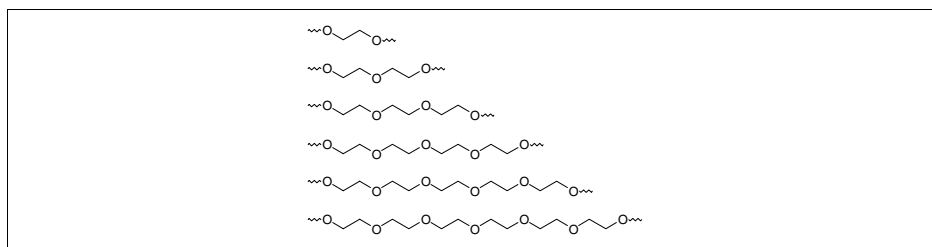


# Engineering Specific Cross-Links in Nucleic Acids Using Glycol Linkers

Simple glycol linkers can be used to cross-link nucleic acid sequences. In the most straightforward approach, such cross-links can be used in place of nucleotide sequences to bridge two domains of higher-order nucleic acid structures. Such linkers can also be viewed as tethers between two independently hybridizing nucleic acid sequences, or between a nucleic acid and some other ligand or reporter group. Although most any carbon chain can be employed to introduce cross-links in nucleic acids, the hydrophilic nature of the ethylene glycol chain gives it one particular advantage. Whereas simple carbon chains may tend to collapse on themselves as the result of the hydrophobic effect, the glycol chains' alternating ethyl and oxygen ether subunits are more likely to be hydrated in aqueous solutions and thus maintain a more extended conformation, which permits them to easily bridge two different sites within the macromolecule. Additionally, a variety of ethylene glycol-based linkers are readily available (Fig. 5.3.1) and only require simple protection reactions in order to be used as cross-linking agents.

Oligo(ethylene glycol) linkers have been used most commonly to replace a portion (Williams and Hall, 1996) or the entirety of the loop structure at the end of DNA (Durand et al., 1990; Altmann et al., 1995) or RNA helices (Benseler et al., 1993; Ma et al., 1993; Thomson et al., 1993; Fu et al., 1994; Hendry et al., 1994; Komatsu et al., 1996), essentially to achieve cross-linking of the terminal residues of the double-stranded helix. However, in some cases ethylene glycol linkers have been used to tether different strands of nucleic acids (Cload and Schepartz, 1991; Amaratunga and Lohman, 1993; Moses and Schepartz, 1996) or even to tether minor groove-binding ligands to the nucleic acid (Robles et al., 1996; Rajur et al., 1997; Robles and McLaughlin, 1997). In most cases, the glycol linker is incorporated as part of the nucleic acid backbone, such that at each terminus the linker is incorporated into a phosphodiester linkage that also incorporates either the 3' or 5' hydroxyl of the adjacent nucleoside residue. It is also possible to incorporate more than a single linker at the same site. Thus, two residues of tri(ethylene glycol) could be used instead of hexa(ethylene glycol) (Benseler et al., 1993; Fu et al., 1994)—in the former case a negatively charged phosphodiester would bridge the two linkers. This approach can be used to generate structures with varying linker lengths via the preparation of only a single linker building block.

In the most common protocol, the linker is protected at one terminus as the 4,4'-dimethoxytrityl derivative (see Basic Protocol 1), and is converted to a phosphoramidite at the second terminus (see Basic Protocol 2). With such derivatives, the linker is simply incorporated into the DNA or RNA sequence by the same procedures as are used for common nucleoside phosphoramidites (see Basic Protocol 3). Preparation of the pro-



**Figure 5.3.1** Varying lengths for readily available ethylene glycol-based linkers.

Contributed by Timothy O'Dea and Larry W. McLaughlin  
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**BASIC  
PROTOCOL 1**

