Reverse-direction (5′→3′) synthesis of oligonucleotides containing a 3′-*S*-phosphorothiolate linkage and 3′-terminal 3′-thionucleosides†

James W. Gaynor,[a] Michael M. Piperakis,[a] Julie Fisher[b] and Richard Cosstick*[a]

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The synthesis of oligodeoxynucleotides containing 3′-thionucleosides has been explored using a reverse-direction (5′→3′) approach, based on nucleoside monomers which contain a trityl- or dimethoxytrityl-protected 3′-thiol and a 5′-*O*-phosphoramidite. These monomers are relatively simple to prepare as trityl-based protecting groups were introduced selectively at a 3′-thiol in preference to a 5′-hydroxyl group. As an alternative approach, trityl group migration could be induced from the 5′-oxygen to the 3′-thiol function. 5′→3′ Synthesis of oligonucleotides gave relatively poor yields for the internal incorporation of 3′-thionucleosides [to give a 3′-*S*-phosphorothiolate (3′-SP) linkage] and multiple 3′-SP modifications could not be introduced by this method. However, the reverse direction approach provided an efficient route to oligonucleotides terminating with a 3′-thionucleoside. The direct synthesis of these thio-terminating oligomers has not previously been reported and the methods described are applicable to 2′-deoxy-3′-thionucleosides derived from thymine, cytosine and adenine.

Introduction

The subtle modification of nucleic acids has proved to be a valuable approach to developing probes for studying nucleic acid structure and function. Analogues which have proved to be successful in this respect include oligonucleotides containing the 3′-*S*-phosphorothiolate (3′-SP) linkage (Scheme 1), in which sulfur replaces the 3′-oxygen atom in the phosphodiester bond.[1] Oligodeoxynucleotides containing this modification have been used in detailed mechanistic investigations into the manipulation of DNA by a number of enzymes including: phosphodiester cleavage catalyzed by the restriction endonuclease Eco RV;[2] exonucleolytic activity of *E. coli* DNA polymerase;[3] the resolution of Holliday junctions by RuvC;[4] DNA repair by *E. coli* DNA T:G mismatch endonuclease;[5] and DNA cleavage by the conjugative relaxase (protein TrwC), which is responsible for DNA processing in plasmid R388 bacterial conjugation.[6] When incorporated into oligoribonucleotides, the 3′-SP linkage has revealed crucial mechanistic information on cleavage processes catalysed by RNA enzymes.[1,7]

The sulfur atom of the 3′-SP linkage also has an effect on the conformation of the sugar to which it is directly attached. Thus, NMR studies have revealed that the 3′-sulfur atom induces a C3′-*endo*/C2′-*exo* or north conformation in the deoxyribose sugar of DNA containing a 3′-SP linkage, which is the conformation normally adopted by ribose sugars in RNA.[8–10] As a consequence, oligodeoxynucleotides bearing this modification show an increased affinity for complementary RNA sequences, in comparison to their unmodified counterparts.[8,9,11] Conversely, these modified DNA oligomers show reduced affinity for complementary DNA sequences.[11] The 3′-SP linkage has also been shown to stabilise an unusual 4-stranded intercalated structure (i-motif) formed by cytidine-rich sequences.[12]

Although several approaches to the synthesis of 3′-SP containing oligonucleotides have been investigated, none are without problems. Dinucleosides joined by a single 3′-SP linkage can be efficiently prepared by a Michael–Arbuzov reaction between nucleoside 5′-phosphites and 3′-*S*-nucleosidyl *S*-aryl disulfides, but apart from the relatively sensitive silyl phosphites, the reaction is slow and this procedure has not so far been adapted to

[a]*Department of Chemistry, University of Liverpool, Crown Street, Liverpool, UK L69 7ZD. E-mail: rcosstic@liv.ac.uk; Fax: +44 (0)151 794 3588; Tel: +44 (0)151 794 3514*
[b]*School of Chemistry, University of Leeds, Leeds, UK LS2 9JT*
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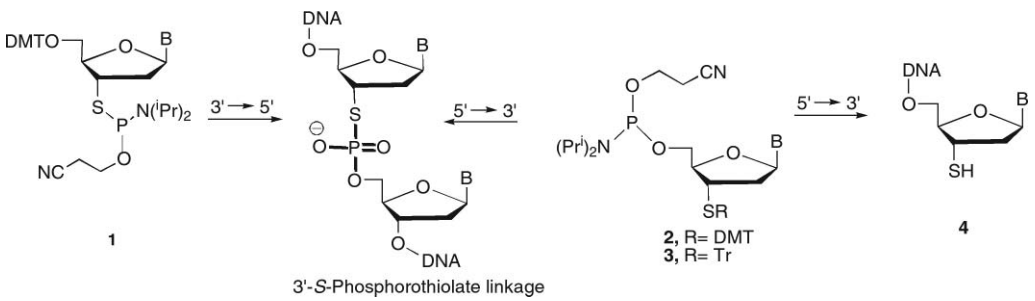


Scheme 1 The 3′-*S*-phosphorothiolate linkage prepared from a nucleoside 3′-*S*-phosphorothioamidite monomer (**1**) or the reverse synthesis monomer (**2**), DMT = 4,4′-dimethoxytrityl, Tr = trityl.

solid-phase procedures.[13] Longer oligonucleotides containing 3′-SP linkages have been prepared exclusively using nucleoside 3′-*S*-phosphorothioamidites (**1**, Scheme 1), although these are significantly less reactive than their 3′-oxygen counterparts and this restricts the number of 3′-SP modifications that can be incorporated into oligonucleotides.[14]

Jahn-Hoffman and Engels[15] have previously demonstrated that oligonucleotides containing a 5′-phosphorothiolate linkage (sulfur atom replaces bridging 5′-oxygen) can be prepared through the reaction of a nucleoside 5′-thiol with a corresponding 3′-phosphoramidite and also shown that the DMT group is suitable for the protection the 5′-thiol in the context of solid-phase synthesis. Their work suggested that the synthesis of 3′-SP linkages should be explored using a reverse-direction approach (5′→3′) utilising a 3′-thiol and 5′-phosphoramidite (**2**, Scheme 1). Although reverse direction synthesis is not widely used, it has been extensively developed and the reagents are commercially available. It is particularly well suited to 3′-modified oligonucleotides,[16] DNA chip and microrray technologies,[17] and it has also proved to be the most efficient method for preparing oligonucleotides containing the related 3′-*N*-phosphoramidate linkages.[18] This reverse approach was particularly attractive for the synthesis of 3′-SP linkages as it avoided the use the relatively unreactive 3′-*S*-phosphorothioamidite (**1**, Scheme 1). As an additional benefit, 5′→3′ synthesis appeared to offer the best route to oligonucleotides terminating with a 3′-thionucleoside (**4**, Scheme 1). The direct synthesis of these thiol-terminating oligomers has not previously been reported although they are the products of enzymatic hydrolysis of 3′-SP linkages, and would thus be useful markers and standards for such cleavage studies. Secondly, they would be interesting substrates for the synthesis of 3′-SP linkages by chemical and enzymatic ligation reactions.

We now describe a reverse approach (5′→3′ direction) for the preparation of 3′-SP linkages in which synthesis is based on the reaction of a 5′-*O*-phosphoramidite with a 3′-thiol and uses monomer (**2**) (Scheme 1). This procedure is shown to be particularly useful for preparing oligomers with a 3′-terminal 3′-thionucleoside.

