

Efficient activation of nucleoside phosphoramidites with 4,5-dicyanoimidazole during oligonucleotide synthesis

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ABSTRACT

A new activator for the coupling of phosphoramidites to the 5'-hydroxyl group during oligonucleotide synthesis is introduced. The observed time to complete coupling is twice as fast with 4,5-dicyanoimidazole (DCI) as the activator, compared with 1*H*-tetrazole. The effectiveness of DCI is thought to be based on its nucleophilicity. DCI is soluble in acetonitrile up to 1.1 M at room temperature and can be used as the sole coupling activator during routine automated solid phase synthesis of oligonucleotides. The addition of 0.1 M *N*-methylimidazole to 0.45 M 1*H*-tetrazole also results in higher product yields during oligonucleotide synthesis than observed with 1*H*-tetrazole alone.

INTRODUCTION

Oligonucleotides and modified oligonucleotides are of great interest as therapeutic candidates, either as inhibitors of gene expression, (1,2) or as inhibitors of protein function (3). This increased interest requires oligonucleotide preparation in ever larger amounts. However, the large scale synthesis of RNA and modified RNA oligonucleotides in particular continues to pose a formidable challenge. The synthesis of oligonucleotides from nucleoside 3'-*N,N*-diisopropylphosphoramidites (4,5) is a common practice. Activation of the phosphoramidites for coupling to a 5'-hydroxyl group of an oligonucleotide is usually achieved by addition of 1*H*-tetrazole. While this activator is the accepted standard for coupling of deoxynucleoside phosphoramidites, alternatives are often sought for coupling of more sterically hindered nucleoside phosphoramidite monomers. For addition of 2'-*O*-methylnucleoside 3'-phosphoramidites to an oligonucleotide by solid phase synthesis, 5-(*p*-nitrophenyl)-1*H*-tetrazole has been suggested (6). Similarly, the coupling of 2'-*t*-butyldimethylsilyl-protected ribonucleoside 3'-phosphoramidites was shown to be accelerated by activation with 5-ethylthio-1*H*-tetrazole (7). Recently, the condensation of otherwise unreactive phosphoramidite analogs with benzimidazolium triflate was demonstrated (8). These activators presumably increase the rate of coupling due to their increased acidity.

The mechanism of phosphoramidite activation and coupling has been studied in detail (9,10). The first step in activation is protonation of the trivalent phosphorus, followed by slow displacement of the *N,N*-diisopropylamine by tetrazolidine. The latter intermediate rapidly reacts with an alcohol, such as the 5'-hydroxyl group of a protected resin-bound oligonucleotide, to yield the phosphite triester product. Thus tetrazole acts both as an acid and as a nucleophilic catalyst. Interestingly, recent activator design has focused mainly on accelerating the protonation step, rather than accelerating the nucleophilic displacement step. In this paper we investigate activation of nucleoside phosphoramidite coupling by less acidic, more nucleophilic activator systems.

MATERIALS AND METHODS

The 4,5-dicyanoimidazole (DCI) was filtered before use. The 2',3'-*O*-isopropylidene uridine was obtained from Sigma Chemical Co. and converted to the 5'-DMT derivative by standard methods. Deoxynucleoside phosphoramidites and oligonucleotide synthesis reagents were obtained from Applied Biosystems, Cruachem Inc., Glen Research or PerSeptive Biosystems. *N*-*t*-Butylphenoxyacetyl- and 2'-TBDMS-protected ribonucleoside phosphoramidites were obtained from PerSeptive Biosystems. The 2'-fluoropyrimidine phosphoramidites (11) and the 2'-trifluoroacetylaminopyrimidine phosphoramidites (12) were prepared as previously described. Beaucage reagent was obtained from Glen Research. Derivatized controlled pore glass solid support was obtained from Prime Synthesis. Derivatized polystyrene support was obtained from Pharmacia Biotech.

