Synthesis and site-specific incorporation of a simple fluorescent pyrimidine

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We describe procedures for the synthesis of a fluorescent pyrimidine analog and its site-specific incorporation into a DNA oligomer. The 5'-protected and 3'-activated nucleoside 4 is synthesized in three steps with an overall yield of 40%. Site-specific incorporation into a DNA oligomer occurs with greater than 88% coupling efficiency. This isosteric fluorescent DNA analog can be used to monitor denaturation of DNA duplexes via fluorescence and can positively detect the presence of abasic sites in DNA duplexes. The total time for synthesis of the phosphoramidite 4 is about 75 h, whereas the total time for site-specific incorporation of nucleoside 2 into an oligonucleotide and purification of the corresponding oligonucleotide is about 114 hours.

INTRODUCTION

When carefully designed and implemented, fluorescent nucleosides and nucleotides can serve as powerful tools for basic research and drug discovery applications that are centered around nucleic acids. Progress in this field has been driven by several fundamental reasons: (i) the natural purines and pyrimidines found in DNA and RNA are practically nonemissive^{1–3}, (ii) naturally occurring fluorescent bases (e.g., Y37 in tRNA^{Phe}) are typically so severely modified that they cannot generally serve as natural base mimics, and (iii) end labeling with common fluorescent tags (e.g., fluorescein), while providing generic tagging, is not necessarily sensitive to remote binding events. Fluorescent nucleobase analogs that are capable of mimicking the structure and function of the natural bases, but possess emission properties, have therefore emerged in recent years as powerful probes for investigating nucleic acids sequence, structure, dynamics and recognition^{4–28}.

Several important features characterize useful emissive nucleobase analogs. These include: (i) high structural similarity to the natural nucleobases, (ii) emission at long wavelengths, preferably in the visible range (this is typically associated with absorption maxima that are red-shifted when compared to the native heterocycles), (iii) sufficient emission quantum efficiency to facilitate their use in real-time fluorescence-based assays and, notably, (iv) susceptibility to the microenvironment, resulting in markedly different photophysical parameters of the chromophore, particularly in emission color or intensity. It is the latter property that is most challenging, yet critical, for fabricating new and useful fluorescent nucleobase probes.

How can environmentally responsive nucleosides be used? One of the contemporary directions represents the design of fluorescent deoxynucleosides that can report the identity of the base opposite to them in a double-stranded helix by yielding different emission wavelengths or intensities. Such base-discriminating fluorescent (BDF) nucleosides, when incorporated into oligonucleotide probes, can find uses in DNA sequence identification and single nucleotide polymorphism (SNP) analysis¹⁹. A more established application of fluorescent bases, is to use nucleobase analogs to interrogate the position and environment of individual bases within oligonucleotide targets. A classical example is 2-aminopur-

ine (2AP), an adenine analog that forms base pairs with thymine or uracil that are isostructural to the corresponding Watson-Crick base pairs. This adenosine mimic displays minimal emission when stacked with other bases, but, upon unstacking and exposure to aqueous environments, a significantly enhanced emission is observed^{29–33}. Many biophysical assays have taken advantage of this feature because base flipping is a rather common event in biologically relevant nucleic acids. Selected examples include: (i) 'real-time' monitoring of the hammerhead ribozyme folding, cleavage and inhibition^{34,35}, (ii) evaluating the dimerization and isomerization of the HIV-1 dimerization initiation site (DIS)³⁶, (iii) examining RNA-protein interactions such as the HIV RRE-Rev binding^{37,38}, and (iv) studying the binding of aminoglycoside antibiotics to the bacterial decoding site, known as the A-site^{39,40}.

Besides base flipping, nucleic acids also undergo a variety of chemical transformations, including depurination and depyrimidination. These abasic sites can be generated either spontaneously or via enzymatic base excision of damaged nucleosides. Several methods have been developed for detecting the presence of these cytotoxic DNA lesions. Most techniques require irreversible modifications of isolated DNA^{41–76}, whereas others use non-natural nucleobase analogs^{77–89} and only a few use 2AP to investigate the stacking ability of a base opposite an abasic site^{90–92}. Fluorescent nucleobase analogs that positively sense the presence of abasic sites could provide useful nondestructive tools for the detection of such important DNA defects.

We have recently reported that conjugating 5-membered aromatic heterocycles to a pyrimidine core generates a family of responsive fluorescent nucleoside analogs with emission in the visible range⁹³. Specifically, conjugating a furan moiety to the 5-position of 2'-deoxyuridine yielded a nucleoside analog **2** (Fig. 1) that emits above 425 nm, and is sensitive to changes in solvent environment, being fairly emissive in polar and rather nonemissive in apolar solvents. When strategically incorporated into DNA oligonucleotides (see Fig. 2) and hybridized to complementary sequences, this furan-modified pyrimidine analog positively signals the presence of DNA abasic sites, manifested via

Figure 1 | Scheme for the synthesis of phosphoramidite **4**.

(a) 2-(tributylstannyl)furan, dichlorobis(triphenylphosphine)Pd(II), dioxane, 94%. (b) 4,4'-dimethoxytrityl chloride, triethylamine, pyridine, 71%; (c) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite, 1*H*-tetrazole, acetonitrile, 65%.

a sevenfold enhancement in emission of a lesion-containing DNA when compared to the emission of a perfect duplex⁹³.

