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Synthesis and Oligonucleotide Incorporation of Fluorescent Cytosine Analogue tC – A Promising Nucleic Acid Probe

Peter Sandin,[‡] Per Lincoln,[‡] Tom Brown[†] and L. Marcus Wilhelmsson[‡,*]

[‡] *Department of Chemical and Biological Engineering/Physical Chemistry, Chalmers University of Technology, SE-41296 Gothenburg, Sweden*

[†] *School of Chemistry, University of Southampton, Highfield, Southampton SO17 1BJ, UK*

[*] Telephone: +46-(0)31-7723051

Fax: +46-(0)31-7723858

email: marcus.wilhelmsson@chalmers.se

Abstract

The tricyclic cytosine, tC, is a fluorescent base analogue with excellent properties for investigating intrinsic characteristics of nucleic acid as well as interactions between nucleic acids and other molecules. Its unique fluorescence properties and insignificant influence on overall structure and dynamics of nucleic acid after incorporation makes tC particularly interesting in fluorescence resonance energy transfer (FRET) and anisotropy measurements. We here describe a straightforward synthesis of the standard monomer form of tC for DNA solid phase synthesis, the tC phosphoramidite, and its subsequent incorporation into oligonucleotides. The total synthesis of the tC phosphoramidite takes approximately eight days and its incorporation and the subsequent oligonucleotide purification an additional day.

Introduction

Fluorescent studies both at the ensemble and single molecule level are at the fore of biochemical as well as biophysical research and will increase in importance in the expanding field of bionanotechnology as the constructs keep decreasing in dimensions. As one of the most sensitive and versatile analytical techniques available for examining biological processes and biochemical mechanisms, fluorescence has found use in *e.g.* single molecule real-time dynamics of nucleic acid as well as protein, cell microscopy, nucleic acid detection and nucleic acid-protein interaction measurements. In these kinds of studies the intrinsically virtually non-fluorescent DNA or RNA has to be covalently or non-covalently modified with a fluorophore. For investigations where the level of detail needs to be high, a modification in which a fluorescent base analogue is covalently incorporated is generally preferable since this minimises perturbations to the native structure and behaviour of the nucleic acid. Furthermore, using fluorescent base analogues enables the probe to be very close to the site of examination compared to fluorescent molecules that are tethered to DNA via long linkers.

Several fluorescent base analogues have been developed and later also used as probes.[1-3] The emission quantum yield and lifetime of a great majority of those, *e.g.* 2-aminopurine,[4, 5] 3- and 6-methylisoxanthopterin (3-MI and 6-MI),[6-8] 6MAP and DMAP,[9, 10] as well as pyrrolo-dC,[11] are highly sensitive to microenvironment. Incorporation of these analogues into B-DNA leads to a significant decrease in quantum yield and excited state lifetime. This has been utilized in studies of both structure and dynamics within DNA and RNA[12, 13] as well as in measurements of kinetics of DNA-protein interaction[14-18]. We have developed a fluorescent base analogue, tricyclic cytosine (tC), that in contrast

has a high emission quantum yield (ϕ_f=0.2) that is virtually insensitive to neighbouring base combination and to changes from single- to double-stranded structures.[19-21] Furthermore, the emission of tC incorporated into DNA is characterized by a single exponential decay in both single and double strands.[21] In combination with a firm stacking, excellent base-pairing, preservation of DNA secondary structure (B-form) and a slight stabilization of duplexes this makes tC unprecedented as a fluorescent probe in nucleic acid containing systems.[22] Since properties like a high quantum yield, a single lifetime as well as a rigid and well-defined geometry considerably simplifies both measuring and evaluation of fluorescence resonance energy transfer (FRET) and fluorescence anisotropy, one can envisage previously practically impossible experiments that have become achievable thanks to tC. One could for example use tC as a probe for very accurately measuring the hydrodynamic radii of various nucleic acid structures as well as the binding of proteins or smaller DNA binding molecules to DNA using anisotropy. For other fluorescent base analogues a low emission quantum yield, a multiexponential emission decay and a weak base-pairing (fast base flipping), resulting in characteristic diffusion times corresponding to the intrinsic mobility of the base analogue, drastically complicates evaluation of this kind of data. As another example, one can also envisage using tC-DNA in combination with a FRET acceptor modified protein to study exact distances within the DNA-protein complex. Exact distance estimates using FRET rely heavily on accurate calculation of the Förster distance (R_0), which in turn depends upon among other things the donor quantum yield and the relative orientation of donor and acceptor. Currently tC is the only fluorescent base analogue exhibiting an environment insensitive emission quantum yield with a low base flipping rate.