Results and discussion

Preparation of monomers for 5′→3′ synthesis

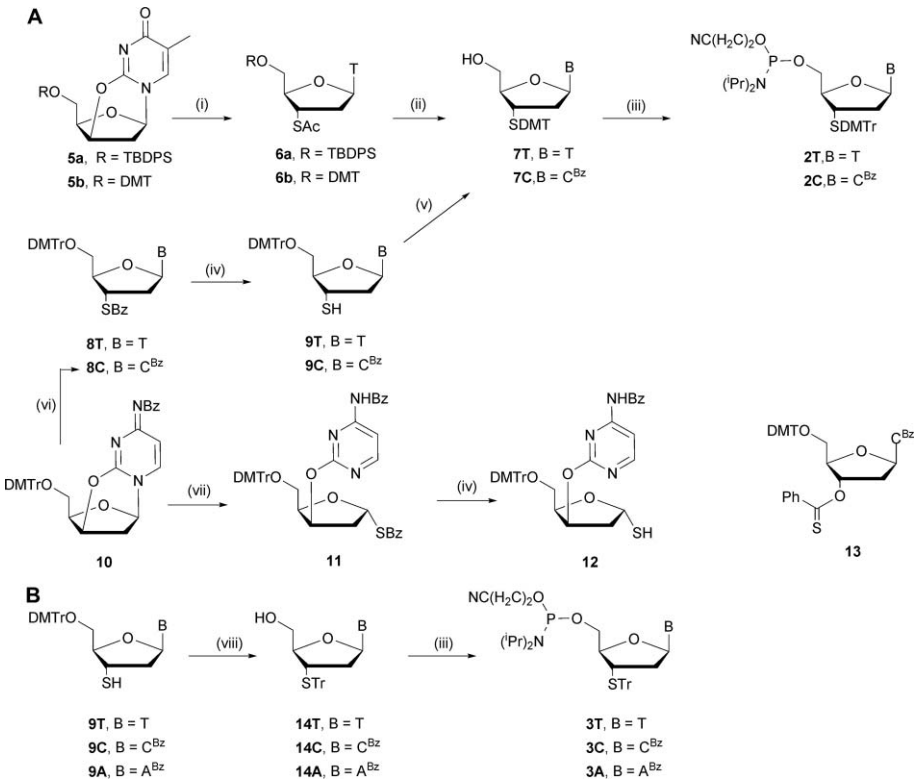
One reason why 5′→3′ DNA synthesis is not widely used for the preparation of unmodified oligonucleotides is that synthesis of the monomers is generally more complex as it requires dimethoxytrityl protection of the less reactive 3′-hydroxyl. It was envisaged that this problem would be alleviated in the preparation of the phosphorothiolate monomers (**2**) as the 3′-thiol would be more reactive towards tritylation than the 5′-hydroxyl group. In addition, there was also precedent for the O to S migration of a simple trityl groups from work conducted on thiophospholipids.[19]

Our initial studies on the synthesis of the reverse-direction monomers were conducted on thymidine and started from the previously reported[20] 5′-*O*-TBDPS-protected anhydrothymidine (**5a**, Scheme 2A). Opening of the anhydronucleoside with potassium thioacetate in DMF gave the thioester (**6a**) in 61% yield. Conveniently, **6a** could be converted into the 3′-*S*-DMT-protected thionucleoside (**7T**) in a one-pot preparation in 45% yield. This procedure involved simultaneous removal of the 5′-*O*-silyl and 3′-*S*-acetyl groups by treatment with HCl–MeOH, followed by direct addition of DMT-Cl to give selective dimethoxytritylation of the secondary thiol over the primary hydroxyl group. The site of dimethoxytritylation was confirmed by ^{13}C NMR, which showed the tertiary trityl carbon resonating at 67.4 ppm when bonded to sulfur, compared to 87–88 ppm when bonded to oxygen. Although the yield of this reaction was not high, the selective *S*-dimethoxytritylation suggested that a route involving 5′-*O*→3′-*S* migration should be feasible. Thus, an alternative procedure was investigated starting from 5′-*O*-DMT-protected anhydrothymidine[14] (**5b**). Following conversion to the corresponding thioester (**6b**, 66%), treatment with HCl–MeOH removed the 5′-*O*-DMT and 3′-*S*-acetyl groups and the resulting DMT cation was quenched by the thiol to give **7T** in 52%.

Although both the above routes to **7T** were convenient, the yields were relatively low. The most efficient route to **7T** started from the thiobenzoate ester (**8T**, Scheme 2A) which was readily available[14] from a previous study. In comparison to the thioacetate ester, the thiobenzoate was not so readily susceptible to acid-catalysed hydrolysis, but under basic conditions **8T** was hydrolysed to the thiol **9T** in almost quantitative yield.[14] A variety of conditions were investigated for the O to S migration of the DMT group that would convert **9T** to **7T** including solutions of acetic acid, tosic acid and HCl. The best results gave **7T** in 89% yield and were obtained using a 0.1 M solution of HCl in *i*-PrOH. Interestingly, this procedure gave much better yields than the analogous method using methanolic HCl and this was attributed to the lower nucleophilicity of *i*-PrOH. This approach to **7T** was also applicable to the synthesis of the corresponding cytidine derivative, and thus **8C**[14] could be converted through to **7C** with similarly good yields (89% over the two steps). Both **7T** and **7C** could be phosphitylated under standard conditions to give **2T** and **2C**, respectively which were the required monomers for reverse direction solid-phase synthesis.

In this and previous studies[14] *N*4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2,3-anhydro-2′-deoxycytidine (**10**, Scheme 2A) has been used as a key intermediate to access 2′-deoxy-3′-thiocytidine derivatives through initial ring opening with thiobenzoate salts to give the thioester **8C**. However, we have found this reaction to be very capricious and although yields above 60% have been reported, they are often only 20–40%. For this reason we have continued to search for procedures that would give consistently good yields for the conversion of **10** to **8C**. In this respect, interesting results were obtained when the anhydrocytidine derivative **10** was treated with thiobenzoic acid in refluxing pyridine. Under these conditions the anhydronucleoside was converted within 10 min to a new product in 81% yield. The new product (**11**) has the same mass as the thioester (**8C**) and the position of H3′ in the proton NMR spectrum (4.42 ppm) initially suggested that the compound could be the 3′-*O*-benzothioate ester **13**.[1] However, its identity as the anomeric thioester (**11**) was confirmed by HMBC coupling from H1′ (6.4 ppm) to the SBz C=O group (191 ppm). Further proof of the structure of **11** was obtained through hydrolysis, which gave thiol **12** in which coupling between the anomeric and SH protons was observed in 2D COSY experiments.

It is noteworthy that anomeric opening of the anhydronucleoside occurred only with the cytidine derivative. Thus, when 5′-*O*-(4,4′-dimethoxytrityl)-2,3′-anhydrothymidine (**5b**) was treated identically with thiobenzoic acid in refluxing pyridine, only the



Scheme 2 Synthesis of monomers for reverse synthesis. *Reagents and conditions*: (i) KSAc, DMAc, 110 °C, 2.5 h; 61% **6a**; 66% **6b**. (ii) HCl–MeOH (1 M), 45 °C, 2 h then (for **6a**→**7T** only) DMT-Cl in AcOH–H_2O (2 : 1), 3 h; 45% **6a**→**7T**; 52% **6b**→**7T**. (iii) $P(NiPr_2)_2(OCH_2CH_2CN)$, tetrazole derivative, MeCN or CH_2Cl_2, 2h; 49% **2T**; 60% **2C**; 56% **3T**; 62% **3C**; 58% **3A**. (iv) Dilute NaOH, see experimental for details; 93% **9T**; >99% **9C**; 94% **9A**; 43% **12**. (v) HCl–*i*PrOH (0.1M) with up to 1 : 1 v/v CH_2Cl_2, 1 h; 89% **7T**/**7C**. (vi) Ref. 14. (vii) HSBz, pyridine, 110 °C, 10 min; 81%. (viii) TrCl, NEt_3, CH_2Cl_2, 0 °C, 1.5 h then HCl–MeOH (1.25M), 0 °C, <15 min; 56% **14T**; 52% **14C**; 51% **14A**. A^{Bz} = *N*6-benzoyladenin-9-yl; Ac = acetyl; Bz = benzoyl; C^{Bz} = *N*4-benzoylcytosin-1-yl; T = thymin-1-yl; TBDPS = *tert*-butyldiphenylsilyl.

expected thioester (**8T**) was formed. The opening of an anhydrocytidine derivative at the anomeric position has been observed with azide[21] and is also implicated in some pyrimidine nucleoside isomerisations observed by Kotick *et al.*[22]

For reasons that will become apparent when the solid-phase synthesis is discussed, it was also necessary to prepare the reverse synthesis monomers protected with the more robust trityl group (**3**, Schemes 1 and 2B). A one-pot procedure was developed for the synthesis of the 3′-*S*-trityl derivatives (**14**), starting from the 5′-*O*-DMT protected 3′-thionucleosides (**9**, Scheme 2B). (The 3′-thioadenosine derivative **9A** was available from the previously reported[23] 3′-*S*-thiobenzoate ester.) Tritylation of the mercapto functions was achieved under standard conditions and was followed by addition of methanolic HCl to selectively remove the 5′-*O*-DMT group. All three 3′-*S*-trityl nucleosides were obtained in yields greater than 50%, although in the case of **14A**, it was necessary to keep the acid treatment brief in order to minimise depurination. Finally, 5′-phosphitylation to give the required monomers (**3**) was performed as described for phosphoramidites **2**.

