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Hydroquinone-O,O'-Diacetic Acid As A More Labile Replacement For Succinic Acid Linkers in Solid-Phase Oligonucleotide Synthesis

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Abstract: Hydroquinone-O,O'-diacetic acid (QDA) can be used to link nucleosides to CPG or polystyrene supports instead of succinic acid. Cleavage of oligodeoxy- or oligoribonucleotides, using ammonium hydroxide, requires only two to five minutes. The QDA linker is stable, easily prepared and does not require any other changes to the reagents, methods, or instrumentation used in automated solid-phase oligonucleotide synthesis. © 1997 Elsevier Science Ltd.

There is a growing need for the fast production of large numbers of oligonucleotides. Biochemical applications now consume more than a million small scale oligonucleotide sequences annually and the growing oligotherapeutic industry will soon require kilogram quantities of oligonucleotides. Consequently, a great deal of attention has been focused on making these products more efficiently. Although faster coupling cycles, improved protecting groups,[1] new solid-phase supports,[2] and higher capacity synthesis instrumentation[3] has been developed, there are still two areas which restrict productivity.

The first area is the time and effort required to manually restart synthesis instrumentation after completion of each batch of oligonucleotides. We are presently working on improvements to the support derivatization chemistry which will alleviate this problem[4] and eventually allow automatic recycling of the solid-phase support. The second bottleneck is the time required to cleave the assembled oligonucleotide from the insoluble support after synthesis. Unlike post-synthesis deprotection,[5] which is performed off the synthesizer, the cleavage step is usually performed by the instrument and subsequent runs cannot be started until cleavage is complete.

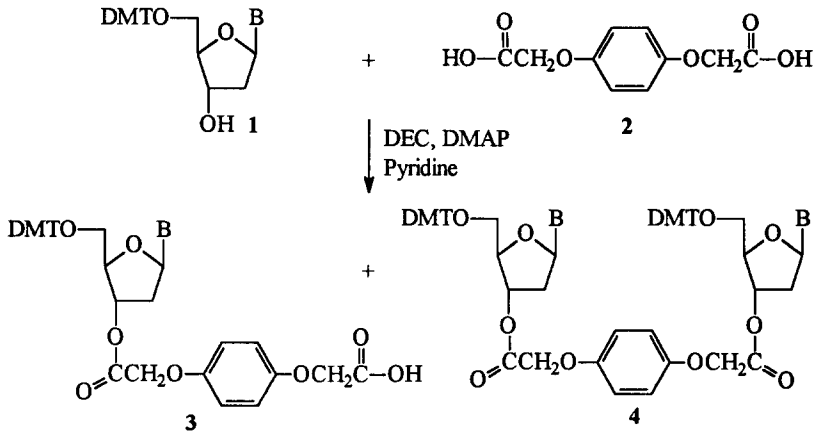
Hydrolysis of commonly used succinic acid linkers with ammonium hydroxide is relatively slow (≥ 1 h)[6] and even a 60 minute cleavage step will be ~30-40% of the total synthesizer time required to produce an average oligonucleotide. Obviously, a faster cleavage would significantly improve productivity. Indeed, to improve throughput, cleavage from the support is sometimes performed manually, off the synthesizer, using either aqueous[7] or gaseous[8] ammonia. However, both labor and chance of error (sample mix-up) are increased.

Faster cleavage reagents (ethanolamine[9] or methylamine[10]) or more easily cleavable linkers (silyl,[11] Fmoc[12] or oxalyl[13]) have been developed. However, the alternative cleavage reagents and the silyl linker are not completely compatible with the preferred protecting groups and there are odour/toxicity problems with the amines, especially when hydrazine is included. The oxalyl linker is a good choice for very base-sensitive sequences but its extreme lability renders it too sensitive for routine use.[14]

We have found that hydroquinone-O,O'-diacetic acid[15] (QDA, **2**), which is more stable than the oxalyl linker but significantly more labile than a succinyl linker is an ideal linker arm. The starting diacid is commercially available and readily coupled onto the 3'-position of protected nucleosides (**1**) by carbodiimides, such as 1-(3-dimethylaminopropyl)ethylcarbodiimide (DEC, Scheme 1). For example, 5'-dimethoxytritylthymidine (2

mmol), QDA (2.4 mmol), DEC (2 mmol), and 4-dimethylaminopyridine (0.2 mmol) in anhydrous pyridine (20 ml) were stirred overnight. The pyridine was concentrated by evaporation, redissolved in $CHCl_3$ (50 ml), and washed successively with water (3X), aqueous NH_4CO_3, and water. Evaporation to dryness yielded a light brown foam containing the product **3** and ~30% dimer **4**, which could be used without further purification. Alternatively, purification by flash chromatography on silica gel using a 0-30% ethanol/ $CHCl_3$ gradient can be performed (52% yield).

