Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 8, pp. 1001-1004, 1994

0960-894X/94 $6.00+0.00

0960-894X(94)E0090-2

The Stereospecific Introduction of Reporter Groups to Oligodeoxynucleotides by the Labeling of Individual Phosphorus Diastereomers

Maryanne J. O'Donnell, Normand Hebert and Larry W. McLaughlin*

Department of Chemistry, Merkert Chemistry Center, Boston College
2609 Beacon St., Chestnut Hill, MA 02167

Abstract: *A procedure is described for the preparation of oligodeoxynucleotides tethering reporter groups or other labels to the phosphate backbone in a stereospecific manner.*

The development of a number of nucleic acid diagnostics and therapeutics may require the ability to covalently attach reporter groups or other entities to specific sequences of DNA.[1] In addition to end-labeling procedures[2] and the incorporation of modified nucleosides[3] to facilitate the covalent attachment of fluorophores and other agents, the phosphodiester backbone[4] offers an attractive site for the introduction of an appropriate tether for such labeling procedures. Backbone labeling does not typically require the synthesis of a modified nucleoside, and since the tether of interest is introduced during the assembly of the DNA sequence, it can be placed at virtually any sequence position. Backbone labeling has been accomplished to date by the formation of a single phosphoramidate linkage at a specific site within an oligodeoxynucleotide of interest, with the nitrogen of the phosphoramidate tethering the reporter group of interest. In some cases the reporter group itself is attached during the assembly of the DNA sequence,[5] while in other reports only the tether is introduced to facilitate post-synthetic modification of the oligodeoxynucleotide.[6] In addition to phosphoramidate linkages, phosphorothioate linkages can also be employed in post-synthetic labeling procedures.[7] Labeling of the internucleotide phosphorus residue results in two diastereomers about phosphorus. In general, the diastereomers are not easily resolved, although in some cases derivatization will enhance chromatographic resolution. In the present report we describe the use of a simple tether that is incorporated into the sequence during assembly, but its presence then permits resolution of the two phosphorus diastereomers during standard isolation procedures.

In the present procedure we have employed N-triphenylacetylcystamine as the backbone tether. Reaction of cystamine with triphenylacetic anhydride produced preferentially the N,N'-bis(triphenylacetyl)cystamine derivative.[8] However, the desired monoamide could subsequently be generated in reasonable yield by a disulfide exchange reaction employing cystamine and catalyzed by 2-aminoethanethiol.[9] After purification, N-triphenylacetylcystamine was converted to its hydrochloride salt and azeotroped from toluene for storage. As needed, small quantities of the hydrochloride salt (30 mg, ~60 μmol) were dissolved in dichloromethane and extracted with sodium carbonate. The organic phase was evaporated to dryness, coevaporated from anhydrous pyridine and finally dissolved in anhydrous pyridine/carbon tetrachloride (1:1, 200 μl).

The current method exploits the chemistry originally reported by Todd and coworkers[10] and is similar to other recent reports.[5,6] An internucleotidic hydrogen phosphonate linkage was generated at the desired sequence position which was subsequently oxidized with carbon tetrachloride in the presence of N-triphenylacetylcystamine and pyridine. After completion of the synthesis, the 5'-DMT protecting group was removed and the sequence underwent standard conc. ammonia deprotection. Sequences containing the triphenylacetamide linker could be easily resolved by HPLC from failed

