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ARTICLE

Progress toward an amplifiable metabolic label for DNA: conversion of 4-thiothymidine (4sT) to 5-methyl-2′-deoxycytidine and synthesis of a 4sT phosphorodiamidate prodrug

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Abstract: The ability to metabolically label DNA in a way that produces a latent change from one nucleobase to another would create a signal that can be amplified by PCR — this in turn would allow studies of newly synthesized DNA using high-throughput sequencing. To function as an amplifiable metabolic label, a nucleotide analogue would need to be taken up by cells and incorporated into cellular DNA; after purification of DNA, it could be converted into a different nucleobase with a different base pairing pattern. We selected 4-thiothymidine (4sT) as a candidate metabolic label: 4sT is readily taken up by a large number of polymerases in vitro, and we present a method that allows 4sT to be converted into 5-methyl-2′-deoxycytidine (5mC) after incorporation into DNA. Encouraged by these results, we treated cells with 4sT nucleoside; however, we found that 4sT is not incorporated into DNA in bacterial, yeast, or mammalian cells to useful levels under the conditions we tested. A phosphorodiamidate prodrug of 4sTMP was successfully synthesized but did not measurably improve incorporation into cellular DNA.

Key words: metabolic labelling, convertible nucleobase, nucleoside prodrug, 4-thiothymidine.

Résumé : La capacité de réaliser le marquage métabolique de l'ADN de manière à produire la transformation latente d'une base nucléique en une autre pourrait permettre de générer un signal amplifiable par PCR, ce qui, par conséquent, permettrait d'étudier de nouveaux ADN synthétisés par séquençage à haut débit. Pour fonctionner comme un marqueur métabolique amplifiable, un analogue de nucléotide doit pouvoir pénétrer dans les cellules et s'incorporer dans l'ADN cellulaire, puis après purification de l'ADN, doit pouvoir être transformé en une autre base nucléique dont l'appariement diffère. Nous avons choisi la 4-thiothymidine (4sT) comme marqueur métabolique potentiel, car elle est facilement intégrée in vitro par un grand nombre de polymérases. Nous présentons une méthode qui permet de transformer la 4sT en 5-methyl-2′-deoxycytidine (5mC) après incorporation dans l'ADN. Encouragés par ces résultats, nous avons traité des cellules avec le nucléoside 4sT. Cependant, nous avons observé que, dans les conditions évaluées, la 4sT n'est pas incorporée dans l'ADN de cellules de bactéries, de levures et de mammifères dans une mesure suffisante pour être utile. Nous avons réussi à synthétiser un promédicament de la 4sTMP à base de phosphorodiamidate, mais nous ne sommes pas parvenus à améliorer de façon mesurable l'incorporation dans l'ADN cellulaire. [Traduit par la Rédaction]

Mots-clés : marquage métabolique, base nucléique convertible, promédicament à base de nucléoside, 4-thiothymidine.

Introduction

Metabolic labeling of cellular biopolymers has opened new frontiers in fields from proteomics[1] to RNA biology[2,3] to glycobiology.[4,5] In this family of approaches, cells or organisms are fed a nucleoside, amino acid, or sugar analogue that they incorporate into newly synthesized cellular biopolymers. In all cases, the analogue is similar enough to its native counterpart to be correctly incorporated into cellular biopolymers — but has a property that allows it to be identified later such as isotope incorporation, recognition by an antibody, or selective reaction with fluorescent dyes.

Metabolic labelling of DNA, in some ways, first dates to John Cairns' 1963 exploration of the origins of DNA replication using radioactive [^{3}H]-labeled thymidine;[6] however, detection via autoradiography is slow and only gives qualitative information. Somewhat more recently, 5-bromo-2′-deoxyuridine (BrdU) has been incorporated and detected by anti-BrdU antibodies,[7] but the approach does not offer base-level resolution. Recently, the use of bioorthogonal chemistry based on copper-catalysed click chemistry has also found extensive use in metabolic labelling approaches.[8,9] For DNA, an alkyne can readily be incorporated at the 5-position of pyrimidines and a bioorthogonal reaction with a probe can then be used, e.g., for visualisation of newly synthesized DNA by reaction with a fluorescent azide[10] in the presence of a Cu(I) catalyst.[8,9] Alternatively azide-modified pyrimidines have been shown to react

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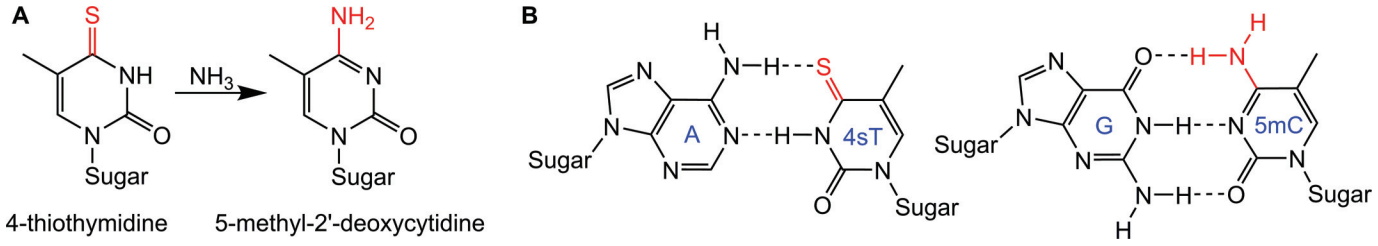
Fig. 1. (A) Treatment of 4sT with ammonia converts it to 5mC. (B) 4sT maintains the base-pairing pattern of T, whereas 5mC maintains the base-pairing pattern of C. [Colour online.]



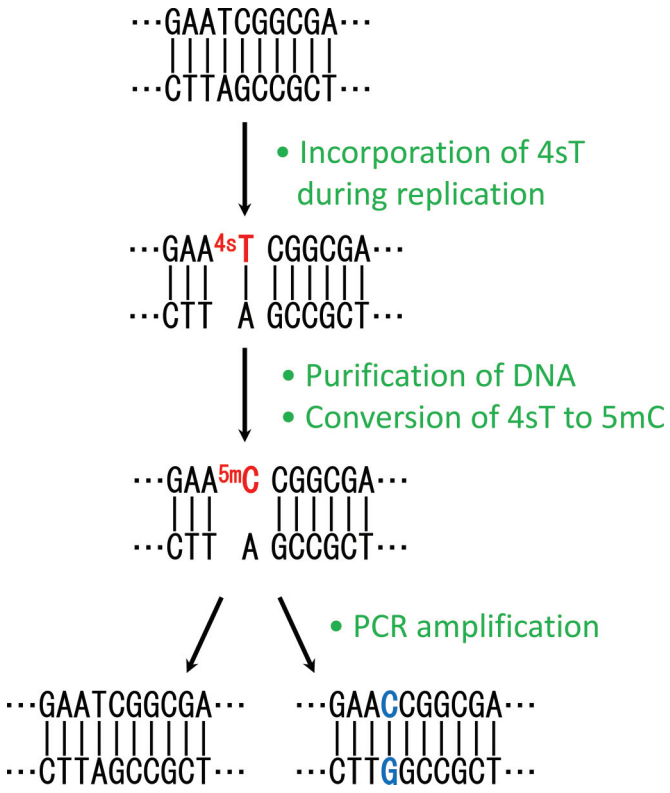
with strained alkynes in a Cu-free environment.[11] A final option in development is to use a single-molecule sequencing technique that can selectively detect alternative bases — either to detect incorporated labels such as BrdU directly or to give a base-level resolution analysis of alkyne- or azide-labeled DNA by conjugating a bulky label after DNA harvest.

Although useful for many applications, these tools do not provide a signal that can be propagated through subsequent PCR amplification steps. This caveat prevents the use of most commonly used high-throughput sequencing technology, significantly slowing progress in the field. We seek to address this unmet need by developing a metabolic labelling approach that produces an amplifiable base-conversion event within labelled DNA.