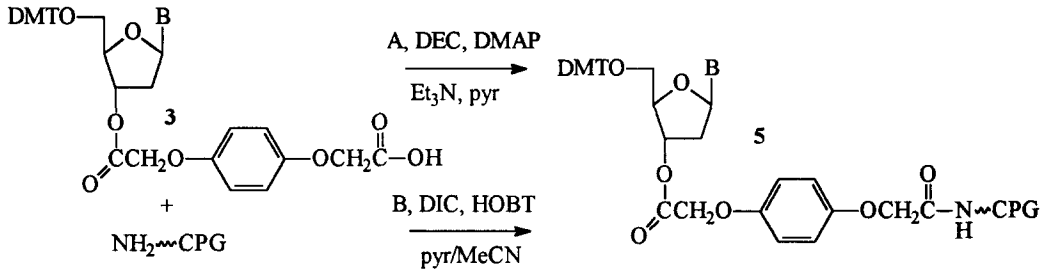
Scheme 1



Derivatization of long chain alkylamine controlled pore glass (LCAA-CPG) beads with **3** was performed in the same way as nucleoside-3'-O-succinates[16,17] (Scheme 2). Although, ~30% dimer (**4**) may be present along with the hemiacetate (**3**) in the unpurified product mixture, the dimer does not interfere with coupling to the support and nucleoside loadings in the optimal 30-40 μmol/g range were obtained. For example (Scheme 2, route A): unpurified nucleoside **3** (0.4 mmol), DEC (1.2 mmol), DMAP (0.2 mmol), and LCAA-CPG (1 g) were combined and shaken in triethylamine (0.1 ml) and anhydrous pyridine (6 ml) overnight; the CPG was filtered off, washed with CH_2Cl_2, and dried; trityl analysis[2] showed a nucleoside (dA^{Bz}, dC^{Bz}, dG^{iBu}, or T) loading of 32-39 μmol/g; unreacted amino groups were then capped with acetic anhydride/methylimidazole.

The purified product (**3**) can also be coupled to the support using diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBT) using a smaller amount of nucleoside.[17] For example (Scheme 2, route B): LCAA-CPG (500 mg), HOBT (0.018 mmol), DIC (0.15 mmol), and anhydrous acetonitrile (1.5 ml) in a septum sealed vial were shaken at room temperature (20 min) before addition, via syringe, of a solution of **3** (B=T, 0.05 mmol) in anhydrous pyridine (0.2 ml) and acetonitrile (0.5 ml). After shaking overnight, the CPG was filtered off and washed with CH_2Cl_2. Trityl analysis showed a nucleoside loading of 56 μmol/g.

Scheme 2



The rate of cleavage of the QDA linker was determined by: 1, synthesis of a d(AGCT)$_5$ test sequence; 2, cleavage from the support with concentrated ammonium hydroxide, with fractions collected at one minute intervals for ten minutes; 3, deprotection of the fractions (50°, 16 h); 4, evaporation to remove ammonia; 5, quantitation by UV at 260 nm; and 6, plotting the cumulative amount recovered vs. time. For comparison, a similar test was also performed using succinyl derivatized support, with fractions collected every 15 minutes for three hours. The cleavage studies were performed on both LCAA-CPG supports and highly crossed-linked aminomethylpolystyrene (PS) using both deoxyribonucleosides and ribonucleosides as the terminal nucleosides (Table 1).

Table 1. Cleavage of QDA and Succinyl Linkers with deoxyribo- and ribonucleosides.

			Amount of Cleavage			
			QDA linker		Succinyl linker	
Nucleoside	Reagent	Support	2 min.	5 min.	60 min.	120 min.
DNA	NH_4OH	LCAA-CPG	93%	98%	79%	92%
DNA	NH_4OH	PS	79%	93%	98%	99%
RNA, 2'-O-Acetyl	3:1 NH_4OH/EtOH	LCAA-CPG	92%	94%	94%	99%
RNA, 2'-O-Acetyl	3:1 NH_4OH/EtOH	PS	92%	95%	98%	99%
RNA, 2'-O-tBDMSi	3:1 NH_4OH/EtOH	LCAA-CPG	42%	92%	72%	91%
RNA, 2'-O-tBDMSi	3:1 NH_4OH/EtOH	PS	43%	85%		

For CPG derivatized with the QDA linker, a two minute hydrolysis released the same amount of oligodeoxynucleotide as a two hour hydrolysis on a succinylated CPG support and 14% more than a conventional 60 minute cleavage. Although, the rate of oligodeoxynucleotide cleavage on the QDA derivatized polystyrene support was slower, possibly due to hydrophobic surface effects, a five minute cleavage time was sufficient for recovery of > 90% of the product.

The rate of cleavage for 2'-O-acetyl protected ribonucleosides was not significantly different from deoxynucleosides and a two minute cleavage was also satisfactory. More hindered ribonucleosides containing a 2'-O-*t*-butyldimethylsilyl (tBDMSi) protecting group were cleaved more slowly for both QDA and succinyl linkers. However, a five minute hydrolysis of the QDA linker was still sufficient for satisfactory cleavage.

Although, the QDA linker can also be cleaved with other milder reagents (work in progress), use of concentrated aqueous ammonium hydroxide is preferred for routine oligonucleotide synthesis. This allows complete compatibility with existing protecting groups, deprotection methods, and instrumentation. The ammonium hydroxide solution released from the support is treated in the same manner as always for oligonucleotide deprotection and work-up. Furthermore, the QDA linker does not affect subsequent coupling steps and no decrease in coupling efficiency or oligonucleotide quality has been observed during the synthesis of over 7,000 different oligonucleotides on QDA derivatized CPG in our core facility.

Replacement of succinic acid with hydroquinone-O,O'-diacetic acid as the preferred linker for solid-phase supports is possible without sacrificing stability or performance and without requiring any modifications to existing methods, reagents, or instrumentation. The reagent is readily available, easily handled, and compatible with the same coupling procedures used to attach succinylated nucleosides to solid-phase supports. However, use of this linker will increase synthesis productivity by at least one or two runs per day because of the time saved by the faster cleavage. This should benefit both smaller core facilities trying to avoid additional instrument purchases as well as larger facilities trying to cope with the heavy demands of projects such as genome sequencing. We also plan to use the time saved during the faster cleavage step for automated on-line nucleoside derivatization of "universal" supports[4] and eventual automated support recycling.

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