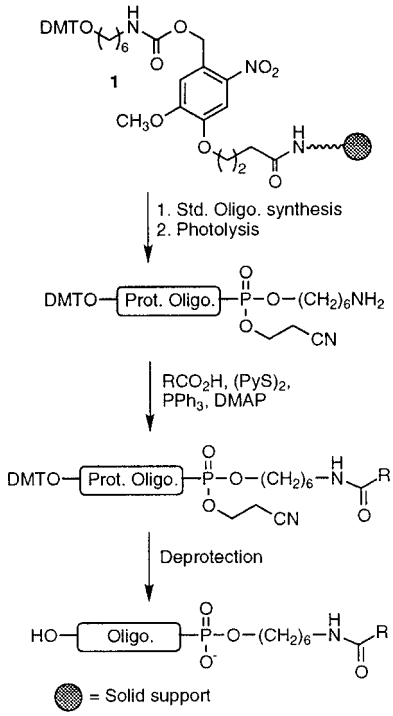
Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

Received August 13, 1997

Modified oligonucleotides are finding increasing application as therapeutic candidates and as mechanistic and diagnostic probes.[1−11] In addition, as the goal of sequencing the human genome approaches reality, the growing need for determining gene function promises to provide further uses for modified oligonucleotides. One general family of modified oligonucleotides that are valuable for the above applications are those biopolymers that are covalently bonded to a variety of molecules including fluorescent tags, intercalators, hydrophobic species, enzyme cofactors, and peptides. These molecules are conjugated at either oligonucleotide terminus, or internally using the phosphates, nucleobases, and carbohydrate components as sites for covalent modification. Current methods for oligonucleotide conjugate synthesis include utilization of specific phosphoramidites, derivatization of support bound biopolymers, and postsynthetic modification of deprotected oligonucleotides. In addition, oligonucleotide−peptide conjugates are preparable in a linear fashion on a single solid phase support.[9] These synthetic methods suffer from a variety of disadvantages that include one or more of the following: nonconvergency, requirement of a large excess of reagents, elaborate substrate synthesis, poor yields, and nonspecific covalent modifications.

We report an orthogonal, convergent strategy for the synthesis of oligonucleotide conjugates that results in high isolated yields of homogeneous products under rapid and mild reaction conditions.[12] Our approach involves solution phase amide bond formation utilizing protected

Scheme 1



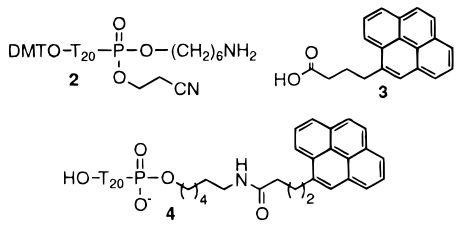
oligonucleotides containing 3′-alkyl amines (Scheme 1).[13] Methods for the modification of the 3′-terminus of oligonucleotides are relatively scarce compared to those available for altering the 5′-terminus.[1c,5] In addition to being a convergent method for oligonucleotide conjugate synthesis, this approach obviates the need for chemically synthesizing specific phosphoramidites for biopolymer modification. Conjugation of oligonucleotides derived from **1** also proceeds with greater chemoselectivity and regioselectivity than comparable reactions on deprotected oligonucleotides. Conjugation of protected oligonucleotides eliminates the possibility for formation of regioisomers via reaction with the exocyclic amines of the nucleobases.[8] Amide protection of purine nucleotides also renders the cyclic nitrogens less nucleophilic than they are in the deprotected oligonucleotides.

When selecting a method for biopolymer conjugation, we anticipated that for many research purposes one would want to utilize submicromole quantities of oligonucleotides. In this way, one could prepare several 3′-terminal modified oligonucleotides from a single standard solid phase synthesis.[14] In order to utilize volumes of (volatile) solvents that are easily manipulated, we anticipated employing millimolar solutions of oligonucleotides. Consequently, we chose to effect amide bond formation using a redox condensation process which has proven very useful for carrying out macrocyclizations, where dilute conditions are necessary.[15,16]

Reaction conditions were optimized using the conjugation of an eicosameric protected polythymidylate (**2**) and 4-pyrenebutanoic acid (**3**). All reactions were carried out on crude photolysate in a 1:1 mixture of acetonitrile and