The protocol outlined below provides a step-by-step synthesis of this useful furan-containing nucleoside and its phosphoramidite, the necessary building block for standard solid-phase DNA synthesis^{94,95}. Detailed procedures for the synthesis (**Fig. 3**) and purification of a modified oligonucleotide are given. Additionally, this protocol provides researchers with experimental procedures for basic photophysical examination of the fluorescently tagged oligonucleotide.

MATERIALS

REAGENTS

- 5-iodo-2'-deoxyuridine (Sigma-Aldrich, cat. no. SA-I7000)
- 2-(tributylstannyl)furan (Sigma-Aldrich, cat. no. 414506) **! CAUTION** 2-(tributylstannyl)furan is toxic and can produce health damage via inhalation, skin contact and/or ingestion
- Dichlorobis(triphenylphosphine)palladium(II) (Alfa Aesar, cat. no. 41245)
- 4,4'-dimethoxytrityl chloride (Sigma-Aldrich, cat. no. 100013)
- 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite (Sigma-Aldrich, cat. no. 305995)
- 0.45M 1*H*-tetrazole in acetonitrile (Glen Research, cat. no. 30-3110-51) **! CAUTION** 1*H*-tetrazole in acetonitrile is flammable and irritating to eyes, respiratory system and skin
- Anhydrous dioxane (Acros, cat. no. 36434) **! CAUTION** Heating of dioxane results in harmful vapors
- Anhydrous pyridine (Fluka, cat. no. 82704) **! CAUTION** Pyridine is flammable and toxic. Exposure via inhalation, skin contact and/or ingestion can cause irreversible health damage
- Anhydrous triethylamine (Sigma-Aldrich, cat. no. 471283) **! CAUTION** Triethylamine is flammable and exposure via inhalation, skin contact and/or ingestion can be harmful
- Anhydrous acetonitrile (Fluka, cat. no. 00695)
- Silica gel (SiliCycle, cat. no. R10030B)
- Thin-layer silica gel plates (EMD Chemicals Inc., cat. no. 5534-3)
- Washed sea sand (Fisher, cat. no. S25)
- Hexanes (Fisher, cat. no. H292)
- Ethyl acetate (Fisher, cat. no. E145)
- Methanol (Fisher, cat. no. A412)
- Chloroform (VWR, cat. no. EM-CX1055)
- Dichloromethane (Fisher, cat. no. D37)
- Acetonitrile for DNA synthesizer (Fisher, cat. no. BP1170)
- Saturated sodium bicarbonate solution
- Saturated sodium chloride solution (brine)
- 1 M sodium hydroxide solution
- 0.1 M *p*-toluenesulfonic acid solution in acetonitrile
- Gel running dye solution (bromophenol blue, 1 × TBE, 7M urea solution)
- Anhydrous sodium sulfate (VWR, cat. no. EM-SX0763)

- Anhydrous calcium sulfate (VWR, cat. no. 22890)
- 10× TBE buffer (0.89 M Tris, 0.89 M boric acid and 20 mM EDTA; pH 8)
- Aqueous ammonium hydroxide (VWR, cat. no. EM-AX1303)

Figure 2 | Key steps in solid phase DNA phosphoramidite synthesis cycle. Step 1: removal of the 5'-DMT protecting group using dichloroacetic acid; Step 2: activation of the phosphoramidite using 1*H*-tetrazole; Step 3: coupling of the 5'-hydroxyl on B₁ to the activated phosphoramidite B₂; Step 4: oxidation of the trivalent phosphorous to the pentavalent phosphorus using iodine in a tetrahydrofuran/pyridine/water solution; Step 5: all unreacted nucleotides (B₁) are capped using acetic anhydride; Step 6: cleavage of the oligonucleotide from the CPG resin and deprotection of protected nucleoside functional groups using concentrated ammonium hydroxide; Step 1': repetition of the cycle continues until the desired DNA length is reached.

- 40% acrylamide solution 19:1 Acryl:Bis (Bio-Rad Laboratories, cat. no. 161-0144)
- Celite 545 (Fisher, cat. no. C212)
- Deblock solution: 2.5% dichloroacetic acid in dichloromethane (Glen research, cat. no. 40-4042-57)
- Oxidizing solution: 0.02 M iodine in tetrahydrofuran/pyridine/water (Glen research, cat. no. 40-4132-55)
- Cap Mix A: tetrahydrofuran/acetic anhydride 9/1 (Glen research, cat. no. 40-4012-50)
- Cap Mix B: 10% 1-methylimidazole in tetrahydrofuran/pyridine 8/1 (Glen research, cat. no. 40-4122-51)
- dA cyanoethyl phosphoramidite (Glen research, cat. no. 10-1000-C2)
- dC cyanoethyl phosphoramidite (Glen research, cat. no. 10-1010-C2)
- dG cyanoethyl phosphoramidite (Glen research, cat. no. 10-1020-C2)
- dT cyanoethyl phosphoramidite (Glen research, cat. no. 10-1030-C2)
- G-terminal 1.0 μ mole controlled pore glass (CPG) column (Glen research, cat. no. 20-2220-41)

