Synthesis of 5-Alkynyl Substituted 2′-Arabinosyl 2′-Halogenated Uridine Nucleosides

Synthesis of 5-Alkynyl Substituted 2'-Arabinosyl 2'-Halogenated Uridine Nucleosides

Authors: David H. Hilko*, Laurent F. Bornaghi, Sally-Ann Poulsen*

Griffith Institute for Drug Discovery, Griffith University, Brisbane, Nathan, Queensland, 4111, Australia

Contact information: D.H. d.hilko@griffith.edu.au, L.B. l.bornaghi@griffith.edu.au S.-A.P. s.poulsen@griffith.edu.au

Significance statement

Chemical probes that are specific for the detection and visualisation of DNA synthesis enable biologists to study cell replication. This protocol unit describes the design and synthesis of novel nucleoside chemical probes with applications for the study DNA synthesis. The synthesis of the target compounds, 2'-halo-arabinosyl derivatives of the nucleoside thymidine, was not accessible using known methods and required the development of new synthetic methodology. Attempted nucleophilic substitution of a 2'-leaving group in pyrimidines leads to formation of a 2,2'-anhydronucleoside instead of the desired product. The key to overcoming this unwanted intramolecular cyclisation was to employ an underexplored protecting group – a *N*-nitro group. This unit reports optimisation of the chemistry of this group to prepare 5-alkynyl-2'-halogenated arabinosyl uridine nucleosides.

ABSTRACT

This unit describes the detailed preparation of 5-alkynyl-2'-halogenated arabinosyl uridine nucleosides (2'-halo-ara-EdU) from uridine. These compounds were synthesised as prospective chemical probes for the detection of DNA synthesis in proliferating cells. Currently, this is the only synthetic methodology reported to access these compounds. The key to success of the synthetic approach was to employ a 3-*N*-nitro protecting group to stabilise the required 2'-triflate nucleoside precursor toward nucleophilic substitution. Several synthetic challenges were overcome to accommodate the combination of a 5-alkyne and 3-*N*-nitro functional group, including facile introduction and removal of the *N*-nitro group, and removal of the sugar acetyl groups under acidic conditions.

BASIC PROTOCOL 1: Synthesis and characterisation of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-triflyl-uridine (7).

BASIC PROTOCOL 2: Synthesis and characterisation of (2'*S*)-chloro-2'-deoxy-5-ethynyl-uridine (Cl-ara-EdU)

BASIC PROTOCOL 3: Synthesis and characterisation of (2'*S*)-bromo-2'-deoxy-5-ethynyl-uridine (Br-ara-EdU)

BASIC PROTOCOL 4: Synthesis and characterisation of (2'*S*)-iodo-2'-deoxy-5-ethynyl-uridine (I-ara-EdU)

Keywords: nucleoside, arabinose, chemical probe, nitro protecting group

INTRODUCTION

5-Ethynyl-2'-deoxyuridine (EdU) is a thymidine analogue employed as a chemical probe to visualise DNA synthesis in proliferating cells. EdU is incorporated into DNA when EdU treated cells undergo mitotic division. The alkyne group of EdU plays an important role as a chemical handle for reaction with an azide functionalised fluorophore using click chemistry, allow detection of the DNA by high content imaging. A small number of nucleoside chemical probes structurally related to EdU have been synthesised and used in varying capacities as chemical probes for DNA (Neef & Luedtke, 2011; Neef et al., 2015; Neef et al., 2012). Of particular note is 2'-fluoro-arabinosyl EdU (F-ara-EdU), which differs to EdU only in the 2' position. F-ara-EdU has reduced cytotoxicity compared to EdU (Neef & Luedtke, 2011) and we were interested to fully explore the structure-activity relationships of halogens at the 2' position of EdU. We thus sought to synthesise 2'-halo-arabinosyl EdU compounds with chlorine, bromine, or iodine at the 2' position (Hilko et al., 2018). The synthesis required the development of an approach to exchange the 2'-hydroxyl of the preformed nucleoside with the halogen that avoided a base-sugar condensation approach that would lead to unwanted mixtures of anomers and lengthy reaction times. An essential feature of this direct methodology is the stabilisation of the requisite 2'-triflate nucleoside precursor (Serra et al., 1998) toward nucleophilic substitution. To accomplish this, facile introduction and removal of a 3-*N*-nitro group was developed in combination with optimisation of protecting group strategies to accommodate the combination of the 3-*N*-nitro and the 5-ethynyl functionality (Hilko et al., 2018).

This unit describes a selective protecting group methodology to synthesise 2'-halo-arabinosyl EdU incorporating chloro (**9**), bromo (**11**), or iodo (**13**) at the 2' position. The overall synthesis (**Figure 1**) is divided into four protocols. The first protocol describes the synthesis of the common 2'-triflate intermediate (**7**). The remaining three protocols describe the synthesis of halogenated derivatives **9**, **11**, and **13**, respectively, from intermediate **7**.

Basic Protocol 1 describes the synthesis of the common 2′-triflate intermediate 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**) from uridine (**1**).

Basic Protocol 2 describes the synthesis of target compound (2′*S*)-chloro-2′-deoxy-5-ethynyl-uridine (Cl-ara-EdU) (**9**) from 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**).

Basic Protocol 3 describes the synthesis of target compound (2′*S*)-bromo-2′-deoxy-5-ethynyl-uridine (Br-ara-EdU) (**11**) from 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**).

Basic Protocol 4 describes the synthesis of target compound (2′*S*)-iodo-2′-deoxy-5-ethynyl-uridine (I-ara-EdU) (**13**) from 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**).

Figure 1 here

BASIC PROTOCOL 1

Synthesis and characterisation of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (7).

Synthesis of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**) was accomplished in seven steps (**Figure 2**). There are two key steps developed for this synthesis: (1) mild conditions for *N*-nitration, and (2) deacetylation conditions compatible with the alkyne and *N*-nitro functionality. The synthesis commences from**1** (uridine), however **3** (5-iodo-per-*O*-acetylated uridine) is commercially available. A modified literature procedure (Claudio-Montero et al., 2015) was employed to synthesise 2′,3′,5′-tri-*O*-acetyl-5-(ethynyl(2-trimethylsilyl))-uridine (**4**) from **3**, then *N*-nitration of intermediate **4** was carried out to give **5**, followed by deacetylation to give **6**. From **6** a one-pot 3′,5′-silylation and 2′-triflylation generated the target 3-*N*-nitro-2′-triflate compound **7**.

Figure 2 here

CAUTION: Some of the chemicals and reagents used are toxic, corrosive, and/or flammable. Refer to material safety data sheets prior to use. All the reactions should be conducted in a well-ventilated fume hood. Use of personal protective equipment is recommended.

NOTE: Anhydrous reaction conditions are required for several steps of this procedure. Anhydrous solvents can be purchased in Sure/Seal bottles (e.g., from Sigma-Aldrich). It is recommended that reaction glassware is oven-dried before use.

Materials

Consumable:

Uridine (**1**; Sigma-Aldrich; cat. no. U3750)

Pyridine, anhydrous (Sigma-Aldrich; cat. no. 270970)

Acetic anhydride (Fluka; cat. no. 45830)

Thin layer chromatography (TLC) plates aluminium backed (silica gel 60 F254) (Merck; cat. no. 1.05554.0001)

Methanol (Merck; cat. no. 1.06009.2500)

Dichloromethane (Sigma-Aldrich; cat. no. 270997)

Vanillin stain (see recipe in Reagents and Solutions)

Amberlite® IR120 hydrogen form acidic resin (Fluka; cat. no. 06428)

Ethyl acetate (Merck; cat. no. 1.09623.2511)

5% Hydrochloric acid aqueous solution (RCI Labscan; cat. no. R1104)

Saturated aqueous sodium bicarbonate solution prepared from (Chem-supply; cat. no. SA001)

Saturated aqueous sodium chloride solution prepared from (Chem-supply; cat. no. SA046)

Magnesium sulfate (powdered, anhydrous) ($MgSO_4$) (Chem-supply; cat. no. ML073)

Iodine (Sigma-Aldrich; cat. no. 270970)

Ceric(IV) ammonium nitrate (Lancaster; cat. no 13970)

Acetonitrile (anhydrous) (Sigma-Aldrich; cat. no. 271004)

5% Sodium thiosulfate aqueous solution prepared from (Chem-supply; cat. no. SA018)

Silica gel 60 (230 to 400 mesh) (Merck; cat. no. 1.09385.1000)

Copper(I) iodide (Sigma-Aldrich; cat. no. 205540)

Tetrakis(triphenylphosphine)palladium(0) (Sigma-Aldrich; cat. no. 216666)

2-Ethynyl-trimethylsilane (Sigma-Aldrich; cat. no. 218170)

Triethylamine (Sigma-Aldrich; cat. no. 471283)

1 M Aqueous ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) solution prepared from (Chem-supply; cat. no. EA023)

Tetrabutylammonium nitrate (AK scientific; cat. no. O468)

Trifluoroacetic anhydride (Sigma-Aldrich; cat. no. 106232)

p-Toluenesulfonic acid monohydrate (Sigma-Aldrich; cat. no. 402885)

1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (AK scientific; cat. no. X2999)

N-Hexane (Merck; cat. no. 1.04391.2500)

1 M Trifluoromethanesulfonic anhydride dichloromethane solution (Sigma-Aldrich; cat. no. 704083)

1 M Aqueous sodium phosphate buffer solution (pH ≈ 7) prepared from NaH_2PO_4 (Sigma-Aldrich; cat. no. S0751) and Na_2HPO_4 (Sigma-Aldrich; cat. no. S9763)

Equipment:

Magnetic stirrer bars

Round bottom flasks – 25 mL, 50 mL, 100 mL, 250 mL

Argon gas supply (high purity >99.9%)

Magnetic stirrer hotplate

Ultraviolet lamp (254 nm)

Separatory funnel – 100 mL, 250 mL

Erlenmeyer flasks

Sintered glass filter funnel (porosity 3)

Rotary evaporator equipped with a both a diaphragm pump and oil pump

Chromatography columns 20 cm (H) × 2 cm (dia.), 20 cm (H) × 3.5 cm (dia.)

Sintered glass filter connector (female to male)

Protocol steps—*Step annotations*

Acetylation of uridine 1 to give 2

1. Add 5 g of uridine and a stirrer bar to an oven dried 50 mL round-bottom flask then apply a vacuum and back flush with argon. Attach a balloon of argon to the reaction vessel.
2. Add 22 mL of pyridine followed by 4.8 mL of acetic anhydride with stirring.

   This reaction is exothermic. If conducted on a larger scale then cooling in an ice bath is recommended during the addition of acetic anhydride.
3. After stirring for 4 hr at RT, TLC analysis typically indicates the reaction is complete. If, however, multiple products are evident, then continue stirring until TLC analysis indicates a single product.

   The starting material should be run alongside the reaction mixture for comparison. The TLC plates are developed using 10% methanol in dichloromethane solution. The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.46 (10% methanol in dichloromethane (v/v)). Pyridine will be highly visible by UV shadowing, this can be removed by treating an aliquot of reaction mixture in a small vial with Amberlite® IR120 H acidic resin in ethyl acetate then applying this mixture to the TLC plate.
4. Add 5 mL of water to the reaction mixture to quench the excess acetic anhydride. Stir for 15 min.