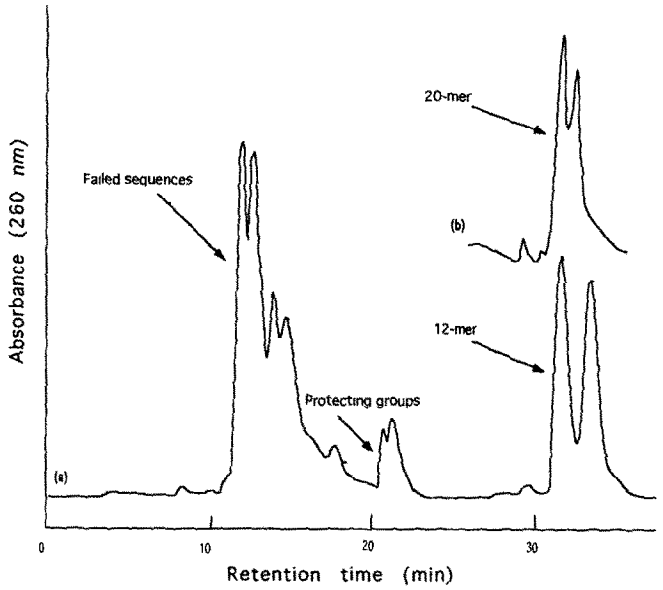

Figure 1. Purification of the diastereomeric sequences (a) d(CGCAXAAAAAGCG), and (b) d(GCGXAAAAGCGC-GCTTTTGCG) where X = N-triphenylacetylcystamine tethered to the internucleotide phosphorus. The isolation employed a 9.4 x 250 mm column of MOS-Hypersil in 20 mM KH_2PO_4 with a 60 min gradient of methanol (0 - 70%).

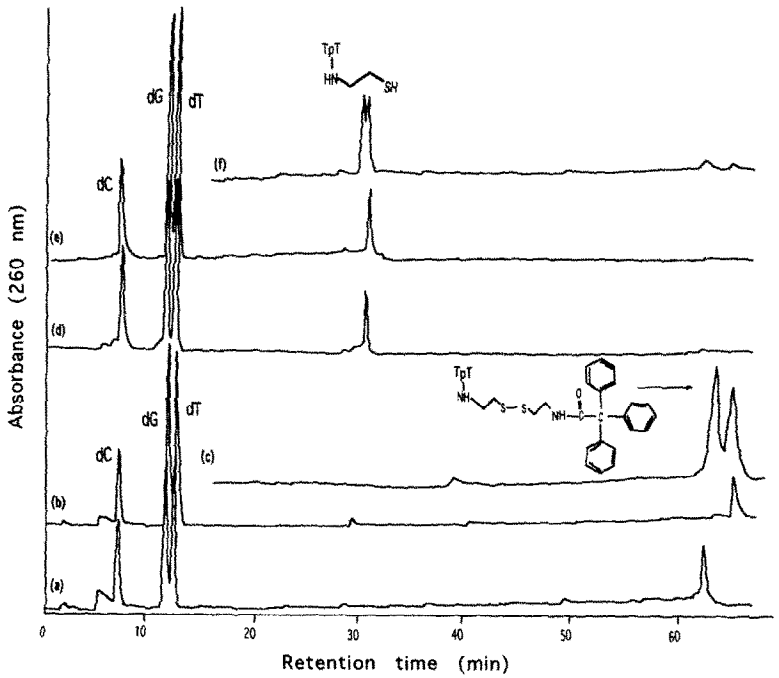

Figure 2. (a, b) HPLC analysis of each diastereomer of d[CGCT(NHCH$_2$CH$_2$SSCH$_2$CH$_2$NHCOCPh$_3$)TTTTTGCG] after treatment with P_1 nuclease, snake venom phosphodiesterase and calf intestinal alkaline phosphatase. (c) Authentic diastereomeric mixture of d[T(NHCH$_2$CH$_2$SSCH$_2$CH$_2$NHCOCPh$_3$)T]. (d, e) Analysis of (a) and (b) after unmasking the thiol-containing tether by treatment with DTT. (f) Authentic mixture of d[T(NHCH$_2$CH$_2$SH)T].

sequences lacking the oxidation product in much the same manner as a 5'-terminal DMT-containing oligonucleotide can be resolved from failed sequences. However, by incorporating the hydrophobic triphenylmethyl moiety at the stereogenic center, the two phosphorus diastereomers could be easily separated (Fig. 1). The ability to resolve the diastereomeric sequences was successful for sequences as long as 20 nucleotides, but with longer fragments such resolution will incrementally diminish.

We initially observed that the yields of oligodeoxynucleotides containing this linker were reduced significantly relative to those obtained simply using the cystamine linker.[6b] Additionally, we noted a peak in the HPLC chromatogram corresponding to an oligodeoxynucleotide containing the N-acetylcystamine linker[6b] - under the described conditions this derivative would not be expected. The presence of this material suggested that the acetic anhydride "capping" step resulted in some transacetylation of the N-triphenylacetylcystamine linker, thus reducing the yield of the desired product. In subsequent syntheses we have eliminated the capping step from the synthesis cycle and this change resulted in enhanced yields of the triphenylacetamide-linker DNAs. By using the triphenylacetamide-containing linker as the basis for HPLC purification (Fig. 1), it is possible that some contaminating materials would copurify with the desired product. Such contaminants would contain the oxidation product (the triphenylmethyl-containing linker), but would not have been fully elongated and would vary in the nature of the 5'-terminus of the sequence. However, after cleavage of the triphenylmethyl-containing linker (see below), we were unable to detect the presence of any DNA materials using HPLC or PAGE analyses, other than the desired product.