Consequently, distances determined using tC will be significantly more accurate than those using other available fluorescent base analogues.

This protocol presents the preparation of the phosphoramidite form of tC and its subsequent incorporation into oligonucleotides. A very convenient synthetic route based on the sodium salt glycosylation procedure[23] is presented (**Fig. 2**, step a), in which the starting materials **2**[24, 25] (**Fig. 1**) and the protected 2-deoxyribosyl chloride are stable and can easily be prepared in gram quantities. 2-deoxyribosyl chloride is commercially available but is expensive and the synthetic procedure from 2-deoxy-D-ribose is uncomplicated and high yielding.[26] From the two starting materials the phosphoramidite, **6**, is achieved in four straightforward steps. The synthetic effort needed to reach the tC phosphoramidite, especially in the purification steps, is significantly reduced from a previous synthetic route.[27] The relatively modest yield of **6** from **2** (~7 %) is made up for by the simplicity by which it can be synthesised and the inexpensive starting materials. Furthermore, the protocol presents details for the activation and incorporation of this specialised phosphoramidite monomer, a piece of information that is of general importance as the need for nucleic acids that contain novel base analogues keeps increasing.

MATERIALS

REAGENTS

- 2-aminothiophenol (Sigma-Aldrich, cat. no. 274240)
- 5-bromouracil (Acros Organics, cat. no. 226440250)
- 2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride (Prepared as described elsewhere.[26] Can be purchase from Sigma-Aldrich, cat. no. D7283-1G)
- 4,4'-dimethoxytrityl chloride (Link technologies, cat. no. 0021-C000)
- *N,N*-diisopropylethylamine (Sigma-Aldrich, cat. no. 387649) **CAUTION** Highly flammable
- 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (Link Technologies, cat. no. 1028-C010)
- Sodium hydride (60 % dispersion in mineral oil) (Sigma-Aldrich, cat. no. 452912) **CAUTION** Highly flammable
- Ethylene glycol (Sigma-Aldrich, cat. no. 324558)
- N,N-Dimethylformamide, over molecular sieve (Fluka, cat. no. 40228)
- Chloroform (Riedel-de Haën, cat. no. 32211)
- Silica gel 60 (0.040-0.063 mm) (Merck, cat. no. 1.09385)
- Sand (Merck, cat. no. 1.07712.1000)
- TLC aluminium sheets, Silica gel 60 F254 (Merck, cat. no. 1.05554.0001)
- Pyridine **CAUTION** Highly flammable
- Ethyl acetate **CAUTION** Highly flammable
- Methanol dry **CAUTION** Highly flammable
- Methanol **CAUTION** Highly flammable

- Dichloromethane
- Dichloromethane, alcohol free
- Aqueous ammonia solution 25%
- Acetic acid, glacial
- Diethyl ether **CAUTION** Highly flammable
- Toluene **CAUTION** Highly flammable
- Tetrahydrofuran **CAUTION** Highly flammable
- Acetonitrile **CAUTION** Highly flammable
- Hydrochloric acid, 37% **CAUTION** Corrosive
- Ethanol 95,5% **CAUTION** Highly flammable
- Ethanol 99,7% **CAUTION** Highly flammable
- Sodium carbonate anhydrous
- Sodium hydroxide **CAUTION** Corrosive
- Potassium hydroxide **CAUTION** Corrosive
- Sodium hydrogen carbonate
- Sodium chloride
- Sodium sulphate anhydrous
- Magnesium sulphate anhydrous
- Potassium chloride
- Calcium hydride anhydrous **CAUTION** Highly flammable
- Sodium metal **CAUTION** Highly flammable
- A, G, C, T phosphoramidite monomers (Proligo: Sigma Aldrich - SAFC)
- Tetrazole coupling catalyst (Applied Biosystems)

- Acetic anhydride and N-methyl imidazole capping reagents (Applied Biosystems)
- 3% trichloroacetic acid in dichloromethane deprotection solution (Applied Biosystems)
- 1% iodine oxidation mixture in tetrahydrofuran (Applied Biosystems)
- Acetonitrile wash solvent (Rathburn Chemical Ltd)
- Aqueous ammonia cleavage solution (Fischer Scientific)
- HPLC elution buffer A: 0.1 M aqueous ammonium acetate (Fischer Scientific)
- HPLC elution buffer B: 0.1 M aqueous ammonium acetate, 25% acetonitrile (Fischer Scientific and Rathburn Chemical Ltd, respectively)

