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Syntheses of DNA Duplexes That Contain a N⁴C-alkyl-N⁴C Interstrand Cross-Link

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Abstract

A simple procedure is described for preparing short DNA duplexes that contain a single N⁴C-alkyl-N⁴C interstrand cross-link. The synthesis is carried out on an automated DNA synthesizer using standard phosphoramidite chemistry. The cross-link is introduced during the synthesis of the duplex. The method can be used to prepare mg quantities of cross-linked duplexes suitable for physical studies and for the preparation of larger DNA molecules that can be used as substrates to study DNA repair in whole cell extracts and in living cells in culture.

The N⁴C-alkyl-N⁴C interstrand cross-link serves as a stable mimic of alkyl interstrand cross-links of the type formed by cancer chemotherapeutic agents such as bis-chloroethylnitrosourea or nitrogen mustards. Figure 1 shows the structure of an N⁴C-ethyl-N⁴C cross-link. The ethyl linker connects the two N⁴-exocyclic amino groups of cytosines located on opposite strands of the DNA duplex. Cross-links with longer alkyl chains can also be synthesized. As shown in Figure 1, the cross-link can be inserted into the DNA duplex in a variety of orientations. For example, in duplex **1** the cross-link joins two mismatched C residues that lie directly opposite each other (Webba da Silva, et al., 2002). Duplexes **2** and **3** contain a cross-link that joins C residues in either a -CG- or -GC- sequence respectively (Noronha, et al., 2002). In the case of duplex **2** the N⁴C-ethyl-N⁴C cross-link is completely compatible with the structure of the B-form helix and its presence creates essentially no distortion of the helix. In contrast, the N⁴C-ethyl-N⁴C cross-link in duplex **3** is too short to span the distance between the N⁴C exocyclic amino groups in the -GC- sequence and consequently the presence of this cross-link creates significant helix distortion. The N⁴C-alkyl-N⁴C cross-link in duplex **4** links C residues in the sequence -CNG-, where N can be any of the four nucleotides. In this case the length of alkyl chain affects the structure of the helix (Noronha, et al., 2002). An ethyl linker creates significant bending deformation of the helix, whereas there is essentially no helix bending when the linker is seven carbons in length.

Short DNA duplexes containing a single N⁴C-alkyl-N⁴C interstrand cross-link can be conveniently synthesized on solid supports using an automated DNA synthesizer and standard phosphoramidite chemistry (Noll, et al., 2001). The synthetic strategy, which is outlined schematically at the bottom of Figure 1, builds each arm of the duplex in a stepwise fashion. The N⁴C-alkyl-N⁴C cross-link is introduced during the synthesis by reaction of an O⁴-triazol-deoxyuridine residue, **U**, located at the 5'-terminus of the first arm of the duplex, **6**, with 5'-O-dimethoxytrityl-3'-O-t-butyl dimethylsilyl-N⁴-alkyl-deoxycytidine, **5a**, whose synthesis has been previously described in detail (Noll, et al., 2001). Arms 2 and 3 are then synthesized simultaneously after selective removal of the 5'-dimethoxytrityl (DMT)

protecting group from **7** to give partial duplex **8**. The 3'-t-butyldimethylsilyl (TBS) protecting group is then removed from **8** and arm 4 is synthesized using 3'-O-dimethoxytrityldexoyribonucleoside-5'-O-cyanoethylphosphoramidites. Following removal from the support and deprotection, the cross-linked duplex is purified by strong anion exchange HPLC. The protocols given below describe the preparation of a 10 base pair DNA duplex containing a N⁴C-ethyl-N⁴C interstrand cross-link, but the same procedures can be used to prepare similar duplexes having cross-links with longer alkyl chains.