Solid-phase synthesis

Compared to standard DNA synthesis, phosphodiester bond formation in the 5′→3′ direction requires the more hindered 3′-hydroxyl to undergo reaction with a nucleoside 5′-phosphoramidite and as a result longer reaction times are required for both the detritylation and coupling steps. As a protocol for 5′→3′ synthesis was not available for our Expedite DNA synthesiser, reaction times for these two steps had to be optimised. Studies conducted on the synthesis of $d(T)_{10}$ established that coupling yields in excess of 98%[24] were generally achievable using a 135 s detritylation (3% TCA) and a 180 s coupling (0.4 M tetrazole); all other steps in the reaction cycle were essentially the same as those used in standard DNA synthesis (see experimental section for full details). With regard to the incorporation of a 3′-SP linkage, initial studies were conducted on the synthesis of the test sequence 5′-TTTTSTT (**ON1**, Table 1) (where TS = 3′-thiothymidine) and coupling of the monomer **2T** was performed using detritylation and coupling conditions described above. Our optimised conditions for the removal of the DMT group from the terminal thiothymidine and subsequent coupling to form the 3′-SP linkage were as follows: a 7 min treatment with 3% TCA, a reduction step (DTT solution) to reduce any oxidised thiol and a 5 min coupling with the next 5′-amidite using the standard tetrazole solution. These reaction conditions are similar to those of Jahn-Hoffman and Engels[15] in their synthesis of 5′-phosphorothiolates, although the removal of the 3′-*S*-DMT group required in this study necessitated a longer treatment with acid. The slow removal of the *S*-DMT group made it difficult to assess the coupling yield using the trityl monitor built into the synthesiser, but based on the HPLC trace of the crude 5′-TTTTSTT-3′-*O*-DMT product, these were generally less than 50%. Using this reverse synthesis procedure, 5′-TTTTSTT could be isolated pure (>90% by HPLC)

Table 1 Monoisotopic ESI mass spectrometry data acquired for oligonucleotide sequences using negative mode ionization. Samples were prepared in aqueous methanol + 0.1% diethylamine. TS, CS or AS indicates 3′-thionucleoside and TSH, CSH or ASH indicates a 3′-thionucleoside with a free thiol group

		Monoisotopic Masses[a]	
#	Sequence	Calculated	Measured
ON1	d(TTT TST)	1778.3871	1778.2976
ON2	d(AAATCTTCC TS)-DMT	3278.6482	3278.6599
	d(AAATCTTCC TS)-Trityl	3218.6270	3218.5750
	d(AAA TCTTCCTSH)[a]	1487.2515	1487.2528
ON3	d(AAATCTTCCCS)-DMT	3263.6484	3263.6326
	d(AAATCTTCCCS)-Trityl	3203.6274	3203.5872
	d(AAA TCT TCC CSH)[a]	1479.7516	1479.7490
ON4	d(AAATCTTCCAS)-Trityl	3227.6386	3227.5901
	d(AAA TCT TCC ASH)[a]	1491.7572	1491.7365

[a] For DMT and trityl-on oligonucleotides, masses are based on the neutral free acid species (M). For oligonucleotides containing a 3′-terminal thiol, the masses are based on the $(M - 2H)^{2-}$ ion.

and was characterised by mass spectrometry. However, yields of the purified oligonucleotide containing a 3′-SP linkage were only 25% of the obtained yields for $d(T)_6$. Unfortunately, we were unable to improve these coupling yields further and were not able to isolate pure product from the preparation of a sequence containing two 3′-SP-linkages 5′-TTTSTSTT. Whilst this 5′→3′ approach clearly offered no advantage for the incorporation of 3′-SP-linkages into an oligomer, it provides an obvious route to oligonucleotides with a terminal 3′-thionucleoside (**4**, Scheme 1).

The synthesis of oligonucleotides with a 3′-terminal 3′-thionucleoside was initially investigated with respect to **ON2** and **ON3**, which have 3′-thiothymidine and 3′-thio-2′-deoxycytidine residues, respectively, at the 3′-terminus. These oligonucleotides were prepared using phosphoramidites **2T** and **2C**, and following HPLC purification of the *S*-DMT-protected oligonucleotides, the thiol group could be released by treatment with $AgNO_3$ followed by dithiothreitol (DTT), as previously described for *S*-trityl derivatives.[25] The *S*-DMT-protected oligonucleotides were shown to be susceptible to detritylation during work-up, HPLC purification and storage in aqueous solution under neutral conditions (Fig. 1A), and suggested that the more robust trityl group would be more suitable for this purpose. Thus, the synthesis of **ON2**, **ON3** and **ON4** were performed using the 3′-*S*-trityl-protected monomers **3T**, **3C** and **3A**, respectively, in the final coupling. After partial deprotection, the crude 3′-*S*-trityl-protected oligonucleotides were shown to be greater than 80% pure by HPLC analysis (Fig. 1b) and could easily be purified by reverse-phase HPLC. The trityl group was removed (Fig. 1c) with silver ions as described above for the *S*-DMT-protected oligonucleotides. In each case both the 3′-*S*-trityl-protected and the 3′-thio-oligonucleotides were characterised by mass spectrometry (Table 1).

The instability of the *S*-DMT-protected oligonucleotides was somewhat surprising given that the acid-catalysed removal of the DMT group from the thiol on the solid-support was clearly much slower than that of the corresponding *O*-DMT group.[26] It is likely that the instability of the *S*-DMT group contributes to the poor coupling yield that was noted above for the introduction of the 3′-SP-linkage in the synthesis of **ON1**; however, there is also a problem with the adventitious oxidation of the released thiol.

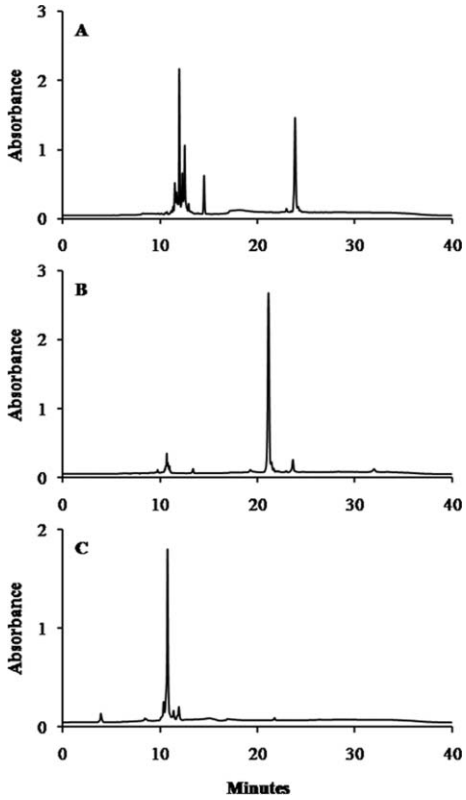


Fig. 1 Reverse-phase HPLC traces for preparations of ON2. (**A**) Crude product retaining a 3′-dimethoxytrityl group. A substantial amount of detritylated material can be seen at ~12 min. (**B**) Crude product retaining a 3′-trityl group. (**C**) Crude ON2 terminating in a 3′-thiol and obtained by treating 3′-trityl–ON2 with $AgNO_3$–DTT (see experimental conditions). *HPLC conditions*: 0–40% acetonitrile in TEAB (0.1 M, pH 7.5) over 25 min.

Conclusions

The first direct synthesis of oligonucleotides containing 3′-terminal 3′-thionucleosides (**4**) is reported and is based on solid-phase DNA synthesis in the 5′→3′ direction using 5′-*O*-phosphoramidites derived from 3′-*S*-trityl-3′-thionucleosides (**3**). These oligomers have been prepared with 3′-thiolated pyrimidine and purine nucleosides, and are isolated as stable *S*-trityl derivatives from which the thiol function can be readily released when required. It is interesting to speculate whether oligonucleotides bearing this 3′-thiol modification could be used to generate 3′-SP linkages either by functioning as a substrate for DNA ligase or by chemical primer extension as demonstrated for 3′-amino primers.[27]

The synthesis of oligonucleotides containing a phosphorothiolate linkage (**ON1**) gave only very low coupling yields (<50%) using the 3′-*S*-DMT protected reverse phosphoramidites (**2**) and unfortunately this method could not be applied to oligomers containing multiple 3′-SP linkages. The exact reasons for this are unclear although there are clearly problems relating to the removal of the 3′-*S*-DMT group and oxidation of the resulting thiol.