EQUIPMENT

- Hotplate magnetic stirrer with temperature controller
- Oil bath
- pH indicator paper
- Water cooled condenser
- Dual nitrogen-vacuum manifold with vacuum pump
- Argon gas
- Glass frit funnels (10-15 μm and 4-10 μm porosity)
- Vacuum desiccator
- Separating funnel (500 ml)
- Rotary evaporator
- Glass and plastic syringes
- Reusable stainless steel syringe needles and disposable syringe needles

- Round-bottom flasks (250 ml, 100 ml, 25 ml and 10 ml)
- Two-neck round-bottom flask (250 ml)
- Pear-shaped flask (5 ml)
- UV-lamp (365nm)
- Columns for chromatography (50 x 1.5 cm, 25 x 4 cm, 55 x 5.5 cm)
- Disposable glass Pasteur pipettes
- 0.45 micron filter
- Automated DNA synthesizer (Applied Biosystems ABI 394)
- Monomer bottles (of types used in Automated DNA synthesizer)
- ABI Aquapore column (C8), 8 mm x 250 mm, pore size 300 Å
- Brownlee Aquapore (C8) reversed-phase column (10 mm x 25 cm) from Perkin Elmer
- NAP-10 column (Sephadex G-25, Pharmacia)

REAGENT SETUP

Where necessary (see relevant steps in the body of the procedure), moisture-free pyridine, dichloromethane, tetrahydrofuran and diisopropylamine were obtained by distillation:

- Pyridine and dichloromethane (alcohol free) are distilled over CaH_2.
- Tetrahydrofuran is distilled over sodium CAUTION Sodium metal is highly flammable
- Diisopropylamine is distilled over KOH pellets

Moisture-free, and degassed ethyl acetate was obtained by adding anhydrous magnesium sulphate (1 g per 100ml) to the ethyl acetate in a suitable sized conical flask. Swirl around the mixture, leave to stand for 5 minutes and then decant the ethyl acetate into a

second conical flask, leaving the magnesium sulphate behind. Degas by bubbling a steady stream of argon gas through the ethyl acetate for 2 minutes. This procedure ensures that excess moisture and oxygen is removed from the ethyl acetate.

PROCEDURE

Synthesis of starting material 1,3-diaza-2-oxophenothiazine (2)

1 Weigh out 8.4 g (67 mmol) 2-aminothiophenol, 9.5 g (50 mmol) 5-bromouracil and 5.3 g (50 mmol) Na_2CO_3 into a 250 ml round-bottom flask. Add 30 ml of ethylene glycol and a magnetic stir bar to the flask.

2 Bubble argon gas through the mixture for 30 s.

3 Fit the flask with the water cooled condenser fitted with a rubber septum at the top. Insert an argon inlet and a vent consisting of a disposable syringe needle into the rubber septum.

4 Turn on a fairly strong argon flow to purge the flask and then reduce the flow to a minimum.

5 Turn on the water to the condenser and the magnetic stirrer. Heat the mixture to 120°C in an oil bath and stir the reaction mixture for 1 h.

CRITICAL STEP The temperature and reaction time are important. Increasing reaction times and temperatures decrease the yield of the reaction.

6 Cool the reaction to room temperature (a precipitate is formed).

7 While stirring, slowly add ~90 ml H_2O to the mixture.

8 Neutralize the mixture with ~3 ml acetic acid (check pH using pH indicator paper).

9 Filter the mixture through a glass frit funnel (porosity 10-16 μm). Wash the precipitate, first with water and then with a large volume of ethanol until the filtrate, initially dark yellow, becomes colourless and finally with a small volume of ether to facilitate drying. The white precipitate will be used in **11**.

10 Dissolve 2 g of sodium hydroxide in 100 ml of water containing a magnetic stir bar and heat the solution to ~50°C while stirring.

11 The white precipitate of the crude product **1** from step 9 is added in portions to the heated solution.

12 When everything is dissolved, the clear solution is neutralized by dropwise addition of acetic acid (check pH ~7 by using pH test paper). A white precipitate is formed.

13 The hot suspension is filtered through a glass frit funnel (porosity 10-16 μm). Wash the precipitate (**1**) well, first with water and then with ethanol and finally with ether and dry it in a vacuum desiccator.

14 Carefully add 7.3 ml HCl (37%) to a 250 ml round-bottom flask containing 73 ml ethanol (95.5%) and a magnetic stir bar. Weigh out 3.6 g (15 mmol) of **1** and while stirring, add it in portions to the flask. Fit the flask with a water cooled condenser, turn the water on, and heat the reaction to 80°C using an oil bath. **PAUSE POINT** Leave the reaction mixture to stir at 80°C for ~24 h. (A white precipitate of **1**·HCl often forms after **1** has dissolved. The precipitate gradually changes colour as the product **2**·HCl is formed as a mass of thin yellow crystal needles.)