A portion of the isolated DNA products (isomer I and isomer II) was analyzed using HPLC,[11] after digestion to the nucleoside constituents by treatment with P1 nuclease, snake venom phosphodiesterase and calf intestinal alkaline phosphatase.[12] Each chromatogram exhibited peaks corresponding to the common nucleosides present and a much later eluting peak corresponding to one of the two isomeric dimers of d[Tp($NHCH_2CH_2SSCH_2CH_2NHCOCPh_3$)T] (Fig. 2a, 2b). The dTpT dimer containing the linker was prepared as a standard essentially as described previously[6b] but using N-

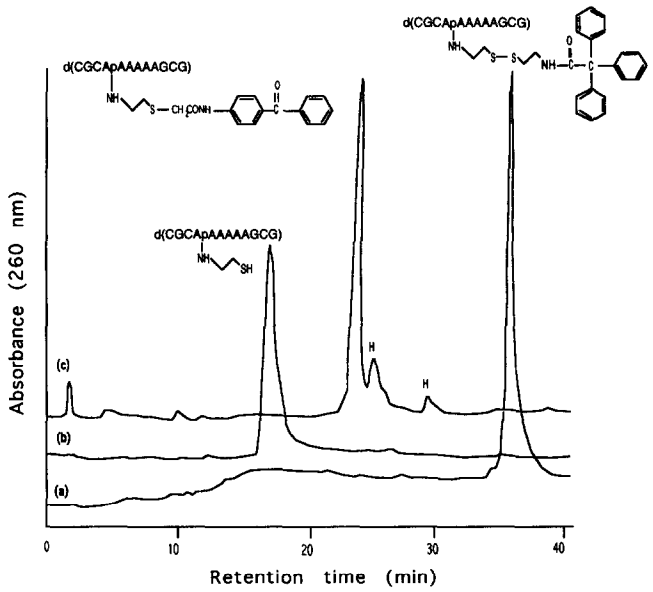

Figure 3. (a) Analysis of dodecamer (Isomer II) tethering a masked thiol. (b) Analysis of the material in (a) after treatment with DTT.[13] (c) Analysis of the material in (b) after treatment with iodoacetamidobenzophenone (retention time > 40 min). HPLC conditions as described in Figure 1. H = hydrolysis products of iodoacetamidobenzophenone.

triphenylacetylcystamine and this diastereomeric mixture was employed as an HPLC standard (Fig. 2c). Treatment of a portion of each digest with DTT altered the retention time of the later eluting peak to a position corresponding to each diastereomer of d[Tp($NHCH_2CH_2SH$)T] (Fig. 2d, 2e) based upon comparison with the appropriate standard (Fig. 2f).

The thiol-containing tether from either of the DNA diastereomers can be unmasked by reaction with DTT and subsequently reacted with an appropriate reporter group.[13] In one example, a dodecamer (isomer II) containing the masked thiol tether (Fig. 3a) was treated with DTT to reduce the disulfide linkage and generate the free thiol (Fig. 3b). The thiol-containing tether was then reacted with a simple benzophenone photoaffinity agent obtained as the iodoacetamido derivative (Fig. 3c).

ACKNOWLEDGEMENTS

This work was supported by a grant from the NIH (GM37065). LWM thanks the American Cancer Society for a Faculty Research Award (FRA-384).

REFERENCES

1 See: Goodchild, J. *Bioconjugate Chemistry* 1990, *1*, 165-187.

2 See: Sinha, N.D. and Striepke, S. in "Oligonucleotides and Analogues," Eckstein, F., Ed., IRL Press, Oxford, 1991, pp 185-210.

3 See: Ruth, J.L. in "Oligonucleotides and Analogues," Eckstein, F., Ed., IRL Press, Oxford, 1991, pp 255-282.

4 For a review see: Conway, N.E., Fidanza, J.A., O'Donnell, M.J., Narekian, N.D., Ozaki, H. and McLaughlin, L.W. in "Oligonucleotides and Analogues," Eckstein, F., Ed., IRL Press, Oxford, 1991, pp 211-240.

