Use of Electrophilic Substitution to Form Site-Specific Cross-Links in DNA

This unit describes the incorporation of a nucleophilic base into an oligodeoxyribonucleotide (ODN), and the use of electrophilic tethers to convert this ODN into an electrophilic probe capable of cross-linking a complimentary DNA strand (see Basic Protocol). The postsynthetic method that generates the electrophilic ODN allows the introduction of functionality not compatible with standard solid-phase synthetic conditions. It also allows the generation of a variety of different electrophilic DNA strands from the same deoxythiouridine-containing ODN by changing the nature of the electrophilic tether. In addition, the site of modification places the tether in intimate contact with the major groove, allowing structural features of the groove to aid in directing the electrophilic attack. The synthesis of the electrophile, *N*,*N*′-bis-bromoacetyl-1,2-diaminobenzene, is also described in this unit (see Support Protocol).

BASIC PROTOCOL

FORMATION OF SITE-SPECIFIC CROSS-LINKS IN DNA

The nucleophilic base used in these studies, 4-thio-2′-deoxyuridine ($d^{S4}U$), is incorporated into an ODN using an S-(2-cyanoethyl)-protected phosphoramidite and standard solid-phase synthesis conditions and reagents (Coleman and Siedlecki, 1991). The DNA is left on the column, and the S-cyanoethyl group is removed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dry acetonitrile. The ODN is then cleaved from the column and purified by a two-step process involving an initial trityl-on purification followed by a trityl-off purification. Cross-linking is performed by derivatizing the modified ODN with the desired electrophilic tether and incubating this probe with a ^{32}P-labeled target (Fig. 5.5.1; Coleman and Kesicki, 1995; Coleman and Pires, 1997).

Materials

S-(2-Cyanoethyl)-protected 4-thio-2′-deoxyuridine (Glen Research)
Dry solvent-grade acetonitrile
1.0 M DBU solution (see recipe)
50 mM NaSH/NH_4OH solution (see recipe)
Nitrogen gas (optional)
HPLC buffer A (see recipe)
HPLC buffer B: HPLC-grade acetonitrile
80% (v/v) distilled glacial acetic acid
Dry ice

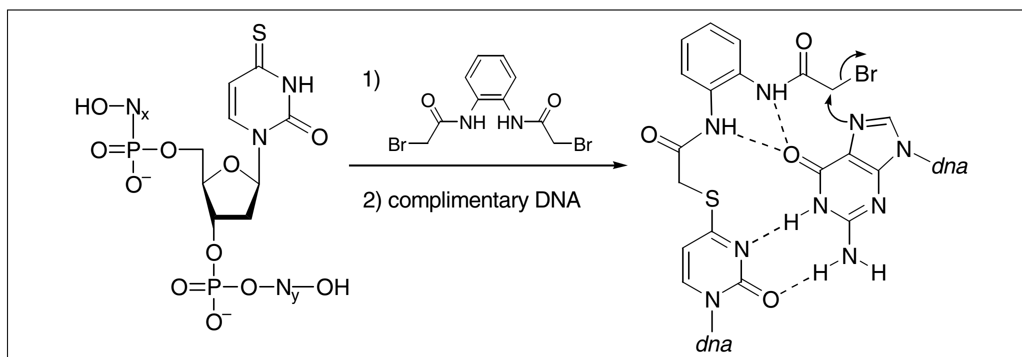

Figure 5.5.1 Cross-linking between 4-thio-2′-deoxyuridine and *N*,*N*′-bis-bromoacetyl-1,2-diaminobenzene.

Contributed by Richard M. Pires and Robert S. Coleman
Current Protocols in Nucleic Acid Chemistry (2001) 5.5.1-5.5.6
Copyright © 2001 by John Wiley & Sons, Inc.

Double-deionized water
2 mg/mL (1.5 U/mg) snake venom phosphodiesterase (SVP) from *Crotalus durissus*
1 U/μL alkaline phosphatase (AP) from calf intestine
0.1 M potassium phosphate buffer, pH 8.0 (*APPENDIX 2A*)
Electrophile: *N*,*N′*-bis-bromoacetyl-1,2-diaminobenzene (see Support Protocol)
Dimethylformamide (DMF)
n-Butanol
Radiolabeled complimentary DNA solution (1 to 5 OD/mL)