EQUIPMENT

- Round-bottom flasks, one and two necks (Kontes)
- Reflux condenser (Kontes)
- Glass-fritted funnel (Chemglass)
- Side neck erlenmeyer (Kontes)
- Rubber vacuum adapter
- Teflon-coated stir bars
- Rotary evaporator (Buchi)
- Hot plate (Corning)
- Filter paper (Whatman)
- Chromatography column: 4.5 cm $\times$ 30 cm (Kontes)
- Balloons (Sigma-Aldrich, cat. no. Z154989)
- Argon gas
- Helium gas
- Sep-Pak C18 cartridges (Waters Corporation, cat. no. wat051910)
- Gel electrophoresis box (Gibco)

- Glass plates for gel box: 36 cm $\times$ 20 cm
- Poly-Prep columns (Bio-Rad laboratories, cat. no. 731-1550)
- DNA synthesizer, Cyclone Plus
- Vacuum pump (Welch)
- Schlenk line
- Disposable pasteur pipets, 9 inch (Fisher, cat. no. 13-678-20C)
- 200 μ l gel-loading pipettor tips (Midwest Scientific, cat. no. mff-2)
- 1,000 μ l ART pipettor tips (VWR, cat. no. 72839-042)
- LabQuake shaker
- Glass funnel
- Cotton
- UV lamp
- Glass rod
- Sharpie marker extra fine point
- Plastic wrap
- Stop cock grease
- Rubber septa, 14/20 (Chemglass, cat. no. CG-3022-24)
- Airline 14/20 adapter (Kontes, cat. no. 276000-0000)
- Luer lock adapter (Popper and sons)
- 8 inch 18-gauge stainless steel needles (Popper and sons)
- Disposable 21-gauge needles (Fisher, cat. no. 14-826-5B)
- Disposable 16-gauge needles (Fisher, cat. no. 14-826-18B)
- 1 ml plastic syringe, slip tip (Fisher, cat. no. 309602)
- 5 ml plastic syringe, luer lock tip (Fisher, cat. no. 14-823-35)
- 5 ml plastic syringe, slip tip (Fisher, cat. no. 14-826-12)

EQUIPMENT SETUP

DNA synthesizer setup Follow directions for the specific instrument being used.

Treat all round-bottom flasks with a 1 M sodium hydroxide solution before drying in the oven.

Before use, place all glassware, including glass syringes and stainless steel needles, in a 110 $^{\circ}$ C oven overnight ($\sim$ 15 h) and then cool to room temperature (18–23 $^{\circ}$ C) in a desiccator filled with anhydrous calcium sulfate.

PROCEDURE

Synthesis of 5-(fur-2-yl)-2'-deoxyuridine 2

1| Weigh out 520 mg (1.47 mmol) of 5-iodo-2'-deoxyuridine and 21 mg (0.03 mmol) of dichlorobis(triphenylphosphine)palladium(II), and add them along with a teflon-coated stir bar to a 100-ml two-neck round-bottom flask.

2| Grease the ground glass joint of the reflux condenser, and insert it into the 100-ml round-bottom flask.

3| Connect water lines to the reflux condenser (inlet in the bottom).

4| Cap the second neck of the 100-ml round-bottom flask with a rubber septum.

5| Connect an airline adapter to the top of the reflux condenser and evacuate the reaction setup for 5 min; then refill the reaction setup with dry argon gas.

6| Repeat Step 5 twice (total of 3 times).

7| With a steady pressure of dry argon gas, remove the airline adapter from the top of the reflux condenser and quickly replace it with a rubber septum.

▲ CRITICAL STEP This reaction is moisture- and oxygen-sensitive, thus its success depends upon the ability to keep the reaction setup anhydrous using dry argon gas.

8| Insert an argon balloon (balloon fitted to the outer portion of a 5-ml plastic syringe) with a 21-gauge needle into the top of the reflux condenser.

9| Transfer 40 ml of dry dioxane via 50 ml glass syringe to the reaction setup via the second septum on the two-neck round-bottom flask.

! CAUTION Heating of dioxane produces harmful vapors.

10| Transfer 2 ml (6.35 mmol) of 2-(tributylstannyl)furan via a 5-ml glass syringe to the reaction flask.

! CAUTION The 2-(tributylstannyl)furan is toxic and can produce health damage via inhalation, skin contact and/or ingestion.