   Quenching of acetic anhydride with water is exothermic. If conducted on a larger scale then cooling in an ice bath is recommended. Some product may precipitate as a solid or gum upon addition of water.
5. Add 10 mL of ethyl acetate to the reaction mixture and stir until any precipitate is redissolved.
6. Transfer this biphasic solution to a separatory funnel and add a further 40 mL of ethyl acetate to the mixture in the separatory funnel.
7. Add 20 mL of 5% hydrochloric acid aqueous solution to the mixture in the separatory funnel and shake thoroughly. Allow the phases to separate then pour the organic (ethyl acetate) and aqueous (5% hydrochloric acid) extracts into separate Erlenmeyer flasks.
8. Back extract the 5% hydrochloric acid aqueous extracts with 20 mL of ethyl acetate to ensure complete recovery of product.
9. Combine the ethyl acetate extracts and return these to the separatory funnel and repeat steps 6 and 7.
10. Add 20 mL of saturated aqueous sodium bicarbonate solution to the ethyl acetate extract and shake to neutralise the residual hydrochloric acid. Allow the phases to separate then pour the layers into separate Erlenmeyer flasks.

Warning: gas is produced during the neutralisation of hydrochloric acid, this may pressurise the separatory funnel. Care should be taken to release the pressure intermittently during shaking by opening of the stopcock with the separatory funnel inverted.

11. Back extract the sodium bicarbonate extracts with 20 mL of ethyl acetate. This helps to ensure complete recovery of product.
12. Combine the ethyl acetate extracts in the separatory funnel and add 20 mL of saturated aqueous sodium chloride solution. Shake to remove excess water from the ethyl acetate extract. Allow the phases to separate then pour the layers into separate Erlenmeyer flasks.
13. Discard the aqueous extract.
14. Dry the ethyl acetate extract over anhydrous $MgSO_4$. Filter and remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C). This will afford a slightly yellow gum that solidifies to white solid on standing.
15. Check purity of the product by ^{1}H NMR and TLC.

2′,3′,5′-Tri-*O*-acetyl-uridine (**2**): 7.6 g, (100%); TLC R_f = 0.46 (10% methanol in dichloromethane (v/v)); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 9.31 (br s, 1H, NH), 7.39 (d, *J* = 8.2 Hz, 1H, H-6), 6.03 (d, *J* = 4.8 Hz, 1H, H-1′), 5.79 (d, *J* = 8.1 Hz, 1H, H-5), 5.37 – 5.29 (m, 2H), 4.39 – 4.28 (m, 3H), 2.13 (s, 3H, AcO), 2.12 (s, 3H, AcO), 2.09 (s, 3H, AcO). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 170.3, 169.78, 169.77, 162.9, 150.4, 139.4, 103.6, 87.6, 80.1, 72.8, 70.3, 63.3, 20.9, 20.6, 20.5.

Iodination of 2 to give 3

16. Add 1.5 g of 2′,3′,5′-tri-*O*-acetyl-uridine (**2**), 1.33 g of iodine, 0.45 g of ceric ammonium nitrate, and a stirrer bar to an oven dried 50 mL round-bottom flask. Apply a vacuum then back flush with argon. Attach a balloon of argon to the reaction vessel.
17. Add 17 mL of acetonitrile, apply stirring and heat to 50 °C.
18. After stirring for 16 hr at 50 °C, TLC analysis typically indicates complete conversion of the starting material to a single product.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 40% ethyl acetate in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until colour change is observed. Typically, the R_f of the desired compound is 0.46 (40% ethyl acetate in dichloromethane (v/v)).
19. Prepare a $MgSO_4$ plug in a sintered glass filter funnel and pre-wet with dichloromethane (50 mL). Dilute the reaction mixture with 25 mL of dichloromethane and filter through the $MgSO_4$ plug. Wash the $MgSO_4$ plug with a further 25 mL of dichloromethane.

20. Transfer the filtrate into a 250 mL separatory funnel. Add 20 mL of 5% sodium thiosulfate aqueous solution and shake until the colouration due to iodine dissipates.
21. Collect the dichloromethane extract into a 250 mL Erlenmeyer flask. Dry over $MgSO_4$, filter, and remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C).
22. Prepare a silica gel flash chromatography column using approximately 70 g of silica in a 20 cm (H) × 3.5 cm (dia.) column.
23. Dissolve the crude product in a minimal amount of dichloromethane (approx. 1 – 2 mL) and apply to the top of the column and elute with a mixture of 20% ethyl acetate in dichloromethane (v/v) using a flow rate of approximately 25 mL/min. Collect 15 mL fractions.
24. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C). A slightly yellow solid remains.
25. Check purity of the product by ^{1}H NMR and TLC.

    2′,3′,5′-Tri-*O*-acetyl-5-iodo-uridine (**2**): 1.88 g, (94%); TLC R_f = 0.35 (5% methanol in dichloromethane (v/v)); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 9.37 (br s, 1H, NH), 7.88 (s, 1H, H-6), 6.10 – 6.03 (m, 1H, H-1′), 5.39 – 5.27 (m, 2H, H-2 and H-3′), 4.44 – 4.28 (m, 3H, H-4′ and H-5′$_{(\alpha \text{ and } \beta)}$), 2.23 (s, 3H, AcO), 2.12 (s, 3H, AcO), 2.10 (s, 3H, AcO). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 170.2 (H_3CCOO), 169.8 (H_3CCOO), 169.8 (H_3CCOO), 159.7 (C-4), 150.1 (C-2), 143.9 (C-6), 87.3 (C-1′), 80.4 (C-4′), 73.2 (C-2′), 70.3 (C-3′), 69.8 (C-5), 63.1 (C-5′), 21.2 (H_3CCOO), 20.6 (H_3CCOO), 20.5 (H_3CCOO). LRMS (ESI): *m/z* = 496 $[M + H]^+$.

Couple 3 with 2-ethynyl-trimethylsilane under Sonogashira conditions to give 4.

26. Add 1.7 g 2′,3′,5′-tri-*O*-acetyl-5-iodo-uridine (**3**), 0.065 g (0.342 mmol) copper(I) iodide, 0.197 g (0.171 mmol) tetrakis(triphenylphosphine)palladium(0), and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.
27. Apply stirring at RT, then add in quick succession by syringe: 10 mL of dichloromethane, 2.4 mL (17.3 mmol) of 2-ethynyl-trimethylsilane, and 1 mL (7.13 mmol) of triethylamine.

    Upon addition of triethylamine, the reaction mixture should clarify and have a orange/yellow colouration. As the reaction proceeds, the solution typically changes colour from yellow to black. The reaction often proceeds faster with older, slightly oxidised catalyst (orange in appearance) as opposed to newer catalyst (bright yellow in appearance).
28. Stir for 2 hr at RT then analyse the reaction mixture by TLC.

The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 °C until a colour change is observed. Typically, the R_f of the desired compound is 0.37 (20% ethyl acetate in dichloromethane (v/v)). The desired product stains red upon heating with vanillin stain and, upon cooling, tends to appear green.

29. Remove the dichloromethane *in vacuo* using a rotary evaporator (water bath temperature 40 °C). Dissolve the residue in 20 mL of ethyl acetate and transfer to a 250 mL separatory funnel.
30. Add 20 mL of 5% hydrochloric acid aqueous solution to the mixture in the separatory funnel. Shake thoroughly. Allow the phases to separate, then pour the layers into separate Erlenmeyer flasks.
31. Back extract the 5% hydrochloric acid aqueous extracts with 20 mL of ethyl acetate. This helps to ensure complete recovery of product.
32. Add 20 mL of saturated aqueous sodium bicarbonate solution to the ethyl acetate extract. Shake thoroughly to neutralise the residual hydrochloric acid in the ethyl acetate extract. Allow the phases to separate, then pour the layers into separate Erlenmeyer flasks.

    Warning: gas is produced during the neutralisation of hydrochloric acid, this may pressurise the separatory funnel. Care should be taken to release the pressure intermittently during shaking by opening of the stopcock with the funnel inverted.
33. Back extract the sodium bicarbonate extracts with 20 mL of ethyl acetate. This helps to ensure complete recovery of product.
34. Combine the ethyl acetate extracts in the separatory funnel. Add 20 mL of 1 M aqueous ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) solution to remove copper salts. Allow the phases to separate then pour the layers into separate Erlenmeyer flasks.
35. Back extract the ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) washing with 20 mL of ethyl acetate to ensure complete recovery of product. Repeat step 34.

    Repeat step 34 until the aqueous extract no longer develops a blue colouration.
36. Combine the ethyl acetate extracts then dry over $MgSO_4$ and concentrate *in vacuo* on a rotatory evaporator (water bath temperature 40 °C).
37. Prepare a silica gel flash chromatography column using approximately 70 g of silica in a 20 cm (H) × 3.5 cm (dia.) column.
38. Dissolve the crude product in a minimal amount of dichloromethane (approx. 1 – 2 mL) and apply to the top of the column and elute with gradient of 10% ethyl acetate in dichloromethane (v/v) to 20% ethyl acetate in dichloromethane (v/v) using a flow rate of approximately 25 mL/min. Collect 15 mL fractions.

39. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C). An off-white foam remains.
40. Check purity of the product by ^{1}H NMR and TLC.

    2′,3′,5′-Tri-*O*-acetyl-5-(ethynyl(2-trimethylsilyl))-uridine (**4**): 1.52 g, (95%); TLC R_f = 0.37 (20% ethyl acetate in dichloromethane (v/v)); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 8.93 (br s, 1H, NH), 7.76 (s, 1H, H-6), 6.10 (d, *J* = 4.9 Hz, 1H, H-1′), 5.36 – 5.28 (m, 2H, H-2′ and H-3′), 4.42 – 4.32 (m, 3H, H-4′ and H-5′$_{(\alpha \text{ and } \beta)}$), 2.21 (s, 3H, AcO), 2.12 (s, 3H, AcO), 2.10 (s, 3H, AcO), 0.21 (s, 9H, $(Si(CH_3)_3)$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 170.2 (H_3CCOO), 169.9 (H_3CCOO), 169.7 (H_3CCOO), 160.8 (C-4), 149.3 (C-2), 142.2 (C-6), 101.6 (C-5), 100.5 (C≡*C*–Si), 95.0 (*C*≡C–Si), 87.3 (C-1′), 80.4 (C-4′), 73.4 (C-2′), 70.3 (C-3′), 63.2 (C-5′), 21.2 (H_3CCOO), 20.7 (H_3CCOO), 20.6 (H_3CCOO), -0.0 ($Si(CH_3)_3$). LRMS (ESI): *m/z* = 465 $[M - H]^-$, 467 $[M + H]^+$, 489 $[M + Na]^+$.