15 Let the reaction cool to room temperature and filter the mixture through a glass frit funnel (porosity 10-16 μm).

16 To prepare the free base of **2** (**2**·HCl→**2**), add the precipitate to a stirred, warm (50°C) aqueous 5% ammonia solution. The yellow crystals turn into a pale yellow microcrystalline precipitate. After 5 min., filter the hot mixture through a glass frit (porosity 4-10 μm).

17 Repeat step 16 with the isolated precipitate from step 16.

18 Wash the yellow product **2** well with water. **PAUSE POINT** Dry product over night in an oven at ~80°C. Product **2** can be stored in room temperature for at least two years.

Synthesis of 3-[2-deoxy-3-O-[2-cyanoethoxy-(*N,N*-diisopropylamino)-phosphino]-5-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-1,3-diaza-2-oxophenothiazine (6).

19 Add 1.0 g (4.6 mmol) of **2** into a 250 ml two-neck, round-bottom flask containing a magnetic stir bar. Add 2.1 g (5.4 mmol) 2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride to a small glass beaker and put it into a vacuum desiccator.

20 Fit the round-bottom flask with a gas-inlet adapter and a rubber septum. Attach the gas-inlet adapter and the vacuum desiccator to a vacuum/nitrogen manifold. **PAUSE POINT** Leave the substances under vacuum over night to dry.

21 Release the vacuum to the round-bottom flask with a flow of nitrogen. Add 60 ml of dry DMF, freshly opened, to the flask using a plastic syringe with a disposable syringe needle. Turn on the magnetic stirrer.

22 Add 0.203 g (5.1 mmol) NaH (60% in mineral oil) to the suspension and wait until a clear solution is obtained. If the suspension has not dissolved in ~1 h, heat the suspension gently with a water-bath containing hot water from the tap. Once a clear solution is obtained, take away the warm water-bath and let the solution cool to room temperature.

23 Increase the flow of nitrogen to the flask and remove the rubber septum. Add the 2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride in portions (~4 portions over

~30 min.) to the solution (recap the flask between each addition). (A precipitate is formed at the end of the addition.)

CRITICAL STEP 2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride is unstable in DMF. Therefore, it should be added as a solid and not dissolved in DMF.

24 Continue stirring for 2 h. Remove the nitrogen inlet and filter the solution through a glass frit funnel. Discard the precipitate on the filter and transfer the filtrate to a 250 ml round-bottom flask and concentrate it to an oil using a rotary evaporator (40°C) connected to a vacuum pump.

25 Add 150 ml of ethyl acetate to the concentrate. After gentle warming (appr. 50°C) and sonication using an ultrasonic bath, filter off any undissolved substance using a glass frit funnel (porosity 4-10 μm). Transfer the filtrate to a separatory funnel and wash the ethyl acetate solution with saturated $NaHCO_3$ (5 x 100 ml) and saturated sodium chloride (1 x 100 ml). Dry the ethyl acetate phase with anhydrous sodium sulphate (add the sodium sulphate to a stirred solution in aliquots of ~0.5g until the organic solution appears to be clear. Leave for at least 5 minutes before filtering).

26 Filter off the sodium sulphate using a frit funnel and transfer the filtrate to a round-bottom flask.

27 Evaporate the ethyl acetate using a rotary evaporator at 40°C.

PAUSE POINT The product can be left overnight in a refrigerator

28 Pack a chromatography column (55 x 5.5 cm) with ~150 g silica gel using 0.5% (vol/vol) methanol in dichloromethane.

29 Add 100 ml of ethyl acetate to dissolve the crude mixture and add silica gel (~45 g). Evaporate the ethyl acetate using a rotary evaporator at 40°C.

30 Load the crude mixture adsorbed to the silica on top of the silica gel column using a long-stemmed powder funnel. Cover the top of the column with a layer of sand (~1 cm thick).

31 Elute the column under gravity or under slight pressure with solvent mixtures with increasing amount of methanol in dichloromethane, 1 l each of 0.5%, 1% and 2% (vol/vol) methanol. Start collecting 50 ml fractions after about ~1.5 l of solvent has run through. The bands can be visualized on the column by fluorescence using a 365 nm UV-lamp (the bands are also coloured yellow but it is easier to identify the different bands by fluorescence).

CAUTION Be careful when using the UV-lamp. UV-light is harmful to eyes.

