Synthesis of Functionally Tethered Oligodeoxynucleotides by the Convertible Nucleoside Approach

Andrew M. MacMillan and Gregory L. Verdine*

Department of Chemistry, Harvard University, 12 Oxford St, Cambridge, Massachusetts 02138

Received August 1, 1990

Summary: A novel approach to the synthesis of oligodeoxynucleotides bearing tethered nonnative functional groups is reported. In this convertible nucleoside approach, the tether is installed at the final step of DNA synthesis, thus allowing the synthesis of a series of functionally tethered oligodeoxynucleotides from a single precursor.

Synthetic oligodeoxynucleotides are able to bind cognate nucleic acid sequences with high affinity and specificity, yet these small DNA molecules are ill-equipped to report binding or to effect chemical modification of a target sequence. In order to overcome this limitation, a number of synthetic avenues have been devised for the attachment of nonnatural ligands to synthetic DNA;[1,2] these routes have furnished hybrid DNA molecules equipped with a wide array of ligands for use as nucleases,[3] detection probes,[4] and photoaffinity reagents.[5] The critical precursors to hybrid DNA molecules are functionally tethered oligodeoxynucleotides (FTOs), synthetic DNA variants having a nonnatural tethered nucleophile that may be used for site-specific ligand attachment. While the known synthetic routes to FTOs vary, virtually all may be described as *nonconvergent*, with the tethered nucleophile being introduced at the monomer level of DNA synthesis.[6] Nonconvergent synthetic approaches suffice for some cases but are limited in two respects: (i) variation of the tether requires a large synthetic investment, involving multistep synthesis of a new monomer and its subsequent use in automated DNA synthesis; and (ii) highly functionalized tethers such as peptides require the employment of complicated protection/deprotection schemes. Ideally, and in contrast to available methods, it would be preferable to access a variety of FTOs, differing in sequence and tether structure, from a single nucleoside precursor—a *convergent* strategy—with the tether being attached to DNA at the final step of FTO synthesis. In this paper we report a new and convergent approach to the synthesis of FTOs and demonstrate the efficiency of this route by synthesis of five structurally distinct FTOs from a single oligonucleotide precursor.

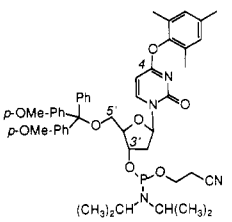
Table I. Reaction of Convertible Nucleoside 1 to dC-Tethered Nucleoside 2 during Aminolysis of the Precursor Oligodeoxynucleotide 5′-d(GCAAG1TTGC)-3′

entry	amine	tether	% convn	T_m, °C
a	H_2NH	-H (none)	95	51.3
b	H_2NCH_3	$-CH_3$	100	45.5
c	$H_2NCH_2CH_2NH_2$	$-CH_2CH_2NH_2$	100	44.4
d	$H_2NCH_2CH_2CH_2CH_2NH_2$	$-CH_2CH_2CH_2CH_2NH_2$	100	45.3
e	$H_2NCH_2CH_2OH$	$-CH_2CH_2OH$	100	41.0
f	$H_2NCH_2CO_2H$	$-CH_2CO_2H$	89	36.7
g	$(H_2NCH_2CH_2S-)_2$	$-CH_2CH_2SSCH_2CH_2NH_2$	100	40.9

[a] All reactions were carried out for 14 h at 65 °C, but the concentration of amine varied: entry a, 14 M; b, 12 M; c, 1 M; d–g, 5 M.

The TMP-dU "phosphoramidite" derivative **3**[7-9] was used in solid-phase DNA synthesis[10] to generate the resin-bound, protected decamer 5′-d(*GCAAG*1*TTGC*)-3′.[11] Mild ammonolysis of the crude product afforded the precursor oligodeoxynucleotide 5′d(GCAACG1TTGC)-3′ without affecting convertible nucleoside **1**. Reaction of this single precursor oligodeoxynucleotide with a variety of aqueous amines[12-14] yielded the respective series of FTOs:

(7) Nucleoside 1 [4-*O*-(2,4,6-trimethylphenyl)-2′-deoxyuridine] was prepared by the general procedure of Zhou and Chattopadyaya[8a] and converted to the corresponding "phosphoramidite" **3** (below) by 5′-tritylation[8b] and 3′-phosphitylation.[8c,9]



TMP-dU "phosphoramidite", 3

(8) (a) Zhou, X.-X.; Chattopadhyaya, J. *Tetrahedron* **1986**, *42*, 5149. (b) Schaller, H.; Weimann, G.; Lerch, B.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 3821. (c) Sproat, B. S.; Lamond, A. I.; Beijer, B.; Neuner, P.; Ryder, U. *Nucleic Acids Res.* **1989**, *17*, 3373.