Experimental

Unless otherwise stated, all general reagents were purchased from either Aldrich, BDH or Fluka and used as supplied. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite was purchased from Chemgenes and 5-ethylthio-1*H*-tetrazole (ETT) (0.25 M) was

supplied from either Link Technologies and/or Applied Biosystems. Analytical thin layer chromatography was performed on UV_{254} sensitive, silica gel 60 coated, aluminium tlc plates purchased from Merck. Ellman's reagent was used to distinguish compounds containing a 3′-thiol group.[14] Flash column chromatography was performed using silica (particle size 40-63 μM, supplied by BDH). All NMR spectra were recorded on a Bruker 400 MHz spectrometer; operating at 400 MHz for ^{1}H and at 162 MHz for ^{31}P. All spectra were recorded in $CDCl_3$, relative to an internal standard of tetramethylsilane (^{1}H NMR) or an external standard of 85% H_3PO_4 (^{31}P). All chemical shifts are reported in ppm and coupling constants (J) in hertz. ^{13}C and ^{31}P spectra were ^{1}H decoupled and were singlets unless otherwise stated. Standard nucleoside numbering systems have been used with additional abbreviations as follows: $^{a/b}$ denotes different diastereoisomers; Ar_2 = 4-methoxyphenyl on DMT; Bz = SBz or NBz; OCH_2 = cyanoethyl; Ph = phenyl on Tr, DMT or TBDPS. Mass spectra were recorded on a MicroMass LCT mass spectrometer using electrospray ionisation (EI) and direct infusion syringe pump sampling. All mass spectra were visualised as a series of multi-charged ions. For the DMT and Trityl-on species, the 'Transform' software was able to transform the spectra into the full length mass. However, for the samples containing a free thiol, the spectra could not be transformed so the monoisotopic masses for the $(M - 2H)^{2-}$ were obtained. Mass spectrometry and HPLC were performed as previously described.[28] Elemental analyses were determined on Thermo FlashEA 1112 series CHNSO instrument.

5′-*O*-(*tert*-Butyldiphenylsilyl)-3′-*S*-acetyl-3′-thiothymidine (6a)

A solution of **5a** (3.00 g, 6.48 mmol) in anhydrous DMF (25 mL) was heated to 110 °C under N_2. Potassium thioacetate (4 × 1.85 g, 16.2 mmol) was then added every 30 min until 10 equivalents had been added. 30 min after the final addition, EtOAc (150 mL) was added and the solution washed with saturated $NaHCO_3$ solution (3 × 150 mL) and water (150 mL). The organic layers were dried over $MgSO_4$ filtered and concentrated under reduced pressure to afford the impure product as a pale brown foam. Purification by flash silica chromatography, eluting with CH_2Cl_2 followed by a gradient of 0–1% MeOH in CH_2Cl_2, afforded the title compound as a white amorphous solid. (2.06 g, 3.82 mmol, 59%). R_f (EtOAc–MeOH 9 : 1) = 0.65; R_f (CH_2Cl_2–MeOH 9 : 1) = 0.63. ^{1}H NMR δ = 1.11(9H, s, CMe_3); 1.58(3H, d, $J_{Me\text{-}H6}$ = 1.2, Me); 2.40(4H, m, H2′, SCOMe); 2.56(1H, m, H2′); 3.89(1H, dd, $J_{H5'\text{-}H5''}$ = 11.5, $J_{H5'\text{-}H4'}$ = 2.6, H5′); 4.02(2H, m, H4′, H5″); 4.27(1H, m, H3′); 6.28(1H, m, H1′); 7.41(6H, m, *m*-Ph, *p*-Ph); 7.49(1H, d, $J_{Me\text{-}H6}$ = 1.4, H6); 7.68(4H, m, *o*-Ph); 9.56(1H, s, NH). ES-HRMS; $C_{28}H_{34}N_2O_5SSiNa$ requires 561.1861; $[M + Na]^+$ = 561.1855 (1.0 ppm).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*S*-acetyl-3′-thiothymidine (6b)

The preparation was analogous to that described above for **6a**, but using **5b**[14] (3 g, 5.7 mmol), anhydrous DMF (30 mL) and potassium thioacetate (4 × 1.63 g, 14.24 mmol). Purification by flash silica chromatography, eluting with 2 : 1 EtOAc–n-hexane, afforded the title compound as a pale brown foam. (2.26 g, 3.74 mmol, 66%). R_f (EtOAc) = 0.5; R_f (CH_2Cl_2–MeOH 9 : 1) = 0.55. ^{1}H NMR δ = 1.45(3H, d, $J_{Me\text{-}H6}$ = 1.1, Me); 2.40(4H, m, H2′, SCOMe); 2.71(1H, ddd, $J_{H2''\text{-}H2'}$ = 13.9, $J_{H2''\text{-}H3'}$ = 8.3, $J_{H2''\text{-}H1'}$ = 5.3, H2″); 3.36(1H, dd, $J_{H5'\text{-}H5''}$ = 10.8, $J_{H5'\text{-}H4'}$ = 3.0, H5′); 3.49(1H, dd, $J_{H5''\text{-}H5'}$ = 10.7, $J_{H5''\text{-}H4'}$ = 2.4, H5″); 3.79(6H, s, OMe); 4.02(1H, m, H4′); 4.28(1H, m, H3′); 6.23(1H, dd, $J_{H1'\text{-}H2'}$ = 6.5, $J_{H1'\text{-}H2''}$ = 5.3, H1′); 6.84(4H, d, $J_{mH\text{-}oH}$ = 8.4, *m*-Ar_2); 7.28(7H, m, *o*-Ar_2, *m*-Ph, *p*-Ph); 7.45-7.68(3H, m, H6, *o*-Ph). ES-HRMS; $C_{33}H_{34}N_2NaO_7S$ requires 625.2011; $[M + Na]^+$ = 625.1984 (4.2 ppm).

3′-*S*-(4,4′-Dimethoxytrityl)-3′-thiothymidine (7T)

From 6b. A solution of **6b** (0.31 g, 0.52 mmol) in HCl–MeOH (10 mL, 1 M) under N_2 was stirred for 2 h at 45 °C. The solution was concentrated *in vacuo*, taken up in CH_2Cl_2 (50 mL) and washed with saturated $NaHCO_3$ (3 × 50 mL). The organic layers were dried with $MgSO_4$ and evaporated *in vacuo* to leave a pale red oil. Purification by flash silica chromatography, eluting with CH_2Cl_2 followed by a gradient of 0–3% MeOH in CH_2Cl_2, afforded the title compound as a white amorphous solid. (0.15 g, 0.27 mmol, 52%).

From 9T. HCl–*i*PrOH (15 mL, 0.1 M, 1.5 mmol) was degassed with nitrogen for 5 min and added to **9T** (0.3 g, 0.54 mmol), along with a small amount of CH_2Cl_2 (*ca.* 1 mL) to aid dissolution. The mixture was stirred for 1 h at RT. The mixture was concentrated *in vacuo* and dissolved in CH_2Cl_2 (20 mL) and washed with saturated $NaHCO_3$ (2 × 20 mL). The combined aqueous layer was back extracted with CH_2Cl_2 (2 × 20 mL), the combined organic layers were dried with $MgSO_4$ and evaporated *in vacuo* to afford the title compound as a white amorphous solid. If further purification was required it could be achieved by flash silica chromatography, eluting with 2 : 1 EtOAc–n-hexane. (0.27 g, 0.48 mmol, 89%). R_f (EtOAc–n-hexane 3 : 1) = 0.25; R_f (EtOAc) = 0.39; R_f (CH_2Cl_2–MeOH 9 : 1) = 0.61. ^{1}H NMR δ = 1.73(1H, ddd, $J_{H2'\text{-}H2''}$ = 14.0, $J_{H2'\text{-}H3'}$ = 7.9, $J_{H2'\text{-}H1'}$ = 3.7, H2′); 1.80(3H, d, $J_{Me\text{-}H6}$ = 1.1, Me); 2.20(1H, ddd, $J_{H2''\text{-}H2'}$ = 14.0, $J_{H2''\text{-}H3'}$ = 8.9, $J_{H2''\text{-}H1'}$ = 7.2, H2″); 2.86(1H, m, H3′); 3.62(1H, dd, $J_{H5'\text{-}H5''}$ = 12.8, $J_{H5'\text{-}H4'}$ = 2.9, H5′); 3.77(6H, s, OMe); 3.83(2H, m, H5″, H4′); 5.99(1H, dd, $J_{H1'\text{-}H2''}$ = 7.1, $J_{H1'\text{-}H2'}$ = 3.7, H1′); 6.81(4H, m, $J_{mH\text{-}oH}$ = 8.9, *m*-Ar_2); 7.20(2H, m, $J_{Me\text{-}H6}$ = 1.2, *p*-Ph, H6); 7.28(2H, m, *m*-Ph); 7.36(4H, m, $J_{oH\text{-}mH}$ = 8.9, *o*-Ar_2); 7.45(2H, m, *o*-Ph). ES-HRMS; $C_{31}H_{32}N_2NaO_6S$ requires 583.1871; $[M + Na]^+$ = 583.1879 (1.3 ppm).