Detritylation of 5'-DMT-2',3'-*O*-isopropylidene uridine by 1*H*-tetrazole and 5-ethylthio-1*H*-tetrazole

A stock solution of 0.2 M 5'-DMT-2',3'-isopropylidene uridine was prepared in dry acetonitrile. To this solution was added the activator solution (acetonitrile) at time 0 and the final concentration of the reaction mixture was adjusted to 0.1 M 5'-DMT-2',3'-isopropylidene uridine. An aliquot was removed at several time points and analyzed by reversed phase HPLC. Rates were determined at three activator concentrations (0.1, 0.2 and 0.5 M).

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Table 1. Automated synthesis cycle for the preparation of oligonucleotide **2**

Step	Reagent	Equivalents ^a	Reaction time (min)
Detritylation	Dichloroacetic acid in CH ₂ Cl ₂ (3% v/v)	200–250	3–15
Coupling	Nucleoside phosphoramidites	4	15–30
	Activator	8 ^b	
Oxidation	0.05 M I ₂ in 95:5 pyridine:water	1.5	4
Capping	Cap A: 1:1:8 Ac ₂ O:2,6-lutidine:THF	32	2
	Cap B: 16% NMI in THF		

^aEquivalents are based on moles of CPG-bound 3'-terminal nucleoside.

^bActivator equivalents are based on moles of nucleoside phosphoramidite.

Rate of phosphoramidite coupling with DCI activation

All experiments were conducted under the same reaction conditions. Phosphoramidite (0.01 mmol), 2',3'-*O*-isopropylidene uridine (0.011 mmol) and activator (0.08 mmol) were dissolved in CD₃CN (0.7 ml) and the reaction rate was followed by ³¹P NMR using D₃PO₄ as an external standard. 2'-*N*-Trifluoroacetamido-5'-DMT-uridine, 2'-*N*-phthalimido-5'-DMT-uridine phosphoramidites were found to couple more than twice as fast when DCI was used as the activator (25 versus >60 min), as was the case with riboguanosine (10 versus >25 min). The coupling time for 2'-*O*-methylguanosine phosphoramidites was also reduced by half, from 6 to 3 min, when activated with DCI.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxythymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite)

A 1 M solution of 4,5-dicyanoimidazole in acetonitrile (1.8 ml, 1.8 mmol) was added to a stirred suspension of 5'-DMT-thymidine nucleoside (1.4 g, 2.57 mmol) in 15 ml dry dichloromethane. 2-Cyanoethyl tetraisopropylphosphorodiamidite (0.98 ml, 3.08 mmol) was then added dropwise via syringe over 4 min. The cloudy reaction was allowed to stir under argon for 3 h then diluted with 10 ml dichloromethane and partitioned twice with 5% sodium bicarbonate. The organic layer was dried with magnesium sulfate, filtered and concentrated to 1.94 g white foam. The solid was dissolved in toluene and added dropwise to rapidly vortexing hexane. A white precipitate was allowed to settle and the supernatant decanted. The precipitation was repeated to give 1.43 g white solid in 75% yield. The analysis agreed with that of an authentic sample.

Preparation of oligonucleotide **2**

The 51mer oligodeoxynucleotide of sequence 5'-TAG-CCA-AGG-TAA-CCA-GTA-CAA-GGT-GCT-AAA-CGT-AAT-GGC-TTC-GGC-TTA-C[3'-3'T]T-3' (oligonucleotide **2**), where [3'-3'] stands for a 3'-3' internucleotide linkage, was prepared at the 0.35 mmol scale on a Millipore 8800 automated synthesizer using standard deoxynucleoside phosphoramidites. The synthesis was carried out using a controlled pore glass (CPG) support of 1000 Å pore size, loaded at 35 mmol/g with 3'-succinylthymidine. The synthesis cycle is shown in Table 1.

The oligonucleotide was cleaved from the support and deprotected by reaction in concentrated ammonia at 55°C for

18 h. The solution was then cooled, filtered and the filtrate concentrated to dryness.

Preparation of (aU)₁₀T oligonucleotides

The 2'-aminouridine oligonucleotides 5'-(aU)₁₀T-3' were prepared at a 0.1 mmol scale on a Pharmacia OligoPilot II automated synthesizer using polystyrene support loaded at 26 mmol/g with 3'-succinylthymidine. The synthesis cycle was as previously described in Table 1. The 2'-aminouridine phosphoramidites were coupled in two aliquots of 3 equiv. each. The oligonucleotide was cleaved from the support and deprotected by reaction in concentrated ammonia at 55°C for 18 h. The solution was then cooled, filtered and the filtrate concentrated to dryness.