BASIC PROTOCOL 1

SYNTHESIS OF AN N⁴C-ETHYL-N⁴C CROSS-LINKED DUPLEX

The procedure outlined below describes the synthesis of a 10 base pair DNA duplex that contains a single N⁴C-ethyl-N⁴C interstrand cross-link in a -CG- sequence context, duplex **9** shown in Figure 1. The synthesis is carried out on an automated DNA synthesizer using commercially available protected 5'-O-dimethoxytrityldexoyribonucleoside-3'-O-cyanoethylphosphoramidites and 3'-O-dimethoxytrityldexoyribonucleoside-5'-O-cyanoethylphosphoramidites and employs synthesis columns that have standard Luer fittings at each end of the column. This procedure has also been used to prepare DNA duplexes with interstrand N⁴C-alkyl-N⁴C cross-links in a C-C mismatch orientation and in -GC- or -CNG- sequence contexts.

Materials—Nucleoside derivatized polystyrene support (PS)

Protected 5'-O-dimethoxytrityldexoyribonucleoside-3'-O-cyanoethylphosphoramidites

Protected 3'-O-dimethoxytrityldexoyribonucleoside-5'-O-cyanoethylphosphoramidites

5'-O-dimethoxytrityl-3'-O-t-butyldimethylsilyl-N⁴-alkyl-deoxycytidine

Anhydrous acetonitrile (ACN)

95% Ethanol (C₂H₅OH, EtOH)

Concentrated ammonium hydroxide solution (NH₄OH)

Anhydrous triethylamine (Et₃N, TEA)

Anhydrous pyridine

Triethylamine trihydrofluoride

Tris(hydroxymethyl)aminomethane hydrochloride (Tris)

Sodium chloride

SAX buffer A - 100 mM Tris (pH 7.8) containing 10% (v/v) acetonitrile

SAX buffer B - 0.5 M NaCl in 100 mM Tris (pH 7.8) containing 10% (v/v) acetonitrile

C-18 buffer A - 50 mM sodium phosphate (pH 5.8) containing 2% (v/v) acetonitrile

C-18 buffer B - 50 mM sodium phosphate (pH 5.8) containing 50% (v/v) acetonitrile

Empty oligonucleotide synthesis column

1 mL disposable syringes

Analytical strong anion exchange (SAX) HPLC column

Analytical C-18 reversed phase HPLC column

CAUTION: Acetonitrile, pyridine, triethylamine, triethylamine trihydrofluoride and ammonium hydroxide are toxic. All procedures and reactions employing these materials should be carried out in a fume hood.

Synthesize Arm 1 of the duplex

1. Load 1 μ mole (usually 25 - 40 mg) of nucleoside-derivatized polystyrene support into an empty DNA synthesis column.
2. Prepare 0.15 M solutions of each of the required 5'-O-dimethoxytrityldexoyribonucleoside-3'-O-cyanoethylphosphoramidites, including 5'-dimethoxytrityl-O⁴-triazole-deoxyuridine- 3'-O-cyanoethylphosphoramidite, in anhydrous acetonitrile.
3. Synthesize Arm 1 (**6**, see Figure 1) on the DNA synthesizer. For best results use a coupling time of 120 sec and program the synthesizer to carry out an additional capping step after the oxidation step.
4. Remove 1 mg of support and treat with 400 μ L of a solution containing 95% EtOH/concentrated NH₄OH (1:3 v/v) for 2.5 hrs at 55°C.
5. Remove the supernatant from the support and evaporate to dryness.
6. Analyze the residue by SAX HPLC using a linear gradient of SAX buffer A to SAX buffer B (see chromatogram A, Figure 2).
7. Analyze the residue by C-18 reversed phase HPLC using a linear gradient of C-18 buffer A to C-18 buffer B (see chromatogram A, Figure 3).

