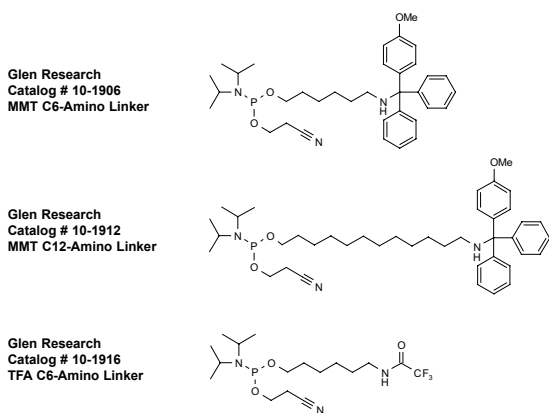


## DNA Linker and Spacer Reagents and Their Utility

By David Combs, Ph.D.; TriLink BioTechnologies

### 5'-Amino Linkers (TFA/MMT)

As DNA-based microarrays and multi-label diagnostic systems increase in their importance, the need for methods to readily prepare functionalized oligonucleotides also grows. Currently the most common modification is a primary amine at the 5'-terminus of the oligonucleotide, which can be introduced using amino-phosphoramidites protected with base labile tri-fluoroacetate (1) or acid labile monomethoxytrityl (2). Both the TFA protected and MMT protected 5'-amino-modifiers can be used in an automated synthesizer or manually coupled (Figure 1). The base labile TFA moiety is employed in cases where the amino-modified oligo is directly isolated from the cleaved and deprotected oligonucleotide. The acid labile MMT protecting group is stable to the basic cleavage and deprotection conditions and can be used as a 'handle' in RP-high performance liquid chromatography to readily isolate the oligonucleotide from failure sequences. The MMT group is subsequently removed using acidic conditions (2). A variety of moieties can be attached to the 5'-primary amine including fluorescent dyes (3,4) biotin (5) and EDTA (6).



**Figure 1:** 5' Amino Linker Structures manufactured by TriLink and sold through Glen Research

### C3, C6 vs C12 Amino Linkers

A wide selection of linker lengths for incorporation between the reactive or diagnostic moiety and the oligonucleotide have been prepared. The shorter carbon chain linkers (C3: (CH<sub>2</sub>)<sub>3</sub>) can be used in instances where the proximity of the oligonucleotide poses no problems. The longer chain linkers (C6 and C12) are typically used when the oligonucleotide must be spaced far enough from the corresponding undesired interactions (7).

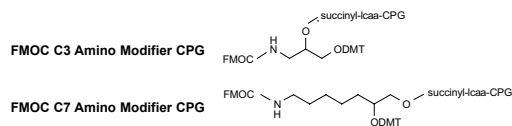
### 3'-Amino-CPG

Introduction of modifications at the 3'-end of an oligonucleotide in concert with various 5'-modifications has become a very powerful tool. One of the more useful modifications at the 3'-end is the introduction of a 3'-amino group which can be used to post-synthetically introduce a variety of products like fluorescent chromophores, biotin, enzymes and polypeptides. In addition to this, some 3'-amino modified oligonucleotides have been found to be more resistant to endonucleases making them candidates for antisense research (8).

Incorporation of a 3'-amino function requires a protecting group that is stable throughout the multi-step oligonucleotide solid phase synthesis. It is also mandatory that the moiety be readily cleaved from the solid support using standard depro-

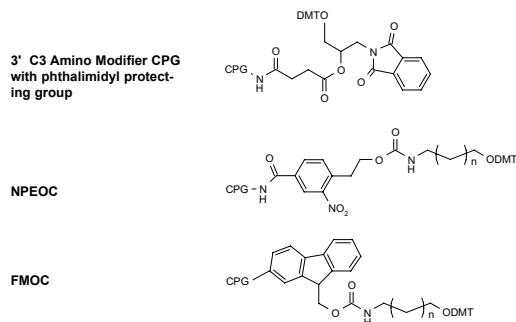
tection conditions. A number of linkers have been developed which introduce the 3'-amino modification.

One of the supports used to introduce the 3'-amino modification is the branched FMOC-protected amino C3 and C7 CPG (Figure 2). The linker is bound to the CPG via a succinate bond through the secondary hydroxyl group while the oligonucleotide is elaborated at the DMT protected hydroxyl group upon removal. A drawback to using this reagent is that the FMOC protecting group is labile to the synthesis conditions of the oligonucleotide, which leads to capping of the amine and reduced yields (9).



**Figure 2:** 3' Amino Linker Structures, FMOC protected

To overcome this inherent problem of the FMOC protection a number of other protecting groups have been developed, with the phthalimidyl derivative giving superior results (8) (Figure 3). However this reagent requires a costly multiple step synthesis.



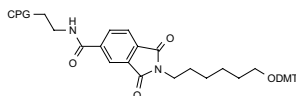
**Figure 3:** Other 3' Amino Linkers

A number of other interesting reagents have been developed using carbamate moieties to attach the linker to the solid supports while acting as a protecting group for the amino functionality (10). The advantage of such a protecting group is that if the protecting group is removed during oligonucleotide synthesis the impurities will be washed away and final purification made considerably easier.