Preparation of oligonucleotide **3**

A 2'-aminopyrimidine-containing oligonucleotide of sequence 5'-TsTsTsTs mGmGaU rGaUrG aUrGrG mArArG mAaCrA rGmGmG mGmGaU mGmGaU aUaC TsTsTsTsT-3' (oligonucleotide **3**), where aU and aC stand for 2'-aminouridine and 2'-aminocytidine, mG and mA stand for 2'-*O*-methylguanosine and 2'-*O*-methyladenosine, rG and rA stand for guanosine and adenosine, T stands for thymidine and s stands for a phosphorothioate internucleotide linkage, was prepared at the 0.4 mmol scale on a Pharmacia OligoPilot II automated synthesizer using a polystyrene support loaded at 26 μmol/g with 3'-succinylthymidine. The synthesis cycle was as previously described in Table 1 using 0.5 M DCI (8 equiv.) with a sulfurization step of 0.5 M Beaucage reagent in acetonitrile (15 equiv., 7.5 min reaction time) following oxidation. The oligonucleotide was cleaved from the resin and the base and phosphate protecting groups were removed by reaction with ammonia-saturated ethanol at room temperature for 72 h. The mixture was filtered and the supernatant evaporated to dryness. The crude material was then subjected to 1 M TBAF in THF for 24 h at room temperature. At that point the reaction was quenched with an equal volume of 1 M Tris-HCl, pH 7.5. The organic solvent was evaporated and the crude oligonucleotide was isolated by ethanol precipitation.

Preparation of oligonucleotide **4**

A 2'-fluoropyrimidine-ribo-purine oligonucleotide of sequence 5'-rGrG rArGfU fCfUfU rArGrG fCrArG fCrGfC rGfUfU fUfUfC rGrArG fCfUrA fCfUfC fC[3'-3'T]-3' (oligonucleotide **4**), where rG and rA stand for guanosine and adenosine, fC and fU stand for

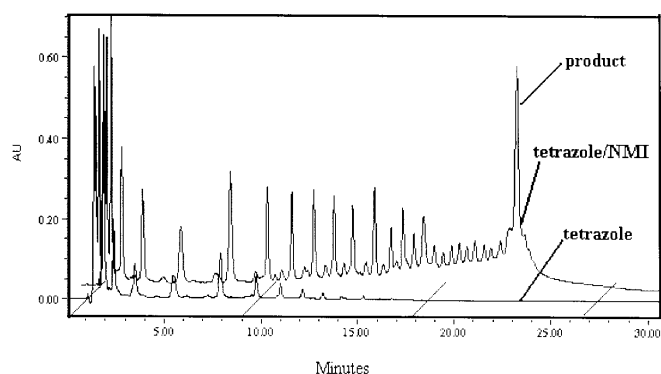


Figure 1. Anion exchange HPLC chromatogram of crude oligonucleotide **4** prepared using tetrazole and tetrazole/NMI respectively. The chromatogram was obtained on a PA-100 4×250 mm Nucleopac column (Dionex) at 80°C eluting from 64% trizma EDTA, pH 7.5 (buffer A):36% trizma EDTA + 1 M NaCl, pH 7.5 (buffer B) to 100% buffer B over 32 min at 1.5 ml/min.

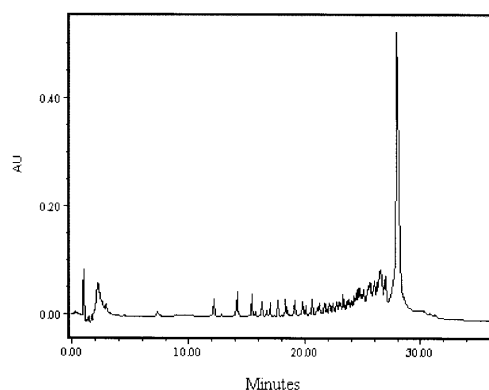


Figure 2. Anion exchange HPLC chromatogram of crude oligonucleotide **2**, a 51mer DNA, using tetrazole/NMI as activator. The chromatogram was obtained on a 4.6×100 mm Gen-Pak Fax column (Waters) at 80°C eluting from 90% trizma EDTA, pH 7.5 (buffer A):10% trizma EDTA + 1 M NaCl, pH 7.5 (buffer B) to 90% buffer B over 30 min at 0.75 ml/min.