(9) See the supplementary material for further details.

(10) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3185. For a recent review, see: Engels, J. W.; Uhlmann, E. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 716.

(11) Italics denote the presence of protecting groups on the oligonucleotide, in addition to a 3′-ester linkage to the controlled-pore glass resin. The synthesis of 5′-d(*GCAAG*1*TTGC*) employed commercially available PAC phosphoramidites (Pharmacia). PAC phosphoramidites possess labile protecting groups on the DNA bases [N^4-isobutyrylcytidine, N^6-(2-phenoxyacetyl)adenine, and N^2-(2-phenoxyacetyl)guanine], which are quantitatively removed by treatment with ammonium hydroxide at room temperature for several hours: Schulhof, J. C.; Molko, D.; Teoule, R. *Tetrahedron Lett.* **1987**, *28*, 51.

(12) The possibility of using O^4-aryl-dU derivatives as precursors to a variety of other pyrimidine derivatives has been previously noted, although in a context unrelated to FTO synthesis: Welch, C. J. Ph.D. Thesis, Uppsala University, 1986.

(13) **General Procedure.** Approximately 15 nmol (determined by A_{260}) of the precursor oligonucleotide was treated with aqueous amine (concentrations given in Table I) in a total reaction volume of 100 μL (1 mL in the cases of ammonium hydroxide and methylamine) in a 65 °C oven for 14 h. The reactions were carried out in a screw-capped Eppendorf tube in order to prevent evaporation. In the cases of ammonia and methylamine, the amine was removed by direct lyophilization; in all other cases, the reaction mixtures were diluted to 1 mL with distilled water and neutralized by the addition of glacial acetic acid. The solutions were then desalted by repeated centrifugal dialysis (Centricon 3, Millipore). Retentates were lyophilized, resuspended in 50 μL of distilled water, and stored at −20 °C.

(1) For a review, see: Goodchild, J. *Bioconj. Chem.* **1990**, *1*, 165.

(2) Haralambidis, J.; Chai, M.; Tregear, G. W. *Nucleic Acids Res.* **1987**, *15*, 4857. Zuckermann, R.; Corey, D.; Schultz, P. G. *Nucleic Acids Res.* **1987**, *15*, 5305. Eshaghour, H.; Soll, D.; Crothers, D. M. *Nucleic Acids Res.* **1979**, *7*, 1485. Agrawal, S.; Christodoulou, C.; Gait, M. J. *Nucleic Acids Res.* **1986**, *14*, 6227. Chu, B. C. F.; Wahl, G. M.; Orgel, L. E. *Nucleic Acids Res.* **1983**, *11*, 6513.

(3) Moser, H. E.; Dervan, P. B. *Science (Washington D.C.)* **1987**, *238*, 645. Strobel, S. A.; Dervan, P. B. *Science (Washington D.C.)* **1990**, *249*, 73. Zuckermann, R. N.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 6592. Chen, C-h. B.; Sigman, D. S. *J. Am. Chem. Soc.* **1988**, *110*, 6592.