32 Identify fractions containing the mixture of the α- and β-anomers of the protected nucleoside **3** by TLC. Use thin-layer silica gel on aluminium support with 5 % (vol/vol) methanol in dichloromethane (R_f = 0.3 for the β-anomer, R_f = 0.2 for the α-anomer). The bands are visualized by fluorescence using a 365 nm UV-lamp. (The anomeric mixture is isolated since the α- and β-anomer separates poorly at this stage. The two anomers are separated after deprotection.)

33 Evaporate the solvent from the pooled fractions containing the α- and β-anomeric mixture using rotary evaporator at 40°C and dry the residual oil in a 100 ml round-bottom flask for a few hours using a vacuum pump.

34 Dissolve a cleanly cut piece of sodium (50 mg) in 50 ml of dry methanol. By using a plastic syringe with a disposable syringe needle, add the sodium methoxid solution (2 eq. MeONa) and a magnetic stir bar to the round-bottom flask containing ~600 mg (~1 mmol) of the α/β-mixture **3**.

CAUTION Sodium metal reacts violently with water and alcohols to produce the highly flammable and explosive hydrogen gas. Use caution when adding sodium to methanol! Carry out this experiment in a fume hood and make sure there are no open flames in the vicinity.

35 Cover the flask with aluminium foil and turn on the stirring. **PAUSE POINT** Stir the solution over night at room temperature.

36 Add ~0.5 ml acetic acid to stop the reaction. Remove the magnetic stir bar and evaporate the reaction to dryness using a rotary evaporator at 40°C.

37 Add 30 ml of methanol to dissolve the crude product and transfer the solution to a 250 ml round-bottom flask. Add another 70 ml methanol together with silica gel (~13 g) and evaporate the methanol using a rotary evaporator at 40 °C.

38 Pack a chromatography column (55 x 5.5 cm) with ~100 g silica gel using 5% (vol/vol) ethanol (99.7%) in chloroform.

39 Load the crude mixture adsorbed to the silica from step 37 on top of the silica gel column using a long-stemmed powder funnel. Cover the top of the column with a layer of sand (~1 cm thick).

40 Run through 200 ml of 5 % (vol/vol) ethanol in chloroform and then increase the ethanol to 9% (vol/vol). Start collecting ~45 ml fractions after running ~1 l of solvent through. The bands are visualized by fluorescence using a 365 nm UV-lamp or by colour (yellow). This provides information of when to start to collect fractions.

41 Identify the fraction containing the β-nucleoside, **4**, by TLC. Use thin-layer silica gel on aluminium support with 15 % (vol/vol) ethanol in chloroform (R_f = 0.25 for the β-

anomer, $R_f = 0.15$ for the α- anomer). The bands are visualized by fluorescence using a 365 nm UV-lamp.

42 Evaporate the solvent from the pooled fractions containing **4** using rotary evaporator at room temperature. **PAUSE POINT** Dry the crystalline product over night using a vacuum pump. Product **4** can be stored at room temperature in the dark for several years.

43 Add 166 mg (0.49 mmol) of 4,4’-dimethoxytrityl chloride to a 5 ml pear-shaped flask.

44 Add 150 mg (0.45 mmol) of **4** to a 10 ml round-bottom flask containing a magnetic stir bar.

45 Fit both flasks with a gas-inlet adapter and attach them to a vacuum/nitrogen manifold. **PAUSE POINT** Leave flasks under vacuum over night.

46 Release the vacuum with a flow of nitrogen.

47 Add 5 ml of freshly distilled pyridine to the 10 ml flask containing **4** using a freshly opened plastic syringe with disposable syringe needle. Evaporate the pyridine using a rotary evaporator at 40 °C.

48 Repeat step 47 twice.

49 Fit the 10 ml flask with a gas-inlet adapter and attach it to a nitrogen flow. Purge the flask with nitrogen and leave it under an atmosphere of nitrogen.

50 Add 4 ml of freshly distilled pyridine to the flask using a freshly opened plastic syringe with a disposable syringe needle. Heating and sonication might be necessary to get **4** into solution.

CRITICAL STEP The reaction yield is strongly dependent on concentration. Use as small amount of solvent as possible.

51 Add 1 ml of freshly distilled (from CaH_2) pyridine to the 5 ml pear-shaped flask containing the 4,4'-dimethoxytrityl chloride using a freshly opened plastic syringe with disposable syringe needle.

52 Using a freshly opened plastic syringe fitted with a 20-gauge stainless steel syringe needle add the 4,4'-dimethoxytrityl chloride solution dropwise to the stirred solution containing **4**.