In this paper, we describe progress toward metabolic labelling of DNA using 4-thiothymidine (4sT, Fig. 1) that retains the size, shape, and hydrogen bonding pattern of thymidine itself but is more reactive toward nucleophilic attack at C4. Treatment of 4sT nucleoside with ammonia converts it into 5-methyl-2′-deoxycytidine (5mC),[12] a common epigenetic mark within cellular DNA and readily recognized by DNA polymerases as equivalent to C. We reasoned that the same reaction might be feasible when 4sT is incorporated into DNA; if so, this would produce a "T" to "C" conversion within newly synthesized DNA, a change that could be propagated by PCR and detected by sequencing (Fig. 2). Therefore, the incorporation and conversion of 4sT might constitute an approach to the metabolic labelling of DNA that produces an amplifiable signal.

We now describe conditions that allow conversion of 4sT to 5mC within oligonucleotides and, to a lesser extent, PCR fragments. 4sT-containing DNA is not as stable to hot concentrated ammonia as is unmodified DNA; thus, the conversion competes to some extent with strand breakage. Consistent with other reports, we find that 4sT triphosphate (4sTTP) is readily incorporated by a variety of polymerases in vitro, but we did not observe efficient incorporation of 4sT into bacterial, yeast, or mammalian cells after treatment of the cells with 4sT nucleoside. Attempting to improve the cellular incorporation of 4sT, we synthesized a novel phosphorodiamidate prodrug of 4sT. The synthesis was successful but use of the prodrug approach did not significantly improve cellular incorporation of 4sT.

Fig. 2. A schematic showing the workflow for 4sT metabolic labelling of DNA. After a single round of DNA replication in the presence of 4sT triphosphate, 4sT would be incorporated by DNA polymerase into one strand of DNA. The cells are then lysed and the DNA is purified, and then, the 4sT is chemically converted into 5mC before PCR amplification. Any PCR products derived from the newly synthesized strand of DNA will contain a mutation (blue) that allows them to be identified by sequencing. [Colour online.]



Materials and methods

General methods

All reactions were carried out under argon atmosphere. Where inert conditions were required, glassware, stir bars, and needles were oven dried overnight. A Schlenk line, syringes, and balloons were used to handle chemicals, and argon gas was first passed through a desiccant column. Solvents were dried using molecular sieves and tested before use on a Karl Fischer titration apparatus. Chemicals were purchased from Sigma Aldrich, Chem-Impex, Glen Research, and Carbosynth. UV light or ninhydrin, H_2SO_4, $KMnO_4$, or molybdenum blue (i.e., Hanessian's cerium ammonium molybdate) stains were used to visualise TLC plates. NMR spectra were recorded on a Bruker Ascend500 500 MHz instrument using both one- and two-dimensional NMR techniques, including ^{1}H, ^{13}C, ^{31}P, DEPT-135, COSY, HSQC, and HMBC, to achieve unambiguous peak assignment. Chemical shifts are referenced to the residual solvent peak for ^{1}H and ^{13}C NMR. Automated column chromatography was carried out on a Biotage Isolera Four, using SNAP Ultra or SNAP Ultra C18 columns. A Biotage-compatible RediSep 5 g SCX ion-exchange column was used for ion exchange of charged final products. Analytical and preparatory HPLC was carried out on an Agilent 1260 Infinity, and small molecule analysis was done using a 50 mm × 4.6 mm Agilent Poroshell 120 EC-C18 2.7 μm column. A 150 mm × 7.5 mm Agilent PL-SAX 8 μm column was used for DNA purification. LRMS was carried out on a direct injection Thermofinnigan LCQ DECA XP Plus, and HRMS for small molecules, as well as all oligonucleotide masses, were obtained on an Agilent 6530 Accurate-Mass Q-TOF/LCMS machine linked to a pre-injection Agilent 1260 Infinity HPLC.

Oligonucleotide synthesis

Oligonucleotides were synthesized in-house at 1 μmol scale on an Applied Biosystems 394 DNA/RNA synthesizer with Universal Unylinker (ChemGenes) support, and with standard detritylation and capping reagents. Activation was achieved with 5-benzylthio-1H-tetrazole (BTT, 0.3 mol/L in acetonitrile). Oxidation was achieved using 0.02 mol/L iodine in THF–water–pyridine. The 4-Thio-dT phosphoramidite (5′-dimethoxytrityl-2′-deoxy-(2-cyanoethyl)–4-thiothymidine, 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]) was purchased from Glen Research and was dissolved to a concentration of 0.15 mol/L in anhydrous acetonitrile immediately before use. Coupling times for all DNA and modified phosphoramidites were 5 min. 4sT-modified oligonucleotides were cleaved from the solid support and deprotected using 50 mmol/L NaSH in concentrated NH_4OH at room temperature (RT) for 24 h. The oligonucleotides were concentrated to dryness in a centrifugal evaporator and then resuspended in RNase-free water for further analysis and use. Preparatory HPLC was used to purify the oligomers using a PL-SAX anion-exchange column, eluting with increasing concentrations of $NaClO_4$ in water containing 30% ACN. Oligonucleotides were then desalted with a NAP-10 Sephadex column using the manufacturer's protocol and stored at –20 °C in MilliQ water until use. Final concentrations were calculated by measuring A_{260}. Extinction coefficients for 4sT-modified oligonucleotides were calculated assuming the A_{260} of 4sT to be negligible, based on the UV spectrum of 4sT nucleoside.[13]

PCR, conversion and electrophoresis of 4sT containing PCR fragments

4sTTP was incorporated by PCR amplification for 5 cycles using Taq polymerase and standard PCR conditions (NEB M0273S). Conversion of the 4sT nucleotide was carried out at 80 °C for 30 min in concentrated aqueous NH_3 and 1 mmol/L DTT. Samples were concentrated using a centrifugal evaporator, diluted in 0.5 mol/L NaCl, and then desalted using 30 KDa Amicon columns (Millipore UFC5030BK). Samples were washed and finally eluted in 10 mmol/L Tris pH 8.0, 0.1 mmol/L EDTA (TLE buffer). After this conversion and clean up, the samples were amplified for up to 10 cycles with Taq polymerase (NEB M0273S) and run on a 2% agarose gel stained with ethidium bromide and referenced using a 100 bp DNA ladder (NEB N3231S). For the AvaI digestion experiments, AvaI (NEB R0152S) was used in NEB Cutsmart Buffer for overnight digestion.

Cell culture conditions

HeLaS3 cells were grown in standard DMEM with sodium puryvate (Gibco #11995073) with 10% heat inactivated foetal bovine serum and 1% PenStrep (Gibco #15140). Cells were seeded in a density of approximately 70 000 cells cm^{-2} and left for 48 h after addition of the compounds or electroporation of compounds. Electroporation was done using the Lonza Amaxa 4D-Nucleofector protocol for HeLa cells, using the SE Cell line X Kit and the CN-114 program. Cells were harvested using StemPro Accutase (Gibco #A1110501) and the percent alive was determined using Trypan Blue staining.

The *Escherichia coli* strain PC5 (F-, *leuB6*(Am), λ-, *thyA47*, *rpsL153*(strR), *dnaA5*(ts), *deoC3*) was obtained from the *E. coli* Genetic Stock Center (strain #5944), maintained in LB medium supplemented with 20 mmol/L thymidine at 25 °C, and tested for growth in LB supplemented with the indicated thymidine or thymidine analogue.

The *Saccharomyces cerevisiae* strain YLV11 (***a*** *ade2–1 ura3–1 his3–11,15 can1–100 cdc21::KanR LEU2::GAL-dNK TRP1::GAL-hENT1*)[14] was maintained in YPG medium supplemented with 20 mmol/L thymidine at 30 °C and tested for growth in YPG medium supplemented with the indicated thymidine or thymidine analog.

