Thiophosphate and thiophosphonate analogues of glucose-1-phosphate: synthesis and enzymatic activity with a thymidylyltransferase

Matthew W. Loranger, Stephen A. Beaton, Katie L. Lines, David L. Jakeman

PII: S0008-6215(13)00240-1
DOI: http://dx.doi.org/10.1016/j.carres.2013.06.007
Reference: CAR 6503

To appear in: *Carbohydrate Research*

Received Date: 6 May 2013
Revised Date: 10 June 2013
Accepted Date: 11 June 2013

Please cite this article as: Loranger, M.W., Beaton, S.A., Lines, K.L., Jakeman, D.L., Thiophosphate and thiophosphonate analogues of glucose-1-phosphate: synthesis and enzymatic activity with a thymidylyltransferase, *Carbohydrate Research* (2013), doi: http://dx.doi.org/10.1016/j.carres.2013.06.007

Thiophosphate and thiophosphonate analogues of glucose-1-phosphate: synthesis and enzymatic activity with a thymidylyltransferase

Matthew W. Loranger,[a] Stephen A. Beaton,[a] Katie L. Lines[a] and David L. Jakeman*[ab]

[a]Department of Chemistry, Dalhousie University, 6274 Coberg Rd., PO Box 15,000, Halifax, Nova Scotia, B3H 4R2, Canada; [b]College of Pharmacy, Dalhousie University, 5968 College St., PO Box 15,000, Halifax, Nova Scotia, B3H, 4R2. Email: david.jakeman@dal.ca;

Abstract

Synthetic methods were investigated for the preparation of *O* and *S*-glucosyl thiophosphates and glucosyl 1C-thiophosphonate. Four protected glucosyl thiophosphate compounds were synthesized and characterized as precursors to glucose 1-thiophosphate. The effect of various reaction conditions and the nature of the carbohydrate and thiophosphate protecting groups and how they impact both the yields and α/β diastereoselectivity of the glucosyl thiophosphate products were explored. A novel isomerization from an *O*-linked to *S*-linked glucosyl thiophosphate was observed. α-D-Glucose-1C-thiophosphonate was synthesized and evaluated as a substrate for the thymidylyltransferase, Cps2L. Tandem mass spectrometric analysis determined the position of sulfur in the sugar nucleotide product.

1. Introduction

Glycosyl phosphates are natural phosphates of importance in biological systems as key metabolic intermediates and as bacterial cell wall constituents.[1,2] Thiophosphates are analogues of natural phosphates in which the P—O bond has been replaced with a phosphorus-sulfur (P—S) bond. The sulfur atom within thiophosphates is capable of mimicking both the binding and nucleophilic properties of the oxygen atom of natural phosphates.[3,4] Many classes of enzymes utilize phosphate or pyrophosphate as substrates or recognition elements, and thiophosphates have provided significant insight into interactions of these enzymes, including determining the stereochemical outcome of phosphoryl transfer at phosphorus.[5] Despite the extensive literature associated with enzymes transferring phosphates, aspects of the mechanism of enzymatic

phosphoryl transfer remain contentious, with metal fluoride complexes providing new insights for those enzymes forming covalent phosphoryl-enzyme intermediates.[6] Phosphonothioates are non-hydrolysable derivatives of phosphates with the structure $RPS(OH)_2$ and have been used to probe receptor signaling. Prestwich and coworkers reported the synthesis and evaluation of several phosphonothioate analogues as antagonists of lysophosphatic acid (LPA) receptors.[7] Sugar phosphorylases are a class of enzyme that use glycosyl phosphates as substrates to furnish oligosaccharides. These enzymes offer complementary approaches to that of chemical synthesis in the preparation of oligosaccharides due to the regio- and stereoselectivity of the glycosidic linkage formed.[8]