5 (a) Letsinger, R.L. and Schott, M.E., *J. Am. Chem. Soc.* 1981, *103*, 7394-7398l, (b) Yamana, K. and Letsinger, R.L., *Nucl. Acids Symp. Ser.* 1985, *16*, 169-173, (c) Letsinger, R.L., Bach, S.A. and Eadie, J.S. *Nucl. Acids. Res.* 1986, 14, 3487-3497. (d) Jäger, A., Levy, M.H. and Hecht, S. M. *Biochemistry* 1988, *27*, 7237-7246, (e) Letsinger, R.L., Guangrong, Z., Sun, D.K., Ikeuchi, T. and Sarin, P. *Proc. Natl. Acad. Sci. USA*, 1989, *86*, 6553.

6 (a) Agrawal, S. and Tang, J.-Y., *Tetrahedron Let.* 1990, *31*, 1543-1546, (b) Fidanza, J.A. and McLaughlin, L.W. *J. Org. Chem.* 1992, *57*, 2340-2346..

7 Fidanza, J.A and McLaughlin, L.W. *J. Am. Chem. Soc.* 1989, *111*, 9117-9120, (b) Agrawal, S. and Zamecnik, P.C. *Nucleic Acids Res.* 1990, *18*, 5419-5426, (c) Fidanza, J.A., Ozaki, H. and McLaughlin, L.W. *J. Am. Chem. Soc.* 1992, *114*, 5509-5517.

8 To a 20 mL solution of triphenylacetic acid (2.60 g; 9.0 mmol) and 1-hydroxy-benzotriazole (HOBT) (1.25 g; 9.2 mmol) was added a 5 mL solution of dicyclohexylcarbodiimide (DCC) (2.06 g; 10.0 mmol). The mixture was stirred for 1 h at room temperature, followed by the addition of a 5 mL solution of cystamine (1.00 g; 4.4 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (1.37 g; 9.0 mmol). The reaction was allowed to stir overnight. The solvent was evaporated, the sample triturated in carbon tetrachloride, and the mixture filtered. The filtrate was evaporated and the N,N'-bistriphenylacetylcystamine was crystallized from methanol (2.90 g, yield - 94%).

9 Cystamine dihydrochloride (1.89 g; 8.4 mmol) and sodium hydroxide (0.32 g; 8.0 mmol) were dissolved in 20 mL methanol and 3 mL water. This solution was added to N,N'-bistriphenylacetylcystamine (2.90 g; 4.2 mmol) followed by 20 mL tetrahydrofuran (THF). Nitrogen was bubbled through the solution for ten minutes after which aminoethanethiol (0.06 g; 0.5 mmol) was added. The reaction was stirred overnight and was purified by chromatography. N-triphenylacetylcystamine was converted to its hydrochloride salt and azeotroped from toluene for storage (1.64 g; 3.6 mmol, yield - 43%).

10 (a) Atherton, F.R., Openshaw, H.T. and Todd, A.R. *J. Chem. Soc.* 1945, 660-663, (b) Blackburn, G.M., Cohen, J.S. and Todd, A.R. *J. Chem. Soc.* 1966, 239-245.

11 Employing a 4.6 x 250 mm column of ODS-Hypersil, 20 mM potassium phosphate (pH 5.5) and a gradient of methanol: 0-70% in 1 h.

12 To 1 A_{260} unit of the oligomer in 25 mM sodium acetate, pH 5.3 was added 2 units of nuclease P1 and the solution incubated for 1 h at 37 °C. The mixture was then rebuffered to pH 8.0 using 200 mM Tris-HCl, 20 mM $MgCl_2$, and 3 units of snake venom phosphodiesterase and 2 units of calf intestine alkaline phosphatase were added and the reaction incubated at 37 °C for 3 h.

13 To 0.8 A_{260} units (~6 nmol, 0.50 mM) of Isomer II of the dodecamer d[CGCA($NHCH_2CH_2SSCH_2CH_2NHCOCPh_3$)AAA-AAGCG] was added DTT (~60 nmol, 5.0 mM) at pH 8.0 and the reaction was heated at 50 °C for 1.5 h. HPLC analysis indicated complete cleavage of the disulfide. To this mixture was added benzophenone-4-iodoacetamide (~325 nmol, 10.5 mM) and the reaction mixture was incubated 2.5 h at 50 °C.

(*Received* 16 *September* 1993)