***N*-Nitration of 4 to give 5.**

41. Prepare a silica gel flash chromatography column using approximately 70 g of silica in a 20 cm (H) × 3.5 cm (dia.) column and condition with 5% ethyl acetate in dichloromethane (v/v).
42. Add 1.52 g of 2′,3′,5′-tri-*O*-acetyl-5-(ethynyl(2-trimethylsilyl))-uridine (**4**), 1.98 g (6.51 mmol) of tetrabutylammonium nitrate, and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon. Attach a balloon of argon to the reaction vessel.
43. Add 7 mL of dichloromethane (anhydrous) and stir at RT for 5 min. Cool in an ice bath.
    Not all the tetrabutylammonium nitrate will dissolve.
44. Keep the reaction vessel in the ice bath. With stirring at high speed (1000 – 1500 rpm) add 0.91 mL (6.51 mmol) of trifluoracetic anhydride and allow to react to for 1-2 min. Next add a few drops of methanol to remove the fuming yellow colouration and quench the reaction.

    Typically, a reaction time between 30 s and 2 min will give quantitative conversion to the desired product, as determined by TLC. The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.91 (20% ethyl acetate in dichloromethane (v/v)) and 0.36 (dichloromethane). The starting material and

desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.

45. Add the reaction mixture to the top of the previously prepared silica flash chromatography column and elute the product using 5% ethyl acetate in dichloromethane (v/v) using a flow rate of approximately 25 mL/min. Collect 7 mL fractions.
46. Check the fractions for the desired compound by TLC. Check these fractions for trifluoroacetic acid contamination using universal indicator pH paper before combining the fractions, neutral pH should be observed for uncontaminated fractions. If acid is detected in the desired product then repeat step 41 and 45 on these fractions, then proceed to step 47.

    Trifluoroacetic acid contamination can occur if excessive methanol is used to quench the reaction or if the column is improperly prepared.
47. Combine the pure fractions of the desired compound and remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C) to give a colourless white solid.
48. Check purity of the product by ^{1}H NMR and TLC.

    2′,3′,5′-Tri-*O*-acetyl-5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-uridine (**5**): 1.66 g (100%); TLC R_f = 0.36 (dichloromethane); mp = 135–137 ˚C; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 7.78 (s, 1H, H-6), 6.08 – 6.05 (m, 1H, H-1′), 5.36 – 5.32 (m, 2H, H-2′ and H-3′), 4.42 (ddd, *J* = 2.6, 0.8 Hz, 1H, H-4′), 4.38 (d, *J* = 2.6 Hz, 2H, H-5′$_{(\alpha \text{ and } \beta)}$), 2.21 (s, 3H, AcO), 2.13 (s, 3H, AcO), 2.12 (s, 3H, AcO), 0.22 (s, 9H, Si(CH_3)$_3$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 169.9 (H_3CCOO), 169.7 (H_3CCOO), 169.7 (H_3CCOO), 153.8 (C-4), 144.7 (C-2), 140.9 (C-6), 102.4 (C≡*C*–Si), 101.3 (C-5), 93.2 (*C*≡C–Si), 88.3 (C-1′), 80.9 (C-4′), 73.4 (C-2′), 70.1 (C-3′), 62.9 (C-5′), 21.0 (H_3CCOO), 20.6 (H_3CCOO), 20.5 (H_3CCOO), -0.2(Si(CH_3)$_3$). LRMS (ESI): *m/z* = 556 $[M + HCOO]^-$, 534 $[M + Na]^+$.

Remove acetate groups from 5 to give 6.

49. Add 0.6 g (1.17 mmol) of 2′,3′,5′-tri-*O*-acetyl-5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-uridine (**5**), 0.127 g (1.17 mmol) *p*-toluenesulfonic acid monohydrate, 0.197 g (0.171 mmol) and a stirrer bar to an oven-dried 25 mL round-bottom flask. Apply a vacuum then flush with argon three times. Attach a balloon of argon to the reaction vessel.
50. Add 3 mL methanol and minimum amount of dichloromethane (c.a. 0.5 to 1 mL) to effect solubilisation of the starting material. Stir the reaction mixture at RT for 36 - 48 h.
51. Analyse the reaction mixture by TLC.

The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.59 (10% methanol in dichloromethane (v/v)). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green. Several by-products and intermediates will be present. The desired product should be second most polar compound above the baseline and should appear most concentrated compared to the other products.

52. Prepare a silica gel flash chromatography column using approximately 40 g of silica gel in a 20 cm (H) × 2 cm (dia.) column and condition with 5% methanol in dichloromethane (v/v).
53. Remove the stirrer bar with a magnetic stirrer bar retriever and rinse it with a few mL of dichloromethane into the reaction vessel. Then add 10 mL of dichloromethane to the reaction mixture followed by approximately 5 g of silica.
54. Adsorb the crude material onto the silica by removing the solvents *in vacuo* using a rotary evaporator (with the rotary evaporator water bath at RT). A free-flowing powder is obtained.

    To prevent damage to the rotary evaporator and vacuum pump, use a sintered glass filter connector in place between the round-bottom flask and the rotary evaporator. Heating of the mixture at this point will cause decomposition of the product. Minimal time should be wasted once the product is adsorbed onto silica as product loss increases with time.
55. Add the compound adsorbed onto silica from step 54 to the top of the silica gel column (step 52). Elute the product with a gradient of 5-8% methanol in dichloromethane (v/v) using a flow rate of approximately 12 mL/min. Collect 10 mL fractions.
56. Using TLC, identify the pure fractions containing the desired product. Combine the pure fractions and remove the solvent *in vacuo* using a rotary evaporator (with the rotary evaporator water bath at RT) to give colourless a gum/glass.

    Either use immediately or store in a freezer (-20 ℃). The compound is stable for at least 2 weeks at -20 ℃ as determined by TLC analysis. It may also be stored in solution as obtained from the chromatography column for 1 -2 days at RT without observable decomposition as determined by TLC analysis. The product decomposes on standing for >2 days at RT.
57. Check purity of the product by ^{1}H NMR and TLC.

    5-(Ethynyl(2-trimethylsilyl))-3-*N*-nitro-uridine (**6**): 0.360 g (80%,); TLC R_f = 0.59 (10% methanol in dichloromethane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 8.66 (s, 1H, H-6), 5.66 (d, *J* = 2.9 Hz, 1H, H-1'), 5.60 (d, *J* = 5.0 Hz, 1H, OH-2'), 5.37 (t, *J* = 4.4 Hz, 1H, OH-5'), 5.11 (d, *J* = 6.3 Hz, 1H, OH-3'), 4.14 (td, *J* = 4.8, 2.7 Hz,

1H, H-2′), 4.01 (q, *J* = 6.2 Hz, 1H, H-3′), 3.92 (dt, *J* = 6.6, 2.4 Hz, 1H, H-4′), 3.78 (ddd, *J* = 12.1, 4.5, 2.6 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 3.61 (ddd, *J* = 12.2, 2.2 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 0.20 (s, 9H, $Si(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 154.8 (C-4), 145.0 (C-6), 144.5 (C-2), 98.9 (C≡*C*–Si), 97.3 (C-5), 95.5 (*C*≡C–Si), 90.9 (C-1′), 84.6 (C-4′), 74.0 (C-2′), 68.1 (C-3′), 59.1 (C-5′), -0.2 ($Si(CH_3)_3$). LRMS (ESI): *m/z* = 384 [M - H]$^-$, 430 [M + $HCOO^-$]$^-$.

Silylate the 3′ and 5′ hydroxyls and triflate the 2′ hydroxyl of 6 in one-pot to give 7.

58. Add 0.300 g (0.778 mmol) of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-uridine (**6**), and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then flush with argon three times. Attach a balloon of argon to the reaction vessel.
59. Add 2 mL of pyridine and stir the mixture until the starting material is dissolved. Cool the mixture in an ice bath.
60. With stirring at high speed (1000 – 1500 rpm) slowly add 0.28 mL (0.875 mmol) of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane.
61. Allow the mixture to warm slowly to RT as the ice in the ice bath melts (c.a. 2-3 hours). Continue stirring until the reaction is complete as evidenced by TLC (c.a. 8 to 16 h).

    The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in hexane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.41 (20% ethyl acetate in hexane (v/v)). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green. One by-product, that is less polar than the desired product, may be present in trace amounts.
62. Cool the mixture in an ice bath. With stirring at high speed (1000 – 1500 rpm) add dropwise 1.2 mL (1.16 mmol) of 1 M trifluoromethanesulfonic anhydride dichloromethane solution.
63. Stir for 10 min then check the reaction progress by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in hexane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.88 (20% ethyl acetate in hexane (v/v)). The intermediate and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green. One by-product, which is more polar than the desired product, may be present in

trace amounts. If the reaction is incomplete add another 0.2 equivalents of 1 M trifluoromethanesulfonic anhydride dichloromethane solution.

64. Once TLC analysis indicates complete conversion of the intermediate to the product, add 20 mL of ethyl acetate and transfer the mixture to a 250 mL separatory funnel.
65. Add 20 mL of 5% hydrochloric acid aqueous solution to the mixture in the separatory funnel. Shake thoroughly. Allow the phases to separate then pour the layers into separate Erlenmeyer flasks.
66. Back extract the 5% hydrochloric acid aqueous extracts with 20 mL of ethyl acetate. This helps to ensure complete recovery of product.
67. Combine the ethyl acetate extracts and return to the separatory funnel. Repeat steps 65 and 66.
68. Add 20 mL of 1 M aqueous sodium phosphate buffer solution (pH ≈ 7) to the ethyl acetate extract in the separatory funnel. Shake to neutralise the residual hydrochloric acid. Allow the phases to separate then pour the layers into separate Erlenmeyer flasks.
69. Back extract the aqueous sodium phosphate buffer solution extracts with 20 mL of ethyl acetate to ensure complete recovery of product.
70. Combine the ethyl acetate extracts in the separatory funnel and add 20 mL of saturated aqueous sodium chloride solution. Shake to remove excess water from the ethyl acetate extract. Allow the phases to separate then pour the layers into separate Erlenmeyer flasks.
71. Dry the ethyl acetate extract over anhydrous $MgSO_4$. Filter and remove the solvent *in vacuo* in a rotary evaporator (with the rotary evaporator water bath at RT). Immediately redissolve the crude product in 1 – 2 mL of dichloromethane.
72. Prepare a silica gel flash chromatography column using approximately 40 g of silica gel in a 20 cm (H) × 2 cm (dia.) column and condition 100% dichloromethane.
73. Apply the crude product from step 71 (dissolved in approx. 1 – 2 mL dichloromethane) to the top of the column. Elute with 100% dichloromethane using a flow rate of approximately 12 mL/min. Collect 7 mL fractions.
74. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C). A waxy white solid remains.
75. Check purity of the product by ^{1}H NMR and TLC.