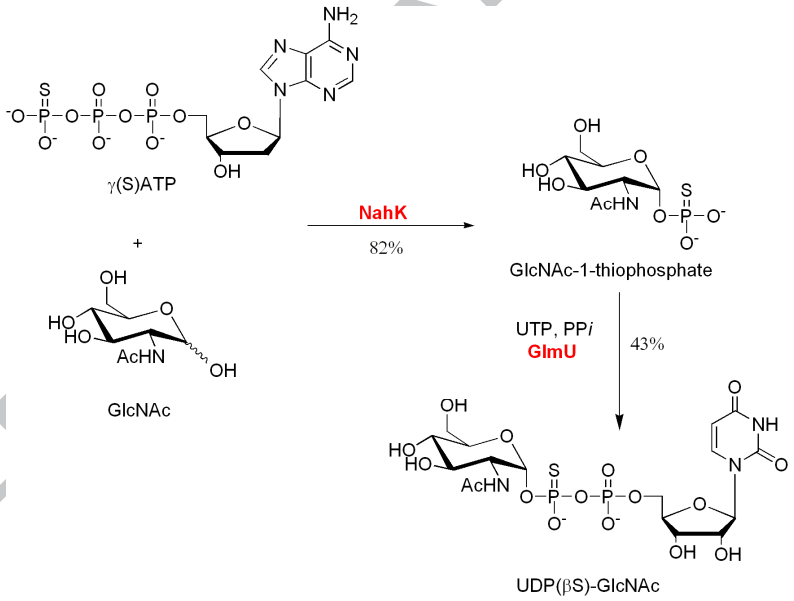
Glycosyl phosphates including β- and α-GlcNAc-1-phosphate, β- and α-glucose 1-phosphate, have various roles in biological pathways, making their thiophosphate or thiophosphonate analogues attractive synthetic targets with which to probe enzyme function. β-D-Glucose 1-phosphate is a substrate for β-phosphoglucomutase and is suspected to be a precursor in cell wall biosynthesis in *Lactococcus lactis*.[9,10] α-D-Glucose 1-phosphate is a substrate for nucleotidylyltransferase enzymes in a number of biosynthetic pathways, including the incorporation of L-rhamnose into the cell wall of harmful pathogens including *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*.[11,12,13]

Glycosyl thiophosphates have been synthesized in both *O* and *S*-glycosylated forms. *O*-glycosyl thiophosphates have been previously synthesized as glycosyl donors for the stereoselective production of glycosides. Hui and coworkers reported the synthesis of acetyl and benzyl-protected glycosyl dimethylthiophosphates in 51% and 86% yields, respectively.[14] They generated several diastereotopic mixtures of fully protected glycosyl thiophosphates with varying α/β selectivity, depending on the nature of the glycosyl protecting groups. More recently, Piekutowska and Pakulski generated a series of benzoyl protected mannose, galactose and glucose *S*-glycosyl thiophosphates by reacting anomeric thiocyanates with *O*-alkyl or *O*-trimethylsilyl phosphites.[15] However, the global deprotection of these glycosyl thiophosphates was not reported.

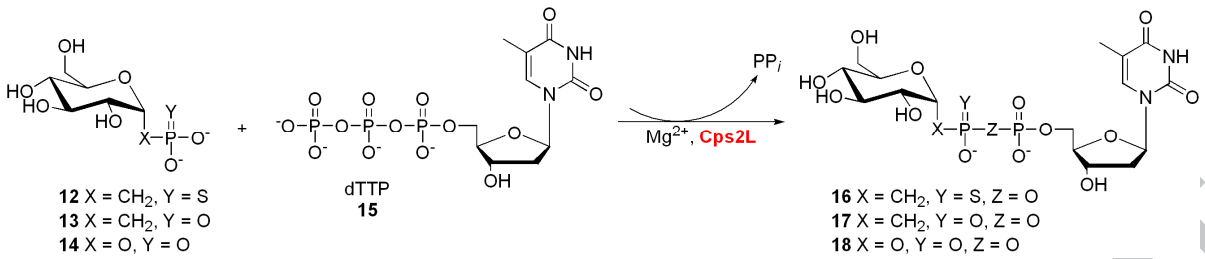
The only example of a free sugar glycosyl thiophosphate was reported by Wang and coworkers in 2011 using enzymatic approaches.[16] Using NahK (EC 2.7.1.162), an *N*-acetylhexosamine 1-kinase, they were able to produce GlcNAc-1-thiophosphate on a milligram scale from GlcNAc and commercially available γ(S)ATP. The GlcNAc-1-P uridyltransferase

GlmU (EC 2.3.1.157) was then utilized to produce UDP(βS)-GlcNAc in a respectable yield (Scheme 1).