3′-*S*-(4,4′-Dimethoxytrityl)-2′-deoxy-4-*N*-benzoyl-3′-thiocytidine (7C)

The preparation was analogous to that described above for conversion of **9T** to **7T**, but using **9C** (0.143 g, 0.215 mmol), HCl–*i*PrOH (6 mL, 0.1 M, 0.6 mmol) and CH_2Cl_2 (*ca.* 2.5 mL) with the product appearing as a pale yellow amorphous solid. (0.127 g, 0.196 mmol, 89%). R_f (EtOAc) = 0.24; R_f (EtOAc–MeOH 98 : 2) = 0.33. ^{1}H NMR δ = 1.93(1H, m, H2′); 2.45(1H, ddd, $J_{H2''\text{-}H2'}$ = 13.9, $J_{H2''\text{-}H3'}$ = 10.3, $J_{H2''\text{-}H1'}$ = 7.0, H2″); 2.68(1H, m, H3′); 3.68(1H, m, H5′); 3.78(6H, s, OMe); 3.89(2H, m, H5″ & H4′); 5.99(1H, dd, $J_{H1'\text{-}H2''}$ = 6.8, $J_{H1'\text{-}H2'}$ = 1.9, H1′); 6.78(4H, m, *m*-Ar_2); 7.25(4H, m, *m*-Ph, *p*-Ph, H5); 7.37(4H, m, *o*-Ar_2); 7.48(4H, m, *o*-Ph, *m*-Bz); 7.61(1H, m, *p*-Bz); 7.87(2H, m, *o*-Bz); 8.24(1H, d, $J_{H6\text{-}H5}$ = 7.2, H6). ES-HRMS; $C_{37}H_{35}N_3NaO_6S$ requires 672.2125; $[M + Na]^+$ = 672.2144 (2.9 ppm).

3′-*S*-(4,4′-Dimethoxytrityl)-3′-thiothymidine-5′-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (2T)

2 - Cyanoethyl - *N*, *N*, *N*′, *N*′ - tetraisopropylphosphordiamidite (0.15 mL, 0.47 mmol) was slowly added to a solution of **7T**

(0.205 g, 0.37 mmol) and 0.25 M ETT solution in MeCN (0.95 mL, 0.24 mmol) in anhydrous MeCN (2 mL). The reaction mixture was then stirred under N_2 at RT for 2 h after which it quenched with MeOH (*ca.* 1 mL, 30 s). The mixture was taken up in CH_2Cl_2 (20 mL) and washed with saturated $NaHCO_3$ (3 × 20 mL) and the organic layer dried over sodium sulfate, filtered and concentrated under reduced pressure to afford the impure product as an off-white foam. Purification by flash silica chromatography, eluting with 1 : 1 EtOAc–n-hexane followed by a gradient of 1 : 1 EtOAc–n-hexane to 3 : 1 EtOAc–n-hexane (with 0.5% NEt_3), afforded the title compound as a white amorphous solid. (0.136 g, 0.18 mmol, 49%). R_f (EtOAc–n-hexane 3 : 1) = 0.40. ^{31}P NMR δ = 150.0 & 150.3. ^{1}H NMR δ = 1.06-1.19(24H, 4 × d, J = 6.6 (×3) & 6.8, ^{a}CHMe_2, ^{b}CHMe_2); 1.44(1H, ddd, $J_{H2'\text{-}H2''}$ = 14.0, $J_{H2'\text{-}H3'}$ = 8.2, $J_{H2'\text{-}H1'}$ = 5.4, ^{a}H2′); 1.73(1H, m, ^{b}H2′); 1.86 & 1.88(6H, 2 × d $J_{Me\text{-}H6}$ = 1.0 & 1.1, ^{a}Me,^{b}Me); 2.03(1H, m, ^{a}H2″); 2.14(1H, ddd, $J_{H2''\text{-}H2'}$ = 13.6, J = 6.0 & 5.0], ^{b}H2″); 2.50 & 2.57(4H, 2 × t, J = 6.3 & 6.4, ^{a}CH_2CN, ^{b}CH_2CN); 2.88(1H, m, ^{b}H3′); 2.98(1H, m, ^{a}H3′); 3.39(1H, m, ^{a}H5′/^{b}H5′); 3.44-3.60(4H, m, ^{a}C$H$$Me_2$, ^{b}C$H$$Me_2$); 3.64-3.80(20H, m, ^{a}OMe, ^{b}OMe, ^{a}H5′/^{b}H5′, ^{a}H5″, ^{b}H5″, ^{a}OCH_2, ^{b}OCH_2); 4.01(2H, m, ^{a}H4′, ^{b}H4′); 6.08(1H, dd, $J_{H1'\text{-}H2'}$ = 5.4, $J_{H1'\text{-}H2''}$ = 6.5, ^{a}H1′); 6.10(1H, m, ^{b}H1′); 6.82(8H, m, m-^{a}Ar_2, m-^{b}Ar_2); 7.20(4H, m, $J_{H6\text{-}Me}$ = 0.8 & 1.1, p-^{a}Ph, p-^{b}Ph, ^{a}H6, ^{b}H6); 7.26-7.37(12H, o-^{a}Ar_2, o-^{b}Ar_2, m-^{a}Ph, m-^{b}Ph); 7.43(4H, m, o-^{a}Ph, o-^{b}Ph); 8.82(2H, s, ^{a}NH, ^{b}NH). ES-HRMS; $C_{40}H_{48}N_4O_7PS$ requires 759.2981; $[M - H]^-$ = 759.2988 (0.9 ppm).

3′-*S*-(4,4′-Dimethoxytrityl)-2′-deoxy-4*N*-benzoyl-3′-thiocytidine-5′-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (2C)

Performed as described for **2T**, but using **7C** (0.148 g, 0.23 mmol), 0.25 M ETT solution in MeCN (0.59 mL, 0.15 mmol), 2 - cyanoethyl - *N*, *N*, *N*′, *N*′ - tetraisopropylphosphordiamidite (0.1 mL, 0.30 mmol) and MeCN (1.3 mL). Purification by flash silica chromatography, eluting with EtOAc (with 0.5% NEt_3), afforded the title compound as an off-white amorphous solid. (0.117 g, 0.14 mmol, 60%). R_f (EtOAc) = 0.32; R_f (EtOAc–MeOH 98 : 2) = 0.43. ^{31}P NMR δ = 150.0 & 150.2. ^{1}H NMR δ = 1.03-1.16(24H, 4 × d, J = 6.8, ^{a}CHMe_2, ^{b}CHMe_2); 1.19(1H, m, ^{a}H2′); 1.70(1H, m, ^{b}H2′); 2.13(1H, m, ^{a}H2″); 2.38(1H, m, ^{b}H2″); 2.58(4H, m, ^{a}CH_2CN, ^{b}CH_2CN); 2.72(2H, m, ^{b}H3′, ^{a}H3′); 3.37(1H, m, ^{a}H5′/^{b}H5′); 3.50(4H, m, ^{a}C$H$$Me_2$, ^{b}C$H$$Me_2$); 3.63-3.81(19H, m, ^{a}OMe, ^{b}OMe, ^{a}H5′/^{b}H5′, ^{a}H5″, ^{b}H5″, ^{a}OCH_2, ^{b}OCH_2); 3.97(2H, m, ^{a}H4′, ^{b}H4′); 5.94(1H, dd, J = 6.6 & 2.8, ^{a}H1′); 6.01(1H, dd, J = 6.4 & 4.8, ^{b}H1′); 6.72(8H, m, m-^{a}Ar_2, m-^{b}Ar_2); 7.12(2H, m, p-^{a}Ph, p-^{b}Ph); 7.17-7.30(14H, o-^{a}Ar_2, o-^{b}Ar_2, m-^{a}Ph, m-^{b}Ph, ^{a}H5, ^{b}H5); 7.35(4H, m, o-^{a}Ph, o-^{b}Ph); 7.44(4H, m-^{a}Bz, m-^{b}Bz); 7.54(2H, m, p-^{a}Bz, p-^{b}Bz); 7.81 (4H, m, o-^{a}Bz, o-^{b}Bz); 8.04 & 8.30(2H, m, ^{a}H6, ^{b}H6); 8.49(2H, s, ^{a}NH, ^{b}NH). ES-HRMS; $C_{46}H_{51}N_5O_7PS$ requires 848.3247; $[M - H]^-$ = 848.3271 (2.8 ppm).