(1) Reviews: (a) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123. (b) Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165.
(2) (a) Perkins, T. A.; Wolf, D. E.; Goodchild, J. *Biochemistry* **1996**, *35*, 16370. (b) Jones, D. S.; Hachmann, J. P.; Osgood, S. A.; Hayag, M. S.; Barstad, P. A.; Iverson, G. M.; Coutts, S. M. *Bioconjugate Chem.* **1994**, *5*, 390.
(3) Manoharan, M.; Ramasamy, K. S.; Mohan, V.; Cook, P. D. *Tetrahedron Lett.* **1996**, *37*, 7675.
(4) Chrisey, L. A.; Lee, G. U.; O'Ferrall, C. E. *Nucleic Acids Res.* **1996**, *24*, 3031.
(5) Hovinen, J.; Guzaev, A.; Azhayeva, E.; Azhayev, A.; Lönnberg, H. *J. Org. Chem.* **1995**, *60*, 2205.
(6) Boutorine, A. S.; Brault, D.; Takasugi, M.; Delgado, O.; Hélène, C. *J. Am. Chem. Soc.* **1996**, *118*, 9469.
(7) Kremsky, J. N.; Pluskal, M.; Casey, S.; Perry-O'Keefe, H.; Kates, S. A.; Sinha, N. D. *Tetrahedron Lett.* **1996**, *37*, 4313.
(8) (a) Ghosh, S. S.; Musso, G. F. *Nucleic Acids Res.* **1987**, *15*, 5353. (b) Bischoff, R.; Coull, J. M.; Regnier, F. E. *Anal. Biochem.* **1987**, *164*, 336.
(9) (a) Truffert, J.-C.; Asseline, U.; Brack, A.; Thuong, N. T. *Tetrahedron* **1996**, *52*, 3005. (b) Bergmann, F.; Bannwarth, W. *Tetrahedron Lett.* **1995**, *36*, 1839.
(10) Fidanza, J. A.; Ozaki, H.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1992**, *114*, 5509.
(11) (a) MacMillan, A. W.; Verdine, G. L. *Tetrahedron* **1991**, *47*, 2603. (b) Xu, Y.-Z.; Zheng, Q.; Swann, P. F. *J. Org. Chem.* **1992**, *57*, 3839.
(12) For orthogonal, convergent methods of the synthesis of peptides and dendrimers see: (a) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, *49*, 11065. (b) Zeng, F.; Zimmerman, S. C. *J. Am. Chem. Soc.* **1996**, *118*, 5326.
(13) McMinn, D. L.; Greenberg, M. M. *Tetrahedron* **1996**, *52*, 3827.
(14) Automated oligonucleotide synthesizers can routinely synthesize oligonucleotides on a 2.5 μmol scale. In this work, an ABI 380B oligonucleotide synthesizer was used. Syntheses were carried out on a 1.0 μmol scale.
(15) Mukaiyama, T. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 94.
(16) (a) Corey, E. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1974**, *96*, 5614. (b) Nicolaou, K. C. *Tetrahedron* **1977**, *33*, 683.

S0022-3263(97)01511-9 CCC: $14.00



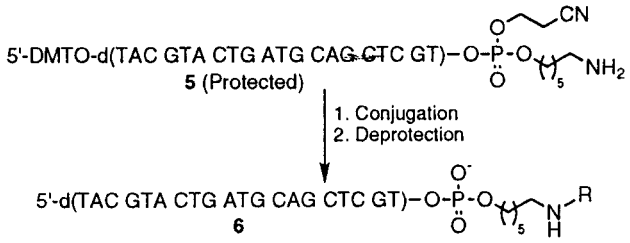
1,2-dichloroethane at 55 °C. Reactions were analyzed by anion exchange HPLC following deprotection of the crude reaction mixture. Conjugate **4** and all other conjugates reported below were characterized by electrospray mass spectrometry. Isolated yields of **4** averaging 91 ± 10% were obtained in 4 h using 10 equivalents of **3**, 2,2′-pyridyl disulfide ($(PyS)_2$), DMAP, and triphenylphosphine (PPh_3), relative to **2** (present at ~3 mM). Further experimentation revealed that comparable yields of **4** were reproducibly obtained using reaction times of 2 h.