MiSeq sample preparation and sequencing

Extraction of genomic DNA in initial experiments on HeLa incorporation was done using the Qiagen DNeasy Blood and Tissue kit (#69504). DNA extractions of the samples submitted for MiSeq sequencing were done by phenol chloroform extraction. DNA was cleaned from RNA using 1 mg/mL RNAse A at 37 °C for 30 min and DNA was digested by NcoI (NEB #R0193S). Both genomic DNA and 4sT containing PCR amplified DNA were converted and cleaned up as described above. The genomic DNA was sonicated in a mean fragment length of 500 bp. Fragments were size selected using Agencourt AMpure XP beads (Beckman Coulter A63881). All samples were prepared for MiSeq sequencing by performing end repair and A-tailing, followed by adaptor ligation and amplification using the TruSeq indexed sequencing adaptors and TruSeq Nano DNA Sample Prep Kit (Illumina FC-121-4001). The samples were cleaned up using Agencourt AMpure XP beads (Beckman Coulter A63881) and sequenced using an Illumina MiSeq machine for paired end 300bp sequencing.

Cyclohexyl (*tert*-butoxycarbonyl)-L-alaninate (1)

Based on the method of Neises and Steglich,[15] a round-bottomed flask was charged with *tert*-butoxycarbonyl)alanine (10.001 g, 53 mmol, 1.0 equiv) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (11.2081 g, 58.3 mmol, 1.1 equiv), then anhydrous CH_2Cl_2 (60 mL) was added under an argon atmosphere and the stirring pale-yellow solution was cooled to 0 °C. After 10 min, 4-dimethylaminopyridine (DMAP) (0.646 g, 5.3 mmol, 0.1 equiv) was added and stirred for a further 5 min. Next, cyclohexanol (8.9 mL, 79.5 mmol, 1.5 equiv) was added, the flask was removed from the ice bath, and the solution was left stirring overnight at RT. The reaction was monitored by TLC (10% EtOAc in CH_2Cl_2 or 20% methanol in CH_2Cl_2, visualized with $KMnO_4$ stain). The crude was concentrated by evaporation and re-dissolved in 150 mL CH_2Cl_2 before being extracted with an aqueous 5% citric acid solution and then brine. The organic phase was dried over $MgSO_4$ and evaporated to an oil. Then, 17.5 g crude oil was purified by column chromatography (6% ethyl acetate in CH_2Cl_2) to afford compound **1** as a colourless oil (9.58 g, 67%). R_f (10% EtOAc in CH_2Cl_2) = 0.85. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.08 (1H, br s, N*H*), 4.83 (1H, tt, J = 9.2 Hz, J = 3.8 Hz, cyclohexyl C*H*-O), 4.25–4.34 (1H, m, C*H*(CH_3)N), 1.80–1.90 (2H, m, cyclohexyl (β to oxygen)), 1.70–1.78 (2H, m, cyclohexyl (β to oxygen)), 1.48–1.60 (2H, m, cyclohexyl (γ to oxygen)), 1.47 (9H, s, tBu CH_3 groups), 1.40–1.46 (2H, m, cyclohexyl (γ to oxygen)), 1.39 (3H, d, J = 7.0 Hz, alanyl CH_3), 1.32 – 1.37 (1H, m, cyclohexyl (δ to oxygen)), 1.25–1.32 (1H, m, cyclohexyl (δ to oxygen)). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.8 (ester), 155.1 (carbamate), 79.7 (tBu quaternary), 73.6 (cyclohexyl *C*H-O), 49.4 (alanyl backbone C), 31.4 (cyclohexyl (β to oxygen)), 28.3 (tBu *C*H_3 groups), 25.3 (cyclohexyl (γ to oxygen)), 23.6 (cyclohexyl (δ to oxygen)), 18.9 (alanyl *C*H_3). ESI-LRMS: *m*/*z* calcd for [$C_{14}H_{25}NO_4$ + H^+], 272.2; observed, 272.8.

Cyclohexyl L-alaninate (2)

Adapting the method of Hartwig et al.,[16] **1** (9.40 g, 34.7 mmol, 1.0 equiv) was dissolved in anhydrous CH_2Cl_2 (30 mL) in a round-bottomed flask under an argon atmosphere. The stirring solution was cooled to 0 °C, and then, trifluoroacetic acid (TFA, 30 mL) was added. The vessel was removed from the ice bath and allowed to warm to RT. The solution turned pale orange, and TLC (10% methanol in CH_2Cl_2, visualized with $KMnO_4$ or ninhydrin stains) indicated completion of the reaction after 15 min. After a further 15 min, the solution was concentrated on a rotary evaporator and then co-evaporated twice with CH_2Cl_2 to remove any residual TFA. The resulting oil was dissolved in CH_2Cl_2 and extracted using 2 × 75 mL saturated sodium bicarbonate and then brine. The organic layer was dried over $MgSO_4$ and again evaporated to a colourless oil. This oil was placed on the high-vac overnight to yield compound **2** as a colourless oil (4.91 g, 83%). R_f (10% methanol in CH_2Cl_2) = 0.42. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.80 (1H, tt, J = 8.9 Hz, J = 3.8 Hz, cyclohexyl C*H*-O), 3.53 (1H, q, J = 7.0 Hz, alanyl C*H*N), 1.81–1.88 (2H, m, H-3, 1), 1.69–1.77 (2H, m, cyclohexyl (β to oxygen)), 1.65 (2H, br s, NH_2), 1.52–1.59 (1H, m, cyclohexyl (δ to oxygen)),

1.36–1.52 (4H, m, cyclohexyl (γ to oxygen)), 1.34 (3H, d, J = 7.0 Hz, alanyl CH_3), 1.23–1.31 (1H, m, cyclohexyl (δ to oxygen)) ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.1 (carbonyl), 73.0 (cyclohexyl *C*H-O), 50.2 (alanyl *C*H(NH_2)CH_3), 31.5 (cyclohexyl (β to oxygen)), 25.3 (cyclohexyl (γ to oxygen)), 23.6 (cyclohexyl (δ to oxygen)), 20.8 (alanyl CH_3). ESI-LRMS: *m*/*z* calcd for [$C_9H_{17}NO_2$ + H^+], 172.1; observed, 171.9.

4-Thiothymidine-5′-phosphorodichloridate (3)

Following a similar method to McGuigan et al.,[17] 4-thiothymidine (150 mg, 0.581 mmol, 5.81×10^{-4} mol, 1.0 equiv) was weighed into a round-bottomed flask and dried overnight on high vacuum. The flask was then sealed with a rubber septum and parafilm and flushed with a continuous supply of dry argon through a bubbler. The yellow solid was dissolved in anhydrous THF (1.5 mL), then triethylamine solution (1.0 mL of 0.64 mol/L in THF, 6.4×10^{-4} moles, 1.1 equiv) was added via syringe, and the reaction was stirred at RT for 30 min. Next, the vessel was cooled to –78 °C on an acetone – dry ice bath, before phosphorous oxychloride solution (1.0 mL of 0.58 mol/L in THF, 5.8×10^{-4} moles, 1.0 equiv) was added drop-wise via syringe over 10 min. After 30 min, the vessel was removed from the ice bath and allowed to warm to RT. The reaction was monitored by ^{31}P NMR and had gone to completion after 2 h, forming **3**. ^{31}P NMR (202 MHz, $CDCl_3$) δ 7.98 (s). This reactive intermediate was used without further characterization or purification.