53 Stir the reaction for 2 h under an atmosphere of nitrogen.

54 Add 0.5 ml of methanol to the reaction using a plastic syringe with disposable syringe needle to stop the reaction.

55 Remove the magnetic stir bar and evaporate the solvent using a rotary evaporator at 40 °C.

56 Dissolve the crude product in 5 ml of toluene and evaporate again using a rotary evaporator at 40 °C.

57 Repeat step 56.

58 Pack a chromatography column (25 x 4 cm) with ~50 g silica gel using 5:1:94 (vol/vol) methanol/pyridine/dichloromethane and cover the top of the column with a layer of sand (~1 cm thick).

59 Dissolve the crude product from step 57 in a minimum volume of the solvent mixture, 5:1:94 (vol/vol) methanol/pyridine/dichloromethane and add this to the top of the column by using a Pasteur pipette.

60 Elute the column under gravity or under slight pressure with 5:1:94 (vol/vol) methanol/pyridine/dichloromethane. The band can be visualized by fluorescence using a 365 nm UV-lamp or simply by the colour (yellow). Start collecting fraction when the yellow band is about to come out. (Not reacted starting material can be eluted after the product has been isolated by running through 8:1:91 (vol/vol) methanol/pyridine/dichloromethane.)

61 Identify fraction containing **5** by TLC. Use thin-layer silica gel on aluminium support with 7:93 (vol/vol) methanol/dichloromethane (R_f = 0.15). The bands are visualized by fluorescence using a 365 nm UV-lamp.

62 Evaporate the solvent from the pooled fractions containing **5** using a rotary evaporator at 40 °C.

63 Coevaporate twice with toluene using a rotary evaporator at 40 °C. **PAUSE POINT** Dry the residual powder over night using a vacuum pump. Product **5** can be stored in the dark at -20°C for several years.

64 Add 199 mg (0.313 mmol) of **5** to a 10 ml round-bottom flask containing a magnetic stir bar.

CRITICAL STEP From this point all glass ware should be dried in an oven for at least 1 h above 100°C before use.

65 Fit the flask with the rubber septum and insert an argon inlet and a vent consisting of a disposable syringe needle.

66 Turn on a fairly strong argon flow to purge the flask and then reduce the flow to a minimum.

CRITICAL STEP Everything should be done and handled under argon from this point.

67 Using a plastic syringe with disposable syringe needle add 5 ml of freshly distilled tetrahydrofuran to the flask. Turn on the magnetic stirrer.

68 Using a plastic syringe with disposable syringe needle (or a Hamilton syringe) first add 0.135 ml (0.785 mmol) of freshly distilled diisopropylethylamine and then 0.07 ml (0.314 mmol) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite.

69 Leave the reaction mixture to stir at room temperature for 30 min.

70 Transfer the reaction under argon pressure using a cannula (double-ended needle) to a separating funnel containing 10 ml dry, degassed ethyl acetate (see Reagent Setup for more details).

71 Wash the solution by extracting it in a seporatory funnel with saturated potassium chloride (2 x 5ml).

72 Dry the ethyl acetate phase with anhydrous sodium sulphate (add the sodium sulphate to a stirred solution in aliquots of ~0.5g until the organic solution appears to be clear. Leave for at least 5 minutes before filtering).

73 Filter off the sodium sulphate using a frit funnel and transfer the filtrate to a 25 ml round-bottom flask.

74 Evaporate the ethyl acetate using a rotary evaporator at 50°C.

75 Purge a chromatography column (50 x 1.5 cm) with argon.

76 Keeping the column under an argon atmosphere pack it with ~50 g silica gel using 1 % (vol/vol) pyridine (distilled from KOH) in ethyl acetate (dried by decanting from anhydrous $MgSO_4$ and then purged with argon before use).

77 Dissolve the crude from step 74 in a minimum amount of 1 % (vol/vol) pyridine in ethyl acetate and load it to the top of the silica gel. Cover the top of the column with a layer of sand (~0.5 cm thick).

78 Elute the column under slight pressure of argon with 1 % (vol/vol) pyridine in ethyl acetate.

79 The product can be visualized as two closely spaced bands on the column by fluorescence using a 365 nm UV-lamp (the bands are also coloured yellow but it is easier to identify the different bands by fluorescence). Start collecting fractions as the first band is approaching. Each fraction eluting from the column was collected in a test tube and a blanket of argon was introduced by having an argon line with a syringe needle attached passing over each individual test tube just after the fraction is collected.

80 Identify fractions containing both stereomers of the product **6** by TLC. Use thin-layer silica gel plates on aluminium support with ethyl acetate (R_f =0.59, 0.47 for the stereomers). The bands are visualized by fluorescence using a 365 nm UV-lamp.