No synthetic route currently exists to access fully deprotected *O*-glycosyl and *S*-glycosyl sugar 1-thiophosphates or glucosyl 1C-thiophosphonate. We report the synthesis of a series of differentially protected *O*-glucosyl and *S*-glucosyl thiophosphates. We looked at the influence of reaction conditions and protecting groups on the diastereotopic ratio of products and explored common deprotection strategies towards the production of *O*- and *S*- glucose 1-thiophosphate. The thiophosphonate analogue, Glucose 1C-thiophosphonate, was synthesized and enzymatically transformed into a sugar nucleotide analogue of deoxythymidine diphosphate-α-D-glucose (dTDP-Glc) using thymidylyltransferase Cps2L.[17] This compound represents the first example of a thiophosphonate containing sugar nucleotide analogue.



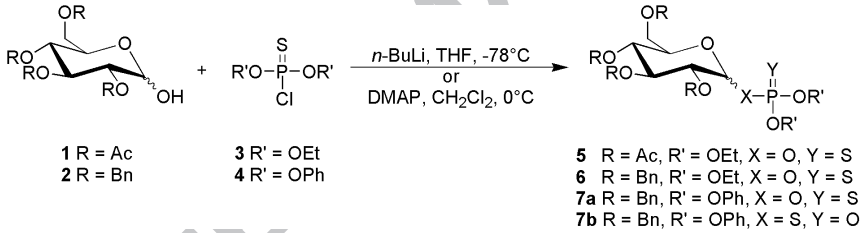
Scheme 1 Enzymatic synthesis of GlcNAc-1TP and UDP(βS)-GlcNAc[16]



Scheme 2 Reactions catalyzed by Cps2L.

2. Results and Discussion

The formation of glucosylated thiophosphates was explored using acetyl (**1**) or benzyl (**2**) protected glucopyranose as the starting sugar together with diethylchlorothiophosphate (**3**) using the conditions described by Hui[14] or diphenylchlorothiophosphate (**4**) using the conditions described[18] for preparation of glycosyl phosphates (Scheme 3). Table 1 summarizes the various reaction conditions and isolated yields for α/β-D-glucopyranosyl thiophosphate synthesis.



Scheme 3 The synthesis of protected α/β-D-glucopyranosyl thiophosphates (**5**–**7**)

Table 1 Reaction conditions for α/β-D-glucopyranosyl thiophosphate synthesis

Entry	Reactants	Conditions	T (°C)	Product(s)	α/β	Yield[c]
1	**1 + 3**	THF/*n*-BuLi	-78	**5**	1:3	57%
2	**2 + 3**	THF/*n*-BuLi	-78	**6**	2:1	67%
3	**2 + 3**	CH_2Cl_2/DMAP	0	N/A	N/A	0%
4	**2 + 4**	CH_2Cl_2/DMAP	0	**7a/7b**	1[a], 4:1[b]	24%
5	**2 + 4**	THF/*n*-BuLi	-78	**7a/7b**	6:1[a], 4:1[b]	16%

[a]Initial ratio for *O*-linked product **7a**. [b]Final ratio for *S*-linked product **7b**. [c]Yields are reported as overall isolated yields for all anomers and isomers.

