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TECHNICAL BULLETIN - 5-FORMYL-dC-CE PHOSPHORAMIDITE

INTRODUCTION

Epigenetics is the study of heritable changes in gene expression and regulation that are not due to variation in the DNA sequence itself. Methylation of deoxycytidine to form 5-methyl deoxycytidine is considered one mechanism involved in the regulation of gene expression. Oxidation of 5-methyl-dC is proposed as a mechanism in the active demethylation pathway and may involve 5-formyl-dC as an intermediate. 5-Formyl-dC is recognized and excised by Thymidine-DNA glycosylase base-excision repair enzymes, leading to the insertion of a non-methylated deoxycytidine.[1]

The incorporation of 5-formyl-dC into an oligonucleotide requires the incorporation of the 5-Formyl-dC-CE Phosphoramidite (1). Following deprotection, 5-(glycolyl)-dC is formed and subsequent oxidation with sodium periodate leads to 5-formyl-dC, as shown in Figure 1.

SYNTHESIS

Use a 3 minute coupling time. No other changes required.

DEPROTECTION

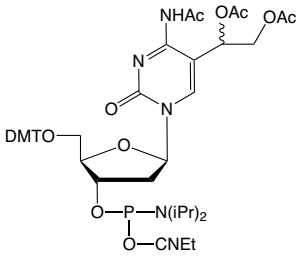
Deprotect with ammonium hydroxide for 17 hours at 55°C, AMA (40% aqueous methylamine/28% ammonium hydroxide (1:1)) for 10 minutes at 65°C, or 0.4 M NaOH in MeOH/water 4:1 (v/v) for 17 hours at room temperature. The deprotection removes all of the protecting groups from the nucleobases and the phosphate backbone.

Note that a side reaction occurs when deprotecting the oligo in ammonia or methylamine solutions leads to an impurity, as described on the second page. 0.4 M NaOH in MeOH/water 4:1 (v/v) for 17 hours at room temperature can be used used to deprotect the oligo without impurity formation.

After deprotection, the 1,2-diol requires oxidation with sodium periodate in water. The oxidation can be performed on the crude or the purified oligo. The oxidation is simple and complete in 30 minutes at 4°C. The best results have been obtained on DMT-ON purified oligos. After oxidation, desalt the oligo using HPLC, Glen-Pak™, or Glen Gel-Pak. Excess sodium periodate can be quenched with ethylene glycol.

REPRESENTATIVE WORKUP (DMT-OFF, CRUDE)

1. Transfer the support to a clean dry vial with screw cap.
2. Add 1mL of deprotection solution (NH4OH or AMA) and deprotect as required for the nucleobases.
3. Filter the supernatant and evaporate the deprotection solution to dryness.
4. Redissolve in 200 µL of cold distilled water (4°C).
5. Add 200 µL of cold (4°C) 50 mM sodium periodate in water for a final 25 mM concentration.
6. React for 30 minutes at 4°C
7. Desalt immediately with a Glen Gel-Pak or a Glen-Pak DNA cartridge, or quench with 5-10 equivalents of ethylene glycol with respect to sodium periodate.



(1) 5-Formyl-dC-CE Phosphoramidite

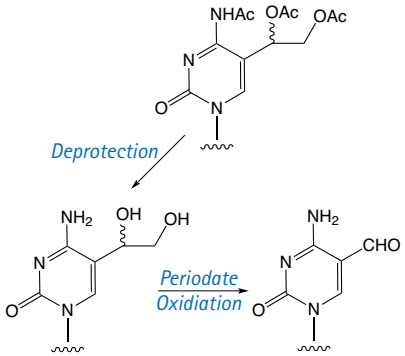


Figure 1: Deprotection and Oxidation to 5-Formyl-dC

REPRESENTATIVE WORKUP (DMT-ON)

1. Synthesize the oligo DMT-ON.
2. Transfer an aliquot of support to a clean dry vial with a screw cap.
3. Add 1 mL of AMA deprotection solution and deprotect for 10 minutes at 65°C.
4. Filter the supernatant.
5. Add an equivalent volume of 100 mg/mL sodium chloride solution and purify on a Glen-Pak DNA cartridge.
6. Elute the product with 50% acetonitrile in water and evaporate the solution to dryness.
7. Redissolve in 200 µL of cold distilled water (4°C).
8. Add 200 µL of cold (4°C) 50 mM sodium periodate in water for a final 25 mM concentration.
9. React for 30 minutes at 4°C.
10. Add 1 mL 0.1M TEAA to the solution.
11. Load on a Glen-Pak DNA cartridge prepared for desalting. Wash with 2 x 1 mL of water.
12. Elute the product oligo with 50% acetonitrile in water.

IMPROVED PROTOCOL FOR DEPROTECTION AND DMT-ON PURIFICATION OF 5-FORMYL-dC

DEPROTECTION WITH SODIUM HYDROXIDE

While 5-Formyl-dC (1) has been a popular product, its use is complicated by a side reaction which occurs when deprotecting the oligo in ammonia or methylamine solutions. Rather than attacking the carbonyl of the acetyl protecting group, the nucleophile attacks the carbon, displacing acetate as a leaving group. Now vicinal to the second acetyl protecting group, it quickly attacks the carbonyl, leading to the formation of a substituted N-acetamide, rather than the desired glycol. The proposed mechanism is shown in Figure 2.