- 11| Turn on the water flow to the condenser, turn on the magnetic stirrer, and then heat the suspension to 90 °C for 2 h.
 - 12| Identify complete consumption of starting material by eluting the reaction mixture along with the starting material on thin-layer silica gel plates with 10% methanol-chloroform (retention factor (R_f) of the starting material is 0.20 and of nucleoside **2** is 0.23). The product glows blue under short wavelength light (254 nm), whereas the starting material does not.
▲ CRITICAL STEP The purification method used will not separate 5-iodo-2'-deoxyuridine from the product, thus it is critical that all starting material (5-iodo-2'-deoxyuridine) be consumed before moving onto the next step.
 - 13| Turn the heat off and allow the reaction setup to cool to room temperature.
 - 14| Disassemble the reaction setup and pour the solution through celite 545 filter setup (celite 545 in a glass fritted funnel connected to a vacuum filtration setup using a rubber vacuum adapter and a side arm Erlenmeyer flask), flushing the celite 545 with 100 ml of methanol.
 - 15| Evaporate the filtrate.
 - 16| Add hexanes (50 ml) to the resulting oil and sonicate the suspension to remove remaining tributyltin species.
 - 17| Remove the liquid via disposable Pasteur pipette.
 - 18| Repeat Steps 16 and 17 twice (3 times total).
■ PAUSE POINT The residue from Step 18 can be left overnight under vacuum at room temperature.
 - 19| Add minimum amount of boiling 1:1 methanol-chloroform solution to completely dissolve the resulting yellow solid.
 - 20| Allow the solution to cool to room temperature.
 - 21| Add hexanes until white precipitate results, and filter off solid.
 - 22| Repeat Steps 19–21 until resulting filtrate contains no desired product, evaporating the filtrate to a solid before each precipitation.
 - 23| Collect all precipitated material (product is a white solid).
■ PAUSE POINT The collected product is stable for over 1 year at room temperature under an argon environment.
- Synthesis of 5'-dimethoxytrityl-5-(fur-2-yl)-2'-deoxyuridine **3****
- 24| Weigh out 160 mg (0.55 mmol) of nucleoside **2** (**Fig. 1**) and 226 mg (0.67 mmol) of 4,4'-dimethoxytrityl chloride, and add them along with a teflon-coated stir bar to a 25-ml single-neck round-bottom flask.
 - 25| Connect an airline adapter to the round-bottom flask and evacuate the reaction setup for 5 min; then refill the reaction setup with dry argon gas.
 - 26| Repeat Step 25 twice (3 times total).
 - 27| With a steady pressure of dry argon gas, remove the airline adapter from the top of the round-bottom and quickly replace it with a rubber septum.
 - 28| Insert an argon balloon with a 21-gauge needle into the top of the round-bottom flask.
▲ CRITICAL STEP This reaction is moisture-sensitive, thus its success depends upon the ability to keep the reaction setup anhydrous using dry argon gas.
 - 29| Transfer 3 ml of dry pyridine via a 5-ml glass syringe equipped with an 8-inch 18-gauge stainless steel needle to the reaction flask.
! CAUTION Pyridine is flammable and toxic. Exposure via inhalation, skin contact and/or ingestion can cause irreversible health damage.
 - 30| Transfer 60 μ l (0.43 mmol) of dry triethylamine via a 100- μ l glass syringe to the reaction flask.
! CAUTION Triethylamine is flammable and exposure via inhalation, skin contact and/or ingestion can be harmful.
 - 31| Turn on the magnetic stirrer and stir the reaction at room temperature for 8 h.
 - 32| Identify complete consumption of starting material by eluting the reaction mixture along with the starting material (nucleoside **2**) on thin-layer silica gel plates with 10% methanol-chloroform (R_f of the nucleoside **3** is 0.5).
 - 33| Evaporate the resulting reaction solution.
■ PAUSE POINT The residue from Step 33 can be left overnight under vacuum at room temperature.

- 34| Pack a chromatography column (4.5 cm × 30 cm) with ~100 ml of silica gel using 7/3 (vol/vol) ethyl acetate/hexanes with 1% triethylamine.
- ▲ **CRITICAL STEP** The addition of triethylamine is essential. The dimethoxytrityl group is acid-labile and can be cleaved by acidic silica gel.
- 35| Add a 1-cm layer of washed sea sand to the chromatography column.
- 36| Dissolve the solid from Step 33 in the minimum amount of dichloromethane with 1% triethylamine, and load the solution onto the chromatography column.
- 37| Elute the column under pressure (1–5 p.s.i.) with 7/3 (vol/vol) ethyl acetate/hexanes 1% triethylamine, collecting 25-ml fractions, until all desired product is collected.
- 38| Identify fractions containing protected nucleoside **3** (**Fig. 1**) by eluting thin-layer silica gel plates with 7/3 (vol/vol) ethyl acetate/hexanes with 1% triethylamine (R_f of the nucleoside **3** is 0.2).
- 39| Combine all fractions that contain nucleoside **3** and evaporate to dryness using a rotary evaporator with a heating bath set at 35–40 °C.
- 40| Place the resulting oil from Step 39 on a Schlenk line connected to a vacuum pump (~0.3 torr).
- 41| Product is an off-white foam (foam is not always seen).
- **PAUSE POINT** The product from Step 41 can be left for several days under vacuum at room temperature.

Synthesis of 3'-(2-cyanoethyl)diisopropylphosphoramidite-5'-dimethoxytrityl-5-(fur-2-yl)-2'-deoxyuridine **4**