Introduce the N⁴C-ethyl-N⁴ C cross-link

1. Transfer the polystyrene support to a 3 mL autosampler vial fitted with a Teflon-lined cap.
2. Add 42 mg (61 μ mol) of 5'-O-dimethoxytrityl-3'-O-t-butyldimethylsilyl-N⁴-ethyl-deoxycytidine (**5a**) and 200 μ L of anhydrous pyridine.
3. Incubate at 45°C for 96 hrs.
4. Dilute the reaction mixture with acetonitrile and transfer the support back to the DNA synthesis column. This transfer can be best accomplished by first fitting the barrel of a 1 mL syringe to a one-holed rubber stopper, which is then fitted to a side-arm filtration flask. Place the empty synthesis DNA synthesis column on the Luer fitting of the syringe barrel and add the slurry of the support to the synthesis column under vacuum.
5. Wash the support with five, 2 mL portions of acetonitrile and dry the support under vacuum.
6. Place the synthesis column on the DNA synthesizer and program the synthesizer to remove the DMT group from oligonucleotide **7**.
7. Remove 1 mg of support and treat with 400 μ L of a solution containing 95% EtOH/concentrated NH₄OH (1:3 v/v) for 2.5 hrs at 55°C.

8. Remove the supernatant from the support and evaporate to dryness.
9. Analyze the residue by SAX HPLC using a linear gradient of SAX buffer A to SAX buffer B (see chromatogram **B**, Figure 2).
10. Analyze the residue by C-18 reversed phase HPLC using a linear gradient of C-18 buffer A to C-18 buffer B (see chromatogram **B**, Figure 3). The retention time of detritylated oligonucleotide **7** is very similar to that of oligonucleotide **6** on the SAX column, but differs significantly from that of **6** on the C-18 reversed phase column due to the presence of the hydrophobic 3'-TBS group.

Synthesize Arms 2 and 3 of the duplex

1. Prepare 0.15 M solutions of each of the required 5'-O-dimethoxytrityldexoyribonucleoside-3'-O-cyanoethylphosphoramidites and include an additional solution that contains only anhydrous acetonitrile.
2. Synthesize Arms 2 and 3 simultaneously (**8**, see Figure 1) on the DNA synthesizer. For best results use a coupling time of 120 sec and program the synthesizer to carry out an additional capping step after the oxidation step. The synthesizer should be programmed to carry out a mock synthesis using anhydrous acetonitrile for the last coupling step. By doing this, a 5'-acetyl group (OAc) will be added to the 5'-ends of Arms 2 and 3 and thus block these ends from further chain extension during the synthesis of Arm 4.
3. Remove 1 mg of support and treat with 400 μ L of a solution containing 95% EtOH/concentrated NH_4OH (1:3 v/v) for 2.5 hrs at 55°C.
4. Remove the supernatant and evaporate to dryness.
5. Analyze the residue by SAX HPLC using a linear gradient of SAX buffer A to SAX buffer B (see chromatogram **C**, Figure 2).

Remove the 3'-O-t-butyldimethylsilyl (TBS) group

1. Remove the DNA synthesis column from the synthesizer and dry the support in the column under vacuum. This can be easily done by attaching the column to a vacuum line for several minutes.
2. Attach an empty 1 mL syringe to one end of the synthesis column.
3. Fill another 1 mL syringe with anhydrous TEA and attach it to the other end of the column.
4. Fill the synthesis column with TEA and pass the solution back and forth several times between the two syringes in order to ensure that the support is completely immersed in TEA. CAUTION: Wear gloves and protective eyewear, and work in the fume hood when carrying out this step.
5. Incubate the TEA-filled column at room temperature for 16 hrs.
6. Remove the 1 mL syringes and use a 10 mL disposable syringe to flush the TEA from the column.
7. Use a 10 mL disposable syringe to wash the support in the column with two, 10 mL portions of dry acetonitrile.
8. Dry the support in the column under vacuum.
9. Use 1 mL syringes as described above to treat the support with 1 mL of triethylamine trihydrofluoride for 3 hrs at room temperature. CAUTION: Wear

gloves and protective eyewear, and work in the fume hood when carrying out this step.

10. Use a 10 mL syringe to flush the triethylamine trihydrofluoride from the support and to wash the support with two, 10 mL portions of dry acetonitrile.
11. Dry the support in the column under vacuum.