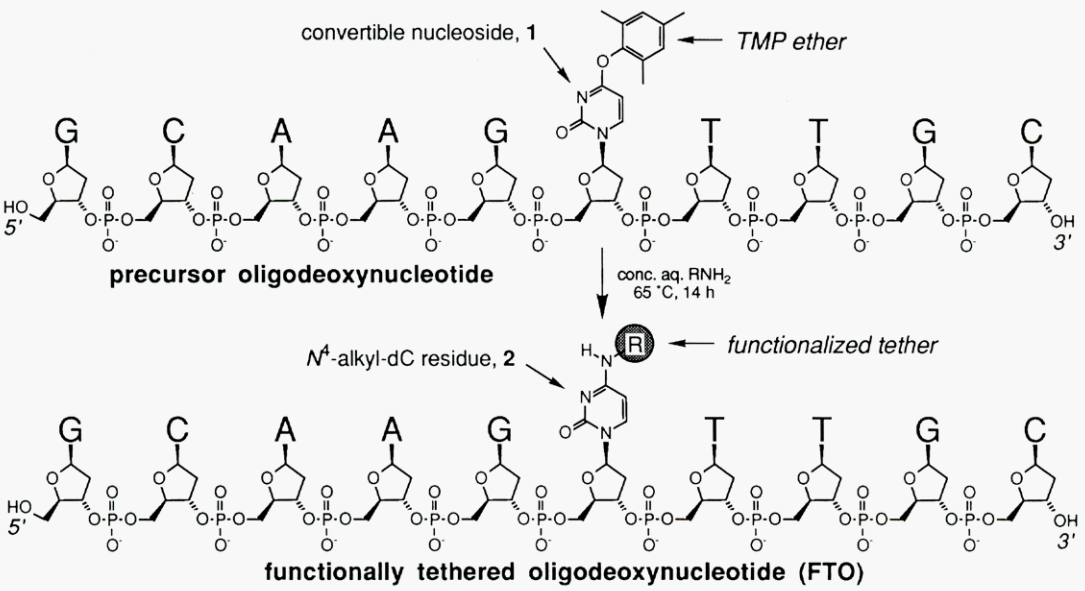
(4) Gillam, I. C.; Tener, G. M. *Anal. Biochem.* **1986**, *157*, 199. Urdea, M. S.; Warner, B. D.; Running, J. A.; Clyne Stempien, M. J.; Horn, T. *Nucleic Acids Res.* **1988**, *16*, 4937.

(5) Gibson, K. J.; Benkovic, S. J. *Nucleic Acids Res.* **1987**, *15*, 6455.

(6) Two exceptions are: Draper, D. E. *Nucleic Acids Res.* **1984**, *12*, 989. Fidanza, J. A.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1989**, *111*, 9117.

0022-3263/90/1955-5931$02.50/0

Scheme I



5′-d(GCAAG**2**TTGC)-3′ (Scheme I). These FTOs were then examined for extent of conversion and overall purity by anion-exchange FPLC chromatography (Pharmacia) and enzymatic digestion to component nucleosides followed by HPLC analysis.[9]

The results of the enzymatic digestions on FTOs 5′-d(GCAAG**2**TTGC)-3′ are summarized in Table I. In all cases, the conversion of **1** to **2** proceeded cleanly, with no evident formation of 2′-deoxyuridine (through hydrolysis of **1**) or other byproducts.[16] In the majority of cases, the conversion of **1** to **2** was quantitative, as judged by the lack of nucleoside **1** in HPLC traces of the enzymatic digests. In the case of aminolysis with NH_4OH and glycine to yield 5′-d(GCAAG**2a**TTGC)-3′ and 5′-d(GCAAG**2f**TTGC)-3′, respectively, small amounts of starting material remained. Although these reactions with nonnucleophilic amines were incomplete, the remaining starting material could easily be removed by FPLC.[9] The delayed elution of 5′-d(GCAAG**1**TTGC)-3′ relative to 5′-d(GCAAG**2**TTGC)-3′ was analytically useful in that it provided a check on the conversion values derived from nucleoside composition analysis.

In B-DNA, the non-base-paired substituent on the N^4-position of dC protrudes directly into the central space of the major groove (Figure 1), thus affording one of the least sterically demanding positions available on a DNA base. Consequently, attachment of a tether at this site should cause little *steric* perturbation of duplex DNA structure; this premise is supported by X-ray crystallographic studies on duplex DNA containing the related *N*-alkyl base N^6-methyladenine.[17] N^4-alkyl-dC residues do weakly destabilize duplex DNA[15] as the result of an *electronic* factor: the need to Watson–Crick base pair via the less stable (anti) rotamer about the C4–N^4 bond (as depicted in Scheme I).[18] The FTOs 5′-d(GCAAG**2**TTGC)-3′ are self-complementary and should therefore spontaneously self-assemble into a B-form duplex:

5′-d(GCAAG**2**TTGC)-3′

3′-d(CGTT**2**GAACG)-5′

in which the tethered nucleosides **2** are diagonally disposed

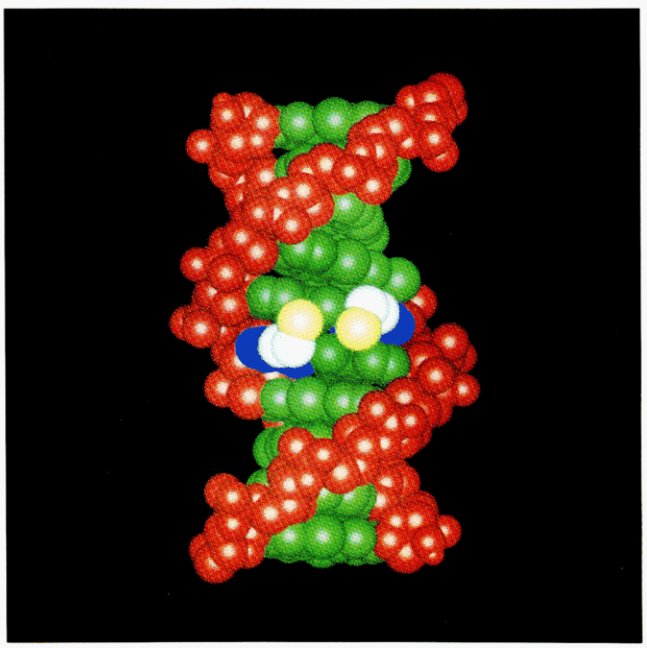


Figure 1. Location of the tether in dC-tethered FTOs. Shown is the free thiol form[21] of the FTO 5′-(GCAATG**2g**TTGC)-3′, with the following color scheme: red, phosphodiester backbone; blue, cytosines to which the tethers are attached; white, methylene units of the tether; yellow, sulfur atoms of the tether; green, all remaining atoms. From this model, it can be seen that the alkyl substituent on the N^4-alkyl-dC residues protrude out into the central major groove space, and thus they provide an excellent location for the attachment of DNA-interacting ligands. In addition, it can be seen that the two thiol residues of the FTO are suitably disposed to form an interstrand disulfide crosslink. The model was constructed in MacroModel (C. Still, Columbia University) using the "grow" mode, normalized for bond lengths, and not further minimized. The atoms of the tether were manually rotated to bring them in an orientation close to that required for disulfide bond formation.

(14) N^4-(Aminoalkyl)-dC residues have previously been incorporated into DNA[4,15] by nonconvergent approaches using tethered monomers.

(15) Telser, J.; Cruickshank, K. A.; Morrison, L. E.; Netzel, T. L. *J. Am. Chem. Soc.* **1989**, *111*, 6966.

(16) The present results on convertible nucleosides in DNA differ significantly from model studies on the free nucleoside **1**, in which up to 10% of dU was formed during aminolysis with non-nucleophilic amines (e.g., NH_4OH): MacMillan, A. M.; Verdine, G. L., manuscript in preparation.

(17) Frederick, C. A.; Quigley, G. J.; van der Marel, G. A.; van Boom, J. H.; Wang, A. H.-J.; Rich, A. *J. Biol. Chem.* **1988**, *263*, 17872.

(18) Engel, J. D.; von Hippel, P. H. *Biochemistry* **1974**, *13*, 4143.

(see Figure 1). The circular dichroism spectra of the FTOs (not shown), as well as their exhibition of discrete, cooperative melting transitions[19] (Table I), indicate that they indeed exist as B-form duplex DNA in solution. Although each of the duplex FTOs bears *two* modified dC residues at adjacent positions, the extent of destabilization caused by the presence of the tethers is modest (Table I). In only one case, 5′-d(GCAAG**2f**TTGC), was duplex stability significantly compromised, likely as the result of charge repulsion between the proximal carboxylate groups of the tether. In most common applications, those in which only one tether would be present in a duplex FTO, the destabilizing effect of the tether alone would be negligible. We believe that the central location of the tether attachment point in these FTOs may make them especially suitable for studies involving attachment of DNA-interactive ligands.[20]

The FTO synthetic technology reported here represents a general, convergent approach to the introduction of tethered functionality into DNA. As demonstrated here, a single phosphoramidite monomer can be used to install a wide variety of tethers, since the tether length and appended functionality are determined solely by the amine employed in the final deprotection step. In this preliminary study, four nonnative functional groups have been tethered to a specific locus in DNA: an aliphatic amine, an alcohol, a thiol (protected as the mixed disulfide),[21] and a carboxylic acid. This base-modification approach leaves the 5′ and 3′ ends free for enzymatic manipulation, a necessity for many molecular biological applications. We expect that this methodology will have significant applicability in the investigation of protein–DNA interactions, the creation of novel, conformationally locked DNA structures, and gene-targeted drug delivery.

Acknowledgment. This work was supported by a DuPont Young Faculty Fellowship and a Camille and Henry Dreyfus Distinguished New Faculty Award. We thank Hewlett-Packard for the generous gift of a 1090M HPLC system. We are greatly indebted to Professor L. McLaughlin (Boston College) for use of his T_m instrument, as well as helpful comments and discussions regarding these measurements.