One solution to this problem is to use hydroxide as the nucleophile rather than ammonia or methylamine. This way, even if the acetate displacement occurs, it is a transparent substitution that still affords the desired 5-glycolyl-deoxycytidine, which, upon oxidation with sodium periodate, yields the desired 5-formyl-dC. Typically, 0.4 M NaOH in MeOH/water 4:1 (v/v) for 17 hours at room temperature is used to deprotect the oligo.

Use of dmf-dG

Quite surprisingly, dimethylformamidine (dmf), a popular protecting group for guanosine, is remarkably resistant to the sodium hydroxide solution. While an isobutyryl-protected dG is cleanly deprotected in 17 hours, the dmf group requires over 72 hours at room temperature.

Use of Ac-dC

In addition, if benzoyl-protected dC is used, nucleophilic displacement of benzamide can occur due to hydroxide attack of the C4 carbon, leading to deamination and a dC to dU mutation. This can be avoided if acetyl-protected dC (Ac-dC) is used during synthesis.

Use of NaOH

A final concern is that the yield of oligos can be highly variable when using 0.4 M NaOH in MeOH/water for deprotection. The CPG will tend to fuse to a solid cake in the bottom of the vial of larger-scale syntheses and the oligo will tend to precipitate onto the CPG. However, we found that by first briefly sonicating the vial to break up the CPG, pipetting off the supernatant and then rinsing the CPG with water, will consistently give good oligo yields.

0.4 M NaOH has to be neutralized or desalted prior to drying down. For neutralization, add 28 µL of glacial acetic acid per mL of 0.4 M NaOH. Neutralization is NOT compatible with DMT-ON purification.

DMT-ON Purification

The use of sodium hydroxide necessitates a desalting step and this can be accomplished, while simultaneously purifying the oligo, by using a Glen-Pak™ purification cartridge. An optimized protocol (for a 1 µmole synthesis scale or smaller synthesized DMT-ON) is given below. With this procedure, multiple incorporations of the 5-formyl-dC can be introduced into an oligo cleanly.

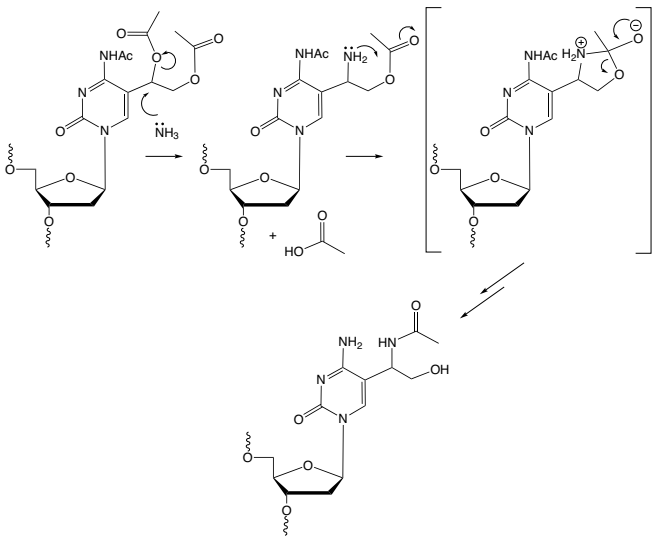


Figure 2: Proposed Mechanism for Impurity Formation with Ammonium Hydroxide

PROTOCOL FOR DEPROTECTION AND DMT-ON GLEN-PAK PURIFICATION

1. Make a fresh solution of 0.4 M NaOH in MeOH/water 4:1 (v/v).
2. To eliminate the cyanoethyl protecting groups on the phosphate backbone, treat the column with 3 mL of 10% DEA in ACN for 2 minutes, pushing the solution back and forth occasionally. Rinse with ACN and air dry the CPG.
3. Transfer the CPG to a vial and add 1 mL of 0.4 M NaOH in MeOH/water 4:1 (v/v).
4. Allow to react for 17 hours at room temperature.
5. Briefly sonicate the vial to break up the CPG.
6. Pipette off the supernatant and transfer to a clean vial. Rinse the CPG with 250 µL of water and combine with the cleaved oligo.
7. Dilute to 10 mL with 100 mg/mL NaCl in water.
8. Load onto a prepped Glen-Pak DNA cartridge (60-5200-xx) fitted with a 10 mL syringe and purify using the standard protocol but beginning with the Salt Wash Step – i.e., the 2 mL rinse with 100 mg/mL NaCl containing 5% ACN.
9. Dry the purified oligo and dissolve it in 250 µL of water. Chill at 4 °C.
10. Make a fresh solution of 50 mM $NaIO_4$ (2.7 mg per 250 µL water)
11. Add 250 µL of 50 mM $NaIO_4$ to the chilled oligo solution, briefly vortex and return it to the refrigerator. Keep at 4 °C for 30 minutes.
12. Add 3 mL 0.1 M TEAA and desalt on a prepped Glen-Pak DNA cartridge.

Reference:

1. Y.F. He, et al., *Science*, 2011, **333**, 1303-7.

http://www.glenresearch.com/Technical/GlenPak_UserGuide.pdf