4-Thiothymidine-5′-*O*-bis-(cyclohexyl-L-alaninyl)-phosphorodiamidate (4)

Leading immediately on from the synthesis of **3**, and based on the method of Pertusati et al.,[18] the solution was diluted with 3 mL anhydrous CH_2Cl_2, and then cyclohexyl L-alaninate **2** (0.50 g, 2.91×10^{-3} moles, 5.0 equiv) was injected to the stirring solution. Next, the vessel was again cooled to –78 °C and triethylamine (0.45 mL, 2.91×10^{-3} moles, 5.0 equiv) was injected drop wise. The stirring solution was left for 16 h in which time the vessel warmed to RT. The reaction was then quenched with 5 mL deionised water, extracted with CH_2Cl_2, dried over $MgSO_4$, and then rotary evaporated to a green–yellow solid. This crude was dissolved in CH_2Cl_2 and purified by column chromatography (20% EtOAc in CH_2Cl_2). The fractions were tested by TLC (20% EtOAc in CH_2Cl_2, visualized by UV, 10% H_2SO_4, $KMnO_4$ or molybdenum blue stains) and mass spectrometry, and fractions containing the pure compound were combined, concentrated, and dried to afford compound **4** as a green–yellow translucent solid (81 mg, 22%). R_f (20% EtOAc in CH_2Cl_2) = 0.56. ^1H NMR (500 MHz, DMSO-d6) δ 12.73 (1H, br s, H-3), 7.69 (1H, br s, H-6), 6.14 (1H, t, J = 6.9 Hz, H-1′), 5.37 (1H, br s, O*H*-3′), 4.87 (1H, t, J = 10.5 Hz, PN*H*), 4.81 (1H, t, J = 10.7 Hz, PN*H*), 4.64 (2H, tt, J = 8.7 Hz, J = 3.8 Hz, cyclohexyl OC*H*), 4.24–4.28 (1H, m, H-3′), 3.92–4.04 (3H, overlapping multiplets, H-5′, 4′), 3.70–3.80 (2H, m, alanyl C*H*CH_3), 2.15 (2H, dd, J = 6.8 Hz, J = 5.1 Hz, H-2′, 2″), 2.01 (3H, s, base CH_3), 1.71–1.78 (4H, m, cyclohexyl (β to O)), 1.61–1.70 (4H, m, cyclohexyl (β to O)), 1.45–1.51 (2H, m, cyclohexyl (δ to O)), 1.28–1.42 (8H, m, cyclohexyl (γ to O)), 1.28–1.33 (2H, m, cyclohexyl (δ to O)), 1.25 (6H, 2 d, J = 7.2 Hz, J = 2.0 Hz, alanyl CH_3) ^{13}C NMR (126 MHz, DMSO-d6) δ 191.3 (C-4), 173.7 (ester C=O), 148.2 (C-2), 133.5 (C6), 118.6 (C-5), 85.9 (C-4′), 85.2 (C-1′), 72.8 (cyclohexyl *C*H-O), 70.7 (C-3′), 64.9 (C-5′), 49.7 (alanyl *C*HCH$_3$), 39.5 (C-2′), 31.4 (cyclohexyl (β to O)), 25.3 (cyclohexyl (γ to O)), 23.5 (cyclohexyl (δ to O)), 20.8 (alanyl CH_3), 17.2 (nucleobase CH_3) ^{31}P NMR (202 MHz, DMSO-d6) δ 12.88 (1P, s). ESI-HRMS: *m*/*z* calcd for [$C_{28}H_{45}N_4O_9PS$ + H^+], 645.2718; observed, 645.2747.

4-Thiothymidine-5′-*O*-mono-(cyclohexyl-L-alaninyl)-phosphoramidate (5)

Using intermediate **3**, the solution was diluted with anhydrous CH_2Cl_2 (3 mL), and then cyclohexyl L-alaninate **2** (0.11 g, 6.39×10^{-4} moles, 1.0 equiv) was injected to the stirring solution. Next, the vessel was again cooled to –78 °C and triethylamine solution (1 mL, 6.39×10^{-4} moles, 1.1 equiv) was injected drop wise. The stirring solution was left for 16 h in which time the vessel warmed to RT. The reaction was then quenched with 5 mL deionised water. The compound was purified using automated reverse phase chromatography (Biotage Isolera, 30 g SNAP Ultra C_{18} column) using gradients of ACN in water containing 0.1 mol/L triethylammonium acetate. The compound-containing fractions were concentrated to yield a green–yellow transparent solid. Next, to exchange triethylammonium ions for sodium ions, a RediSep 5 g SCX cationic ion exchange column was fitted to the Isolera instrument and flushed with aqueous NaOH (50 mL, 0.5 mol/L), before being flushed with deionised water until pH 7–8 was achieved. The target compound was then dissolved in deionised water and applied to the column at a flow rate of 1 mL min^{-1}, eluting with water. Compound-containing fractions were combined, concentrated, and dried to afford the title compound as a green–yellow translucent solid (14 mg, 4.7%). ^1H NMR (500 MHz, DMSO-d6) δ 7.79 (1H, br s, H-6), 6.17 (1H, t, J = 1.0 Hz, H-1′), 4.64 (1H, tt, J = 8.9 Hz, J = 3.8 Hz, cyclohexyl C*H*-O), 4.21–4.26 (1H, m, H-3′), 3.68–3.83 (4H, overlapping multiplets, H-4′, 5′, alanyl C*H*CH_3), 2.83 (1H, br s, 3′-OH), 2.05 (2H, t, J = 6.3 Hz, H-2′, 2″), 2.01 (3H, s, nucleobase CH_3), 1.70–1.78 (2H, m, cyclohexyl (β to O)), 1.60–1.64 (2H, m, cyclohexyl (β to O)), 1.43–1.49 (1H, m, cyclohexyl (δ to O)), 1.30–1.40 (4H, m, cyclohexyl (γ to O)), 1.23–1.26 (1H, m, cyclohexyl (δ to O)), 1.16 (3H, d, J = 7.0 Hz, alanyl CH_3) ^{13}C NMR (126 MHz, DMSO-d6) δ 190.1 (C-4), 175.4 (ester C=O), 150.5 (C-2), 133.0 (C-6), 118.5 (C-5), 86.8 (C-4′), 84.7 (C-1′), 72.2 (cyclohexyl C-O), 71.6 (C-3′), 64.0 (C-5′), 50.8 (alanyl *C*HCH$_3$), 40.2 (C2′), 31.4 (cyclohexyl (δ to O)), 25.3 (cyclohexyl (γ to O)), 23.5 (cyclohexyl (β to O)), 21.8 (alanyl CH_3), 18.0 (nucleobase CH_3) ^{31}P NMR (202 MHz, DMSO-d6) δ 3.58 (1P, s, P-1) ESI-HRMS: *m*/*z* calcd for [$C_{19}H_{29}N_3O_8PS^-$], 490.1418; observed, 490.1511.

4-Thiothymidine-5′-monophosphate (6)