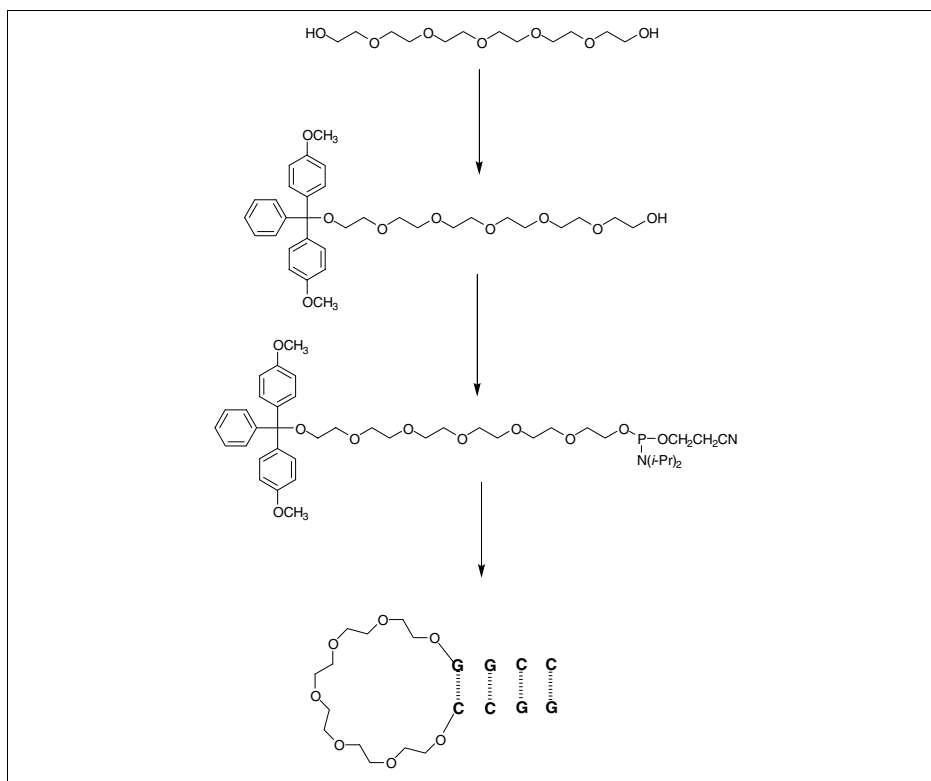
ected linker-phosphoramidites follows a common procedure regardless of length; protocols for the hexa(ethylene glycol) linker are presented here.

**PROTECTION OF THE GLYCOL CHAIN WITH A TRITYL GROUP**

The following protocol outlines the protection of one terminus of an ethylene glycol chain with a trityl group. The first reaction, illustrated in Figure 5.3.2, promotes monoprotection of the ethylene glycol chain with 4,4'-dimethoxytrityl chloride. Although the specific protocol for hexa(ethylene glycol) follows, this protocol has also been successful with glycol chains of various lengths: 1,3-propanediol, tri(ethylene glycol), the tetra- and penta-compounds, and so on. The monoprotected ethylene glycol product can be purified by silica-gel column chromatography.

**Materials**

- Hexa(ethylene glycol) (HEG)
- Anhydrous pyridine (preferably freshly distilled)
- Nitrogen or argon gas
- 4,4'-Dimethoxytrityl chloride (DMT-Cl)
- 5% (v/v) methanol in dichloromethane
- 10% (v/v) aqueous sulfuric acid (H<sub>2</sub>SO<sub>4</sub>; Table A.2A.1)
- Triethylamine (Et<sub>3</sub>N, TEA)
- Dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>, DCM; preferably freshly distilled)
- 5% (w/v) aqueous sodium hydrogen carbonate (NaHCO<sub>3</sub>)
- Sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>)
- Methanol (CH<sub>3</sub>OH, MeOH)



**Figure 5.3.2** Reaction pathway for the preparation of a glycol linker and a sample nucleic acid sequence containing the linker.

Engineering  
Specific  
Cross-Links in  
Nucleic Acids  
Using Glycol  
Linkers

5.3.2

Current Protocols in Nucleic Acid Chemistry

Non-acid-generating desiccant: e.g., sodium hydroxide or calcium carbonate  
100-mL round-bottom flask and rubber stopper  
Device for maintaining nitrogen or argon atmosphere (e.g., balloon, syringe, and rubber stopper; see step 2)  
Needle and syringe  
Separatory funnel  
Silica gel  
Column for chromatography  
Rotary evaporator  
Thin-layer chromatography (TLC) apparatus (see APPENDIX 3D)

**CAUTION:** Pyridine and its vapors are toxic; exposure to pyridine must be minimal. The reaction should be performed in a fume hood.