DNA synthesizer with bottle (and septum) that fits a phosphoramidite inlet port
Vacuum pump
Desiccator
Centrifugal evaporator
Binary high-performance liquid chromatograph (HPLC) equipped with UV detector and recorder
Polystyrene reversed-phase (PRP-1) HPLC column (Hamilton)
Spectrophotometer
Small spin column containing 0.3 ml Sephadex G-25 equilibrated with double-deionized water

Additional reagents and equipment for DNA synthesis (*APPENDIX 3C*), SVP/AP digestion (*UNIT 10.6*), and denaturing polyacrylamide gel electrophoresis (PAGE; *APPENDIX 3B*)

Synthesize $d^{S4}U$-containing ODN

1. Dissolve 50 mg S-(2-cyanoethyl)-protected 4-thio-2′-deoxyuridine in 2 mL dry solvent-grade acetonitrile in a bottle that fits a phosphoramidite inlet port of a DNA synthesizer.

   Use an empty bottle that previously contained a phosphoramidite standard. Wash and dry bottle thoroughly before reuse.

2. Place a septum on the bottle, and evaporate sample to dryness using a vacuum pump. Repeat this azeotropic drying procedure two times.

3. Add enough dry solvent-grade acetonitrile to prepare a 0.1 M phosphoramidite solution, and place the bottle on phosphoramidite port no. 5 of the synthesizer. Set the synthesizer to perform a standard synthesis (*APPENDIX 3C*), except do not remove the trityl group of the last base, and leave the DNA attached to the column.

   A 200-nmol or 100-μmol synthesis is appropriate.

4. After synthesis is complete, evaporate remaining phosphoramidite solution to dryness as in step 2, and store at −20°C in a desiccator (stable indefinitely).

   This storage method substantially increases the shelf life of the phosphoramidite.

5. Remove the column containing DNA from the synthesizer, and cleave the S-cyanoethyl group by passing 2 mL of 1.0 M DBU solution through the column over 2 hr using the double-syringe technique.

   The double-syringe technique is performed by placing a syringe at each end of the column and passing the reagent back and forth slowly.

6. Flush column two times with 3 mL dry solvent-grade acetonitrile.

   Residual DBU interferes with the purification of DNA.

7. Cleave DNA from column with 5 mL of 50 mM $NaSH/NH_4OH$ solution over 3 hr at room temperature using the double-syringe technique.

8. Allow sample to sit 16 hr at room temperature in 50 mM $NaSH/NH_4OH$ solution to remove the base-protecting groups.

9. Evaporate sample to dryness, either with a stream of nitrogen gas or under vacuum using a centrifugal evaporator.

Purify $d^{S4}U$-containing ODN

10. Redissolve sample in 100 µL HPLC buffer A and inject onto a PRP-1 column. Perform reversed-phase HPLC using 95% HPLC buffer A to 50% HPLC buffer A over 30 min at a flow rate of 1 mL/min.

    NaSH elutes from the column in ~3 min and has a horrible odor. The untritylated failure sequences elute at ~10 min, whereas the full-length tritylated product elutes at ~20 min.

11. Collect purified product and evaporate to dryness using a centrifugal evaporator. Redissolve in 1 mL of 80% distilled glacial acetic acid. Allow sample to sit 20 min at room temperature.

12. Freeze sample using dry ice and evaporate to dryness under vacuum using a centrifugal evaporator.

13. Redissolve sample in 100 µL HPLC buffer A.

    The solution may turn cloudy, because the trityl alcohol produced is relatively insoluble in HPLC buffer A. If it turns cloudy, centrifuge to remove solid material.

14. Inject sample onto the PRP-1 column. Perform reversed-phase HPLC using 92% HPLC buffer A to 75% HPLC buffer A over 30 min at a flow rate of 1 mL/min.

    The purified ODN elutes at ~12 min.

15. Evaporate sample to dryness with a centrifugal evaporator, redissolve in double-deionized water, and determine the concentration by measuring A_{260}.

16. Check sample for correct base composition by SVP/AP digestion (*UNIT 10.6*).

17. Perform HPLC using 95% HPLC buffer A to 90% HPLC buffer A over 12 min at a flow rate of 1 mL/min. Monitor dA, dG, dC, and dT at A_{254}, and monitor $d^{S4}U$ at A_{332}.

Derivatize ODN with N,N′-bis-bromoacetyl-1,2-diaminobenzene

18. Dilute 5 µL $d^{S4}U$-containing ODN stock solution obtained in step 17 to 95 µL with 0.1 M potassium phosphate buffer, pH 8.0.

    The ODN stock solution is usually close to 50 OD/mL.