- 42| Weigh out 58 mg (0.10 mmol) of nucleoside **3** and place it in a 25 ml round-bottom flask.
- 43| Add 3 ml of dry acetonitrile via a 5-ml glass syringe equipped with an 8-inch 18-gauge stainless steel needle to the reaction flask, and evaporate the solution to dryness using a rotary evaporator with a heating bath set at 35–40 °C.
- 44| Repeat Step 43 four times (5 times total). The coevaporation of nucleoside **3** with dry acetonitrile helps remove any residual water.
- 45| Connect an airline adapter to the round-bottom flask, and evacuate the reaction setup for 5 min; then refill the reaction setup with dry argon gas.
- 46| Repeat Step 45 twice (3 times total).
- 47| With a steady pressure of argon gas, remove the airline adapter from the top of the round-bottom flask and quickly replace it with a rubber septum.
- 48| Insert an argon balloon with a 21-gauge needle into the top of the round-bottom flask.
- 49| Add 2 ml of dry acetonitrile via a 5-ml glass syringe equipped with an 8-inch 18-gauge stainless steel needle to the reaction flask.
- 50| Add 215 µl (0.097 mmol) of 0.45M 1*H*-tetrazole solution via a 100-µl glass syringe to the reaction flask.
- ! **CAUTION** The 1*H*-tetrazole in acetonitrile is flammable and irritating to eyes, respiratory system and skin.
- 51| Add 37 µl (0.12 mmol) of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite via a 100-µl glass syringe to the reaction flask.
- ▲ **CRITICAL STEP** The purity of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite is very important for the success of this reaction. We used new unopened vials for each reaction.
- 52| Turn on the magnetic stirrer and stir the reaction at room temperature for 4 h.
- 53| Identify complete consumption of starting material by eluting the reaction mixture along with the starting material (nucleoside **3**) on thin-layer silica gel plates with 1/1 (vol/vol) ethyl acetate/hexanes with 1% triethylamine (R_f of the nucleoside **4** is 0.6). Note that the product is a mixture of diastereomers, resulting in two closely migrating spots on TLC.
- 54| Add 100 ml of cold 1% (vol/vol) triethylamine/dichloromethane solution to the reaction mixture and transfer the solution to a 250-ml separatory funnel.

PROTOCOL

- 55| Wash the organic solution using a separatory funnel with 10 ml of 1 M sodium bicarbonate solution twice and with 10 ml of brine solution twice.
- 56| Dry the resulting organic layer by adding anhydrous sodium sulfate until a portion of the added sodium sulfate no longer clumps. Swirl the flask by hand to properly mix the suspension.
- 57| Filter the mixture through a glass funnel plugged with cotton, and collect the filtrate.
- 58| Evaporate the filtrate to dryness using a rotary evaporator with a heating bath set at 35–40 °C.
- 59| Pack a chromatography column (4.5 cm × 30 cm) with ~100 ml of silica gel using 1/1 (vol/vol) ethyl acetate/hexanes with 1% triethylamine.
- 60| Add a 1-cm layer of washed sea sand to the chromatography column.
- 61| Dissolve the resulting solid from Step 58 in the minimum amount of 1/1 (vol/vol) ethyl acetate/hexanes with 1% triethylamine and load the solution onto the chromatography column.
- 62| Elute the column under pressure (1–5 p.s.i.) with 1/1 (vol/vol) ethyl acetate/hexanes 1% triethylamine, collecting 10–15-ml fractions, until all desired product is collected.
- 63| Identify fractions containing nucleoside **4** (**Fig. 1**) by eluting thin-layer silica gel plates with 1/1 (vol/vol) ethyl acetate/hexanes with 1% triethylamine (R_f of the nucleoside **4** is 0.6).
- ▲ **CRITICAL STEP** The diastomeric mixture can be separated chromatographically (thin-layer and column). Both diastomers are combined and used in the next step.
- 64| Combine all fractions that contain nucleoside **4** and evaporate to dryness using a rotary evaporator with a heating bath set at 35–40 °C.
- 65| Place the resulting film or foam from Step 64 on a Schlenk line connected to a vacuum pump (~0.3 torr).
- **PAUSE POINT** Residue from Step 65 can be left for one or two days under vacuum at room temperature, but should be used as soon as possible as degradation of the product will adversely affect its incorporation into a DNA oligomer.
- Site-specific incorporation of nucleoside 4**
- 66| Dissolve each amidite (dC, dA, dG and dT cyanoethyl phosphoramidite) in 10 ml of dry acetonitrile.
- ▲ **CRITICAL STEP** Solid phase oligonucleotide synthesis is very moisture sensitive.
- 67| Attach each amidite bottle to the DNA synthesizer.
- 68| Following manufacturer instructions, prime all reagents for start up.
- 69| Enter in the desired DNA sequence (sequence from 3' to 5' until the desired point of modified nucleoside incorporation including dimethoxytrityl removal of the final base).
- ▲ **CRITICAL STEP** Greater than 99% coupling efficiency of the purchased amidites is extremely important. A short test sequence can be run (using each amidite), collecting each 4,4'-dimethoxytrityl cation containing deprotection fraction to ensure proper coupling efficiency.
- 70| Begin DNA sequence, collecting each DMT deprotection fraction for coupling efficiency determination (see below).
- 71| At the point of desired modification, remove CPG column from synthesizer.
- 72| Connect a 1-ml plastic syringe filled with 100 µl of nucleoside **4** solution (see **Box 1** for preparation) to one end of the CPG column.
- 73| Invert the column-syringe with the syringe on the bottom and push the nucleoside solution into the column (some acetonitrile previously remaining in the column will be pushed out).
- 74| Attach a 1-ml syringe with 200 µl of activator solution to the open end of the CPG column.
- 75| Using the syringes attached to either end of the CPG column, gently push the solutions back and forth through the CPG column in order to completely mix the solutions.
- 76| Continue to push back and forth for 2–3 min.