Synthesize Arm 4 of the duplex

1. Prepare 0.30 M solutions of each of the required 3'-O-dimethoxytrityldexoyribonucleoside-5'-O-cyanoethylphosphoramidites.
2. Synthesize Arm 4 (9, see Figure 1) on the DNA synthesizer. For best results use a coupling time of 120 sec and program the synthesizer to carry out an additional capping step after the oxidation step.
3. Remove all of the support from the column and transfer to a 3 mL autosampler vial fitted with a Teflon-lined cap.
4. Treat with 400 μ L of a solution containing 95% EtOH/concentrated NH_4OH (1:3 v/v) for 3.5 hrs at 55°C.
5. Remove the supernatant from the support and wash the support with five, 200 μ L aliquots of 50% aqueous acetonitrile.
6. Combine the supernatant and washings, and evaporate to dryness under vacuum.
7. Dissolve the residue in 500 μ L of 50% aqueous acetonitrile.
8. Analyze an aliquot of the solution by SAX HPLC using a linear gradient of SAX buffer A to SAX buffer B (see chromatogram D, Figure 2).

BASIC PROTOCOL 2

PURIFICATION OF THE CROSS-LINKED DUPLEX

The cross-linked duplex can be readily purified by high performance liquid chromatography employing a strong anion exchange column. Because the capacity of these columns is relatively high, an analytical column is adequate for purifying cross-linked duplexes that have been synthesized on a 1 μ mol scale. A linear sodium chloride gradient is used to separate the cross-linked duplex from shorter failure sequences and a reversed phase desalting column is used to remove buffer from the purified duplex.

Materials—SAX buffer A - 100 mM Tris (pH 7.8) containing 10% (v/v) acetonitrile

SAX buffer B - 0.5 M NaCl in 100 mM Tris (pH 7.8) containing 10% (v/v) acetonitrile

C-18 buffer A - 50 mM sodium phosphate (pH 5.8) containing 2% (v/v) acetonitrile

Acetonitrile

Deionized water

50% (v/v) aqueous acetonitrile

Analytical strong anion exchange (SAX) HPLC column

C-18 reversed phase desalting column or cartridge

Purify the cross-linked duplex by SAX HPLC

1. Evaporate aliquots containing 20 OD₂₆₀ units each of the crude cross-linked duplex and dissolve each residue in 200 μ L of SAX buffer A.
2. Inject each sample onto the analytical SAX HPLC column, elute the column with a linear gradient of SAX buffer A to SAX buffer B, and monitor the column at 290 nm.
3. Collect the product and combine fractions from each HPLC run that contain the pure cross-linked duplex.
4. Evaporate the combined fractions on a vacuum centrifuge (Speed Vac) and dissolve the residue in 3 mL of C-18 buffer A.
5. Pre-equilibrate a C-18 reversed phase desalting column or cartridge with the following solutions: 3 mL of acetonitrile, 3 mL of 50% aqueous acetonitrile; and 3 mL of C-18 buffer A.
6. Pass the solution containing the cross-linked duplex obtained from step 4 through the desalting column and collect the flow-through.
7. Pass the flow-through back through the desalting column two more times. Passing the flow-through over the desalting column ensures that all of the cross-linked duplex is absorbed by the column.
8. Wash the column with three, 3 mL aliquots of deionized water.
9. Elute the cross-linked duplex from the column with 3 mL of 50% aqueous acetonitrile.
10. Measure the absorbance of the solution using a UV spectrophotometer and determine the total amount of material obtained.
11. Store the cross-linked duplex in 50% aqueous acetonitrile at 4°C.