2'-deoxy-2'-fluorocytidine and 2'-fluorouridine and [3'-3'] stands for a 3'-3' internucleotide linkage, was prepared at the 1 mmol scale on a Millipore 8800 automated synthesizer. Two equivalents of 5'-DMT-2'-*O*-TBDMS-*N*²-*t*-butyl-phenoxyacetylguanosine and 5'-DMT-2'-*O*-TBDMS-*N*⁶-*t*-butyl-phenoxyacetyladenosine 3'-*N*,*N*-diisopropyl-(2-cyanoethyl) phosphoramidites and 2'-deoxy-2'-fluoro-5'-DMT-*N*⁴-acetylcytidine and 2'-deoxy-2'-fluoro-5'-DMT-uridine 3'-*N*,*N*-diisopropyl-(2-cyanoethyl) phosphoramidites were used. The activator formulations are described in the text. The syntheses were carried out using a CPG support of 600 Å pore size, 80–120 mesh and 60–70 μmol/g loading with 5'-succinylthymidine. The synthesis cycle was as previously described in Table 1 with 5.2 equiv. oxidizer.

RESULTS AND DISCUSSION

The acidity of 1*H*-tetrazole (13) is similar to that of acetic acid, a common reagent for removal of the 5'-dimethoxytrityl protecting group from nucleosides and oligonucleotides. Thus, at the longer coupling times typically required for condensation of sterically demanding nucleoside 3'-phosphoramidites, such as 2'-*t*-butyldimethylsilyl-protected ribonucleoside phosphoramidites, detritylation of the phosphoramidite monomer by the activator becomes a potentially bothersome side reaction. We found that detritylation of 5'-DMT-2',3'-isopropylideneuridine **1** by 1*H*-tetrazole and 5-ethylthio-1*H*-tetrazole increases with the decrease in pK_a between these activators. Under conditions that are typical for a solid phase coupling reaction the second order detritylation rate of **1** was found to be $k = 0.08/\text{M}/\text{min}$ for 1*H*-tetrazole and $k = 0.32/\text{M}/\text{min}$ for 5-ethylthio-1*H*-tetrazole.

Therefore, we were interested in exploring less acidic and more nucleophilic activators for coupling of nucleoside phosphoramidites in oligonucleotide synthesis. It has already been shown that a mixture of dimethylaminopyridine (DMAP) and 5-(*p*-nitrophenyl)-1*H*-tetrazole can catalyze coupling of nucleoside phosphoramidites (14). However, it was later discovered that DMAP can cause unwanted side product formation by attack on the O⁶ position of guanosine (15). We opted to increase the effective concentration

of nucleophilic catalyst in the activation mixture by adding *N*-methylimidazole (NMI) to 1*H*-tetrazole.

A mixture of 0.1 M NMI in 0.45 M tetrazole resulted in a significant increase in oligonucleotide product yield during solid phase synthesis of oligonucleotides with a variety of monomers. In our hands 2'-deoxy-2'-trifluoroacetyl-amino-5'-dimethoxytritylpyrimidine 3'-*N*,*N*-diisopropyl-2-cyanoethylphosphoramidites are the least efficient monomers for oligonucleotide synthesis. We were interested to test whether this observation is dependent on the activator. By addition of a mixture of 0.1 M NMI in 0.45 M tetrazole (8 equiv.) the stepwise average coupling efficiency for preparation of a deca-2'-aminouridylylate-thymidine [(aU)₁₀-T] oligonucleotide was increased from 96.4 to 98.0%. Addition of >0.1 M NMI resulted in decreased product yield, presumably due to basicity of the medium.