5-(Ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**): 0.472 g (80%,); TLC R_f = 0.88 (20% ethyl acetate in *n*-hexane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 7.92 (s, 1H, H-6), 5.89 (s, 1H, H-1′), 5.23 (d, *J* = 4.2 Hz, 1H, H-2′), 4.45 (dd, *J* = 9.5, 4.3 Hz, 1H, H-3′), 4.33 (d, *J* = 13.9 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 4.17 (dd, *J* = 9.5, 2.5 Hz, 1H, H-4′), 4.03 (dd, *J* = 14.0, 2.7 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 1.18 – 0.97 (m, 28H, (Si(*i*-Pr)$_2$)$_2$), 0.22 (s, 9H, Si(CH_3)$_3$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 153.9 (C-4), 144.2 (C-2), 140.1 (C-6), 102.8

(C≡*C*–Si), 100.9 (C-5), 92.9 (*C*≡C–Si), 88.8 (C-1′), 87.3 (C-2′), 82.7 (C-4′), 66.9 (C-3′), 58.6 (C-5′), 17.6 (SiCH(*C*H_3)$_2$), 17.6 (SiCH(*C*H_3)$_2$), 17.4 (SiCH(*C*H_3)$_2$), 17.3 (SiCH(*C*H_3)$_2$), 17.0 (SiCH(*C*H_3)$_2$), 16.9 (SiCH(*C*H_3)$_2$), 16.9 (SiCH(*C*H_3)$_2$), 16.8 (SiCH(*C*H_3)$_2$), 13.7 (Si*C*H(CH$_3$)$_2$), 13.0 (Si*C*H(CH$_3$)$_2$), 12.9 (Si*C*H(CH$_3$)$_2$), 12.7 (Si*C*H(CH$_3$)$_2$), -0.2 (Si(CH$_3$)$_3$). LRMS (ESI): *m/z* = 688 [M-TMS+H]$^+$, 564 [M –TfO + H]$^+$.

BASIC PROTOCOL 2

Synthesis and characterisation of (2′*S*)-chloro-2′-deoxy-5-ethynyl-uridine (Cl-ara-EdU) (9).

The synthesis of (2′*S*)-chloro-2′-deoxy-5-ethynyl-uridine (Cl-ara-EdU) (**9**) is accomplished by displacement of the 2′-*O*-triflyl group of **7** with chloride to give **8**, followed by global deprotection of **8** (**Figure 3**). The product, compound **9**, is purified by silica gel flash chromatography. The key chemical step in this synthesis was the development of mild, one-pot desilylation/de-*N*-nitration methodology for deprotection of **8**. ^{1}H NMR 2D NOESY (Nuclear Overhauser Effect Spectroscopy) of product **9** confirmed the desired α-configuration at the 2′ position.

Figure 3 here

CAUTION: Some of the chemicals and reagents used are toxic, corrosive, and/or flammable. Refer to material safety data sheets prior to use. All the reactions should be conducted in a well-ventilated fume hood. Use of personal protective equipment is recommended.

NOTE: Anhydrous reaction conditions are required for several steps of this procedure. Anhydrous solvents can be purchased in Sure/Seal bottles (e.g., from Sigma-Aldrich). It is recommended that reaction glassware is oven-dried before use.

Materials

Consumables

Tetrabutylammonium chloride (Sigma-Aldrich; cat. no. 86870)

Dichloromethane (anhydrous) (Sigma-Aldrich; cat. no. 270997)

Thin layer chromatography (TLC) plates aluminium backed (silica gel 60 F254) (Merck; cat. no. 1.05554.0001)

Ethyl acetate (Merck; cat. no. 1.09623.2511)

N-Hexane (Merck; cat. no. 1.04391.2500)

Vanillin stain (see recipe in Reagents and Solutions)

Silica gel 60 (230 to 400 mesh) (Merck; cat. no. 1.09385.1000)

Acetonitrile (anhydrous) (Sigma-Aldrich; cat. no. 271004)

Triethylamine trihydrofluoride (Sigma-Aldrich; cat. no. 344648)

Methanol (Merck; cat. no. 1.06009.2500)

Acetic acid (anhydrous) (Chem-supply; cat. no AA221)

Zinc activated (see recipe in Reagents and Solutions)

Cotton wool (ThermoFisher; cat. no. S-MSM-10102412)

Equipment

Magnetic stirrer bars

Round bottom flasks – 25 mL, 50 mL, 100 mL, 250 mL

Argon gas supply (high purity >99.9%)

Magnetic stirrer hotplate

Ultraviolet lamp (254 nm)

Chromatography columns 20 cm (H) × 2 cm (dia.), 15 cm (H) × 1.7 cm (dia.)

Rotary evaporator equipped with both a diaphragm pump and oil pump

Pasteur pipettes

Sintered glass filter connector (female to male)

Protocol steps—*Step annotations*

Displace the 2'-*O*-triflyl group of 7 with the chloride ion to give 8

1. Add 0.300 g (0.395 mmol) of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-triflyl-uridine (**7**), 0.132 g (0.474 mmol) of tetrabutylammonium chloride and a stirrer bar to an oven dried 25 mL round-bottom

flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.

2. Add 2 mL of dichloromethane and stir at RT for 30 min.
3. Analyse the reaction mixture by TLC.

   The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in hexane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 °C until a colour change is observed. Typically, the R_f of the desired compound is 0.68 (20% ethyl acetate in hexane (v/v)). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.
4. Prepare a silica gel flash chromatography column using approximately 40 g of silica gel in a 20 cm (H) × 2 cm (dia.) column and condition with 100% dichloromethane.
5. Add the reaction mixture directly to the top of the column and elute with 100% dichloromethane using a flow rate of approximately 12 mL/min. Collect 7 mL fractions.
6. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C) until a white solid remains.
7. Check purity of the product by ^{1}H NMR and TLC.

   (2′*S*)-2′-Chloro-2′-deoxy-5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-tetraisopropyldisiloxane-1,3-diyl)-uridine (**8**): 0.111 g (100%,); TLC R_f = 0.68 (20% ethyl acetate in *n*-hexane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 7.77 (s, 1H, H-6), 6.27 (d, *J* = 6.4 Hz, 1H, H-1′), 4.57 (dd, *J* = 8.1, 6.4 Hz, 1H, H-2′), 4.39 (t, *J* = 8.2 Hz, 1H, H-3′), 4.15 (dd, *J* = 13.2, 2.5 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 4.06 (dd, *J* = 13.3, 3.1 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 3.84 (dt, *J* = 8.2, 2.7 Hz, 1H, H-4′), 1.17 – 0.95 (m, 28H, (Si(*i*-Pr)$_2$)$_2$), 0.23 (s, 9H, Si(CH$_3$)$_3$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 154.3 (C-4), 144.8 (C-2), 141.3 (C-6), 102.4 (C≡*C*–Si), 100.5 (C-5), 93.5 (*C*≡C–Si), 84.6 (C-1′), 83.0 (C-4′), 75.4 (C-3′), 62.7 (C-2′), 60.2 (C-5′), 17.84 (SiCH(*C*H$_3$)$_2$), 17.75 (SiCH(*C*H$_3$)$_2$), 17.6 (SiCH(*C*H$_3$)$_2$), 17.5 (SiCH(*C*H$_3$)$_2$), 17.3 (SiCH(*C*H$_3$)$_2$), 17.24 (SiCH(*C*H$_3$)$_2$), 17.22 (SiCH(*C*H$_3$)$_2$), 14.1(Si*C*H(CH$_3$)$_2$), 13.3 (Si*C*H(CH$_3$)$_2$), 12.75 (Si*C*H(CH$_3$)$_2$), 12.67 (Si*C*H(CH$_3$)$_2$), 0.0 (Si(CH$_3$)$_3$). LRMS (ESI): *m/z* = 690, 692 [M + HCOO, ^{35}Cl, ^{37}Cl]$^-$, 468 [M + H]$^+$, 668, 670 [M + Na, ^{35}Cl, ^{37}Cl].

Deprotect the 3′,5′-*O*-hydroxyls, 5-ethynyl-TMS group and the *N*-nitro group of 8 in a one-pot desilylation and reductive denitration reaction to give 9.

8. Add 0.080 g (0.123 mmol) of (2′*S*)-2′-chloro-2′-deoxy-5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-tetraisopropyldisiloxane-1,3-diyl)-uridine (**8**) and a stirrer bar to an oven

dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.

9. Add 2.6 mL of acetonitrile (anhydrous) and stir until the starting material is dissolved.
10. Add 0.2 mL (1.23 mmol) triethylamine trihydrofluoride and stir at RT for 18 h.
11. Analyse the reaction mixture by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the intermediate desilylated compound is 0.62 in 10% methanol in dichloromethane (v/v). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.
12. Cool the reaction flask in an ice bath. With stirring add 0.14 mL (2.4 mmol) of acetic acid followed by approximately 100 mg of silica gel. Wait for bubbling to cease.
13. Add 0.041 g of activated zinc and stir the reaction mixture for 5 min at 0 °C.

    If no reaction has occurred do not heat the reaction mixture or allow it to react at RT whilst in the presence of zinc, this will cause decomposition of the starting material and/or product. To reinitiate the reaction, add additional quantities of silica (100 mg) and zinc (0.041 g) then monitor the reaction by TLC.
14. Analyse the reaction mixture by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.32 10% methanol in dichloromethane (v/v). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.
15. Plug a Pasteur pipette with cotton wool and pack with silica. Filter the reaction mixture through the silica in the pipette. Rinse the silica with acetonitrile.
16. Prepare a silica gel flash chromatography column using approximately 12 g of silica gel in a 15 cm (H) × 1.7 cm (dia.) column and condition 10% methanol in dichloromethane (v/v).
17. Add 0.5 g of silica to filtrate from step 15. Adsorb the crude product onto the silica by then removing the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C) until a free-flowing powder is obtained.

    To prevent damage to the rotary evaporator and vacuum pump, use a sintered glass filter connector in place between the round-bottom flask and the rotary evaporator. Heating of the mixture at this point will cause decomposition of

the product. Minimal time should be wasted once the product is adsorbed onto silica as product loss can occur with time.

18. Add the silica from step 17 to the top of the silica gel column and elute the product with 10% methanol in dichloromethane (v/v) using a flow rate of approximately 5-10 mL/min. Collect 5 mL fractions.
19. Check the purity of the product.

(2′*S*)-Chloro-2′-deoxy-5-ethynyl-uridine (Cl-ara-EdU) (**9**): 0.0345 g, (97%); TLC R_f = 0.32 (10% methanol in dichloromethane (v/v)). ^{1}H NMR (500 MHz, DMSO-d_6) δ_H = 11.80 (s, 1H, NH), 8.31 (s, 1H, H-6), 6.19 (d, *J* = 5.9 Hz, 1H, H-1′), 6.11 (br s, 1H, OH-3′), 5.37 (br t, *J* = 5.1 Hz, 1H, OH-5′), 4.62 (t, *J* = 6.1 Hz, 1H, H-2′), 4.19 (t, *J* = 6.5 Hz, H-3′), 4.13 (s, 1H, ≡CH), 3.82 – 3.70 (m, 2H, H-4′ and H-5′$_{(\alpha \text{ or } \beta)}$), 3.63 (dt, *J* = 12.3, 4.1 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$). ^{13}C NMR (125 MHz, DMSO-d_6) δ_C = 161.5 (C-4), 149.2 (C-2), 144.3 (C-6), 97.3 (C-5), 83.8 (C≡*C*H), 83.7 (C-4′), 83.4 (C-1′), 76.1 (*C*≡CH), 74.3 (C-3′), 64.0 (C-2′), 59.0 (C-5′). LRMS (ESI): *m/z* = 285, 287 [M - H, ^{35}Cl, ^{37}Cl]$^-$, 287, 289 [M + H, ^{35}Cl, ^{37}Cl]$^+$.