COMMENTARY

Background Information

A variety of bifunctional agents such as nitrogen mustards, bis-chloroethylnitrosourea, mitomycin C and cisplatin can react with bases in complementary strands of DNA to form interstrand cross-links (Rajski and Williams, 1998, Noll, et al., 2004, Noll, et al., 2006). The presence of an interstrand cross-link acts as an absolute block to both DNA replication and transcription, and if left unrepaired triggers the cell to undergo apoptosis. This process forms the mechanistic basis for many of the cancer chemotherapeutic agents currently used today. It is also becoming evident that DNA interstrand cross-links can be formed in cells as a consequence of reactions with the products of lipid peroxidation such as malondialdehyde and other electrophilic aldehydes. The formation of such cross-links has been implicated in premature aging syndromes and in neurodegenerative disease.

There has been considerable interest in studying the mechanisms by which DNA interstrand cross-links are repaired (Guainazzi and Scharer, 2010, Hlavin, et al., 2010, Muniandy, et al., 2010). Furthermore, it is becoming evident that the structure of the interstrand cross-link and its effect on the structure of the DNA helix are important parameters to consider when studying the mechanism of such repair (Smeaton, et al., 2008, Smeaton, et al., 2009, Hlavin, et al., 2010). Because reactions of bifunctional agents with DNA often create side products, such as monoadducts and abasic sites, in addition to the desired interstrand cross-link, synthetic methods are required that allow the preparation of DNA molecules that contain a single defined interstrand cross-link. The procedures described above enable the preparation

of mg quantities of short, blunt-ended DNA duplexes containing a single N^4C -alkyl- N^4C interstrand cross-link. These quantities are sufficient for studying the effects of the cross-link on the structure of the DNA helix by methods such as high resolution nmr spectroscopy (Webba da Silva, et al., 2002, Noll, et al., 2005) and by X-ray crystallography (Swenson, et al., 2007).

Although not described here, the same synthetic procedures can be used to prepare short DNA cross-linked duplexes that have 3'-overhanging ends with unique sequences. Such ends enable ligation of the cross-linked duplex to other duplexes to create longer, linear DNA molecules or to be ligated into DNA reporter plasmids. DNA molecules of these types provide substrates that can be used to study DNA repair both in whole cell extracts (Smeaton, et al., 2008, Smeaton, et al., 2009) and in living cells in culture (Hlavin, et al., 2010).

Critical Parameters

As is the case with any procedure involving phosphoramidite chemistry, it is absolutely imperative to maintain anhydrous conditions during the synthesis of the various arms of the duplex. Although suitably dry acetonitrile is commercially available and is adequate for general washing procedures during the synthesis, we have found that it is helpful to use dry acetonitrile that has been stored over calcium hydride to prepare the phosphoramidite solutions that go on the synthesizer. In this regard, we only prepare enough of the phosphoramidite solution to carry out the synthesis at hand and we discard any unused solution. Furthermore, we have found that using phosphoramidites at a concentration of 0.15 M in the case of the nucleoside-3'-O-cyanoethylphosphoramidites and 0.3 M in the case of the nucleoside-5'-O-phosphoramidites helps to ensure maximum coupling yields. These concentrations are somewhat higher than those recommended by the manufacturers of nucleoside phosphoramidites. Finally, we have found that adding an additional capping step after the oxidation step facilitates coupling yields. We believe this additional capping step, which involves exposure of the support to acetic anhydride, helps to remove residual moisture from the support that remains after the aqueous iodine oxidation step.

The cross-link conversion step (6 to 7, see Figure 1) proceeds essentially quantitatively when arm 1 is 8 nucleotides or less in length. However, we have found that the yield of this conversion reaction drops off rather dramatically as the length of arm 1 increases beyond 8 nucleotides. The reason for this is not clear.

Treatment of oligonucleotide **8** with triethylamine prior to removal of the TBS protecting group is critical for maintaining good yields. This treatment removes the phosphate cyanoethyl groups thus preventing subsequent cleavage of the internucleotide bonds at the side of the cross-link when the TBS group is removed. It is important to use anhydrous triethylamine for this step in order to avoid cleaving the oligonucleotide from the support.

