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-

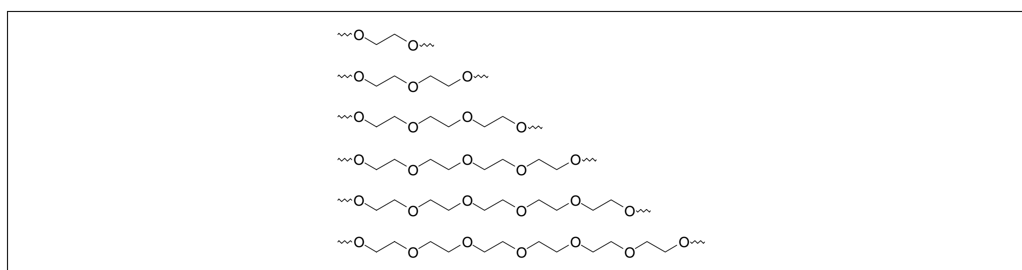

Figure 5.3.1 Varying lengths for readily available ethylene glycol–based linkers.

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

BASIC PROTOCOL 1

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_2SO_4; Table A.2A.1)
Triethylamine (Et_3N, TEA)
Dichloromethane (CH_2Cl_2, DCM; preferably freshly distilled)
5% (w/v) aqueous sodium hydrogen carbonate ($NaHCO_3$)
Sodium sulfate (Na_2SO_4)
Methanol (CH_3OH, MeOH)

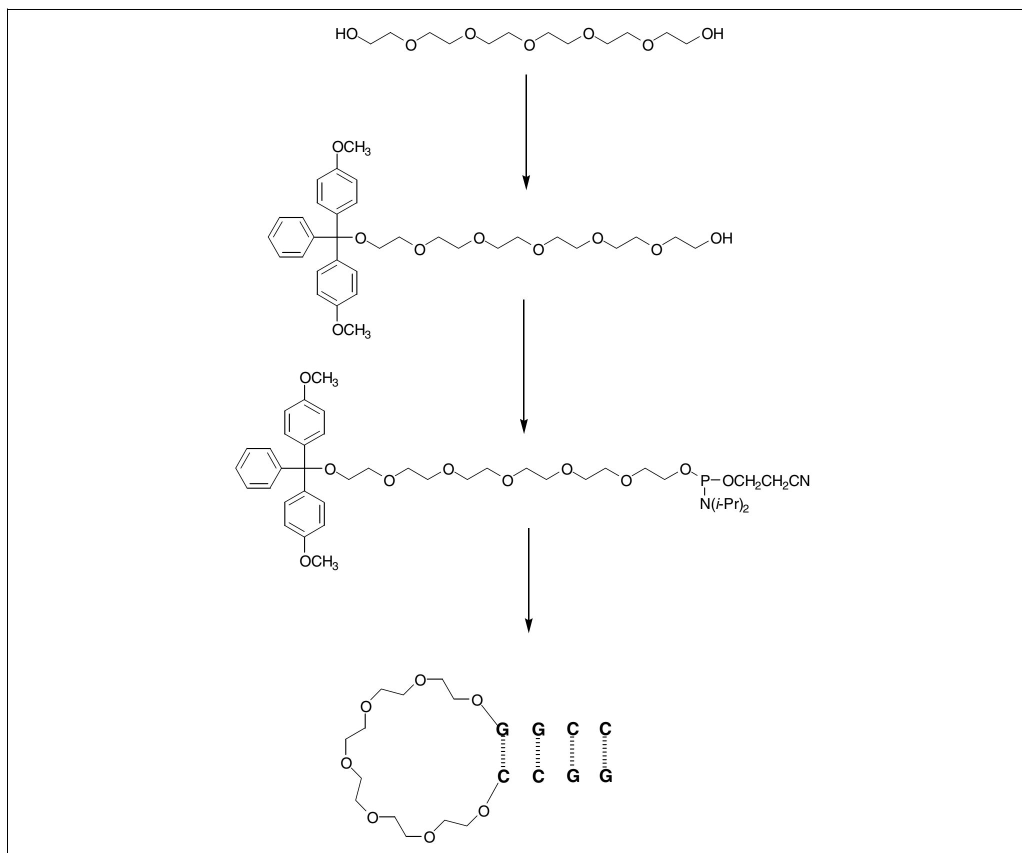

Figure 5.3.2 Reaction pathway for the preparation of a glycol linker and a sample nucleic acid sequence containing the linker.

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).

BASIC PROTOCOL 2

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–phosporamidite

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°C

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

PREPARATION OF ETHYLENE GLYCOL LINKERS FOR INCORPORATION INTO OLIGONUCLEOTIDES

BASIC PROTOCOL 3

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 µmol) in 250 µL anhydrous acetonitrile.

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

6. Place the bottle on the automated DNA synthesizer and purge as recommended by the manufacturer.

7. The ethylene glycol linker can now be incorporated into oligonucleotides by solid-support synthesis using standard phosphoramidite protocols.

   The oligonucleotide can be synthesized with the DMT group on and then purified by HPLC analysis, or with the DMT group off and then purified by gel electrophoresis.

   If poor coupling occurs, see Critical Parameters for possible solutions.

COMMENTARY

Background Information

The cross-linking agents described in this unit are those that are employed during the assembly of the DNA sequences—in this respect they are introduced at very specific sites. Other simple carbon-based linkers can also be employed in a similar manner, but as noted earlier, simple carbon chains may tend to collapse on themselves in an aqueous environment, while the glycol chains are more likely to be hydrated and thus maintain a more extended conformation. Other approaches to cross-linking are also available, most notably the introduction of thiol-based linkers, which upon oxidation form a disulfide cross-link between two sites within a higher-order nucleic acid complex (Ferentz and Verdine, 1991; Wolfe and Verdine, 1993; Goodwin and Glick, 1994; Cain and Glick, 1998; UNITS 5.1 & 5.4).