19. Mix ~1 mg electrophile in 30 µL DMF.

20. Add electrophilic solution to ODN stock solution and allow mixture to sit 1.5 hr at room temperature.

21. Terminate reaction by passing solution through a small spin column containing 0.3 mL Sephadex G-25 equilibrated with double-deionized water.

22. Concentrate sample to dryness by precipitating with *n*-butanol.

23. Place sample briefly under vacuum to remove any trace of *n*-butanol.

    Use derivatized ODN immediately after preparation.

Cross-link target DNA using electrophilic ODN

24. Dissolve electrophilic ODN in 4 µL of 0.1 M potassium phosphate buffer, pH 8.0.

25. Add 2.0 µL radiolabeled complimentary DNA solution.

    The radiolabeled ODN stock solution is usually between 1 and 5 OD/mL.

26. Incubate sample 15 min to 16 hr at room temperature.

    Maximum cross-linking usually requires an overnight incubation.

27. Analyze cross-linking using denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B*).

SUPPORT PROTOCOL

SYNTHESIS OF *N,N′*-BIS-BROMOACETYL-1,2-DIAMINOBENZENE

To synthesize the electrophilic tether, the appropriate diamine reacts with bromoacetyl bromide (Skinner et al., 1967).

Materials

1,2-Diaminobenzene
Nitrogen gas
Tetrahydrofuran (THF), freshly distilled
Triethylamine (TEA), freshly distilled
Bromoacetyl bromide
Saturated aqueous $NaHCO_3$
Ethyl acetate
Saturated NaCl
5% (v/v) HCl
$MgSO_4$
Silica
9:1 (v/v) dichloromethane/methanol (CH_2Cl_2/CH_3OH)

Flame-dried 100-mL flask
3 × 15–cm glass column

Additional reagents and equipment for flash column chromatography (*APPENDIX 3E*)

1. Place 500 mg 1,2-diaminobenzene in a flame-dried 100-mL flask under nitrogen gas.
2. Dissolve diamine in 50 mL freshly distilled THF.
3. Add 1.6 mL freshly distilled TEA.
4. Place flask under nitrogen gas and cool sample 5 min at −78°C.
5. Add 1 mL bromoacetyl bromide with a syringe over a 10-min period.
6. Stir mixture 30 min at −78°C, then 90 min at room temperature.
7. Add 50 mL saturated aqueous $NaHCO_3$ to stop the reaction.
8. Dilute sample with 50 mL ethyl acetate. Extract organic layer with saturated NaCl, then with 5% HCl.
9. Dry organic phase over $MgSO_4$.
10. Evaporate sample onto 1 to 2 g silica for loading, and purify in a 3 × 15–cm glass column by flash column chromatography (*APPENDIX 3E*) using 9:1 CH_2Cl_2/CH_3OH.

    Column-purified samples result in a higher percentage of cross-linking than recrystallized samples. The product should be used immediately.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX.***

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) solution, 1.0 M

Dissolve 15.2 g DBU in 75 mL solvent-grade acetonitrile that has been freshly distilled from CaH_2. Allow DBU to dissolve, then add acetonitrile to 100 mL final volume. Store up to 12 months at 25°C in a desiccator.

HPLC buffer A

Dissolve 69.7 mL triethylamine that has been freshly distilled from CaH_2 in 850 mL double-deionized H_2O. Adjust to pH 6.5 using distilled acetic acid. Adjust volume to 1 L. Then dilute 100 mL to 500 mL (final 0.1 M triethylamine). Store up to 6 months at 5°C.

If acetic acid is not distilled, impurities may appear in the HPLC.

$NaSH/NH_4OH$ solution, 50 mM

Add 0.28 g $NaSH{\cdot}H_2O$ to 75 mL concentrated NH_4OH from a freshly opened bottle. Allow NaSH to dissolve, then add NH_4OH to 100 mL final volume. Store up to 6 months at 4°C.

COMMENTARY

Background Information

$d^{S4}U$ is a versatile modified DNA base that can be used to accomplish a variety of postsynthetic transformations in synthetic ODNs. However, if the 4-thio group is left unprotected during solid-phase synthesis of $d^{S4}U$-containing ODNs, the yield of product is typically near 20% per step. The 2-cyanoethyl group is a convenient protecting group for sulfur that can be readily removed by treatment with a non-nucleophilic base such as DBU. Yields from ODN syntheses utilizing the 2-cyanoethyl protecting group are equivalent to those containing only the four naturally occurring bases (Coleman and Kesicki, 1994). In addition to its reaction with electrophiles, as described above, oxidation-reduction chemistry can be used to form disulfide-constrained hairpins and duplexes. $d^{S4}U$ can also be modified by strong nucleophiles.