The use of triethylamine trihydrofluoride results in essentially quantitative removal of the TBS group from oligonucleotide **8**. This reagent is much more efficient than is the more commonly used silyl deprotection reagent, tetra-*n*-butylammonium fluoride. However, triethylamine trihydrofluoride dissolves controlled pore glass, the support that is usually used for oligonucleotide synthesis. Therefore it is essential to carry out the synthesis on a polystyrene support, a material that is impervious to dissolution by triethylamine trihydrofluoride.

Although not absolutely necessary, we have found that it is very helpful to follow the progress of the synthesis by using either SAX or C-18 reversed phase HPLC to analyze the products after each step. Although such analyses add to the time required to complete the

synthesis, doing so simplifies identification of the final product and also flags steps that have not gone to completion.

Anticipated Results

The yield of a purified, 10 base pair cross-linked duplex from a 1 μ mol scale synthesis is typically 25 -50 OD₂₆₀ units, or approximately 1 to 2 mg of duplex. This amount is more than sufficient for preparing longer DNA duplexes and reporter plasmids for biochemical and biological studies. If larger amounts of cross-linked duplex are required for physical studies, multiple 1 μ mol scale reactions can be run on DNA synthesizers that are capable of simultaneously carrying out multiple syntheses. We have not tried to scale up the procedure beyond 1 μ mol.

Time Considerations

The lengthiest step in the procedure is the conversion reaction that creates the interstrand cross-link (6 to 7), which requires 96 hrs to complete. To reduce “dead time”, it is advisable to start this reaction in such a manner that it includes incubation over the weekend. The synthesis of each arm and subsequent deprotection and HPLC analysis can be accomplished in approximately one day (per arm). Purification and desalting of the duplex generally takes one day. All told, the preparation of the interstrand cross-linked duplex requires approximately 9 days.

Acknowledgments

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Figure 1.

Structure of the N^4C -ethyl- N^4C interstrand cross-link. The wavy lines indicate the two strands of the DNA helix. The cross-link can be inserted in the DNA duplex as a C-C mismatch (**1**); in a -CG- sequence (**2**); in a -GC- sequence (**3**); or in a -GNC- sequence (**4**) where N is A, G, C or T. The reagents used to prepare the interstrand cross-link are: 5'-O-dimethoxytrityl-3'-O-t-butyldimethylsilyl- N^4 -aminoalkyl-2'-deoxycytidine (**5a**), where DMT and TBS are dimethoxytrityl and t-butyldimethylsilyl protecting groups, and 5'-dimethoxytrityl-O4-triazole-deoxyuridine-3'-O-cyanoethylphosphoramidite (**5b**). Steps in the preparation of the cross-linked duplex on the polystyrene support (**PS**) include: synthesis of arm 1 (**6**), where $\underline{U}$ is O4-triazole-deoxyuridine; introduction of the N^4C -alkyl- N^4C interstrand cross-link (**7**); simultaneous synthesis of arms 2 and 3 (**8**); and synthesis of arm 4 (**9**).

Figure 2.

Strong anion exchange (SAX) HPLC analyses of deprotected oligonucleotide intermediates **6** (panel A), detritylated **7** (panel B) and **8** (panel C) created during the synthesis of the N⁴C-ethyl-N⁴C cross-linked duplex **9** (panel D). For each chromatogram, the 0.46 × 25 cm SAX column was eluted with a 30 mL linear gradient of 0.0 M to 0.5 M sodium chloride in 100 mM Tris buffer (pH 7.8) containing 10% acetonitrile at flow rate of 1.0 mL/min. The column was monitored at 260 nm and the X-axis shows time in min.

Figure 3.

C-18 reversed phase HPLC analyses of deprotected oligonucleotide intermediates **6** (panel A) and detritylated **7** (panel B). For each chromatogram, the 0.46×15 cm C-18 reversed phase column was eluted with a 20 mL linear gradient of 2% to 50% acetonitrile in 50 mM sodium phosphate buffer (pH 5.8) at flow rate of 1.0 mL/min. The column was monitored at 260 nm and the X-axis shows time in min.