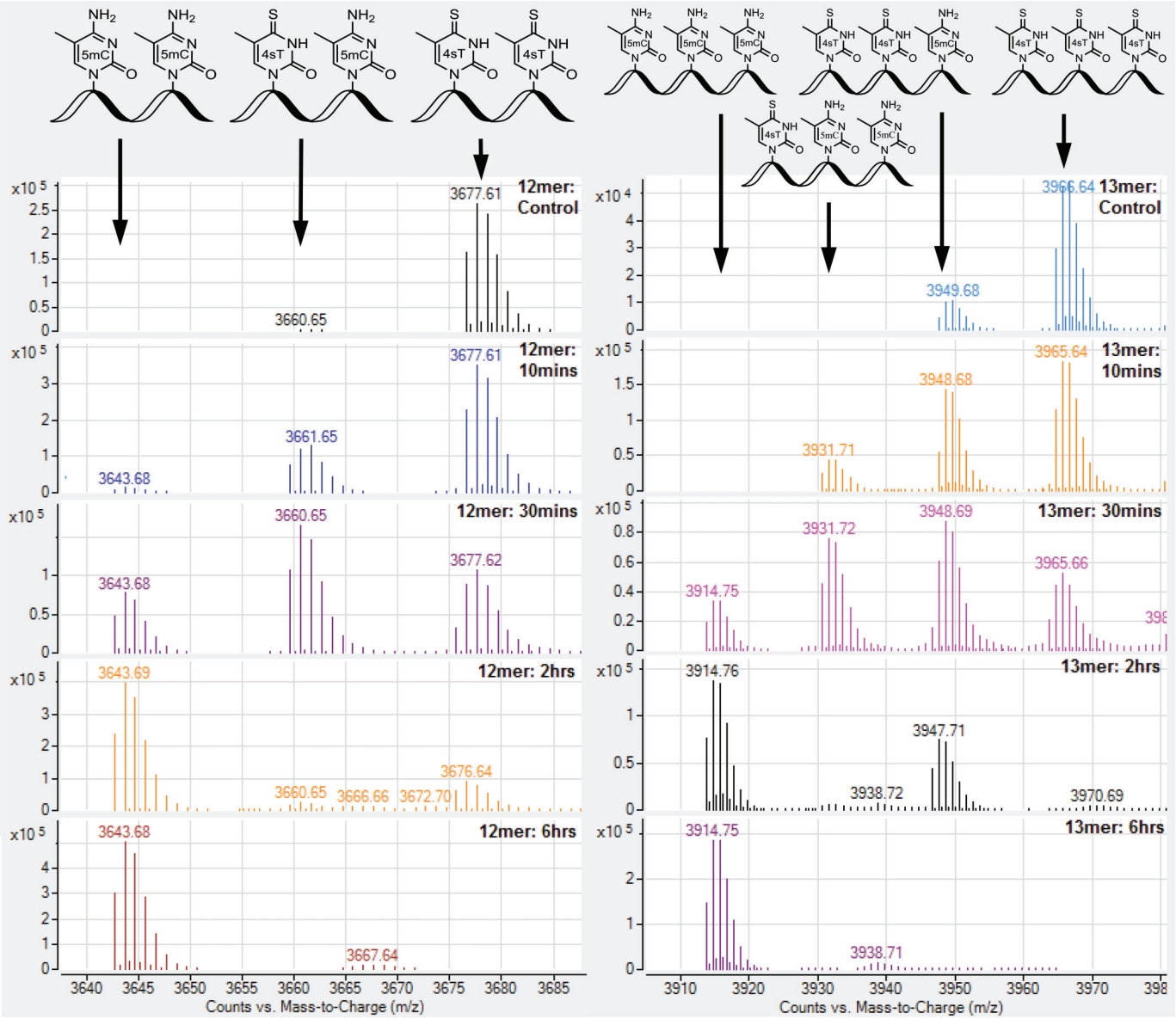
Leading immediately on from the synthesis of **3**, the solution was diluted with anhydrous CH_2Cl_2 (3 mL) and then quenched with deionised water (5 mL). The compound was purified using automated reverse phase chromatography (Biotage Isolera, 30 g SNAP Ultra C_{18} column) using gradients of ACN in water containing 0.1 mol/L triethylammonium acetate. The compound-containing fractions were concentrated to yield a green–yellow transparent solid. Next, to exchange triethylammonium ions for sodium ions, a RediSep 5 g SCX cationic ion exchange column was fitted to the Isolera instrument and flushed with aqueous NaOH (50 mL, 0.5 mol/L), before being flushed with deionised water until pH 7–8 was achieved. The target compound was then dissolved in deionised water and applied to the column at a flow rate of 1 mL min^{-1}, eluting with water. Compound-containing fractions were combined, concentrated, and dried to afford compound **6** as a green–yellow glass (34 mg, 11.4%) ^1H NMR (500 MHz, methanol-d4) δ 7.92 (1H, s, H-6), 6.31 (1H, t, J = 6.8 Hz, H-1′), 4.53–4.58 (1H, m, H-3′), 4.04–4.07 (3H, m, H-4′, 5′), 2.24–2.39 (2H, m, H-2′), 2.14 (3H, s, CH_3) ^{13}C NMR (126 MHz, methanol-d4) δ (ppm) = 193.0 (C-4), 150.4 (C-2), 134.2 (C-6), 121.2 (C-5), 88.4 (C-4′), 86.7 (C-1′), 72.8 (C-3′), 65.6 (C-5′), 41.1 (C-2′), 17.7 (CH_3) ^{31}P NMR (202 MHz, methanol-d4) δ (ppm) = 3.24 (s). ESI-HRMS: *m*/*z* calcd for [$C_{10}H_{14}N_2O_7PS]^-$, 337.0625; observed, 337.0564.

Results and discussion

Conversion of 4sT to 5mC within oligonucleotides

We synthesized two DNA oligonucleotides modified with 4sT. The first sequence was the Dickerson–Drew dodecamer, d[CGCGAAT*T*CGCG], which is self-complementary and adopts a B-form helix. The second sequence was a non-self-complementary 13mer, d[CCAT*CGT*AAT*AGC]. This allowed us to test 4sT conversion in different contexts, including single- and double-stranded sequences, and adjacent and non-adjacent 4sT modifications.

Fig. 3. Stacked high-resolution mass-spectrometry data shows base conversion of 4sT-modified 12mer (left) and 13mer (right) oligonucleotides by treatment with concentrated aqueous ammonia at 80 °C. Conversion of each 4sT base to 5mC is seen as a mass loss of 17 Da. Peaks are split due to differences in isotopic abundances. [Colour online.]



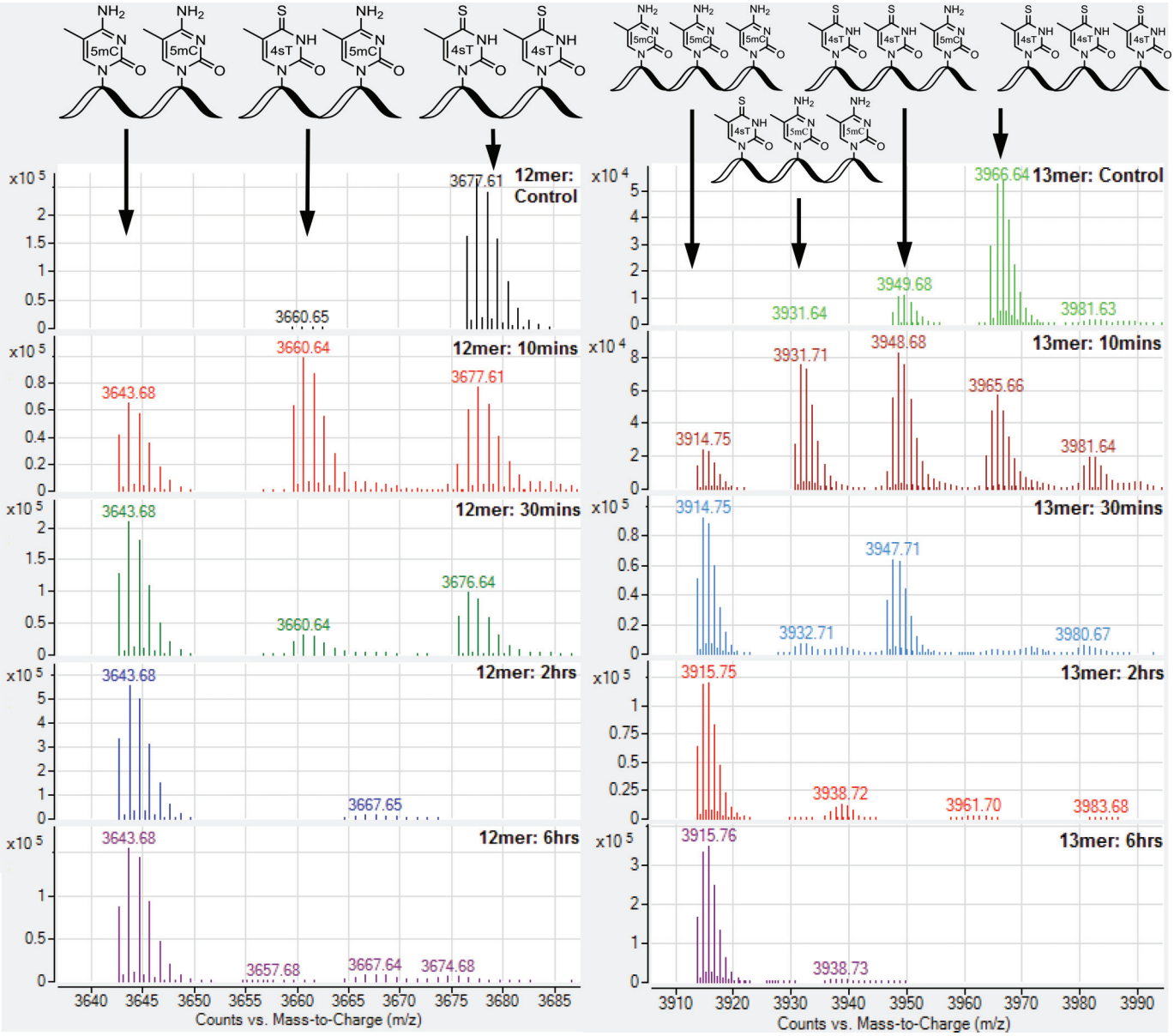
The conversion conditions employed must be strong enough to convert the thiocarbonyl into a primary amine; however, they must be selective enough not to cause damage to the DNA or react with other functional groups present in the polymer.