BASIC PROTOCOL 3

Synthesis and characterisation of (2′*S*)-bromo-2′-deoxy-5-ethynyl-uridine (Br-ara-EdU) (11).

The synthesis of (2′*S*)-bromo-2′-deoxy-5-ethynyl-uridine (Br-ara-EdU) (**11**) is accomplished by displacement of the triflyl group of the triflate intermediate **7** with bromide to give **10**, followed by global deprotection of **10** (**Figure 4**). The product, compound **11**, is purified by silica gel flash chromatography. The key chemical step in this synthesis was the development of mild a one-pot desilylation/de-*N*-nitration methodology for deprotection of **10**. ^{1}H NMR 2D NOESY (Nuclear Overhauser Effect Spectroscopy) of product **11** confirmed the desired α-configuration at the 2′ position.

Figure 4 here

CAUTION: Some of the chemicals and reagents used are toxic, corrosive, and/or flammable. Refer to material safety data sheets prior to use. All the reactions should be conducted in a well-ventilated fume hood. Use of personal protective equipment is recommended.

NOTE: Anhydrous reaction conditions are required for several steps of this procedure. Anhydrous solvents can be purchased in Sure/Seal bottles (e.g., from Sigma-Aldrich). It is recommended that reaction glassware is oven-dried before use.

Materials

Consumables

Tetrabutylammonium bromide (Sigma-Aldrich; cat. no. 193119)

Dichloromethane (anhydrous) (Sigma-Aldrich; cat. no. 270997)

Thin layer chromatography (TLC) plates aluminium backed (silica gel 60 F254) (Merck; cat. no. 1.05554.0001)

Ethyl acetate (Merck; cat. no. 1.09623.2511)

N-Hexane (Merck; cat. no. 1.04391.2500)

Vanillin stain (see recipe in Reagents and Solutions)

Silica gel 60 (230 to 400 mesh) (Merck; cat. no. 1.09385.1000)

Acetonitrile (anhydrous) (Sigma-Aldrich; cat. no. 271004)

Triethylamine trihydrofluoride (Sigma-Aldrich; cat. no. 344648)

Methanol (Merck; cat. no. 1.06009.2500)

Acetic acid (anhydrous) (Chem-supply; cat. no AA221)

Zinc activated (see recipe in Reagents and Solutions)

Cotton wool (ThermoFisher; cat. no. S-MSM-10102412)

Equipment

Magnetic stirrer bars

Round bottom flasks – 25 mL, 50 mL, 100 mL, 250 mL

Argon gas supply (high purity >99.9%)

Magnetic stirrer hotplate

Ultraviolet lamp (254 nm)

Chromatography columns 20 cm (H) × 2 cm (dia.), 15 cm (H) × 1.7 cm (dia.)

Rotary evaporator equipped with both a diaphragm pump and oil pump

Pasteur pipettes

Sintered glass filter connector (female to male)

Protocol steps—*Step annotations*

Displace the 2′-*O*-triflyl group of 7 with the bromide ion to give 10

1. Add 0.334 g (0.439 mmol) of 5-(Ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-triflyl-uridine (**7**), 0.212 g (0.660 mmol) of tetrabutylammonium bromide and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.
2. Add 2 mL of dichloromethane and stir at RT for 30 min.
3. Analyse the reaction mixture by TLC.

   The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in hexane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.67 (20% ethyl acetate in hexane (v/v)) and 0.35 (10% ethyl acetate in hexane (v/v)). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.
4. Prepare a silica gel flash chromatography column using approximately 40 g of silica gel in a 20 cm (H) × 2 cm (dia.) column and condition with 100% dichloromethane.
5. Add the reaction mixture directly to the top of the column and elute with 100% dichloromethane (v/v) using a flow rate of approximately 12 mL/min. Collect 7 mL fractions.
6. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C). A white solid remains.
7. Check purity of the product by ^{1}H NMR and TLC.

   (2′*S*)-2′-Bromo-2′-deoxy-5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**10**): 0.296 g, (98%); TLC R_f = 0.35 (10% ethyl acetate in *n*-hexane (v/v)), 0.67 (20% ethyl acetate in *n*-hexane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 7.77 (s, 1H, H-6), 6.24 (d, *J* = 6.4 Hz, 1H,

H-1′), 4.58 (dd, *J* = 8.4, 6.5 Hz, 1H, H-2′), 4.50 (t, *J* = 8.3 Hz, 1H, H-3′), 4.16 (dd, *J* = 13.3, 2.4 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 4.06 (dd, *J* = 13.3, 3.0 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 3.82 (dt, *J* = 8.1, 2.7 Hz, 1H, H-4′), 1.17 – 0.99 (m, 28H, (Si(*i*-Pr)$_2$)$_2$), 0.23 (s, 9H, Si(CH$_3$)$_3$). ^{13}C NMR (125 MHz, CDCl$_3$) δ_C = 154.3 (C-4), 144.8 (C-2), 141.2 (C-6), 102.4 (C≡*C*–Si), 100.5 (C-5), 93.5 (*C*≡C–Si), 84.5 (C-1′), 83.7(C-4′), 75.5(C-3′), 60.2 (C-5′), 53.1 (C-2′), 17.9 (SiCH(*C*H$_3$)$_2$), 17.7 (SiCH(*C*H$_3$)$_2$), 17.6 (SiCH(*C*H$_3$)$_2$), 17.5 (SiCH(*C*H$_3$)$_2$), 17.4 (SiCH(*C*H$_3$)$_2$), 17.32 (SiCH(*C*H$_3$)$_2$), 17.30 (SiCH(*C*H$_3$)$_2$), 17.28 (SiCH(*C*H$_3$)$_2$), 14.3 (Si*C*H(CH$_3$)$_2$), 13.3 (Si*C*H(CH$_3$)$_2$), 12.70 (Si*C*H(CH$_3$)$_2$), 12.67 (Si*C*H(CH$_3$)$_2$), 0.0 (Si(CH$_3$)$_3$). LRMS (ESI): *m/z* = 688, 690 [M - H, ^{79}Br, ^{81}Br]$^-$, 712, 714 [M + Na, ^{79}Br, ^{81}Br]$^+$.

Deprotect the 3′,5′-*O*-hydroxyls, 5-ethynyl-TMS group, and the *N*-nitro group of 10 in a one-pot desilylation and reductive denitration reaction to give 11.

8. Add 0.100 g (0.145 mmol) of (2′*S*)-2′-bromo-2′-deoxy-5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**10**) and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.
9. Add 2.6 mL of acetonitrile (anhydrous) and stir until the starting material is dissolved.
10. Add 0.23 mL (1.44 mmol) triethylamine trihydrofluoride and stir at RT for 18 h.
11. Analyse the reaction mixture by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the intermediate desilylated compound is 0.63 in 10% methanol in dichloromethane (v/v). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.
12. Cool the reaction flask in an ice bath then with stirring add 0.17 mL (2.96 mmol) of acetic acid followed by approximately 100 mg of silica gel. Wait for bubbling to cease.
13. Add 0.047 g of activated zinc and stir for 5 min at 0 °C.

    If no reaction has occurred do not heat the reaction mixture or allow it to react at RT whilst in the presence of zinc, this will cause decomposition of the starting material and/or product. To reinitiate the reaction, add additional quantities of silica (100 mg) and zinc (0.041 g) then monitor the reaction by TLC.
14. Analyse the reaction mixture by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in

vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.33 in 10% methanol in dichloromethane (v/v). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.

15. Plug a Pasteur pipette with cotton wool and pack with silica. Filter the reaction mixture through the silica in the pipette. Rinse the silica with acetonitrile.
16. Prepare a silica gel flash chromatography column using approximately 12 g of silica gel in a 15 cm (H) × 1.7 cm (dia.) column and condition 10% methanol in dichloromethane (v/v).
17. Add 0.5 g of silica to filtrate from step 15. Adsorb the crude product onto the silica by then removing the solvent *in vacuo* using a rotary evaporator (water bath at RT) until a free-flowing powder is obtained.

    To prevent damage to the rotary evaporator and vacuum pump, use a sintered glass filter connector in place between the round-bottom flask and the rotary evaporator. Heating of the mixture at this point will cause decomposition of the product. Minimal time should be wasted once the product is adsorbed onto silica as product loss can occur with time.
18. Add the compound adsorbed onto silica from step 17 to the top of the silica gel column. Elute the product with 10% methanol in dichloromethane (v/v) using a flow rate of approximately 5-8 mL/min. Collect 5 mL fractions.
19. Check the purity of the product.

    (2′*S*)-2′-Bromo-2′-deoxy-5-ethynyl-uridine (Br-ara-EdU) (**11**): 0.0350 g, (73%); TLC R_f = 0.33 (10% methanol in dichloromethane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 11.80 (s, 1H NH), 8.30 (s, 1H, H-6), 6.13 (d, *J* = 6.0 Hz, 1H, H-1′), 6.11 – 6.08 (br S, 1H, OH-3′), 5.39 – 5.35 (br m, 1H, OH-5′), 4.65 (dd, *J* = 6.8, 6.0 Hz, 1H, H-2′), 4.30 (t, *J* = 6.7 Hz, 1H, H-3′), 4.13 (s, 1H, ≡CH), 3.79 – 3.71 (m, 2H, H-4′ and H-5′$_{(\alpha \text{ or } \beta)}$), 3.64 (dt, *J* = 13.3, 4.2 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$). ^{13}C NMR (125 MHz, DMSO-d_6) δ_C = 161.4 (C-4), 149.2 (C-2), 144.2 (C-6), 97.4 (C-5), 84.1 (C-4′), 83.8 (C≡*C*H), 83.2 (C-1′), 76.1 (*C*≡CH), 74.4 (C-3′), 59.1 (C-5′), 55.8 (C-2′). LRMS (ESI): *m/z* = 328, 330 [M - H, ^{79}Br, ^{81}Br]$^-$, 330, 332 [M + H] ^{79}Br, ^{81}Br]$^+$.

BASIC PROTOCOL 4

Synthesis and characterisation of (2′*S*)-iodo-2′-deoxy-5-ethynyl-uridine (I-ara-EdU) (13).

The synthesis of (2′*S*)-iodo-2′-deoxy-5-ethynyl-uridine (I-ara-EdU) was accomplished by displacement of the triflyl group of the triflate **7** with iodide from tetrabutylammonium iodide, followed by deprotection of the iodide intermediate **12** (**Figure 5**). The key chemical step in this synthesis was the development of mild a one-pot desilylation/de-*N*-nitration

conditions. Unlike the chloro and bromo analogues, care was needed during the displacement of the triflyl group of **7** to avoid decomposition of both the intermediate and the starting material. The product **13** was purified by silica gel flash chromatography. ^{1}H NMR 2D NOESY (Nuclear Overhauser Effect Spectroscopy) confirmed the desired α-configuration at the 2' position of **13**.