The effect of NMI upon phosphoramidite activation is illustrated by the attempted preparation of a 34mer 2'-fluoropyrimidine-ribopurine oligonucleotide. Two syntheses (1 mmol scale) were run in parallel, one using 0.1/0.4 M NMI to 1*H*-tetrazole, the other using just 0.45 M tetrazole as activator. Rather stringent conditions were chosen, with a 2-fold excess of phosphoramidite per coupling and a 20 or 30 min coupling time for the 2'-fluoropyrimidine phosphoramidites or ribopurine phosphoramidites respectively. After complete deprotection no product was observed by anion exchange HPLC chromatography for the tetrazole-activated synthesis. In contrast, 13% product was observed in the case of NMI-buffered tetrazole (Fig. 1). The latter activation mixture also promotes efficient preparation of oligodeoxynucleotides. A DNA 51mer was prepared in 41% yield using 0.1 M NMI in 0.45 M tetrazole as the activator and 4 equiv. monomer per coupling at a 0.35 mmol scale (Fig. 2). This corresponds to an average stepwise coupling efficiency of 98.3%. At larger scales, particularly in column reactors, chromatographic separation of the NMI from the tetrazole and the nucleoside phosphoramidite limits the utility of this activation system (data not shown). Therefore, we were interested in identifying a single compound which would facilitate efficient nucleoside phosphoramidite activation while being less acidic and more nucleophilic than tetrazole.

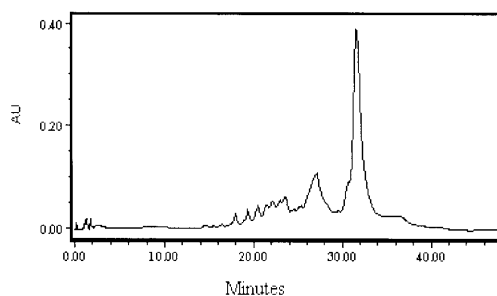


Figure 3. Anion exchange HPLC chromatogram of crude oligonucleotide **3**. The chromatogram was obtained on a 4.6 × 100 mm Gen-Pak Fax column (Waters) at 80°C eluting from 90% trizma EDTA, pH 7.5 (buffer A):10% trizma EDTA + 1 M NaCl, pH 7.5 (buffer B) to 90% buffer B over 30 min at 0.75 ml/min.

The screening of several candidates resulted in identification of DCI as the activator of choice. The DCI was prepared as described previously (US patent 2,534,331, 1949). It is important that highly pure DCI is used for oligonucleotide synthesis. We have observed that commercially available technical grade DCI can result in errant contaminant peaks in crude oligonucleotides. This activator has a pK_a of 5.2. In addition, DCI is soluble up to 1.1 M in acetonitrile, allowing for higher effective concentrations of activated phosphoramidite during the coupling step in solid phase oligonucleotide synthesis. Compared with tetrazole, DCI increases the rate of activation and coupling of the nucleoside phosphoramidite to an alcohol by a factor of two as determined by ^{31}P NMR. The time to complete coupling of 2'-TBDMS-protected riboguanosine 3'-phosphoramidite to alcohol **1** at near stoichiometric conditions, for example, decreased from 25 to 10 min. Similar decreases in reaction time were measured for a variety of 2'-substituted nucleoside phosphoramidites.

Activation with DCI proved the most efficient way to assemble oligonucleotides containing 2'-deoxy-2'-aminopyrimidines. A typical preparation of a 2'-aminopyrimidine-containing modified oligonucleotide, oligonucleotide **3** (sequence given in Materials and Methods), of 36 nt length with DCI activation is shown in Figure 3. This oligonucleotide is unique since it not only contains 2'-aminopyrimidines, but also a mixture of 2'-*O*-methylpurines, ribopurines and a stretch of 3'- and 5'-terminal phosphorothioate linkages. Conversely, activation with tetrazole did not give satisfactory yields (data not shown).