81 Transfer the pooled fractions containing **6** (under argon) to a 250 ml round bottom flask and evaporate using a rotary evaporator at 50°C.

82 Re-dissolve the residual oil in 5 ml dry tetrahydrofuran (destilled over sodium).

83 Transfer the tetrahydrofuran solution (under argon) to a pre-weighed 50 ml round bottom flask with a septum fitted.

84 Evaporate using a rotary evaporator at 50°C, re-fit the septum and quickly weigh the flask. **CRITICAL STEP** Do not purge this flask with argon as it will affect weight.

85 Put three disposable syringe needles in the septum, purge the flask with argon and remove the needles.

PAUSE POINT Product **6** can be stored in the dark at -20°C for at least 1 year.

Preparing the tC phosphoramidite 6 for DNA synthesis

86 Dissolve the phosphoramidite (**6**) in 8 ml of dry synthesis grade acetonitrile under argon.

87 Take up in a syringe and pull excess amidite back into the syringe body so that the needle is empty.

88 Put a 0.45 micron filter on the syringe and reattach the needle.

89 Filter the monomer into a weighed clean dry round-bottom flask which has been purged with argon.

CAUTION It is quite difficult to push the solution through the syringe. Make sure the syringe needle is fitted securely.

90 Evaporate off the acetonitrile from the amidite solution on a rotary evaporator at 50°C and let argon gas in when the flask is removed.

91 Redissolve the amidite in freshly distilled dichloromethane (alcohol free) under argon. Use 1 ml for every 100 mg of amidite.

92 Syringe the amidite into several dry monomer bottles (of the type used on the DNA synthesizer) under argon, 100 mg of amidite per bottle.

93 Put the monomer bottles in a desiccator, fit each with a septum and put a needle in each septum.

94 Evacuate under high vacuum and leave for 2 hours.

95 Carefully repressurise the desiccator with argon, remove the bottles from the desiccator and purge each with argon.

PAUSE POINT Monomer bottles can be stored in the dark at -20°C for at least 1 year.

Oligonucleotide synthesis and purification – general description

96 Oligonucleotides are synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser using a 0.2 μmole phosphoramidite cycle of acid-catalysed detritylation, coupling, capping and iodine oxidation. The synthesis is performed according to standard protocols (for details see Brown, T. and Grzybowski, J.)[28] except that the coupling time is increased to 325 s for the tC monomer (A, G, C and T coupling time is 25 s).

97 Purification of oligonucleotides containing tC is performed according to standard procedures (for details see Brown, T. and Brown D.J.S.)[29].

ANTICIPATED RESULTS

Absorption (ε=4000 $M^{-1}cm^{-1}$ at long wavelength maximum of tC) and emission spectra of derivatives of tC and tC incorporated into oligonucleotides as well as duplex stabilisation properties and examples of using tC can be found elsewhere.[19-22]

Typical yields

Typical isolated yields of the starting material, **2**, will be 20-25% over two steps. Typical yield of the tC nucleoside, **4**, is 10-15 % over the two steps. Typical yield of the DMT-protection and the phosphytilation is 60-70% and 90-95% respectively.

Analytical data

Characterization of intermediate **3** is not performed; identification is made by TLC (condition given in the procedure section).

2,4-dihydroxy-5-(2-amino-phenylthio)pyrimidine (1)

Yield, 30-40%; white powder. UV absorption (0.1 M NaOH) λ_{max} 294 nm, λ_{min} 265 nm

3*H*-1,3-diaza-2-oxophenothiazine (2)

Yield, ~70%; yellow powder. UV absorption (0.1 M NaOH) λ_{max} 235 nm, 309 nm, λ_{min} 282 nm, $\lambda_{shoulder}$ 253 nm, 352 nm.

3-(2-deoxy-β-D-ribofuranosyl)-1,3-diaza-2-oxophenothiazine (4).

Yield, 10-15% from **2**; yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.86 (s, 1H), 7.07 (m, 2H), 6.92 (m, 2H), 6.08 (t, J=6.6 Hz, 1H), *5.22 (1H, OH-not always visible-), 5.09 (1H, OH -not always visible-)*, 4.20 (m, 1H), 3.78 (m, 1H), 3.65-3.50 (m, 2H), 2.12 (m, 1H), 2.02 (m, 1H).