5-*O*-(4,4-Dimethoxytrityl)-3-*O*-(4*N*-benzoyl-4-aminopyrimidin-2-yl)-2-deoxy-α-D-*threo*-pentofuranosyl-1-*S*-thiobenzoate (11)

Thiobenzoic acid (1.14 mL, 9.74 mmol) was slowly added to a stirred solution of **10** (3 g, 4.87 mmol) in refluxing anhydrous pyridine (30 mL). After 10 min the sample was concentrated *in vacuo* and the residue taken up in EtOAc (100 mL) and washed with saturated $NaHCO_3$ (3 × 100 mL). The organic layer dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the impure product as a white foam. Purification by flash silica chromatography, eluting with 3 : 1 EtOAc–n-hexane, afforded the title compound as a white amorphous solid. (2.98 g, 3.95 mmol, 81%). R_f (EtOAc) = 0.66; R_f (EtOAc–n-hexane 1 : 1) = 0.44. ^{1}H NMR δ = 2.63(1H, m, H2′); 2.79(1H, ddd, $J_{H2''\text{-}H2'}$ = 14.8, $J_{H2''\text{-}H1'}$ = 7.3, $J_{H2''\text{-}H3'}$ = 2.1, H2″); 3.44 (1H, dd, $J_{H5'\text{-}H5''}$ = 9.4, $J_{H5'\text{-}H4'}$ = 6.9, H5′); 3.49(1H, dd, $J_{H5''\text{-}H5'}$ = 9.3, $J_{H5''\text{-}H4'}$ = 5.3, H5″); 3.70(6H, s, OMe); 4.42(1H, ddd, $J_{H4'\text{-}H5'}$ = 6.6, $J_{H4'\text{-}H5''}$ = 5.2, $J_{H4'\text{-}H3'}$ = 3.8, H4′); 5.92 (1H, ddd, $J_{H3'\text{-}H2'}$ = 5.6, $J_{H3'\text{-}H4'}$ = 3.7, $J_{H3'\text{-}H2''}$ = 2.0, H3′); 6.35(1H, m, H1′); 6.71(4H, m, m-Ar_2); 7.13(1H, m, p-Ph); 7.20(6H, m, m-Ph, o-Ar_2); 7.35(2H, m, o-Ph); 7.41-7.62(6H, m, m-NBz, m-SBz, p-NBz, p-SBz); 7.92(4H, m, o-NBz, o-SBz); 7.96(1H, d, $J_{H5\text{-}H6}$ = 5.7, H5); 8.39(1H, d, $J_{H6\text{-}H5}$ = 5.7, H6); 8.51(1H, s, NH). ES-HRMS; $C_{44}H_{39}N_3NaO_7S$ requires 776.2386; $[M + Na]^+$ = 776.2406 (2.6 ppm).

5-*O*-(4,4-dimethoxytrityl)-3-*O*-(4*N*-benzoyl-4-aminopyrimidin-2-yl)-2-deoxy-α-D-*threo*-pentofuranosyl-1-mercaptan (12)

N_2 was bubbled through a stirred solution of **11** (0.5 g, 0.66 mmol) in THF (25 mL), methanol (17 mL) and water (4 mL) at 0 °C. A degassed solution of 0.5 M NaOH (0.08 g, 4 mL, 1.99 mmol) was added and the mixture allowed to stir at 0 °C for 45 min. 1 M citric acid (2.09 g, 10 mL, 9.95 mmol) was added and reaction stirred for a further 10 min, after which saturated $NaHCO_3$ (75 mL) was added and the mixture was subsequently extracted with ethyl acetate (3 × 75 mL). The organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash silica chromatography, eluting with CH_2Cl_2 followed by a gradient of 0–2% MeOH in CH_2Cl_2, resulted in a white amorphous solid. (0.19 g, 0.285 mmol, 43%). R_f (EtOAc) = 0.66; R_f (EtOAc–n-hexane 1 : 1) = 0.44. ^{1}H NMR δ = 2.26(1H, m, H2′); 2.34(1H, d, $J_{SH1'\text{-}H1'}$ = 9.4, SH1′); 2.59(1H, m, H2″); 3.28(1H, dd, $J_{H5'\text{-}H5''}$ = 9.2, $J_{H5'\text{-}H4'}$ = 6.7, H5′); 3.37(1H, dd, $J_{H5''\text{-}H5'}$ = 9.2, $J_{H5''\text{-}H4'}$ = 5.9, H5″); 3.64(6H, s, OMe); 4.46(1H, m, H4′); 5.69(1H, m, H1′); 5.92(1H, m, H3′); 6.65(4H, m, m-Ar_2); 7.12-7.29(9H, m, p-Ph, m-Ph, o-Ph, o-Ar_2); 7.46(2H, m, m-Bz); 7.55(1H, m, p-Bz); 7.85(3H, m $J_{H5\text{-}H6}$ = 5.6, o-Bz, H5); 8.30(1H, d, $J_{H6\text{-}H5}$ = 5.6, H6); 8.38(1H, s, NH). ES-HRMS; $C_{37}H_{35}N_3NaO_6S$ requires 672.2144; $[M + Na]^+$ = 672.2139 (0.8 ppm).

General procedure for 3′-*S*-trityl-3′-thionucleosides (14)

A solution of trityl chloride (0.42 g, 1.5 mmol) in CH_2Cl_2 (2 mL) was added to a solution of the appropriate 3′-thionucleoside (**9**) (1.0 mmol) and triethylamine (0.28 mL, 2.0 mmol) in degassed CH_2Cl_2 (3 mL) at 0 °C. The mixture was stirred at 0 °C for 90 min. 1.25 M HCl–MeOH (5 mL) was added and the reaction quenched (after 15 min for pyrimidines or 5 min for deoxyadenosine) by dilution with CH_2Cl_2 (40 mL) and extraction with saturated $NaHCO_3$ (2 × 50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash silica chromatography, eluting with 3 : 1 EtOAc–n-hexane, resulted in a white amorphous solid.

3′-*S*-Trityl-3′-thiothymidine (14T). (0.28 g, 0.56 mmol, 56%). R_f (EtOAc–n-hexane 3 : 1) = 0.39. ^{1}H NMR δ = 1.67(1H, ddd, $J_{H2'\text{-}H2''}$ = 14.1, $J_{H2'\text{-}H3'}$ = 8.0, $J_{H2'\text{-}H1'}$ = 3.6, H2′); 1.80(3H, d, $J_{Me\text{-}H6}$ = 0.8, Me); 2.21(1H, ddd, $J_{H2''\text{-}H2'}$ = 14.1, $J_{H2''\text{-}H3'}$ = 9.0, $J_{H2''\text{-}H1'}$ = 7.4,

H2″); 2.83(1H, m, H3′); 3.61(1H, dd, $J_{H5'-H5''}$ = 13.0, $J_{H5'-H4'}$ = 3.2, H5′); 3.83(2H, m, H5″ & H4′); 6.00(1H, dd, $J_{H1'-H2''}$ = 7.3, $J_{H1'-H2'}$ = 3.5, H1′); 7.16-7.34(10H, m *o*-Ph, *p*-Ph, H6); 7.49(6H, m, *m*-Ph); 8.85(1H, s (br), NH). ES-HRMS; $C_{29}H_{28}N_2NaO_4S$ requires 523.1667; $[M + Na]^+$ = 523.1666 (0.3 ppm). Elemental analysis: Calculated for $C_{29}H_{28}N_2O_4S$: C, 69.58; H, 5.64; N, 5.60. Found: C, 69.36; H, 5.92; N, 5.48.