BOX 1 | PREPARATION OF NUCLEOSIDE 4 FOR DNA INCORPORATION

1. Weigh out 21.8 mg of nucleoside 4 and add it to a 5 ml round-bottom flask.
 2. Cap the round-bottom flask with a rubber septum.
 3. Evacuate the reaction setup for 5 min using a 16-gauge disposable needle connected to a luer-lock hose adapter and inserted into the septa; then refill the reaction setup with argon gas.
 4. Repeat Step 3 twice (a total of three times).
 5. Insert an argon balloon (balloon fitted to the outer portion of a 5 ml plastic syringe) with a 21-gauge needle into the top of the round-bottom flask.
 - ▲ **CRITICAL STEP** Solid phase oligonucleotide synthesis is very moisture sensitive and thus any moisture will result in low coupling efficiency of nucleoside 4 into a growing DNA strand.
 6. Add 100 μ l of dry acetonitrile via 100 μ l glass syringe to the reaction flask.
 - ▲ **CRITICAL STEP** The resulting solution should be made directly before use, as nucleoside 4 will degrade over time.
 - **TIMING**
- Steps 1 and 2, 6 min; Steps 3–5, 30 min; Step 6, 3 min.

- 77| Remove the syringes from the CPG column and place the column back on the DNA synthesizer.
- 78| Run the oxidizing and capping cycles on the DNA synthesizer (note: a wash cycle is included in the beginning of the oxidizing cycle).
- 79| Enter the second half of the sequence into the instrument and continue with the DNA synthesis.
- 80| Upon completion of the DNA synthesis, remove the CPG column from the instrument.
- 81| Connect an empty 5-ml slip tip syringe to the CPG column.
- 82| Connect another 5-ml slip tip syringe filled with 3 ml of concentrated ammonium hydroxide to the open end of the CPG column.
- 83| Gently push the concentrated ammonium hydroxide solution back and forth through the CPG column for 1 min every 5 min over a 15-min period.
- 84| Repeat Step 83 every hour for 2 h.
- 85| Push all the solution into one syringe and remove it from the CPG column.
- 86| Repeat Steps 82 and 83 using only 1 ml of concentrated ammonium hydroxide.
- 87| Push all the solution into one syringe and remove it from the CPG column.
- 88| Consolidate all ammonium hydroxide solutions.
- **PAUSE POINT** Cap the consolidated ammonium hydroxide solutions and allow them to remain at room temperature for 24 h.
- 89| Use a steady stream of air to evaporate the ammonia gas.
- 90| Remove the resulting water via SpeedVac or freeze-dry techniques.
- **PAUSE POINT** Residue from step 90 can be left for several days at -20°C .

Purification of DNA oligonucleotide with nucleoside 2 incorporated

- 91| Add 200 μ l of water and 25 μ l of gel running dye solution to the resulting dry crude oligonucleotide mixture using a 200- μ l pipettor.
- 92| Load 225 μ l of the above solution into two wells on a 20% acrylamide (19:1 poly:bis), $1\times$ TBE, 7 M urea gel (36 cm $\times$ 20 cm) using a 200- μ l pipettor equipped with a gel-loading tip and run at 25 watts for 4.5 h.
- ! **CAUTION** High voltage is used during the gel running process.
- 93| Turn off gel electrophoresis power unit and allow gel to cool to room temperature.
- 94| Remove the glass plates and cover the gel in plastic wrap.
- 95| Identify product band by UV shadowing (placing the gel inside a UV box on top of a fluorescent TLC plate).
- 96| Trace the highest running band with a Sharpie marker, and cut it out using a razor blade.

BOX 2 | SEP-PAK CARTRIDGE EQUILIBRATION

1. Using a plastic 5 ml luer-lock tip syringe, flush 15 ml (5 ml $\times$ 3) of acetonitrile through the Sep-Pak cartridge.
2. Flush 10 ml (5 ml $\times$ 2) of water through the Sep-Pak cartridge.

● TIMING

Steps 1 and 2, 10 min.

- 97| Place the cut band, being sure to remove the plastic wrap, into a Poly-Prep column, and crush the band with a glass rod.
- 98| Add 2.5 ml of 1 $\times$ TBE buffer solution using a 1,000 μ l pipettor and cap the Poly-Prep column.
■ **PAUSE POINT** Attach the Poly-Prep column to the shaker and mix the tube at 4 °C for 24–36 h.
- 99| Break off the tip of the Poly-Prep column and place the column into a 15-ml falcon tube.
- 100| Poke a hole in the cap of the Poly-Prep column using a 16-gauge disposable needle.
- 101| Centrifuge the falcon tube and Poly-Prep column setup for 10 minutes at room temperature (18–23 °C).
- 102| Load the resulting liquid onto an equilibrated Sep-Pak cartridge (see **Box 2** for equilibration instructions).
- 103| Flush the Sep-Pak cartridge with 5 ml of water and then elute the DNA with 40% acetonitrile/water collecting 1-ml fractions in 1.7-ml Eppendorf tubes.
- 104| Measure the absorption spectra of each fraction in order to identify those fractions that contain DNA.
- 105| Combine each tube that contains DNA and remove water/acetonitrile via SpeedVac or freeze drying techniques. To determine coupling efficiency, see **Box 3**.
■ **PAUSE POINT** Samples from Step 105 can be left for several months at –20 °C.