The main difference for the α-anomer in the ^{1}H NMR under the same conditions are: δ 6.01 (d, J=7.2 Hz, 1H), 4.20 (m, 2H), 1.89 (d, 1H). Furthermore, the multiplet at 3.65-3.50 in the β-spectrum has shifted and is presumably covered by the water peak at 3.33 ppm and one of the hydrogen at 2.12 and 2.02 is shifted and presumably covered by the solvent residual peak at 2.50 ppm.

3-[2-deoxy-5-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-1,3-diaza-2-oxophenothiazine (5).

Yield, 60-70%; yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.59 (s, 1H), 7.20-7.40 (m, 9H), 7.07 (m, 1H), 6.89-6.93 (m, 7H), 6.09 (t, J=6.4 Hz, 1H), *5.32 (1H, OH-not always visible-),* 4.26 (m, 1H), 3.91 (m, 1H), 3.71 (s, 6H), 3.23 (m, 1H), 3.14 (m, 1H), 2.11-2.26 (m, 2H).

3-[2-deoxy-3-O-[2-cyanoethoxy-(*N,N*-diisopropylamino)-phosphino]-5-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-1,3-diaza-2-oxophenothiazine (6)

Yield, 90-95% from **5,** ^{31}P NMR spectrum: δ_P(162 MHz, $CDCl_3$) 149.7, 149.2.

LRMS (ES+, CH_3CN) (*m/z*) [M+Na^+] calcd for $C_{45}H_{50}N_5O_7PSNa$, 858, found: 858 (M+Na^+, 100%). HRMS (ES+, CH_3CN) (*m/z*) [M+H^+] calcd for $C_{45}H_{51}N_5O_7PS$, 858.3061, found 858.3073.

TIMING

The starting materials **2** and the 2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride are both done in ~2 days and with these at hand the tC nucleoside, **4**, will require ~3 days and the phosphoramidite, **6**, another 2 days. Oligonucleotide synthesis takes 1 day and purification takes 3 hours.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests**. [I have added this competing Interests Statement on your behalf based on what the authors declared in our web-based system. Please review.]**

Figures and Figure Legends

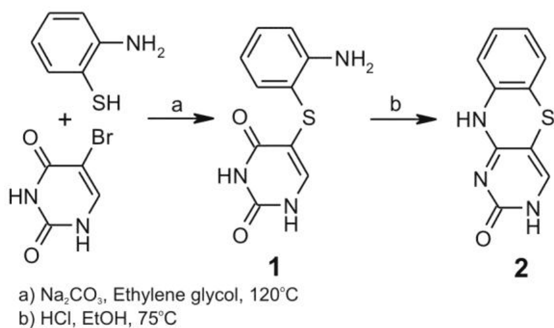

Figure 1 Scheme for the synthesis of starting material **2**, the tC base.

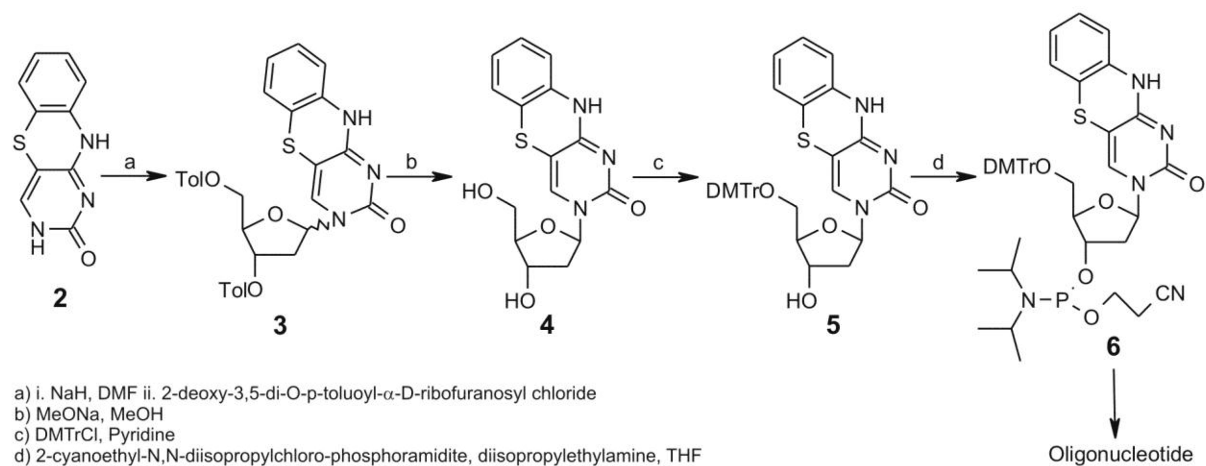

Figure 2 Scheme for the synthesis of the tC phosphoramidite.

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