Oligonucleotides containing 4sT residues were subjected to various conditions and were analysed by high-resolution mass spectrometry; successful conversion of 4sT to 5mC is observed as a mass loss of 17 Da per converted base. A potential competing reaction is attack by water in the presence of base, which would lead to loss of label (4sT to T) and would correspond to a lost mass of 16 Da per converted base.

Successful conversion was observed when reacting the oligonucleotides with concentrated aqueous ammonia at elevated temperatures (Figs. 3 and 4). The 12mer has a mass of 3678.58, and the 13mer has a mass of 3966.61 (both uncharged). After full conversion of both 4sT residues on the 12mer, the final expected mass would be 3644.49. For the 13mer, full conversion of all three 4sT residues would give a final expected mass of 3915.48. Intermediate peaks are seen at separations of 17 Da, which is explained by oligonucleotides that have undergone conversion of a subset of their 4sT residues to 5mC.

Treatment of 4sT-containing oligonucleotides with concentrated aqueous ammonia at 80 °C began to show conversion between 10 and 30 min, and conversion was complete between 2 and 6 h (Fig. 3). Increasing the temperature to 95 °C showed faster conversion, with significant conversion visible by 10 min and full conversion of 4sT by 2 h (Fig. 4). The quicker conversion at 95 °C may be due to a combination of increased reaction kinetics and more complete denaturation of double-stranded DNA (such as the melting of the self-complementary Dickerson–Drew dodecamer). Interestingly, almost identical reaction profiles are seen for the 12 and 13mer oligonucleotides at both temperatures. This indicates that the position of the 4sT residues relative to each other in the DNA polymer has no effect on their conversion to 5mC, which is advantageous for metabolic labelling applications.

Fig. 4. Higher temperature conversion allows more complete conversion of 4sT to 5mC within oligonucleotides. Stacked high-resolution mass-spectrometry data shows base conversion of 4sT-modified 12mer (left) and 13mer (right) oligonucleotides by treatment with concentrated aqueous ammonia at 95 °C. Peaks are split due to differences in isotopic abundances. [Colour online.]



Conversion of 4sT residues within PCR products

Next, work was carried out to see if 4sT could be converted to 5mC after incorporation into a 700 bp PCR fragment. Preliminary 4sTTP incorporation tests were carried out using PCR and Taq polymerase. 4sT has a characteristic UV absorption peak at 335 nm, which can be used to unambiguously identify incorporation into DNA samples. The plasmid sequence was subjected to 5 cycles of PCR in the presence of 0%, 10%, 50%, 90%, and 100% 4sTTP (relative to the concentration of the natural thymidine triphosphate, TTP) to assess incorporation and conversion. PCR samples were desalted and analysed by UV spectrometry (Fig. 5).

The 0% 4sTTP sample acts as a PCR control with no 4sT present, and as expected, no 335 nm peak is seen in the UV spectrum. A linear, dose-dependent incorporation of 4sT is clear across the full range of concentrations, indicating no strong preference by the polymerase for one nucleotide over the other (Fig. 5*b*).

We treated 4sT-modified PCR products with various conversion conditions similar to those above. We observed loss of amplified product at longer conversion reaction times (data not shown). We tried including dithiothreitol (DTT, 1 mmol/L) in the PCR and conversion reactions to ensure that this product loss was not caused by the formation of disulfide bonds between different 4sT residues in their thiol tautomers that could subsequently block amplification. Nevertheless, we observed some loss of signal for the 4sT-containing PCR fragments at long conversion times, which suggests that 4sT-modified DNA may be subject to a degradation reaction that competes with conversion.

Because the PCR products are too large for mass spectrometry, we carried out restriction digest experiments as a sensitive method of monitoring conversion of 4sT to 5mC. The AvaI restriction enzyme cleaves DNA at CYCGRG sequences, where Y represents a pyrimidine base and R a purine. We used a PCR fragment that contains no CYCGRG sites but multiple YYYRRR sites. If 100% incorporation and conversion took place, all YYYRRR sites within the fragment would become CCCGGG, which is competent for AvaI digest. Conversion of a subset of T residues to C residues

Fig. 5. UV spectra from PCR amplified DNA in the presence of 4sTTP. PCR was carried out for five cycles, with varying ratios of 4sTTP to natural thymidine triphosphate (TTP). 4sT has a characteristic 335 nm absorbance peak, which correlates linearly with the % 4sTTP present in the PCR solution. (A) UV–vis spectra of PCR products containing 4sT and (B) ratio of 335 nm peak to 250 nm peak. [Colour online.]

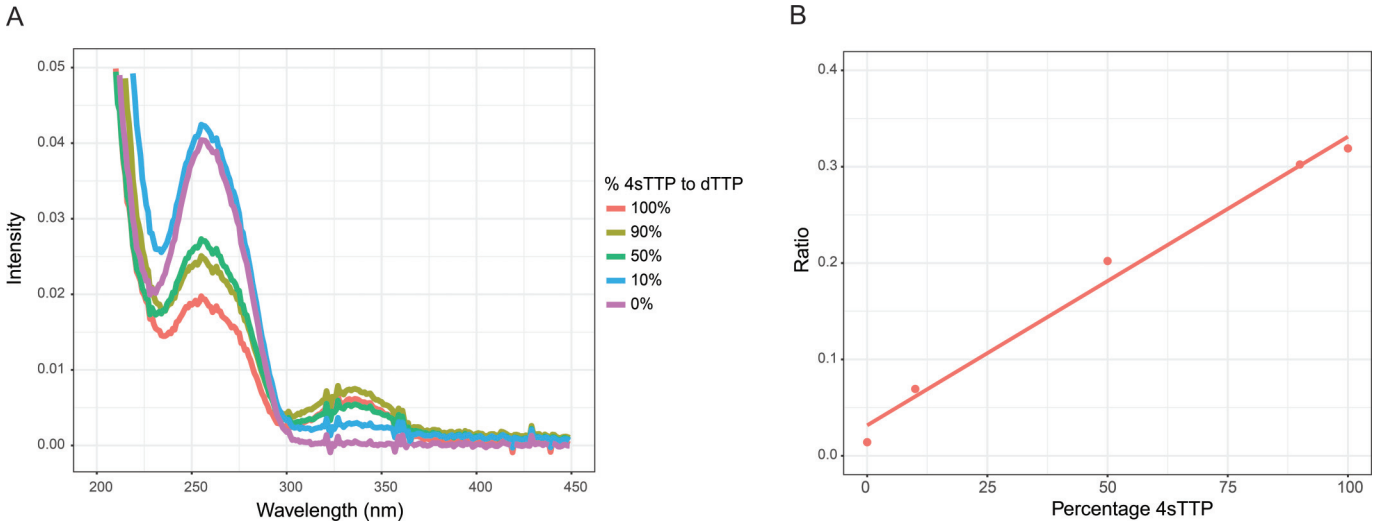
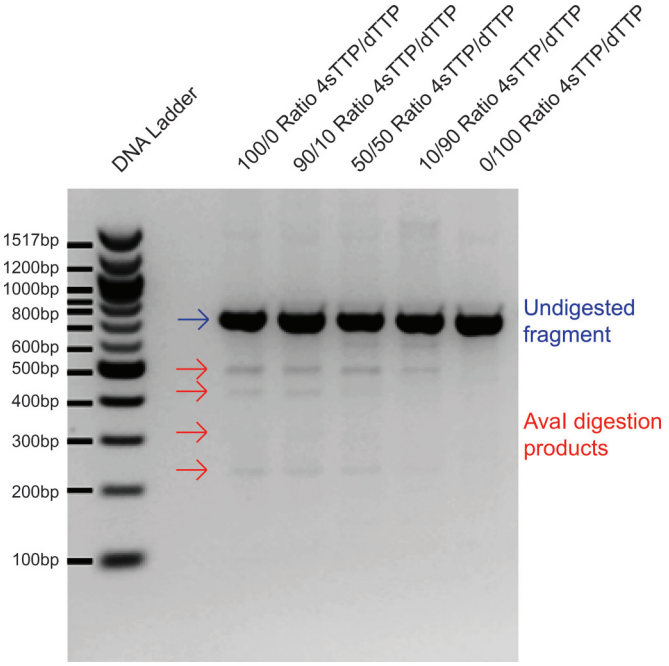