The efficiency of activation with DCI allows for a significant reduction in nucleoside phosphoramidite excess during coupling. With only 2 equiv. phosphoramidite per monomer addition a 2'-fluoropyrimidine-ribopurine 34mer oligonucleotide, oligonucleotide **4**, was prepared in 54% yield after complete deprotection (Fig. 4). This was achieved on a 1 mmol synthesis scale using 1.0 M DCI as activator. Under the same stringent conditions activation with 0.45 M tetrazole or a mixture of 0.45 M tetrazole and 0.1 M NMI did not give satisfactory product yields (Fig. 1). In comparison, the product yield increased with increasing nucleophilicity of the activator. Our laboratory now employs a 1.0 M solution of DCI in acetonitrile as the standard activator for solid phase synthesis of substituted and unsubstituted oligonucleotides.

Since it is known that nucleophilic attack at the O^6 position of guanosine followed by deprotection in ammonia results in formation of 2,6-diaminopurine contamination (15), it was

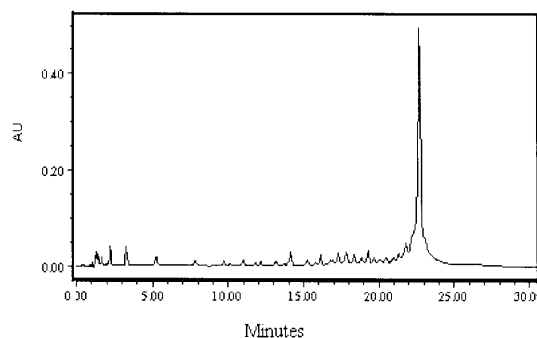


Figure 4. Anion exchange HPLC chromatogram of crude oligonucleotide **4** prepared with DCI activation. The chromatogram was obtained on a PA-100 4 × 250 mm Nucleopac column (Dionex) at 80°C eluting from 64% trizma EDTA, pH 7.5 (buffer A):36% trizma EDTA + 1 M NaCl, pH 7.5 (buffer B) to 100% buffer B over 32 min at 1.5 ml/min.

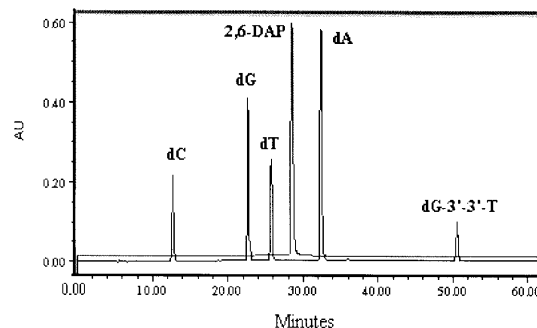


Figure 5. Reversed phase HPLC chromatogram of digested DNA overlaid with 2,6-diaminopurine. A DNA oligonucleotide, prepared as described for oligonucleotide **2** but with 1 M DCI as activator, of length 28mer was digested into nucleobases as described in Materials and Methods. The chromatogram was obtained on a 4.6 × 250 mm 100 A C18 Symmetry column (Waters) eluting from 98% triethylammonium acetate, pH 6.5 (100 mM, buffer A):2% methanol (buffer B) to 30% buffer B over 61 min at 0.5 ml/min.

important to test whether DCI is nucleophilic enough to undergo this reaction. For this purpose a 28mer oligodeoxynucleotide was prepared with DCI activation and deprotected in concentrated ammonia. An enzymatic digest of the crude product to its nucleoside components did not show contamination by 2,6-diaminopurine (Fig. 5).

In addition to activation of nucleoside phosphoramidites during coupling to oligonucleotides, activation with DCI is also useful in phosphorylation of nucleosides with 2-cyanoethyl tetraisopropylphosphorodiamidite. 5'-DMT-thymidine was efficiently converted to the 3'-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite derivative using DCI.

CONCLUSION

We have shown that phosphoramidite coupling during oligonucleotide synthesis proceeds rapidly and with no observable side reaction when DCI is used as the coupling activator. This activator increases the rate of coupling in comparison with tetrazole while being less acidic. It is highly soluble in acetonitrile

and thus allows for higher effective concentrations of nucleoside phosphoramidites during solid phase synthesis. A higher effective concentration in turn allows for lower phosphoramidite excess during coupling.

Finally, the utility of DCI is not only recognized in oligonucleotide synthesis, but also in the preparation of nucleoside phosphoramidites from 3'-hydroxynucleosides and bis-(*N,N*-diisopropyl)-2-cyanoethyl phosphoramidite.

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