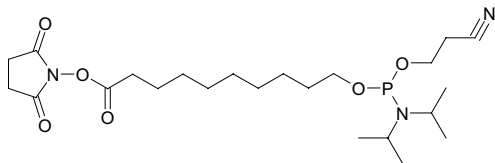


# DADE: A Pre-Activated Carboxyl Linker, Applications and Methods

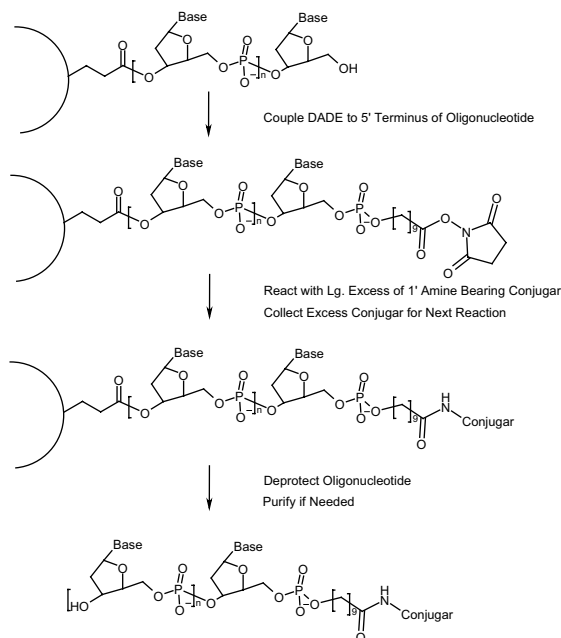
By Richard Hogrefe, Ph.D.; TriLink BioTechnologies

TriLink's DADE (decanoic acid diester) linker (Figure 1) is unique on the market. It offers a novel way of preparing conjugates more economically and with much more flexibility. It was originally designed to allow high throughput screening of a large number of conjugates for therapeutic application. However, it has several other inherent advantages that make it a powerful tool for the scale up of oligonucleotide conjugates as well.



**Figure 1:** Structure of DADE linker

The linker is used as shown in Figure 2. After coupling to the 5' terminus of an oligonucleotide, it is now ready to be coupled to any primary amine. This greatly increases the number of conjugates that can be readily prepared. Previously, either an activated carboxylic acid or maleimidyl derivative had to be laboriously prepared in order to prepare a conjugate to an oligonucleotide with a primary amine or thiol. The only alternative was to react the oligonucleotide with either a heterobifunctional or homobifunctional linker, which added costly steps to the process and was often difficult to reproduce. Now, for the same price as a 5' amino linker, researchers can quickly screen through a number of interesting conjugates that were chemically difficult if not impossible to synthesize and do it in a controlled, single step fashion that enhances the probability of success.



**Figure 2:** A typical application of the DADE linker

The fact that the conjugation chemistry is accomplished while the oligonucleotide is still support bound adds several advantages of its own. The choice of conjugator is often limited by the requirement that some water (20-50%) is needed to dissolve the oligonucleotide in the conjugation mixture. Solid phase conjugations allow the use of completely organic systems, even dichloromethane. This allows the ready conjugation of very lipophilic compounds to oligonucleotides. These fatty conjugators can then act as purification handles for RP-purification of the conjugate.

Another advantage of this system is that the reagent used in excess, the amine, is not affected by the reaction and is therefore recoverable. In fact, the reagent can be used exactly as is for the next reaction, especially if the excess is in the range of 100 fold or more. The use of 1% of the conjugator will not be noticeable kinetically for at least many reactions. At that time the reaction mix can be replenished. Besides the obvious cost savings, this will also improve overall efficiency in that much larger excesses are now feasible even in large-scale conjugations. By driving the reaction further to completion, the need for downstream operations, such as purifications, are reduced or even eliminated.

All in all, DADE offers enough advantages that it should be worthy of consideration for your high throughput or scale-up conjugation operation.

## Sample Applications of DADE

### 1. Conjugation of the Lipid Octadecylamine to a Phosphorothioate

Octadecylamine was conjugated to a phosphorothioate oligonucleotide.

- The DADE linker was coupled to the support bound oligonucleotide (5  $\mu$ mole) using 50  $\mu$ moles as per the synthesis method supplied with the linker. The sulfurization was carried out using 10 equivalents of Beaucage reagent.
- After the linker modified support-bound oligonucleotide was washed and dried, the beads were added to a vial containing 10 equivalents of 1-octadecylamine (stearylamine) dissolved in 2 mL of dichloromethane with 10% triethylamine. The reaction was allowed 4 hours at room temperature with continuous mixing. The beads were then washed well with dichloromethane and dried.
- The oligonucleotide conjugate was then deprotected and cleaved from support using 2 mL of conc. ammonium hydroxide for 5 hours at 65° C, after which time the reagent was decanted and the beads rinsed with 4 mL of 25% acetonitrile in water. The combined solutions were dried *in vacuo*. The resulting residue was reconstituted in 1 mL of 25% acetonitrile in water and the yield determined (965 OD<sub>260</sub> units).
- The crude product was analyzed and purified on a Waters (Milford, MA)  $\mu$ Bondapak C-18 RP-column (4.6 x 305 mm) using a gradient of acetonitrile (5% to 70% over 70 minutes) in 100 mM triethylammonium acetate, pH 7, at a flow rate of 1 mL/min. The conjugation occurred with 80% efficiency. The product was isolated using the same method with injection of 200 OD<sub>260</sub> units of the crude sample per run. The isolated oligonucleotide conjugate was analyzed by electrospray mass

spectroscopy and found to have the correct mass (6585 au). The final yield was 435 OD<sub>260</sub> units; 2.25 μmoles; 45% of overall theoretical yield from the starting scale of 5 μmoles.