Fig. 6. Electrophoresis (2% agarose) of the AvaI digest products of PCR fragments amplified with varying ratios of 4sTTP to TTP. All sequences were subjected to conversion at 80 °C with concentrated aqueous ammonia and DTT, re-amplified with 10 rounds of PCR using normal dNTPs, and then digested with AvaI. The fragment containing no 4sT showed no AvaI cleavage as expected. A small amount of successful conversion, dependent on the presence and amount of 4sT in the original dNTP mix, is evidenced by smaller bands due to AvaI cleavage at newly introduced restriction sites. [Colour online.]



within the YYYRRR sites in the PCR fragment would produce other AvaI-competent CYCGRG sites. Therefore, digestion of the converted and amplified sequence into smaller DNA fragments by AvaI was used to indicate successful conversion.

We amplified the sequence in the presence of different ratios of 4sT and then subjected the PCR product to concentrated aqueous ammonia containing 1 mmol/L DTT at 80 °C for 30 min. The PCR product was then desalted, digested by AvaI, and analysed by agarose gel electrophoresis (Fig. 6). Most of the product remained undigested (main band, ~700 bp); however, for samples containing 4sT, a series of smaller bands became visible. Samples containing more 4sT showed a greater extent of digestion, whereas the sample with no 4sT was not digested. Nevertheless, for all samples, even those containing 100% 4sT, the digested products were minor relative to the undigested PCR product. This suggests that the conversion is much more challenging in the context of long double-stranded DNA (such as a PCR fragment or genomic DNA) than a short oligonucleotide, even at high temperature where the DNA is expected to be fairly dynamic. It may be that in the context of a duplex, the electrophilic C4 atom is less susceptible to the nucleophilic attack required for conversion because of the steric hindrance associated with being in a stack of base pairs. Thus further work may be required to develop conversion conditions that allow efficient conversion without strand cleavage.

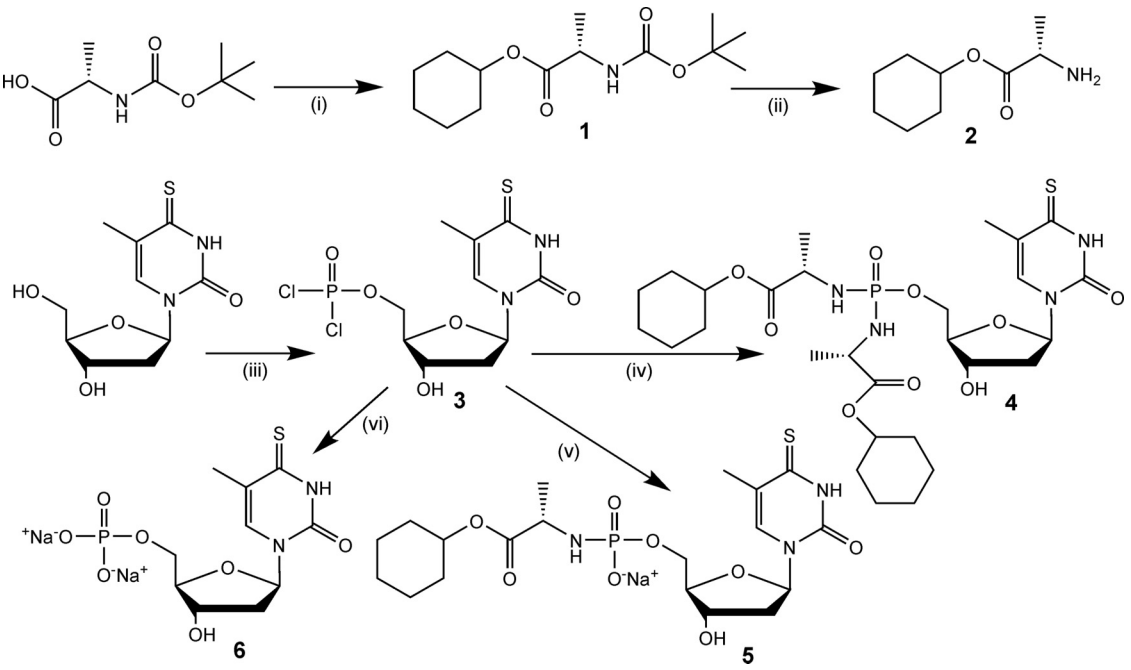
Table 1. Dose dependence of survival of a thymidylate synthase mutant of *S. cerevisiae* expressing a nucleoside transporter.

Concentration (μmol/L)	Thymidine	4sT
100	Grows to saturation	Grows to saturation
10	Grows to saturation	Grows to saturation
1	Moderate growth	Fails to saturate
0.1	Fails to saturate	Fails to saturate
0 (control)	Fails to saturate	Fails to saturate

Attempted 4sT incorporation in cultured mammalian cells

Other researchers have published the idea that 4sT can be incorporated into cellular DNA after treating mammalian cells with 4sT nucleoside.[19–21] As such, we grew HeLa cells in media containing a concentration series ranging from 50 μmol/L to 2 mmol/L 4sT nucleoside. We extracted genomic DNA and attempted to detect 4sT incorporation by UV–vis. However, we did not observe a peak for 4sT in the UV–vis spectrum above baseline. In addition to this, we converted and amplified the genomic DNA using the method used for the 4sT containing PCR products, followed by AvaI digestion. If 4sT was successfully incorporated and converted many additional AvaI restriction sites would have been introduced and the 4sT containing genomic DNA should have been digested more

Scheme 1. Synthetic pathway from 4sT starting nucleoside to the reactive phosphorodichloridate intermediate **3** and then to the corresponding target compounds: phosphorodiamidate prodrug **4**, mono-protected prodrug **5**, and 4sT 5′-phosphate **6**. Reagents and conditions: (*i*) cyclohexanol, EDC, DMAP, 67%; (*ii*) TFA, CH_2Cl_2, 83%; (*iii*) 1 equiv $POCl_3$, 1.1 equiv trimethylamine, –78 °C; (*iv*) 5 equiv cyclohexyl-L-alaninate (**2**), 5 equiv triethylamine, CH_2Cl_2, –78 °C, 22% over two steps; (*v*) 1 equiv cyclohexyl-L-alaninate (**2**), 1.1 equiv triethylamine, CH_2Cl_2, –78 °C, then H_2O, over two steps; (*vi*) 3:5 CH_2Cl_2/H_2O, 11% over two steps.



compared with the control genomic DNA. However, this was not observed.

Attempted 4sT incorporation by yeast and bacterial thymidine auxotrophs

To further explore whether other cell types could incorporate 4sT after treatment with the nucleoside, we used thymidine auxotrophs (i.e., strains that are defective in thymidine biosynthesis and require an external source of thymidine for survival and replication) of both *E. coli* (thyA mutant) and *S. cerevisiae* (thymidylate synthase mutant expressing a nucleoside transporter[14]). We grew both strains in the absence of thymidine but the presence of 100 μmol/L 4sT nucleoside. Treatment of both types of cells allowed the cells to grow to saturation. Nevertheless, to our surprise, DNA extracted from these cells showed no spectroscopic evidence of 4sT incorporation. To follow up further, we grew the yeast in decreasing concentrations of thymidine or 4sT (Table 1). The mutant yeast required about an order of magnitude higher concentration of 4sT relative to thymidine for survival and growth. This could be explained if our 4sT sample was contaminated with a small amount of thymidine (the supplier guaranteed >98% purity), though we also cannot rule out a conversion of 4sT to thymidine by the cells prior to DNA incorporation, as also speculated by others.[22] Either way, the clear conclusion from these experiments is that both types of cells select against incorporation of 4sT when fed the nucleoside, in contrast to the lack of selection noted above when using *Taq* polymerase with 4sTTP (Fig. 5*b*).