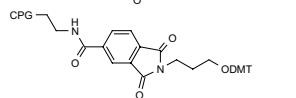
We currently sell a C6 variant of 3'-amino CPG support through Glen Research which was first published several years ago (Figure 4) (11,12) along with a novel C3 analog. These new supports take advantage of the stability of the phthalimidyl group as an amino protecting group with the additional advantage as the bridging unit between the linker and CPG support.

A comparison of the commonly used 3'-amino modifier C7 (purchased from Glen Research, Sterling, VA.) with the phthalimidyl-3'-C6-Amino-CPG was done. It was found that the phthalimidyl-3'-C6-Amino-CPG was a more reliable reagent giving oligo product with a higher conjugation efficiency.

Glen Research  
Catalog # 20-2956  
Pth C6-Amino Linker CPG



Glen Research  
Catalog # 20-2954  
Pth C3-Amino Linker CPG



**Figure 4:** 3' Amino Linker Structures manufactured by TriLink and sold through Glen Research

### 5'-Aldehyde Modification

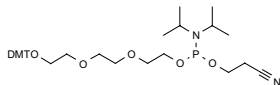
Another approach for introducing reactive functionality at the 5'-end that can be further modified post synthetically is the 5'-aldehyde. In contrast to the 5'-amino and 5'-thiol modifications, which are nucleophilic substitutions, the aldehyde modifier is an electrophilic substitution. The aldehyde moiety can be reacted with a variety of substituted hydrazinos and semicarbazides to form stable hydrazones and semicarbazides, respectively (13). The 5'-Aldehydes can also react with amines to form Schiff's base, but the Schiff's base must be reduced to form a stable linkage.

Two phosphoramidites that incorporate an aldehyde moiety on the 5'-terminus are available. The amidites introduce the aldehyde via a benzaldehyde functional group but vary in the linker length (C6 vs C2). Both aldehyde modifiers are incorporated using standard solid phase synthesis methodologies. However, the 5'-aldehyde modifier C2 is protected and the aldehyde must be freed using detritylation conditions with 80% acetic acid or 2% aqueous trifluoroacetic acid after purification.

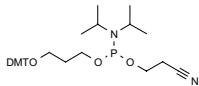
### 5'-Spacers

A number of different spacer phosphoramidites are available (Figure 5). These spacer amidites differ in the number of atoms and are typically used to bridge sections of oligonucleotides. In addition spacers can be used in conjunction with 3' and 5'-amino-modifiers and/or additional spacers to place tags at greater distances from the oligonucleotide and reduces interaction between the oligonucleotide and the fluorescent dye (14). They can also increase hybridization to a support bound oligonucleotide by reducing steric interaction between support and bound oligo (15). The C3 spacer can be used to mimic the three carbon spacing between the 3'- and 5'-hydroxyls of the oligonucleotide (16) or replace a base within a sequence when the base is unknown. The dSpacer can be used to mimic abasic sites within an oligonucleotide and is known to undergo  $\beta$ -elimination reactions and lead to single

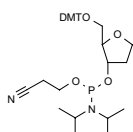
Glen Research  
Catalog # 10-1909  
Spacer 9



Glen Research  
Catalog # 10-1913  
C3/Propyl Linker Amidite



Glen Research  
Catalog # 10-1914  
dSpacer



**Figure 5:** Spacers

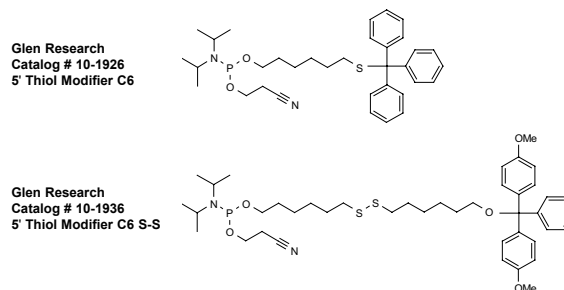
strand scission of DNA (17,18).

### 5'-Thiol-Modifiers

As the number of thiol specific dyes continue to increase so does the interest in 3'- and 5'-thiol modified oligonucleotides. Like the 3'- and 5'- amino modifiers the thiol modification can be used to introduce biotin (19). In addition to this powerful tool, thiols can be conjugated with a variety of fluorescent probes (20) via reactions of the thiol with iodoacetate and maleimide derivatives of the dye to form thioether linkages and enzymes such as horseradish peroxidase, and peptides via a disulfide linkage (21), and bound to metal surfaces (22).

Thiol modification at the 5'-end of the oligonucleotide is typically done using the 5'-thiol-modifier C6 or the 5'-thiol modifier C6 S-S (Figure 6). Like the MMT protected amino linker the trityl protecting group on the 5'-thiol-modifier C6 is usually kept on after cleavage of the oligonucleotide to aid in purification. However, because the trityl group is not as acid labile, unlike MMT or DMT, it must be removed by oxidation with silver nitrate. Although successfully used, oxidative detritylation can be a problem.

An alternative to this reagent is the 5'-thiol-modifier C6 S-S. As with other reagents the trityl protecting group can be kept to aid in purification of the oligonucleotide. The thiol is then freed by treatment with dithiothreitol (DTT).



**Figure 6:** 5' Thiol Linkers

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