● TIMING

Synthesis of nucleoside **2**: Steps 1–7, 25 min; Steps 8–10, 10 min; Step 11, 2 h; Step 12, 5 min; Step 13, 30 min; Step 14, 20 min; Step 15, 20 min (evaporation depends upon the volume and equipment used); Steps 16–18, 30 min; Steps 19–23, 40 min (for one precipitation; but several will be required).

Synthesis of nucleoside **3**: Steps 24–28, 25 min; Steps 29 and 30, 8 min; Step 31, 8 h; Step 32, 5 min; Step 33, 20 min (evaporation depends upon the volume and equipment used); Steps 34–36, 20 min; Step 37, 30 min; Step 38, 5 min; Step 39, 20 min (evaporation depends upon the volume and equipment used); Steps 40 and 41, 1 min.

Synthesis of nucleoside **4**: Steps 42–44, 1 h; Steps 45–48, 22 min; Steps 49–51, 15 min; Step 52, 4 h; Step 53, 5 min; Steps 54 and 55, 25 min; Steps 56 and 57, 15 min; Step 58, 20 min (evaporation depends upon the volume and equipment used), Steps 59–61, 20 min; Step 62, 30 min; Step 63, 5 min; Step 64, 20 min (evaporation depends upon the volume and equipment used), Step 65, 1 min.

Site-specific incorporation of nucleoside **4**: Steps 66–69, 40 min; Step 70, time varies depending upon the DNA synthesizer used; Steps 71–73, 10 min; Steps 74–77, 7 min; Steps 78 and 79, time varies depending upon the DNA synthesizer used; Steps 80–82, 7 min; Steps 83 and 84, 2 h; Steps 85–87, 20 min; Step 88, 24 h; Step 89, 30 min; Step 90, time varies depending upon the technique and instrument used.

Purification of DNA oligonucleotide with nucleoside **1** incorporated: Steps 91 and 92, 5 h; Step 93, 30 min; Step 94, 10 min; Steps 95 and 96, 10 min; Steps 97 and 98, 36 h; Steps 99–101, 20 min; Steps 102 and 103, 15 min; Step 104, 15 min; Step 105, time varies depending upon the technique and instrument used.

BOX 3 | QUANTIFICATION OF DNA COUPLING REACTIONS

1. Add the entire DMT fraction to a 25 ml volumetric flask, and fill to the line with 0.1 M *p*-toluenesulfonic acid solution in acetonitrile.
2. Remove 5 ml of the DMT solution from Step 1 and add it to another 25 ml volumetric flask.
3. Dilute again to the fill line with 0.1 M *p*-toluenesulfonic acid solution.

4. Acquire a UV spectrum of each DMT fraction diluted, monitoring at 498 nm.

Coupling efficiencies (each step or overall) can be determined by the ratio of the absorption of the current step over that of the previous step. Quantification can also be determined after purification of the oligonucleotide (Step 105) via UV spectrometer, using the following extinction coefficients for each base: dA = 15,400 cm^{–1} M^{–1}, dC = 7,300 cm^{–1} M^{–1}, dG = 11,700 cm^{–1} M^{–1}, dT = 8,800 cm^{–1} M^{–1} and 2 = 13,000 cm^{–1} M^{–1}.

● TIMING

Step 1, 5 min; Steps 2 and 3, 5 min; Step 4, 5 min per sample.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reasons	Solution
Low yield for synthesis of nucleoside 2	Poor technique or wet reagents	Repeat reaction with fresh reagents, being sure that all are anhydrous. Use extra caution to remove and maintain oxygen-free reaction conditions
Bilayer formed upon precipitation of nucleoside 2	Too little chloroform to solubilize the added hexanes	Repeat precipitation using more chloroform
Low yield or slow reaction for synthesis of nucleoside 4	Poor technique or wet reagents	Repeat reaction with fresh reagents, being sure that all are anhydrous (1 <i>H</i> -tetrazole in acetonitrile is hygroscopic). Use extra caution to maintain anhydrous reaction conditions
Poor coupling efficiencies of modified nucleoside seen by DMT monitoring	Moisture in the reaction or degraded phosphoramidite	Repeat the coupling with fresh acetonitrile Add molecular sieves to the phosphoramidite and 1 <i>H</i> -tetrazole solutions Confirm the purity of the phosphoramidite via phosphorous NMR

ANTICIPATED RESULTS

Typical yields

For yields of nucleosides **2–4**, see **Figure 1**. For DNA synthesis, typical unoptimized coupling efficiencies of greater than 88% for incorporation of nucleoside **4** were obtained.

Spectroscopy

Absorption spectra (**Fig. 4**) were observed on a Hewlett Packard 8452A Diode Array Spectrometer, and the extinction coefficient of nucleoside **2** was determined to be $13,000 \text{ cm}^{-1} \text{ M}^{-1}$ at 260 nm. Absorption and steady state fluorescence spectra of nucleoside **2** (**Fig. 4**; 1% DMSO to solubilize the nucleoside) were measured in 10 mM sodium phosphate, 100 mM sodium chloride, pH = 7.0. Steady state fluorescence spectra of nucleoside **2** (**Fig. 4**) were measured on a Perkin Elmer LS 50B luminescence spectrometer.