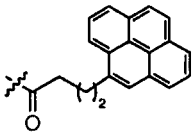
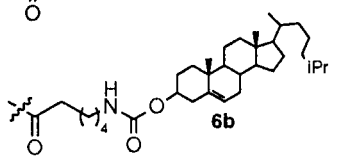
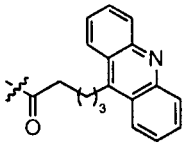
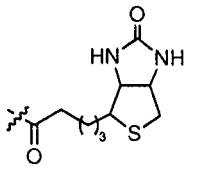
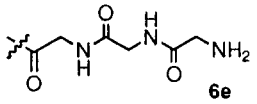
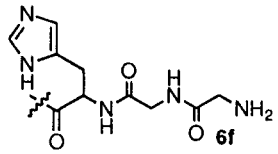
The generality of the conjugation method was demonstrated using an eicosameric heteropolymer (**5**). The protected heteropolymer **5** was synthesized using commercially available *N*-isobutyryl protected phosphoramidites in order to avoid transamidation of nucleobase protecting groups to the 3′-alkyl amine.[17] Using the optimized conditions found for preparing **4**, isolated yields of 89% of **6a** were obtained (Table 1). The syntheses of **6b**−**d** proceeded in comparable efficiency (Table 1). Reactions yielding **6c** and **6d** were carried out in DMF in order to solvate the respective carboxylic acid substrates. Conjugates from **5** were isolated in yields comparable to those obtained from **2** using the same number of equivalents of reagents (Table 1). In addition, we were unable to detect alkaline labile lesions such as those resulting from acylation of nucleophilic positions of purine bases (e.g. N^3-adenine, N^7-guanine). These observations indicate that neither cyclic or protected exocyclic amines are reactive under the conditions required to effect efficient conjugation.

Additional support for the versatility and generality of this method is gleaned by preparing oligonucleotide−peptide conjugates (**6e**, **6f**, Table 1). Recently, a number of reports concerning the linear synthesis of oligonucleotide-peptide conjugates have appeared.[9] These methods also require the stepwise incorporation of a spacer molecule between the two types of biopolymers during the solid phase synthesis and typically result in low yields. Conjugations of **5** to *N*-FMOC-protected tripeptides were carried out in DMF at room temperature, under otherwise identical conditions as discussed above. These reactions were carried out at room temperature in order to prevent loss of the peptide FMOC group and subsequent oligomerization of the tripeptide. The oligonucleotide−peptide conjugates, **6e** and **6f**, were obtained in 99% and 89% yields, respectively. The synthesis of **6f** is noteworthy in that the Gly·Gly·His tripeptide has potential nuclease activity.[9a,18,19] The synthesis of this conjugate is unobtainable using previous methods unless the *N*-terminus of the peptide is modified.[9a]

In conclusion, we have developed a general, efficient, high-yielding, solution phase method for the conjugation of protected oligonucleotides. This is the first method for conjugating protected oligonucleotides in solution. The protected oligonucleotides are obtained in a straight forward manner using commercially available reagents and standard oligonucleotide synthesis protocols. These results represent a general method for the synthesis of oligonucleotide conjugates that are not limited to DNA, or the type of linkage described above.

Table 1. Isolated Yields of Oligonucleotide Conjugates Obtained from 5



R	Isolated Yield (%)[a,b]
 6a	89 ± 1[c]
 6b	83[c]
 6c	99 ± 2[d]
 6d	88 ± 2[d]
 6e	99 ± 2[d]
 6f	89 ± 3[d]

[a]Isolated yields were determined via comparing the amount of oligonucleotide obtained from a comparable amount of unconjugated material subjected to the identical deprotection, purification, isolation conditions. [b]Average yields represent a minimum of two reactions. [c]Solvent, acetonitrile:1,2-dichloroethane (1:1). [d]Solvent, DMF.

Acknowledgment. This research was generously supported by the National Science Foundation (CHE-9424040), with partial support from CATI Center for RNA Chemistry. We are grateful for an unrestricted gift from Glen Research Inc. M.M.G. is an Alfred P. Sloan Research Fellow.

Supporting Information Available: General procedure for conducting conjugation reactions and electrospray mass spectra of **4** and **6a**−**f** (5 pages).

JO971511F

(17) McMinn, D. L.; Greenberg, M. M. *Tetrahedron Lett.* **1997**, *38*, 3123.
(18) Shullenberger, D. F.; Eason, P. D.; Long, E. C. *J. Am. Chem. Soc.* **1993**, *115*, 11038.
(19) Mack, D. P.; Dervan, P. B. *Biochemistry* **1992**, *31*, 9399.