3′-*S*-Trityl-2′-deoxy-4*N*-benzoyl-3′-thiocytidine (14C). (0.31 g, 0.52 mmol, 52%). R_f (EtOAc–n-hexane 3 : 1) = 0.38. ^{1}H NMR δ = 1.86(1H, ddd, $J_{H2'-H2''}$ = 13.9, $J_{H2'-H3'}$ = 7.0, $J_{H2'-H1'}$ = 1.9, H2′); 2.45(1H, ddd, $J_{H2''-H2'}$ = 14.0, $J_{H2''-H3'}$ = 10.5, $J_{H2''-H1'}$ = 7.0, H2″); 2.70(1H, m, H3′); 3.70(1H, m, H5′); 3.88(2H, m, H5″, H4′); 6.00(1H, dd, $J_{H1'-H2''}$ = 6.9, $J_{H1'-H2'}$ = 1.8, H1′); 7.20-7.30(10H, m, *o*-Ph, *p*-Ph, H5); 7.45-7.54(9H, m, *m*-Ph, *m*-Bz, *p*-Bz); 7.88(2H, m, *o*-Bz); 8.22(1H, d, J_{H6-H5} = 7.4, H6); 8.66(1H, s (br), NH). ES-HRMS; $C_{35}H_{31}N_3NaO_4S$ requires 612.1933; $[M + Na]^+$ = 612.1918 (2.4 ppm).

3′-*S*-Trityl-2′-deoxy-6*N*-benzoyl-3′-thioadenosine (14A). (0.31 g, 0.51 mmol, 51%). R_f (EtOAc–n-hexane 3 : 1) = 0.27. ^{1}H NMR δ = 1.99(1H, m, H2′); 2.32(1H, m, H2″); 3.34(2H, m, H3′, H5′); 3.73(1H, m, H5″); 4.01(1H, m, H4′); 6.10(1H, m, H1′); 7.13-7.23(9H, m, *o*-Ph, *p*-Ph); 7.38-7.58(9H, m, *m*-Ph, *m*-Bz, *p*-Bz); 7.88(1H, s, H2); 7.94(2H, m, *o*-Bz); 8.57(1H, s, H8); 9.11(1H, s (br), NH). ES-HRMS; $C_{36}H_{31}N_5NaO_3S$ requires 636.2045; $[M + Na]^+$ = 636.2047 (0.3 ppm).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxy-6*N*-benzoyl-3′-thioadenosine (9A)

Performed as described for **12**, but using **8A**[23] (0.863 g, 1.11 mmol), THF (42 mL), methanol (28 mL), water (6.5 mL), 0.5 M NaOH (0.133 g, 6.7 mL, 3.33 mmol) and 1 M citric acid (3.5 g, 16.7 mL, 16.7 mmol). Flash silica chromatography was not required. (0.75 g, 1.11 mmol, >99%). R_f (EtOAc) = 0.39. ^{1}H NMR δ = 1.60(1H, d, *J* = 7.0, SH3′); 2.45(1H, ddd, $J_{H2'-H2''}$ = 13.8, $J_{H2'-H3'}$ = 10.6, $J_{H2'-H1'}$ = 7.1, H2′); 3.06(1H, ddd, $J_{H2''-H2'}$ = 13.4, $J_{H2''-H3'}$ = 7.0, $J_{H2''-H1'}$ = 2.2, H2″); 3.35(1H, dd, $J_{H5'-H5''}$ = 10.9, $J_{H5'-H4'}$ = 3.9, H5′); 3.50 (1H, dd, $J_{H5''-H5'}$ = 10.8, $J_{H5''-H4'}$ = 3.0, H5″); 3.70-3.83(7H, m, OMe, H3′); 3.97(1H, m, H4′); 6.34(1H, dd, $J_{H1'-H2'}$ = 7.3, $J_{H1'-H2''}$ = 2.3, H1′); 6.70-6.75(4H, m, *m*-Ar_2); 7.18-7.23(7H, m, *o*-Ar_2, *m*-Ph & *p*-Ph); 7.30-7.40(2H, m, *o*-Ph); 7.45-7.55(3H, m, *m*-Bz, *p*-Bz); 7.97(2H, m, *o*-Bz); 8.25(1H, s, H2); 8.75 (1H, s, H8); 8.95(1H, s (br), NH). ES-HRMS; $C_{38}H_{35}N_5O_5NaS$ required 696.2241; $[M + Na]^+$ found = 696.2257 (2.2 ppm).

3′-*S*-Trityl-3′-thiothymidine-5′-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (3T)

Performed as described for **2T**, but using **14T** (0.489 g, 0.98 mmol), 0.25 M ETT solution in MeCN (2.54 mL, 0.64 mmol), 2 - cyanoethyl - *N*, *N*, *N*′, *N*′ - tetraisopropylphosphordiamidite (0.4 mL, 1.27 mmol) and MeCN (3.6 mL). Purification by flash silica chromatography, eluting with 2 : 1 EtOAc–n-hexane (with 0.5% NEt_3), afforded the title compound as a white amorphous solid. (0.382 g, 0.54 mmol, 56%). R_f (EtOAc–n-hexane 3 : 1) = 0.49. ^{31}P NMR δ = 149.3 &149.0. ^{1}H NMR δ = 0.98-1.11(24H, 4 × d, *J* = 6.7 (×3) & 6.8, ^{a}CHMe_2, ^{b}CHMe_2); 1.21(1H, ddd, $J_{H2'-H2''}$ = 14.0, *J* = 8.4 & 5.2, ^{a}H2′); 1.59(1H, m, ^{b}H2′); 1.79 & 1.80(6H, 2 × d, J_{Me-H6} = 1.0 & 0.9, ^{a}Me, ^{b}Me); 1.90(1H, m, ^{a}H2″); 2.04(1H, ddd, $J_{H2''-H2'}$ = 14.0, *J* = 6.0 & 5.0, ^{b}H2″); 2.41 & 2.50(4H, 2 × t, *J* = 6.3 & 6.4, ^{a}CH_2CN, ^{b}CH_2CN); 2.79(1H, m, ^{b}H3′); 2.91(1H, m, ^{a}H3′); 3.28(1H, m, ^{a}H5′/^{b}H5′); 3.46(4H, m, ^{a}C*H*Me_2, ^{b}C*H*Me_2); 3.56-3.76(7H, m, ^{a}H5′/^{b}H5′, ^{a}H5″, ^{b}H5″, ^{a}OCH_2, ^{b}OCH_2); 3.92(2H, m, ^{a}H4′, ^{b}H4′); 6.01(2H, m, ^{a}H1′, ^{b}H1′); 7.09-7.25(20H, m, *o*-^{a}Ph, *o*-^{b}Ph, *p*-^{a}Ph, *p*-^{b}Ph, ^{a}H6, ^{b}H6); 7.40(12H, *m*-^{a}Ph, *m*-^{b}Ph); 8.73(2H, s, ^{a}NH, ^{b}NH). ES-HRMS; $C_{38}H_{45}N_4NaO_5PS$ requires 723.2746; $[M+Na]^+$ = 723.2746 (1.0 ppm). Elemental analysis: Calculated for $C_{38}H_{45}N_4O_5PS$: C, 65.12; H, 6.47; N, 7.99. Found: C, 64.93; H, 6.54; N, 8.01.