Steady state fluorescence spectra of 5'-GCGATG2GTAGCG-3' (**Fig. 5**) were measured in 10 mM sodium phosphate, 100 mM sodium chloride (pH 7.0) on a Horiba Fluoromax-3 luminescence spectrometer (**Fig. 5**) and a Perkin Elmer LS 50B luminescence spectrometer (**Fig. 3**). The minimum concentration of a singly modified oligonucleotide containing nucleoside **2** required for adequate fluorescence readings depends upon the sensitivity of the luminescence spectrometer. For example, adequate fluorescence readings using the Horiba Fluoromax-3 are obtained at $0.1 \mu\text{M}$ (**Fig. 5**), whereas the lower limit for the Perkin Elmer LS 50B is $0.5 \mu\text{M}$.

Analytical data

5-(fur-2-yl)-2'-deoxythymidine (**2**).

^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.63 (s, 1H), 8.33 (s, 1H), 7.62 (s, 1H), 6.86 (d, $J = 2.8 \text{ Hz}$, 1H), 6.52 (m, 1H), 6.22 (t, $J = 6.6 \text{ Hz}$, 1H), 5.27 (d, $J = 4.4 \text{ Hz}$, 1H), 5.08 (t, $J = 4.4 \text{ Hz}$, 1H), 4.28 (m, 1H), 3.84 (m, 1H), 3.61 (m, 2H), 2.18 (t, $J = 5.4 \text{ Hz}$, 2H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 160.1, 149.4, 146.5, 141.5, 134.7, 111.6, 107.9, 105.6, 87.6, 84.7, 70.4, 61.1, 40.1; HR-FAB calculated for $\text{C}_{13}\text{H}_{15}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$ 295.0925, found: 295.0928; UV (buffer): $\lambda_{\text{max}} = 252 \text{ nm}$ ($\epsilon = 13,800 \text{ cm}^{-1} \text{ M}^{-1}$); $\lambda_{\text{max}} = 316 \text{ nm}$ ($\epsilon = 11,000 \text{ cm}^{-1} \text{ M}^{-1}$); $\epsilon_{260} = 13,000 \text{ cm}^{-1} \text{ M}^{-1}$.

5'-dimethoxytrityl-5-(fur-2-yl)-2'-deoxythymidine (**3**).

^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.70 (s, 1H), 7.96 (s, 1H), 7.27–7.16 (m, 10H), 6.84–6.81 (m, 5H), 6.46–6.44 (m, 1H),

Figure 3 | Fluorescence spectra of an oligonucleotide containing **2**, hybridized to its perfect complement (red dashed line) and an oligonucleotide containing an abasic site (green solid line) at $1.0 \mu\text{M}$.

Figure 4 | Absorption spectra at 50 μM (red) and fluorescence spectra at 10 μM (blue) of nucleoside **2**.

Figure 5 | Typical fluorescence spectra of 5'-GCGATG2GTAGCG-3' at 1.0 μM (red), 0.5 μM (blue) and 0.1 μM (green).

6.18 (t, $J = 6.6$ Hz, 1H), 5.36 (d, $J = 4.4$ Hz, 1H), 4.23 – 4.19 (m, 1H), 3.96 – 3.93 (m, 1H), 3.69 (s, 6H), 3.24 – 3.15 (m, 2H), 2.27 – 2.24 (m, 2H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 160.1, 158.0, 149.3, 146.1, 144.7, 141.2, 135.5, 133.9, 129.6, 127.7, 127.6, 126.6, 113.1, 111.4, 107.9, 105.6, 85.8, 85.1, 70.3, 63.6, 55.0, 40.2; ESI-MS calculated for $\text{C}_{34}\text{H}_{32}\text{N}_2\text{NaO}_8$ $[\text{M}+\text{Na}]^+$ 619.21, found 619.08.

3'-2-cyanoethyl-diisopropylphosphoramidite-5'-dimethoxytrityl-5-(fur-2-yl)-2'-deoxythymidine (**4**)

^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.60 (s, 1H), 7.99 (s, 1H), 7.39 – 7.17 (m, 10H), 6.82 – 6.79 (m, 5H), 6.45 – 6.43 (m, 1H), 6.17 (t, $J = 6.4$ Hz, 1H), 4.50 – 4.44 (m, 1H), 4.11 – 4.08 (m, 1H), 3.69 – 3.39 (m, 10H), 3.24 – 3.19 (m, 2H), 2.64 – 2.61 (m, 2H), 2.46 – 2.34 (m, 2H), 1.23 – 1.08 (m, 12H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 160.1, 158.1, 149.2, 146.1, 144.6, 141.2, 135.3, 134.0, 129.7, 127.7, 127.6, 126.6, 118.7, 113.1, 111.5, 108.0, 105.7, 85.9, 85.2, 72.3, 63.5, 58.5, 55.0, 42.6, 42.5, 40.2, 24.3, 24.3, 24.2, 19.8; ^{31}P NMR (162 MHz, $\text{DMSO}-d_6$, referenced to H_3PO_4): δ 148.7, 148.3; ESI-MS calculated for $\text{C}_{43}\text{H}_{49}\text{N}_4\text{NaO}_9\text{P}$ $[\text{M}+\text{Na}]^+$ 819.31, found 819.14.

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