It has been difficult to design effective protocols to confirm the presence of the linker within the nucleic acid sequence. With other types of modified sequences, DNA digests can often be used to confirm the presence of the modification. In the present case, the linkers are not easily identifiable, and such digests only confirm the presence of the nucleoside components. However, when the linker is present in RNA, it is possible to treat a small quantity of the nucleic acid fragment with T2 RNase and an alkaline phosphatase, which results in cleavage of all linkages save that between the linker and the 5′ terminus of the nucleotide (there is no requisite 2′-OH at this linkage). The resulting nucleoside attached to the linker can then be identified after the appropriate standard is prepared (Fu et al., 1994).

Careful analysis of the digest by HPLC, with the use of an appropriate standard, can confirm the presence of the linker in the sequence of interest (Fu et al., 1994). However, this procedure can be tedious, and requires the preparation of the necessary standard(s). Recent work in the authors' lab (D.J. Fu, G. Xiang, and L. McLaughlin, unpub. observ.) has indicated that MALDI-TOF (UNIT 10.1) analyses of such nucleic acid analogues are much simpler and are effective in providing evidence for the presence of the linker in both DNA and RNA target sequences.

Critical Parameters

The synthesis of these linkers should not present significant problems for anyone with even a moderate level of laboratory experience. As can be noted with the protocol, the glycol linkers tend to be quite inexpensive and are used in excess over the DMT-Cl reagent to ensure that only the mono-protected product results. These reactions can be performed with stoichiometries of 1:1, but usually some of the bis-protected DMT-linker results. This unwanted product can be removed during the purification step.

For these protocols to succeed, the reactions must be performed under anhydrous conditions. Because of DMT-Cl's sensitivity to water, great care must be taken to maintain a dry environment for the reaction. All ethylene glycol reagents should be coevaporated with pyridine and kept under vacuum before use. A nitrogen or argon atmosphere for the reaction helps maintain the anhydrous conditions while the reaction is being run. The simplest apparatus is a balloon fixed to a syringe body and filled with dry argon/nitrogen. The reaction flask is sealed with a rubber septum and a needle affixed to the syringe is pushed through the septum. This simple apparatus keeps the reaction mixture under a slight positive pressure with an anhydrous inert gas.

High yields require that one be aware of the acid lability of the DMT-protecting group. To limit decomposition as a result of trace quantities of acid, a small amount of the organic base triethylamine (TEA) is added during work-up. It is also critical to have some TEA present (~0.5%) during the chromatographic purification step employing a silica-gel column. TEA neutralizes the slight acidity of the silicic acid, promoting greater stability of the product when

it is adsorbed on the column support. The ~0.5% TEA does not significantly alter chromatographic mobility, and its presence results in a greater overall yield of recovered product.

For the phosphitylation protocol, taking into consideration the lability of the phosphoramidite is critical for a successful experiment. Once synthesized, effort must be taken to minimize the exposure of the phosphoramidite to air and acidic compounds of any kind. Both the reagent and the isolated product should be stored in parafilm-sealed vials at –20°C. When the phosphoramidite reagent loses its pale-yellow color and becomes a deeper yellowish-orange color, the reagent has typically degraded and should not be used.

Preparation of the linker for use with a DNA synthesizer is only complicated by the fact that it is an oil rather than a solid. The authors have found the simplest procedure is one in which some of the oil is transferred to a suitable flask, weighed, and dissolved in sufficient anhydrous solvent. From this solution, an aliquot corresponding to ~30 µmol of linker (per coupling) is transferred to the DNA synthesis bottle. The solvent is then removed from the bottle under vacuum and the residue is kept under high vacuum overnight. The requisite amount of acetonitrile can then be added to the bottle before the latter is attached to the DNA synthesis machine—ensure that the residue in the bottle completely dissolves first.

To obtain efficient coupling of the linker to a DNA strand, the synthesizer programming need not to be altered. However, if efficient coupling is not achieved, several parameters can be changed to attain better coupling. First, extended "wait" periods can be added to the cycle—these are typically the time periods during which the coupling reaction takes place. A second option is to perform two coupling steps in sequence without any intervening capping or oxidation steps.

Anticipated Results

Yields for the protection of the glycol linker with DMT should be >70%. When isolated by column chromatography the DMT-ethylene glycol product is a pale-orange oil with R_f = 0.45 (5% MeOH in DCM). The yields expected for the phosphitylation protocol should be >70% when isolated from the column as a clear oil (R_f = 0.8, 1:9 TEA/ethyl acetate). Successful phosphitylation can be achieved without the column chromatography step. In this case, simply perform the aqueous work-up, dry the solution, and evaporate to an oil. ^{31}P NMR will confirm the ratio of the phosphitylated product to any phosphorus contaminants. So long as the latter are minimal in quantity, effective incorporation of the linker can be obtained with material prepared in this manner.

Time Considerations

Monoprotection of ethylene glycol and its isolation can be accomplished in <5 hr. The phosphitylation protocol can be done in <2 hr when the DMT-ethylene glycol product is prepared ahead of time (see Basic Protocol 1). Incorporation of the ethylene glycol linker into the oligonucleotide will not require more than a half-hour beyond the normal coupling time required of a standard phosphoramidite.

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