### **Monoprotect ethylene glycol**

1. Coevaporate 1.25 g (5 eq, 4.43 mmol) HEG twice with ~10 mL anhydrous pyridine in a 100-mL flask.
2. Under an anhydrous nitrogen or argon atmosphere, add 10 mL anhydrous pyridine and a dry stir bar, and seal the 100 mL-flask with a rubber stopper.

*The easiest means to create a nitrogen or argon atmosphere is via a balloon sealed to a syringe with a needle. To construct: Remove plunger from syringe, cut off the now opened end, slip a balloon onto this end, and seal well with parafilm. Fill balloon with gas, attach needle, and punch needle through rubber stopper.*

3. Begin stirring at ambient temperature.
4. In a separate flask under nitrogen, dissolve 300 mg (1 eq, 0.885 mmol) DMT-Cl in ~3 mL anhydrous pyridine.
5. Using a syringe, puncture the rubber stopper and gradually add the DMT-Cl solution to the reaction flask.

*Useful increments are 0.5 mL every 5 min over a 30-min period.*

*The reaction can be monitored by TLC (silica gel, 60 Å, see APPENDIX 3D) using 5% methanol in DCM as eluant. The  $R_f$  is 0.45. The product is visible under UV and turns orange when reacted with 10% aqueous  $H_2SO_4$ .*

6. After 2 hr, add 2 mL TEA and dilute with ~25 mL DCM.

*TEA neutralizes the acid that has been generated, which otherwise will cleave the mono-DMT derivative of the ethylene glycol linker.*

7. Extract the organic layer twice with 5%  $NaHCO_3$  (~40 mL) and once with distilled water (~40 mL) using a separatory funnel.
8. Dry the organic layer over  $Na_2SO_4$  and remove solvent with a rotary evaporator.

*The product remains as a clear or slightly colored oil.*

### **Purify mono-DMT–ethylene glycol product**

9. Pack a silica-gel column (~15 g, roughly 10× expected solute amount), using 0.5% TEA in DCM as eluant.

*Again, TEA reduces the acidic nature of the silica gel, thus reducing decomposition of the mono-DMT–ethylene glycol during chromatography.*

10. Dissolve the mono-DMT–ethylene glycol product (from step 8) in a minimum quantity of DCM/TEA and pour onto the column. Elute with at least 400 mL of 0.5%

TEA in DCM, followed by a step gradient using 400-mL aliquots of 0.5% TEA/DCM containing from 0.5% to 3% MeOH.

*The product will elute in <3% MeOH.*

11. Test fractions by TLC (APPENDIX 3D;  $R_f = 0.45$ ) using 5% MeOH in DCM as the eluant.
12. Combine fractions containing the correct product and remove solvent by rotary evaporation (high vacuum is needed to remove excess TEA in product).
13. Store in a sealed vial at ambient temperature over a desiccant.

*The 4,4'-dimethoxytrityl-protected hexa(ethylene glycol) product (DMT-HEG) is stable for several months with minimal decomposition provided it is not stored over a desiccant that liberates acid (e.g.,  $P_2O_5$ ).*

### PHOSPHITYLATION OF THE MONOPROTECTED GLYCOL LINKER

The following protocol details the phosphitylation of a 4,4'-dimethoxytrityl-protected glycol linker with 2-(cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite. For an efficient reaction with high yield, conditions must be kept scrupulously anhydrous. While the following procedure outlines the use of a monoprotected hexa(ethylene glycol) linker, the protocol has been successful with monoprotected glycol compounds of various lengths. The reaction is illustrated in Figure 5.3.2.

#### Materials

- 4,4'-Dimethoxytrityl-protected hexa(ethylene glycol) (DMT-HEG; see Basic Protocol 1)
- Anhydrous pyridine (preferably freshly distilled; UNIT 3.2)
- Non-acid-generating desiccant: e.g., sodium hydroxide or calcium carbonate
- Nitrogen or argon gas
- Anhydrous dichloromethane ( $CH_2Cl_2$ , DCM; preferably freshly distilled)
- Diisopropylethylamine
- 2-(Cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite
- Ethyl acetate
- 10% (v/v) triethylamine ( $Et_3N$ , TEA) in ethyl acetate
- 5% (w/v) aqueous  $NaHCO_3$
- Saturated aqueous NaCl
- Sodium sulfate ( $Na_2SO_4$ )
- 25-mL round-bottom flask and rubber stopper

**CAUTION:** Pyridine and its vapors are toxic; exposure to pyridine must be minimal. The reaction should be performed in a fume hood.