### 2. High Throughput Screening of Conjugates

A number of amine bearing conjugates can be rapidly prepared using a variation of the following concept. This method could also be used to screen a number of different conditions to optimize the formation of a conjugate if you already have a chosen one.

a. Prepare a 15 μmole scale synthesis of the oligonucleotide sequence of interest. Remove a very small amount of support from the synthesis column and reseal the column. This sample will be deprotected and serve as the unmodified standard.

b. Couple DADE to the oligonucleotide using the procedures supplied with the reagent. This scale of synthesis will require 100 μmoles of reagent.

c. Dry the support and divide into 15 equal aliquots, approximately 1 μmole each. For most syntheses, this will give the equivalent of 0.3-0.5 μmole of isolatable full-length product.

d. Place the support in 15 separate 2 mL glass screw-cap vials in which the conjugations will be carried out.

e. Dissolve the amines to be conjugated separately in the solvents of choice to a concentration of approximately 0.05 M. The conjugar will not be affected by the reaction and is therefore recoverable, allowing you to use large excesses of the reagent. We commonly will use 100 fold excesses (200 μL of solution). As to preferred solvents, we have successfully carried out conjugations in acetonitrile, dichloromethane (methylene chloride), DMSO, high quality DMF (free of amine contaminants), and water. We add 10% volume of triethylamine to organic reactions, although this may not be necessary when large excesses of conjugar are used, and 10% volume of freshly prepared 0.5 M sodium bicarbonate to aqueous reactions.

f. Add the dissolved conjugars to the vials containing the support. Up to 14 reactions can be done simultaneously from this scale. The 15th will be kept as a retainer. A small aliquot of this material should be deprotected to use as a control during analysis of the oligonucleotide conjugates. Analysis of this material by PAGE or HPLC will also establish the efficiency of the DADE coupling.

g. Place the conjugation reactions on a rotary mixer and allow the reactions at least 4 hours. We recommend overnight reactions to ensure high coupling yields.

h. Decant the reagents from the beads and store for later use. Rinse the beads with fresh solvent, the same as used for the conjugation, followed by a rinse with acetone. Allow the beads to dry, then deprotect with conc. aqueous ammonium hydroxide as usual.

i. The oligonucleotide conjugates are now ready for use if crude samples are sufficient. Of course, they can be purified by HPLC or PAGE at this time. We recommend that the products are all analyzed by PAGE or HPLC and compared to the controls.

### 3. Scale up of Oligonucleotide Conjugates

This process is essentially the same as that described for the high throughput application above. The only difference is scale.

a. Synthesize the sequence of interest on solid support. For this example we will work with a 15 μmole scale synthesis. Remove a very small amount of support from the synthesis column and reseal the column. This sample will be deprotected and serve as the unmodified control.

b. Couple DADE to the oligonucleotide using the procedures supplied with the reagent. This scale of synthesis will require 100 μmoles of reagent.

c. Dissolve 100 fold excess of the amine (1500 μmoles for this example) to be conjugated in the solvent of choice. See Example 1 for ideas regarding solvent choice. Add 10% volume of triethylamine to organic reactions, although this may not be necessary when large excesses of conjugar are used, and 10% volume of freshly prepared 0.5 M sodium bicarbonate to aqueous reactions. Attempt to make your conjugation solution at approximately 0.1 M. A 0.1 M solution will result in 1.5 mL of solution (0.15 mL of which is triethylamine.)

d. Place the support bound oligonucleotide in a vial that can be sealed and is big enough to contain the support and the reaction mix. A small aliquot of this material should be deprotected to use as a control during analysis of the oligonucleotide conjugates. Analysis of this material by PAGE or HPLC will also establish the efficiency of the DADE coupling.

e. Follow steps g, h and i from section 2.

### 4. Preparation of Oligonucleotides with 5' Carboxylic Acid Linkers

Merely using the following deprotection scheme allows the use of DADE to directly prepare 5' COOH linkers on oligonucleotides.

a. Prepare the sequence of interest on solid support in the scale desired. Remove a very small amount of support from the synthesis column and reseal the column. This sample will be deprotected and serve as the unmodified control.

b. Couple the appropriate amount of DADE linker to the oligonucleotide using the procedures supplied with the reagent. DADE can be added to the modified reagent port of any DNA synthesizer like 5' amino linkers or 5' biotin phosphoramidites.

c. Deprotect the oligonucleotide at room temperature for 48 hours with the following reagent:

0.4 M NaOH in Methanol/H<sub>2</sub>O (4:1)

(The time can be reduced if fast deprotecting monomers are used. Refer to the manufacturer of your phosphoramidites for specific deprotection requirements using the 0.4 M NaOH solution if you are uncertain.)

d. Isolate the oligonucleotide by first passing the reaction through the desalting process of your choice, and then purify as usual. The product can also be used crude after desalting.

DADE offers many opportunities to discover new solutions to old problems. TriLink now sells the DADE DNA Synthesis Reagent through Glen Research.