Figure 5 here

CAUTION: Some of the chemicals and reagents used are toxic, corrosive, and/or flammable. Refer to material safety data sheets prior to use. All the reactions should be conducted in a well-ventilated fume hood. Use of personal protective equipment is recommended.

NOTE: Anhydrous reaction conditions are required for several steps of this procedure. Anhydrous solvents can be purchased in Sure/Seal bottles (e.g., from Sigma-Aldrich), while glassware needs to be oven-dried.

Materials

Consumables

Tetrabutylammonium iodide (Sigma-Aldrich; cat. no. 270997)

Toluene (anhydrous) (Sigma-Aldrich; cat. no. 244511)

Thin layer chromatography (TLC) plates aluminium backed (silica gel 60 F254) (Merck; cat. no. 1.05554.0001)

Ethyl acetate (Merck; cat. no. 1.09623.2511)

N-Hexane (Merck; cat. no. 1.04391.2500)

Vanillin stain (see recipe in Reagents and Solutions)

Silica gel 60 (230 to 400 mesh) (Merck; cat. no. 1.09385.1000)

Dichloromethane (anhydrous) (Sigma-Aldrich; cat. no. 270997)

Acetonitrile (anhydrous) (Sigma-Aldrich; cat. no. 271004)

Triethylamine trihydrofluoride (Sigma-Aldrich; cat. no. 344648)

Methanol (Merck; cat. no. 1.06009.2500)

Acetic acid (anhydrous) (Chem-supply; cat. no AA221)

Zinc activated (see recipe in Reagents and Solutions)

Cotton wool (ThermoFisher; cat. no. S-MSM-10102412)

Equipment

Magnetic stirrer bars

Round bottom flasks – 25 mL, 50 mL, 100 mL, 250 mL

Argon gas supply (high purity >99.9%)

Magnetic stirrer hotplate

Ultraviolet lamp (254 nm)

Rotary evaporator equipped with both a diaphragm pump and oil pump

Chromatography columns 20 cm (H) × 2 cm (dia.), 15 cm (H) × 1.7 cm (dia.)

Pasteur pipettes

Sintered glass filter connector (female to male)

Protocol steps—*Step annotations*

Displace the 2'-*O*-triflyl group of 7 with the iodide ion to give 12

1. Add 0.150 g (0.778 mmol) of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-triflyl-uridine (**7**), 0.0875 g (0.237 mmol) of tetrabutylammonium iodide and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.
2. Preheat a 25 mL heating mantle to 50 °C.
3. Add 1 mL of toluene to the mixture, lower the mixture into the heating mantle and for 20 min.
4. Remove the flask from the heating mantle and cool in a water bath.
5. Analyse the reaction by TLC.

   The starting material should be run alongside the reaction for comparison. The plates are developed using 20% ethyl acetate in hexane (v/v). The bands are

visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 °C until a colour change is observed. Typically, the R_f of the desired compound is 0.71 (20% ethyl acetate in hexane (v/v)). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.

6. Evaporate the toluene *in vacuo* using a rotary evaporator (water bath at RT) without heating.
7. Prepare a silica gel flash chromatography column using approximately 40 g of silica gel in a 20 cm (H) × 2 cm (dia.) column and condition with 100% dichloromethane.
8. Dissolve the crude product in a minimal amount of dichloromethane (c.a. 1 – 2 mL) then add directly to the top of the column and elute with 100% dichloromethane using a flow rate of approximately 12 mL/min. Collect 7 mL fractions.
9. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath temperature 40 °C) to give a white solid.
10. Check purity of the product by ^{1}H NMR and TLC.

(2′*S*)-2′-Deoxy-5-(ethynyl(2-trimethylsilyl))-2′-iodo-3-*N*-nitro-3′,5′-*O*-tetraisopropyldisiloxane-1,3-diyl)-uridine (**12**): 0.0871 g, (60%); TLC R_f = 0.71 (20% ethyl acetate in *n*-hexane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 7.75 (s, 1H, H-6), 6.11 (d, *J* = 6.6 Hz, 1H, H-1′), 4.63 – 4.52 (m, 2H, H-2′ and H-3′), 4.17 (dd, *J* = 13.4, 2.0 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 4.06 (dd, *J* = 13.4, 3.0 Hz, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 3.77 (ddd, *J* = 8.0, 2.9, 1.9 Hz, 1H, H-4′), 1.18 – 0.99 (m, 28H, (Si(*i*-Pr)$_2$)$_2$), 0.24 (s, 9H, Si(CH$_3$)$_3$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 154.2 (C-4), 144.6 (C-2), 140.6 (C-6), 102.3 (C≡*C*–Si), 100.6 (C-5), 93.4 (*C*≡C–Si), 85.1 (C-1′), 84.6 (C-4′), 76.2 (C-3′), 59.8 (C-5′), 29.4 (C-2′), 17.7 (SiCH(*C*H$_3$)$_2$), 17.5 (SiCH(*C*H$_3$)$_2$), 17.4 (SiCH(*C*H$_3$)$_2$), 17.4 (SiCH(*C*H$_3$)$_2$), 17.3 (SiCH(*C*H$_3$)$_2$), 17.3 (SiCH(*C*H$_3$)$_2$), 17.2 (SiCH(*C*H$_3$)$_2$), 17.2 (SiCH(*C*H$_3$)$_2$), 14.4 (Si*C*H(CH$_3$)$_2$), 13.1(Si*C*H(CH$_3$)$_2$), 12.5 (Si*C*H(CH$_3$)$_2$), 12.4 (Si*C*H(CH$_3$)$_2$), -0.2 (Si(CH$_3$)$_3$). LRMS (ESI): *m/z* = 735 [M - H]$^-$, 781 [M + HCOO]$^-$, 759 [M + Na]$^+$.

Deprotect the 3′,5′-*O*-hydroxyls, 5-ethynyl-TMS group and the *N*-nitro group of 12 in a one-pot desilylation and reductive denitration reaction to give 13.

11. Add 0.0870 g (0.118 mmol) of (2′*S*)-2′-deoxy-5-(ethynyl(2-trimethylsilyl))-2′-iodo-3-*N*-nitro-3′,5′-*O*-tetraisopropyldisiloxane-1,3-diyl)-uridine (**12**) and a stirrer bar to an oven dried 25 mL round-bottom flask. Apply a vacuum then back flush with argon three times. Attach a balloon of argon to the reaction vessel.
12. Add 2.6 mL of acetonitrile (anhydrous) and stir until the starting material is dissolved.
13. Add 0.2 mL (1.17 mmol) triethylamine trihydrofluoride and stir at RT for 18 h.

14. Analyse the reaction mixture by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the intermediate desilylated compound is 0.67 10% methanol in dichloromethane (v/v). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.

15. Cool the reaction flask in an ice bath then with stirring add 0.14 mL (2.5 mmol) of acetic acid followed by approximately 100 mg of silica gel and wait for bubbling to cease.
16. Add 0.039 g of activated zinc and stir for 5 min at 0 °C.

    If no reaction has occurred do not heat the reaction mixture or allow it to react at RT whilst in the presence of zinc, this will cause decomposition of the starting material and/or product. To reinitiate the reaction, add additional quantities of silica (100 mg) and zinc (0.041 g) then monitor the reaction by TLC.

17. Analyse the reaction mixture by TLC.

    The starting material should be run alongside the reaction for comparison. The plates are developed using 10% methanol in dichloromethane (v/v). The bands are visualised by UV shadowing using 254 nm light and dipping the plate in vanillin stain solution followed by heating with a heat gun to 300 ℃ until a colour change is observed. Typically, the R_f of the desired compound is 0.37 10% methanol in dichloromethane (v/v). The starting material and desired product both stain red upon heating with vanillin stain and upon cooling tend to appear green.

18. Plug a Pasteur pipette with cotton wool and pack with silica. Filter the reaction mixture through the silica in the pipette. Rinse the silica with acetonitrile.
19. Prepare a silica gel flash chromatography column using approximately 12 g of silica gel in a 15 cm (H) × 1.7 cm (dia.) column and condition 10% methanol in dichloromethane (v/v).
20. Add 0.5 g of silica to filtrate from step 18. Adsorb the crude product onto the silica by then removing the solvent *in vacuo* using a rotary evaporator (water bath at RT) until a free-flowing powder is obtained.

    To prevent damage to the rotary evaporator and vacuum pump, use a sintered glass filter connector in place between the round-bottom flask and the rotary evaporator. Heating of the mixture at this point will cause decomposition of the product. Minimal time should be wasted once the product is adsorbed onto silica as product loss can occur with time.

21. Add the compound adsorbed onto silica from step 20 to the top of the silica gel column and elute the product with 10% methanol in dichloromethane (v/v) using a flow rate of approximately 5-8 mL/min. Collect 5 mL fractions.
22. Combine the fractions that contain the pure product, as determined by TLC. Remove the solvent *in vacuo* using a rotary evaporator (water bath at RT) to give a white solid.

23. Check the purity of the product.

    (2′*S*)-2′-Deoxy-5-ethynyl-2′-iodo-uridine (l-ara-EdU) (**13**): 0.0221 g, (50%); TLC R_f = 0.37 (10% methanol in dichloromethane (v/v)). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H = 11.80 (br s, 1H, NH), 8.29 (s, 1H, H-6), 6.02 (br s, 1H, OH-3′), 5.96 (d, *J* = 6.5 Hz, 1H, H-1′), 5.38 (br s, 1H,OH-5′), 4.58 (dd, *J* = 8.0, 6.5 Hz, 1H, H-2′), 4.35 (t, *J* = 7.6 Hz, 1H, H-3′), 4.14 (s, 1H, ≡CH) 3.78 – 3.72 (m, 1H, H-5′$_{(\alpha \text{ or } \beta)}$), 3.70 – 3.62 (m, 2H, H-4′ and H-5′$_{(\alpha \text{ or } \beta)}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C = 161.4 (C-4), 149.2 (C-2), 144.0 (C-6), 97.6 (C-5), 84.8 (C-4′), 83.8 (C-1′ and C≡*C*H), 76.2 (*C*≡CH), 75.3 (C-3′), 58.9 (C-5′), 33.0 (C-2′). LRMS (ESI): *m/z* = 376 $[M - H]^-$, 422 $[M + HCOO]^-$, 378 $[M + H]^+$

REAGENTS AND SOLUTIONS

Vanillin stain

Add 340 mL of ethanol (Merck; cat. no. 1.00983.2511) to an Erlenmeyer flask. With stirring, slowly add 11 mL of concentrated sulfuric acid (Merck; cat. no. 1.12080.2500) followed by 40 mL of water. Finally, add 21 g of vanillin (Sigma-Aldrich; cat. no. V1104).