#### Phosphitylate DMT-ethylene glycol

1. Coevaporate 300 mg (1 eq, 0.51 mmol) of DMT-HEG twice with ~10 mL anhydrous pyridine. Place under high vacuum over a non-acid-generating desiccant and leave overnight.
2. In a rubber-stoppered 25-mL round-bottom flask with a dry stir bar under an anhydrous nitrogen or argon atmosphere, dissolve the DMT-HEG in 1 mL anhydrous DCM and 0.22 mL (3 eq, 1.54 mmol, 157 mg) anhydrous diisopropylethylamine.

*A balloon sealed to a syringe provides an easy means to create a nitrogen or argon atmosphere (see Basic Protocol 1, step 2, for details).*

*TEA may be used here as an alternative to diisopropylamine, if preferred.*

3. While stirring, add 0.115 mL (1 eq, 0.51 mmol, 121 mg) 2-(cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite to the reaction flask using a syringe.

*The reaction can be monitored via TLC using 9:1 (v/v) ethyl acetate/TEA as the eluant. The  $R_f$  of DMT-HEG is 0.50 and that of DMT-HEG-phosphoramidite is 0.80. The product is visible under UV and turns orange when treated with 10%  $H_2SO_4$ .*

4. After 25 min, dilute reaction with ~20 mL ethyl acetate.
5. Extract the organic layer twice with 5% aqueous  $NaHCO_3$  and once with saturated aqueous NaCl.
6. Filter organic layer over  $Na_2SO_4$  and evaporate solvent with rotary evaporator.

#### **Purify DMT-ethylene glycol-phosphoramidite**

7. Pack a silica-gel TLC column with 1% (v/v) TEA in ethyl acetate.
8. Elute the product with increasing percentages of TEA (1% to 5%) in ethyl acetate.
9. Test fractions by TLC ( $R_f = 0.80$ ) using 10% TEA in ethyl acetate as the eluant.
10. Combine fractions containing the correct product and remove solvent using rotary evaporator (high vacuum is needed to remove the TEA).
11. Store in a sealed vial at  $-20^\circ C$

*The DMT-HEG-P will remain stable for several weeks.*

### **PREPARATION OF ETHYLENE GLYCOL LINKERS FOR INCORPORATION INTO OLIGONUCLEOTIDES**

The DMT-protected and phosphitylated glycol linkers can be inserted into DNA sequences using standard automated phosphoramidite synthesis. Since the glycol linker is an oil, several preparative steps facilitate its incorporation using an automated synthesizer.

For an overview of oligonucleotide synthesis, see *APPENDIX 3C*.

#### **Materials**

Dimethoxytrityl-protected hexa(ethylene glycol) phosphoramidite (DMT-HEG-P)  
(see Basic Protocol 2)  
Anhydrous dichloromethane ( $CH_2Cl_2$ , DCM; preferably freshly distilled)  
Anhydrous acetonitrile (preferably freshly distilled)  
Bottle from DNA synthesizer, tared

1. Dissolve 266 mg DMT-HEG-P (0.34 mmol) in 1.00 mL anhydrous  $CH_2Cl_2$  under an anhydrous nitrogen or argon atmosphere.

*A balloon sealed to a syringe provides an easy means to create a nitrogen or argon atmosphere (see Basic Protocol 1, step 2, for details).*

2. Using a syringe, transfer 0.100 mL DMT-HEG-P solution to a suitable tared DNA synthesizer bottle.
3. Remove solvent on rotary evaporator and dry under high vacuum overnight.
4. Weigh DNA synthesis bottle to determine exact amount of DMT-HEG-P.
5. Dissolve 24 mg DMT-HEG-P (~30  $\mu$ mol) in 250  $\mu$ L anhydrous acetonitrile.

*Care must be taken to ensure DMT-HEG-P is dissolved completely.*

**BASIC  
PROTOCOL 3**

**Methods for  
Cross-Linking  
Nucleic Acids**

**5.3.5**

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