Supplementary Material Available: Detailed procedures for the synthesis, purification, and digestion of modified oligodeoxynucleotides, FPLC analysis of modified oligodeoxynucleotides, and HPLC analysis of enzymatic digestion products (13 pages). Ordering information is given on any current masthead page.

(19) Experimental conditions for the T_m measurements were as follows: samples with initial O.D.$_{260}$ of 0.4 A.U. were prepared in 1 M NaCl, 10 mM KH_2PO_4 pH 7.0. T_m's (±0.1 °C) were obtained from first and second derivative plots of absorbance vs. temperature curves. Data were collected on a Perkin-Elmer Lambda 3B spectrophotometer equipped with an immersible temperature probe and digital temperature controller interfaced to an IBM-XT personal computer using ASYST (version 1.53) data collection software.

(20) Telser et al.[15] have reported that a duplex FTO containing a single N^4-(3-aminopropyl)-dC residue is weakly destabilized. Attachment of a pyrenebutyrate moiety to the tether, however, causes a dramatic perturbation of duplex structure. This result may be interpreted as evidence that the pyrenebutyrate interacts strongly (perhaps by intercalation) with the DNA molecule to which it is tethered.

(21) The mixed-disulfide FTO 5′-d(GCAAG**2g**TTGC)-3′ can be quantitatively reduced to the free thiol form by overnight treatment with dithiothreitol (10 mM) at 55 °C in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The Synthesis of the First Perfluorocryptand

W. D. Clark, T. Y. Lin, S. D. Maleknia, and R. J. Lagow*

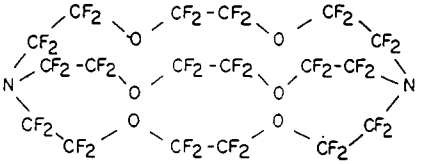
Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712

Received May 7, 1990

Summary: Using carefully controlled reactions of elemental fluorine, we have prepared and characterized the first perfluorocryptand [222], specifically perfluoro-4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane. This is a very stable, inert, high boiling clear oil.

In 1985, Lagow and co-workers prepared the first perfluoro crown ethers.[1] The compounds perfluoro 12-crown-5, perfluoro 15-crown-5, and perfluoro 18-crown-6, were prepared in good yield using controlled direct fluorination elemental fluorine reactions. Cryptands were prepared by Lehn and co-workers[2] and have been studied extensively by Lehn and others. As is well-known, these are a very useful class of ligands which make very stable complexes with numerous metal cations.

We report in this paper the synthesis of the first perfluorocryptand, perfluoro-4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane, which is the perfluorocryptand [222]. This is a very stable, inert, high-boiling clear oil and was obtained in 28% yield by direct fluorination of the starting hydrocarbon cryptand [222].



The substitution of fluorine into cryptand systems is sure to produce some interesting effects. Indeed, the presence of fluorocarbon groups in crown ethers has been shown to increase the rate of ion transport through a polymer membrane.[3] The presence of fluorine in partially fluorinated cyclams has been shown to reduce the basicities of such compounds.[4]

The hydrocarbon compound 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane, (cryptand [222]), was purchased from Aldrich Chemical Co. Fluorine was

(1) (a) Lin, W. H.; Bailey, W. I., Jr.; Lagow, R. J. *J. Chem. Soc., Chem. Commun.* **1985**, 1385. (b) Lin, W. H.; Bailey, W. I., Jr.; Lagow, R. J. *Pure Appl. Chem.* **1988**, *60*, 473.

(2) (a) Dietrich, B.; Lehn, J. M.; Sauvage, J. P. *Tetrahedron Lett.* **1969**, *34*, 2885. (b) Lehn, J. M.; Sauvage, J. P.; Dietrich, B. *J. Am. Chem. Soc.* **1970**, *92*, 2916. (c) Lehn, J. M. *Angew. Chem.* **1988**, *100*(1), 91.

(3) Kimura, E.; Shionoya, M.; Okamoto, M.; Nada, H. *J. Am. Chem. Soc.* **1988**, *110*, 3679.

(4) Shinkai, S.; Torigoe, K.; Manabe, O.; Kajiyama, T. *J. Am. Chem. Soc.* **1987**, *109*, 4458.

0022-3263/90/1955-5933$02.50/0