Critical Parameters

The location of the 4-thio group near the base-pairing atoms of the duplex places the electrophilic tether in intimate contact with the major groove. Because of this positioning of the tether, the efficiency of cross-linking depends not only on the nucleophilicity of the target base but also on the local steric and electronic environment of the duplex. The steric environment can significantly influence the accessibility of the target to the nucleophile. For instance, when deoxythiouridine is placed in the sequence 5′-d(TAATACGACCXACTATA)-3′ (X = $d^{S4}U$), lower than expected levels of cross-linking to the complimentary target occur (≤35%). Modeling studies indicate that the methyl group of the T that is base paired to the dA in the 3′ direction blocks the approach of electrophiles to N7 of dG in this duplex. This is supported by studies where this T was replaced by dU, resulting in higher levels of cross-linking (Coleman and Kesicki, 1994). The cross-linking efficiency also seems to mirror the local charge density of the major groove. Guanines that are expected to be in a more negative environment based on calculations also appear to cross-link more readily (Coleman and Pires, 1997).

In addition, the linker design is of critical importance in cross-linking efficiency. Considerations important for linker design include the tether geometry, H-bonding ability, and reactivity. The most effective electrophile found has been *N*,*N*′-bis-bromoacetyl-1,2-diaminobenzene. Using the optimal sequence with this electrophile, >90% cross-linking of a duplex can be achieved (Coleman and Pires, 1997).

One final consideration is duplex stability. As the cross-linking occurs in the context of the duplex, factors that destabilize the duplex are expected to reduce the efficiency of cross-linking. Melting studies have shown that substitution of dC with $d^{S4}U$ destabilizes a 17-mer duplex by 9°C. Melting studies on the same 17-mer containing a non-cross-linking model

of the electrophilic tether show a transition temperature that is an additional 3°C lower. Interpretation of data from melting experiments performed on the cross-linked duplex are complicated by the rapid depurination of the target strand that occurs as the temperature is raised, although the duplex appears to be intact up to ≥10°C above the transition temperature for the non-modified duplex (Coleman and Pires, 1997).

Anticipated Results

The percent cross-linking obtained depends on the parameters discussed above. With the appropriate sequence and the most effective bis-electrophile, cross-linking efficiencies of between 80% and 95% are routinely obtained. Cross-linking levels of 30% to 80% have been obtained with less effective bis-electrophiles (e.g., *N*,*N′*-bis-bromoacetyl-1,3-diaminobenzene).

Time Considerations

Synthesis of DNA, including cleavage and deprotection, generally takes 1 day. Purification is limited by the time required to remove solvent from samples, and generally requires 1.5 to 2 days. The cross-linking reaction takes ~2 hr to set up for an overnight reaction. To prepare the electrophilic tether, 3 hr are required to set up and run the reaction, and another 2 hr are needed to purify the product.

Literature Cited

Coleman, R.S. and Kesicki, E.A. 1994. Synthesis and postsynthetic modification of oligodeoxynucleotides containing 4-thio-2′-deoxyuridine ($d^{S4}U$). *J. Am. Chem. Soc.* 116:11636-11642.

Coleman, R.S. and Kesicki, E.A. 1995. Template-directed cross-linking of oligonucleotides: Site specific covalent modification of dG-N7 within duplex DNA. *J. Org. Chem.* 60:6252-6253.

Coleman, R.S. and Pires, R.M. 1997. Covalent cross-linking of duplex DNA using 4-thio-2′-deoxyuridine as a readily modifiable platform for introduction of reactive functionalities into oligonucleotides. *Nucl. Acids Res.* 25:4771-4777.

Coleman, R.S. and Siedlecki, J.M. 1991. Synthesis and stability of S-cyanoethyl protected thiouridine and 2′-deoxy-4-thiouridine. *Tetrahedron Lett.* 32:3033-3034.

Skinner, W.A., Cory, M., Shellenberger, T.E., and Degraw, J.I. 1967. Effect of organic compounds on reproductive processes. V: Alkylating agents derived from aryl-, aralkyl-, and cyclohexylmethylenediamines. *J. Med. Chem.* 10:120-121.

Contributed by Richard M. Pires and Robert S. Coleman
The Ohio State University
Columbus, Ohio