Activated zinc (dust)

Add approximately 10 g of zinc dust (Sigma-Aldrich; cat. no. 209988) to a sintered glass funnel. Add enough 5% hydrochloric acid aqueous solution (prepared from RCI Labscan; cat. no. R1104) to cover the zinc then agitate with a glass rod and allow to react for approximately 1 – 2 min. Attach a rubber septum to the top of the filter funnel and apply a positive pressure of argon or nitrogen to the mixture, filter off the acid. Next, add enough 100% ethanol (Merck; cat. no. 1.00983.2511) to cover the zinc dust, then filter off the ethanol while under argon or nitrogen, repeat (×2). Next, add enough diethyl ether (Merck; cat. no. 1.00921.2500) to cover the zinc dust, then filter off the diethyl ether under argon or nitrogen, repeat (×2).

COMMENTARY

Background Information

Halogenation at the 2′ position of nucleosides is known to modulate biological activity, with the arabinosyl (2′-up) configuration demonstrating the most pronounced difference in activity when compared to the parent nucleoside (Watanabe et al., 1979; Wright et al., 1970). 2′-Fluorinated arabinosyl nucleosides are well known and have reported syntheses. Two classical approaches are used in nucleoside chemistry to produce 2′-fluorinated arabinosyl nucleosides: either direct substitution of the 2′-hydroxyl, once converted into a suitable leaving group (i.e. triflate or imidazylate), or condensation of the requisite nucleobase with the 2′-fluoro-arabinosyl sugar. When attempting to synthesise arabinosyl pyrimidine nucleosides incorporating higher halogens (chlorine, bromine, and iodine) at the 2′ position, direct substitution of a 2′-leaving group leads instead to intramolecular cyclisation, attributed to nucleophilic attack at the 2′ position by the O-2 carbonyl of the pyrimidine. This yields a 2,2′-anhydronucleoside (Fukukawa et al., 1983; Pankiewicz & Watanabe, 1993). Alternatively, attempts to synthesise 5-substituted-2′-halogenated arabinosyl pyrimidine nucleosides using sugar-base condensation has only ever been demonstrated employing thymine as the nucleobase and chlorine or bromine at the 2′ position, furthermore it relies on crystallisation to separate the mixture of anomers that form (Watanabe et al., 1983). 2′-Iodo-arabinosyl pyrimidine analogues have not been reported using the sugar-base condensation approach. 5-Substituted-2′-halogenated arabinosyl pyrimidine nucleosides are rarely reported.

To enable a general synthesis of 5-substituted-2′-halogenated-arabinosyl pyrimidine nucleosides, we have further developed the work of Vilarrasa and co-workers (Serra et al., 1998). They showed that the 2′-triflate pyrimidine nucleoside is stabilised toward nucleophilic substitution using a 3-*N*-nitro group. To accommodate the 5-alkynyl substituent of our target compounds, milder *N*-nitration and *N*-denitration conditions were necessary. Rapid and mild *N*-nitration was achieved with tetrabutylammonium nitrate and trifluoroacetic anhydride, while an equally rapid and mild reductive *N*-denitration procedure was achieved, employing zinc in acetic acid (Hilko et al., 2018). This methodology is fully compatible with 5-alkynyl arabinosyl uridines with chloro, bromo, or iodo functionality at the 2′ position (Hilko et al., 2018).

CRITICAL PARAMETERS

Troubleshooting

For the synthesis of Cl-ara-EdU **9** and Br-ara-EdU **11** there are two steps that warrant close attention: (i) deacetylation of **5** to give **6**, and (ii) reductive *N*-denitration of **8** and **10**, respectively.

(i) For deacetylation, the crude reaction mixture is directly adsorbed onto silica gel. Once adsorbed, the yield of the product decreases if left in this dry state for a prolonged period. To achieve a maximum yield, it is therefore recommended to perform column chromatography immediately after adsorption of the crude product onto silica.

(ii) Reductive *N*-denitration may fail to initiate. This is usually related to the condition/age of the activated zinc. When performing the *N*-denitration procedure it is critical to keep the reaction mixture cold and if no reaction has occurred after 2 min, as determined by TLC, then additional silica gel and zinc should be added. The reaction is heterogeneous and sensitive to the rate of stirring. A fast rate of stirring should be applied.

In addition to the abovementioned caveats, for synthesis of I-ara-EdU **13**, displacement of the triflate group with iodide may give highly variable outcomes. The reasons for this are twofold. Firstly, displacement with iodide on a sterically hindered substrate is kinetically unfavourable, and secondly, the molecular iodine (I_2) produced during the reaction of the triflate and tetrabutylammonium iodide causes decomposition of both the triflate and the iodinated intermediate. Prolonged reaction times should be avoided. Proceed to workup the reaction even if starting material is still present. The crude product should be purified as soon as possible to minimise decomposition.

UNDERSTANDING RESULTS

Anticipated Results

The purity of isolated nucleoside products is typically >98%. The overall expected yields for Cl-ara-EdU (**9**), Br-ara-EdU (**11**) and I-ara-EdU (**13**) over eight steps from uridine (**1**) are 51%, 41%, and 17%, respectively. Starting from 1 g of uridine (**1**) for each synthesis the mass of Cl-ara-EdU (**9**), Br-ara-EdU (**11**) and I-ara-EdU (**13**) is expected to be 0.598 g, 0.554 g, and 0.318 g, respectively. The α-configuration of the 2′ position for all arabinosyl nucleosides was established by two-dimensional nuclear Overhauser effect spectroscopy (2D-NOESY) NMR experiments. **Figure 6** shows the 2D-NOESY ^{1}H NMR spectrum of 2′-Cl-ara-EdU (**9**) as an exemplar. Strong correlations between H-6 and H-3′ and between H-4′ and H-2′ are indicative of the arabinosyl configuration (Wilds & Damha, 2000). A further feature of chloro- and bromo- compounds is a distinctive mass spectrum that results from the isotopic abundance ratio of chlorine and bromine, respectively. Additionally, compounds containing the *N*-nitro protecting group are observed using negative mode electrospray ionisation mass spectrometry. Positive mode electrospray ionisation of the *N*-nitro compounds often resulted in fragmentation of the *N*-nitro group [M – NO_2 + H] and the expected molecular ion may not be observed $[M+H]^+$.

Figure 6 here

TIME CONSIDERATIONS

The synthesis of the triflate intermediate **7** (Basic protocol 1) requires approximately 4-5 days. Synthesis of each of the 2′-halo-ara-EdU compounds **9**, **11**, and **13** (Basic protocols 2-4) from **7** takes a further 2 days.

ACKNOWLEDGEMENT

This research was financed by the Australian Research Council (DP180102601). We thank Griffith University for an Australian Postgraduate Award Scholarship to D.H. We are grateful to the Cancer Therapeutics CRC, whose activities are funded by the Australian Government's Cooperative Research Centre Programme for a PhD Top-Up Scholarship to D.H. We thank Mr. Jun Ma for high resolution mass spectrometry measurements.

LITERATURE CITED

Claudio-Montero, Pinilla-Macua, Fernandez-Calotti, Sancho-Mateo, Lostao, Colomer, Grandas, & Pastor-Anglada. (2015). Fluorescent Nucleoside Derivatives as a Tool for the Detection of Concentrative Nucleoside Transporter Activity Using Confocal Microscopy and Flow Cytometry. *Mol. Pharm., 12*(6), 2158-2166. doi:10.1021/acs.molpharmaceut.5b00142

Fukukawa, Uedao, & Hirano. (1983). Synthesis of 2'-Substituted Derivatives of Neplanocin A (Nucleosides and Nucleotides. XLIV). *Chem. Pharm. Bull. (Tokyo), 31*(5), 1582-1592. doi:10.1248/cpb.31.1582

Hilko, Bornaghi, & Poulsen. (2018). Stereoselective Synthesis of Highly Functionalized Arabinosyl Nucleosides through Application of an N-Nitro Protecting Group. *J. Org. Chem, 83*(19), 11944-11955. doi:10.1021/acs.joc.8b01834

Neef, & Luedtke. (2011). Dynamic metabolic labeling of DNA in vivo with arabinosyl nucleosides. *Proc. Natl. Acad. Sci. U. S. A., 108*(51), 20404-20409. doi:10.1073/pnas.1101126108

Neef, Pernot, Schreier, Scapozza, & Luedtke. (2015). A Bioorthogonal Chemical Reporter of Viral Infection. *Angew. Chem., 127*(27), 8022-8025. doi:doi:10.1002/ange.201500250

Neef, Samain, & Luedtke. (2012). Metabolic labeling of DNA by purine analogues in vivo. *ChemBioChem, 13*(12), 1750-1753. doi:10.1002/cbic.201200253

Pankiewicz, & Watanabe. (1993). Synthesis of 2′-β-fluoro-substituted nucleosides by a direct approach. *J. Fluor. Chem., 64*(1), 15-36. doi:10.1016/S0022-1139(00)80060-3

Serra, Aragones, Bessa, Farras, & Vilarrasa. (1998). Stabilisation of pyrimidine nucleoside triflates by N-nitro groups. *Tetrahedron Lett., 39*(41), 7575-7578. doi:10.1016/S0040-4039(98)01616-5

Watanabe, Reichman, Hirota, Lopez, & Fox. (1979). Nucleosides. 110. Synthesis and antiherpes virus activity of some 2'-fluoro-2'-deoxyarabinofuranosylpyrimidine nucleosides. *J. Med. Chem., 22*(1), 21-24. doi:10.1021/jm00187a005