3′-*S*-Trityl-2′-deoxy-4*N*-benzoyl-3′thiocytidine-5′-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (3C)

Performed as described for **2T**, but using **14C** (0.256 g, 0.43 mmol), 0.25 M ETT solution in MeCN (1.1 mL, 0.28 mmol), 2 - cyanoethyl - *N*, *N*, *N*′, *N*′ - tetraisopropylphosphordiamidite (0.18 mL, 0.56 mmol) and CH_2Cl_2 (1.7 mL) instead of acetonitrile. Purification by flash silica chromatography, eluting with 3 : 2 EtOAc–n-hexane (with 0.5% NEt_3), afforded the title compound as a white amorphous solid. (0.214 g, 0.23 mmol, 62%). R_f (EtOAc–n-hexane 3 : 1) = 0.52. ^{31}P NMR δ = 149.4 & 149.1. ^{1}H NMR δ = 1.02-1.24(25H, 4 × d, *J* = 6.8, ^{a}CHMe_2,^{b}CHMe_2, ^{a}H2′); 1.59(1H, m, ^{b}H2′); 2.05(1H, ddd, $J_{H2''-H2'}$ = 14.3, $J_{H2''-H3'}$ = 9.2, $J_{H2''-H1'}$ = 6.9, ^{a}H2″); 2.34(1H, m, ^{b}H2″); 2.58(4H, m, ^{a}CH_2CN, ^{b}CH_2CN); 2.75(2H, m, ^{b}H3′, ^{a}H3′); 3.36(1H, m, ^{a}H5′/^{b}H5′); 3.50(4H, m, ^{a}C*H*Me_2, ^{b}C*H*Me_2); 3.63-3.85(7H, m, ^{a}H5′/^{b}H5′, ^{a}H5″, ^{b}H5″, ^{a}OCH_2, ^{b}OCH_2); 3.96(2H, m, ^{a}H4′, ^{b}H4′); 5.91(1H, dd, $J_{H1'-H2''}$ = 6.7, $J_{H1'-H2'}$ = 2.7, ^{a}H1′); 6.00(1H, dd, *J* = 6.1 & 4.8, ^{b}H1′); 7.18(20H, m, *o*-^{a}Ph, *o*-^{b}Ph, *p*-^{a}Ph, *p*-^{b}Ph, ^{a}H5, ^{b}H5); 7.36-7.47(16H, m, *m*-^{a}Ph, *m*-^{b}Ph, *m*-^{a}Bz, *m*-^{b}Bz); 7.53(2H, *p*-^{a}Bz, *p*-^{b}Bz); 7.81(4H, m, *o*-^{a}Bz, *o*-^{b}Bz); 8.01 & 8.26(2H, 2 × d, J_{H6-H5} = 7.4 (×2), ^{a}H6, ^{b}H6); 8.51(2H, s, ^{a}NH, ^{b}NH). ES-HRMS; $C_{44}H_{48}N_5O_5NaPS$ requires 812.3012; $[M + Na]^+$ = 812.3051 (4.9 ppm).

3′-*S*-Trityl-2′,3′-dideoxy-6*N*-benzoyl-3′thioadenosine-5′-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (3A)

Performed as described for **2T**, but using **14A** (0.285 g, 0.47 mmol), 0.25 M ETT solution in MeCN (1.2 mL, 0.30 mmol), 2 - cyanoethyl - *N*, *N*, *N*′, *N*′ - tetraisopropylphosphordiamidite (0.19 mL, 0.60 mmol) and CH_2Cl_2 (1.7 mL) instead of acetonitrile. (0.22 g, 0.27 mmol, 58%). R_f (EtOAc–n-hexane 3 : 1) = 0.52. ^{31}P NMR δ = 148.7 & 149.0. ^{1}H NMR δ = 0.98-1.10(24H, 4 × d, *J* = 6.8 (×2), 6.7 (×2), ^{a}CHMe_2, ^{b}CHMe_2); 1.41(1H, ddd, $J_{H2'-H2''}$ = 14.1, $J_{H2'-H3'}$ = 7.2, $J_{H2'-H1'}$ = 2.3, ^{a}H2′); 1.86(1H, ddd, $J_{H2'-H2''}$ = 13.7, $J_{H2'-H3'}$ = 7.3, $J_{H2'-H1'}$ = 4.1, ^{b}H2′); 2.03(1H, ddd, $J_{H2''-H2'}$ = 14.1, $J_{H2''-H3'}$ = 9.6, $J_{H2''-H1'}$ = 7.0, ^{a}H2″); 2.17(1H, m, ^{b}H2″); 2.54(4H, m, ^{a}CH_2CN, ^{b}CH_2CN); 3.20(2H, m, ^{b}H3′, ^{a}H3′); 3.34(2H, m, ^{a}C*H*Me_2/^{b}C*H*Me_2); 3.47(3H, m, ^{a}C*H*Me_2/^{b}C*H*Me_2, ^{a}H5′/^{b}H5′); 3.60-3.84(7H, m, ^{a}H5′/^{b}H5′, ^{a}H5″, ^{b}H5″, ^{a}OCH_2, ^{b}OCH_2); 4.05(2H, m, ^{a}H4′, ^{b}H4′); 6.14(1H, dd, $J_{H1'-H2''}$ = 6.8, $J_{H1'-H2'}$ = 2.5, ^{a}H1′); 6.20(1H, dd, $J_{H1'-H2''}$ = 6.8, $J_{H1'-H2'}$ = 3.9, ^{b}H1′); 7.10-7.22(18H, m, *o*-^{a}Ph, *o*-^{b}Ph, *p*-^{a}Ph, *p*-^{b}Ph); 7.41-7.54(18H, m, *m*-^{a}Ph, *m*-^{b}Ph, *m*-^{a}Bz, *m*-^{b}Bz, *p*-^{a}Bz, *p*-^{b}Bz); 7.93(4H, m, *o*-^{a}Bz, *o*-^{b}Bz); 7.99 & 8.05(2H, 2 × s, ^{a}H2, ^{b}H2); 8.63 & 8.93(2H, 2 × s, ^{a}H8, ^{b}H8); 8.86 & 8.93(2H, 2 × s, ^{a}NH, ^{b}NH). ES-HRMS; $C_{45}H_{48}N_7O_4NaPS$ requires 836.3124; $[M + Na]^+$ = 836.3160 (4.3 ppm).

Synthesis and characterisation of oligonucleotides

All DNA synthesis reagents, including DNA synthesis grade acetonitrile, were supplied from either Link Technologies or Applied Biosystems. **ON1** was prepared on an Expedite™ 8909 DNA synthesizer, which was equipped with standard Expedite™ Workstation Software and utilizing 1*H*-tetrazole (0.4M) as the activator. All other sequences were prepared on a MerMade 4 (MM4) synthesiser (BioAutomation, Plano, Texas), utilizing 5-ethylthio-1*H*-tetrazole (0.25M) as the activator. Standard Expedite™ or MerMade synthesis reagents were used as purchased. All oligonucleotides were prepared on 1.0 μmol controlled-pore glass (CPG) columns and in all cases, the final 3′-DMT or 3′-trityl group was left on the oligonucleotide strands at the end of the synthesis (trityl-on).

5′-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite-3′-*O*-(4,4′-DMT)-nucleosides were supplied from either Link Technologies or Proligo and diluted with acetonitrile, and used at the recommended concentration of 0.1 M. The 3′-*S*-protected 5′-*O*-phosphoramidites (**2** and **3**) were dissolved in acetonitrile (0.15 M) and dithiothreitol [DTT, 0.1 M in THF–water (9 : 1)] was used as the reductant. For the coupling of all 5′-*O*-phosphoramidites with support-bound 3′-hydroxyl moieties, a 180 s coupling time was implemented. For the coupling of 5′-*O*-phosphoramidites with support-bound 3′-sulfhydryl groups, a coupling time of 8 min was used.

Detritylation, capping and oxidation conditions on the Expedite 8909 synthesiser were as follows: a 135 s 3′-*O*-DMT detritylation using 3% trichloroacetic acid (TCA), a 465 s 3′-*S*-DMT detritylation using 3% trichloroacetic acid (TCA) followed by a reduction step with DTT (10 min) and a thorough wash with dichloromethane and acetonitrile (both 60 s). When compared to standard 3′→5′ DNA synthesis, slightly prolonged capping (15 s), oxidation (15 s) and second capping (5 s) conditions were used.

Detritylation, capping and oxidation conditions on the MM4 synthesiser were as follows: a 180 s 3′-*O*-DMT detritylation using TCA and standard MM4 oxidation and capping conditions were utilised.

The oligonucleotides were then cleaved from the solid-support, purified by reverse-phase HPLC.[14]

Oligonucleotides containing terminal 3′-*S*-trityl or 3′-*S*-DMT moieties were detritylated using the following procedure (adapted from a previously reported method[25]). An oligonucleotide solution (200μL, ~20 OD), treated with silver nitrate (4μL, 1M, final concentration ~20 mM) and incubated at 30 °C for an hour. Dithiothreitol (DTT, 8μL, 1M, final concentration ~40 mM) was added and the resultant yellow precipitate vortexed. After centrifugation (3 min, 9000 rpm), the supernatant was washed with ethyl acetate (5 × 250μL), to remove excess DTT.[25] The resultant solution was analysed by HPLC and or ES-HRMS.

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