Prodrug design and synthesis

The relatively poor incorporation of 4sT into cellular DNA after feeding cells the 4sT nucleoside could be due to failure at a number of steps. In particular, the incorporation of modified nucleoside analogues into DNA requires the corresponding nucleoside triphosphate. Three separate kinases, each with their own substrate specificities, add the first, second, and third phosphate groups.[23–26] Nevertheless, the first phosphorylation event is often the rate-limiting step in the biosynthesis of modified nucleoside triphosphates.[26–28] The addition of nucleotides or dNTPs to cells directly is generally unhelpful because the charged phosphate blocks diffusion across the cell membrane. Therefore, to overcome the (potentially rate limiting) initial phosphorylation of 4sT nucleoside, without the delivery limitations of nucleotides, we have employed a nucleotide prodrug approach to maximise the intracellular bioavailability of 4sT 5′-phosphate (4sTMP).

Many different nucleotide prodrug motifs have been reported in the literature, including the diaryl ester,[29,30] phosphoramidate,[31] phosphorodiamidate,[17,32] S-acylthioethyl ester (SATE),[33] and cycloSAL[34,35] approaches. The hepatitis C drug Sovaldi (sofosbuvir), approved in 2013, is a clinically successful example of a phosphoramidate prodrug. Many ester groups and amino acids can be incorporated into phosphoramidate and phosphorodiamidate prodrugs.[17,32] Among the most promising motifs in terms of high potency (in the context of antiviral nucleoside prodrugs) and low cytotoxicity is the cyclohexyl-L-alanyl motif (Scheme 1).[17] Therefore, we selected this motif for the development of a 4sT prodrug.

Accordingly, we coupled Boc-L-alanine with cyclohexanol in the presence of EDC and DMAP. The resulting amino acid ester was deprotected with TFA followed by a basic workup to isolate the neutral amine. This amino acid ester was used to produce both mono and bis protected 4sT prodrugs.

We selectively phosphorylated the primary 5′-OH group in the presence of an unprotected 3′-OH group, by treating the 4sT nucleoside with 1 equiv of $POCl_3$ in the presence of triethylamine at –78 °C. The resulting 4sT 5′-phosphorodichloridate was used as a common intermediate to generate the phosphorodiamidate prodrug **4**, as well as two controls: the mono-protected prodrug **5** and the unprotected nucleotide 4sTMP **6** (Scheme 1).

Prodrug fails to improve 4sT incorporation

We treated HeLa cells with the nucleoside, mono- and bis-protected prodrugs. In addition, we tried electroporating 4sT tri-

Fig. 7. The phosphorodiamidate prodrug of 4sT causes marked cell death at concentrations over 25 μmol/L. (A) Survival curve for non-electroporated samples; (B) survival curve for electroporated samples. [Colour online.]

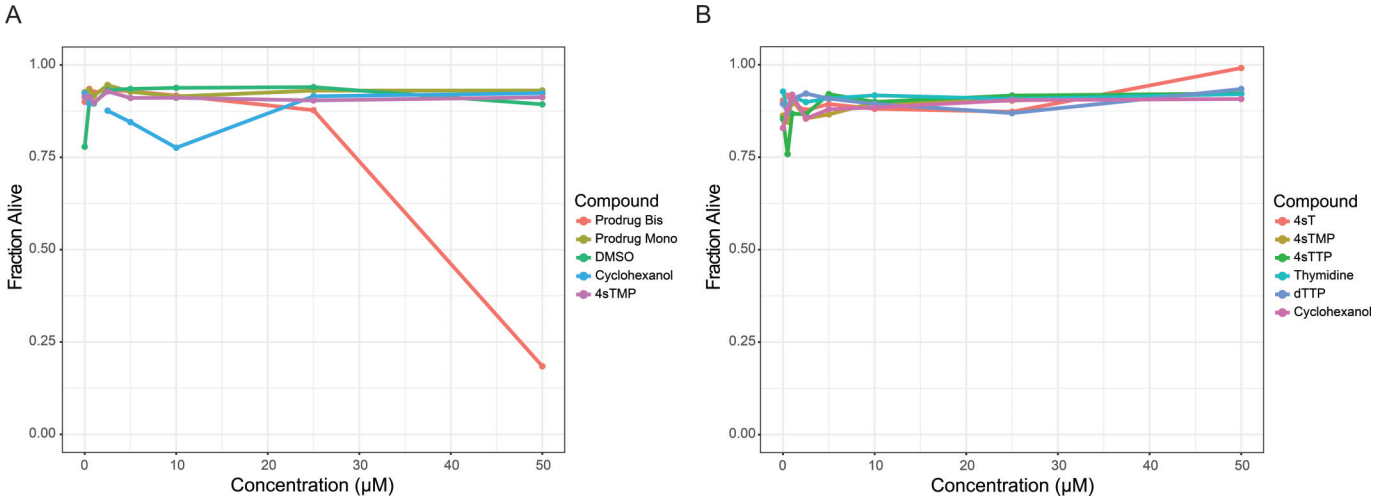


Table 2. GC content from high-throughput DNA sequencing.

	GC content (%)
PCR fragment amplified with 4sTTP[a]	
Ratio 4sTTP/TTP 0:100	62
Ratio 4sTTP/TTP 10:90	62
Ratio 4sTTP/TTP 50:50	63
Ratio 4sTTP/TTP 90:10	64
Ratio 4sTTP/TTP 100:0	63
Samples tested by electroporation	
4sTTP 50 μmol/L	45
4sTTP 25 μmol/L	44
4sTTP 10 μmol/L	45
4sTMP 50 μmol/L	45
4sTMP 25 μmol/L	45
4sT 50 μmol/L	46
4sT 25 μmol/L	45
Prodrug (bis) 50 μmol/L	45
Prodrug (bis) 25 μmol/L	45
No compound added	45
Samples tested without electroporation	
Prodrug (bis) 50 μmol/L	45
Prodrug (bis) 25 μmol/L	45
Prodrug (bis) 10 μmol/L	45
Prodrug (mono) 50 μmol/L	45
Prodrug (mono) 25 μmol/L	43
4sT 2 mmol/L	46
No compound added	45

[a]The actual GC content of this PCR fragment is 63%.

phosphate to induce uptake. Significant cell death was observed after treatment with the 4sT phosphorodiamidate prodrug at a concentration of 50 μmol/L after 48 h, whereas no other compounds caused measurable toxicity (Fig. 7). We harvested DNA from treated cells and looked for evidence of 4sT incorporation by UV spectrometry but failed to see a peak at 335 nm under any of these conditions.

To look more deeply, we subjected the harvested DNA to the conversion conditions described above (concentrated aqueous NH_3 containing 1 mmol/L DTT, 80 °C, 30 min) and PCR-amplified with the introduction of sequencing barcodes. We then carried out a run of Illumina sequencing to confirm whether incorporation of 4sT could be measured at the sequence level. Incorporation and conversion of 4sT would be expected to lead to an increase in the ratio of GC to AT bases in the sequenced DNA, but no such increase was observed (Table 2).

As a positive control, we included the PCR fragment in the sequencing experiment. However, even in this context and even for the PCR fragment amplified with 100% 4sTTP, we saw no measurable increase in the GC ratio relative to unlabelled PCR fragment (Table 2). This shows that our conversion method is very inefficient in the context of double-stranded DNA, consistent with the AvaI digest results described above.

Conclusions

We attempted to use 4sT labeling and conversion to 5mC as a base-resolution, amplifiable metabolic label for DNA. The amination of 4sT to 5mC was successful in the context of oligonucleotides, but in the context of PCR products, it produced a low yield of 5mC-modified DNA and under the same conditions we observed some breakdown of the 4sT-modified DNA. Moreover, we did not observe significant cellular incorporation of 4sT after treatment of cells with either 4sT nucleoside or a phosphorodiamidate prodrug of 4sT. Because of these challenges, we are now focusing on an alternative method for amplifiable metabolic labelling of cellular DNA, which will be published in due course.

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