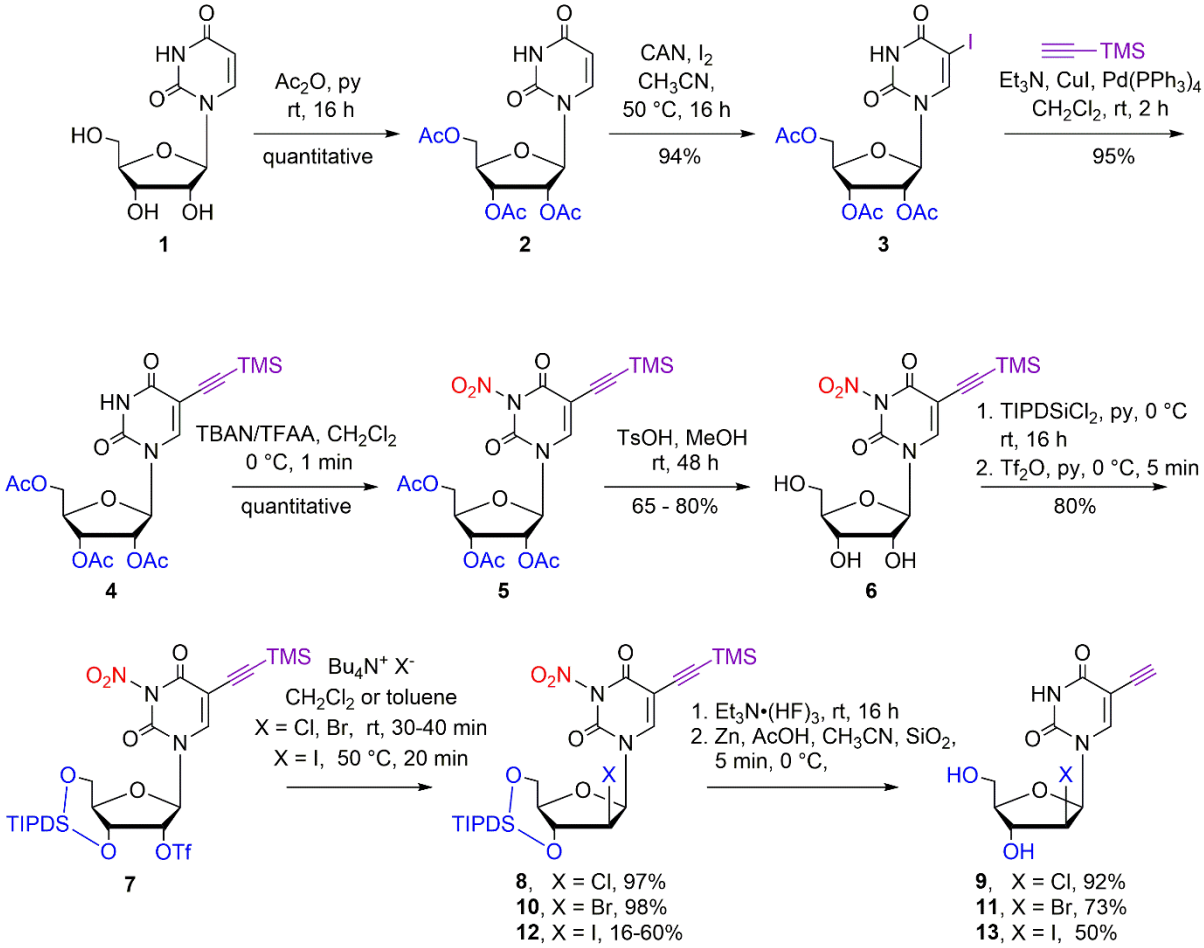
Watanabe, Su, Klein, Chu, Matsuda, Chun, Lopez, & Fox. (1983). Nucleosides. 123. Synthesis of antiviral nucleosides: 5-substituted 1-(2-deoxy-2-halogeno-β-D-arabinofuranosyl)cytosines and -uracils. Some structure-activity relationships. *J. Med. Chem., 26*(2), 152-156. doi:10.1021/jm00356a007

Wilds, & Damha. (2000). 2′-Deoxy-2′-fluoro-β-D-arabinonucleosides and oligonucleotides (2′ F-ANA): synthesis and physicochemical studies. *Nucleic Acids Res., 28*(18), 3625-3635. doi:10.1093/nar/28.18.3625

Wright, Wilson, & Fox. (1970). Nucleosides. LXIV. Fluoro sugar analogs of arabinosyl- and xylosylcytosines. *J. Med. Chem., 13*(2), 269-272. doi:10.1021/jm00296a024

FIGURE LEGENDS

Figure 1. Synthetic scheme for 2'-halo-arabinosyl EdU analogues **9**, **11** and **13**.



Reprinted (adapted) with permission from (Hilko, David; Bornaghi, Laurent; Poulsen, Sally-Ann. Stereoselective synthesis of highly functionalised arabinosyl nucleosides through application of a *N*-nitro protecting group. *J. Org. Chem* **2018,** *83* (19), 11944-11955). Copyright (2018) American Chemical Society

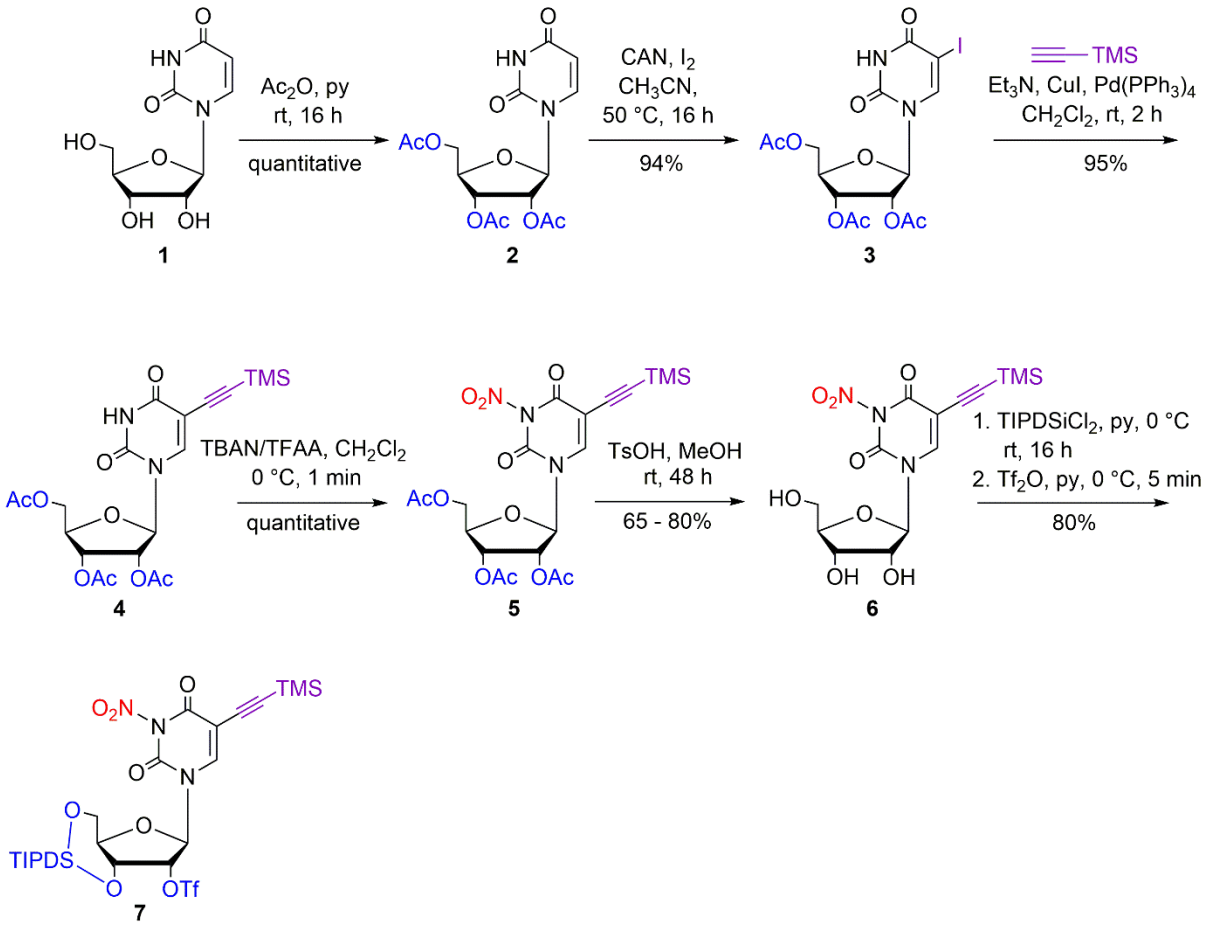


Figure 2. Synthesis of 5-(ethynyl(2-trimethylsilyl))-3-*N*-nitro-3',5'-*O*-(tetraisopropyl disiloxane-1,3-diyl)-2'-*O*-triflyl-uridine.

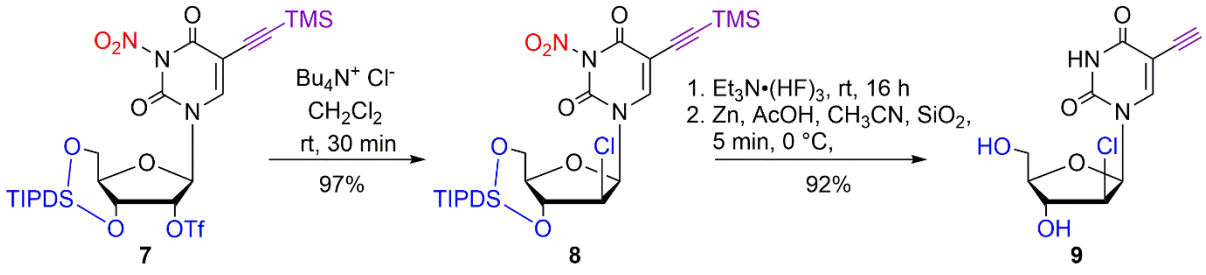


Figure 3. Synthesis of (2'*S*)-chloro-2'-deoxy-5-ethynyl-uridine (Cl-ara-EdU) from **7**.

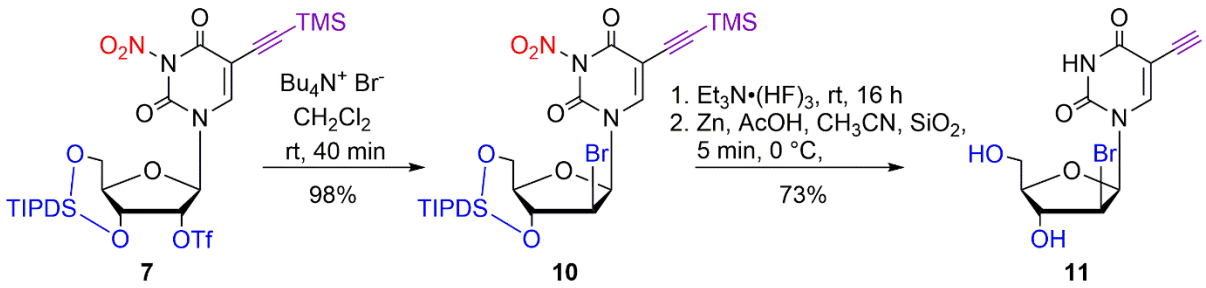


Figure 4. Synthesis of (2′*S*)-bromo-2′-deoxy-5-ethynyl-uridine (Br-ara-EdU) from **7**.

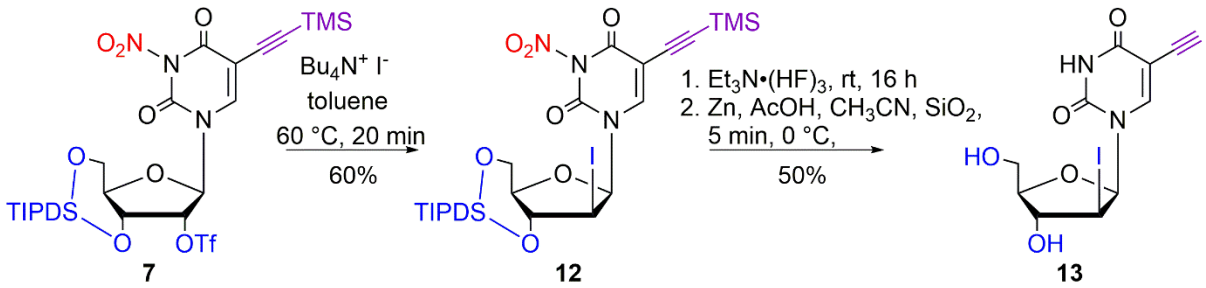


Figure 5. Synthesis of (2′*S*)-iodo-2′-deoxy-5-ethynyl-uridine (I-ara-EdU) from **7**.

Figure 6. Diagnostic ^{1}H NMR NOESY correlations for compound **9**.