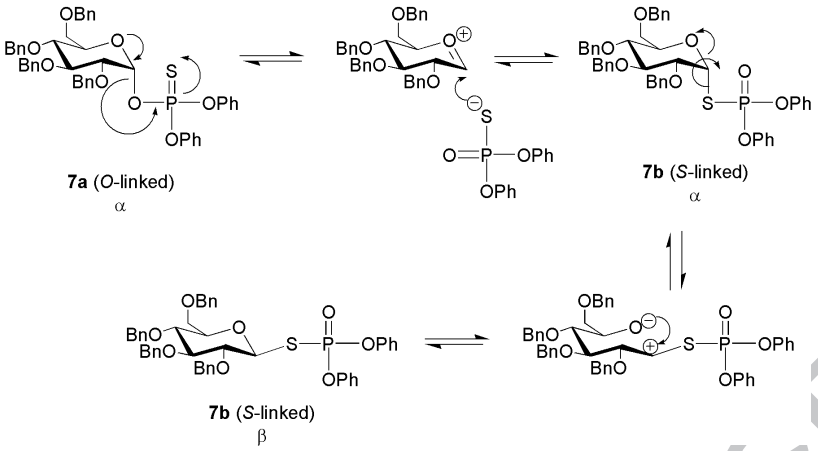
The reaction solvent and base were investigated in an attempt to enhance yield and selectivity. When diethylchlorothiophosphate (**3**) was used as the electrophile (entries 1-3), both *n*-

butyllithium (*n*-BuLi) and lithium diisopropylamide (LDA) were comparably effective bases in tetrahydrofuran (THF), however 4-dimethylaminopyridine (DMAP) in dichloromethane (CH_2Cl_2) was ineffective in promoting glycosyl coupling. The yields for **5** and **6** (entry 1-2) were comparable will analogous methyl protected glycosyl thiophosphates reported by Hui (51-86%).[14] For ease of preparation, *n*-BuLi was chosen as the base of choice for the production of **5** and **6**. When diphenyl chlorothiophosphate (**4**) was used as the electrophile (entries 4-5), either *n*-BuLi/THF or DMAP/CH_2Cl_2 conditions proved to be sufficient for the production of **7a**. The complete structures of **7a** and **7b** can be found in Scheme 4. The reactions conditions used affected both the diastereotopic ratio of α/β products as well as the overall yield. When *n*-BuLi /THF conditions were used the resultant α/β ratio of **7a** was found to be ~6:1 with a 16% overall yield (entry 5). When DMAP/CH_2Cl_2 conditions were used, only the α isomer was obtained with a 24% overall yield (entry 4). The yields of **7a/7b** (entry 4) were significantly less than analogous glycosyl phosphates synthesized using DMAP/CH_2Cl_2 conditions reported previously (55-82%).[18] While reduced coupling efficiency can be partially attributed to the steric bulk of the phenyl protecting groups of **4** relative to the ethyl protected **3**, the driving factor in the reduced yields is believed to be the excellent leaving group capacity of the diphenylthiphosphate moiety, leading to decomposition during purification. The DMAP/CH_2Cl_2 method was preferred in the synthesis of **7a** due to its stereospecificity and improved yield relative to the *n*-BuLi/THF coupling method.

The diastereotopic ratios of the starting sugars **1** (β only) and **2** (α/β ~3:1) were not retained in the production of any of the glucosyl thiophosphates (**5**–**7**). Varying temperature conditions from -78°C to -40°C did not affect the diastereotopic ratio of products for compounds **5** and **6**. Using the benzyl-protected sugar (**2**) moderate improvement in overall yield was observed relative to the acetyl-protected sugar (**1**), which can be attributed to the acetyl groups of **1** deactivating the anomeric hydroxyl group's ability to act as a nucleophile in the coupling reaction with the chlorothiophosphate. Reactions involving the chlorothiophosphate **3** produced an α/β ratio of 1:3 (entry 1) with respect to coupling with the acetyl-protected sugar (**1**) and an α/β ratio of 2:1 (entry 2) with respect to coupling with the benzyl-protected sugar (**2**). In the absence of neighboring group participation, the anomeric effect drives the ratio of axial α products for the benzyl-protected sugar and accounts for the formation of the dominant α product in **6**. The C2 acetate group of **1** effectively increases the proportion of the equatorial (β) hydroxyl

group in the free sugar by way of blocking the axial site via neighboring group participation, which consequently drives the reaction to the preferential β form in **5**. Complete α selectivity was achieved with the coupling of **4** and benzyl-protected sugar (**2**) using the DMAP/CH_2Cl_2 conditions (entry 4), although the yields were significantly reduced relative to the *n*-BuLi/THF coupling conditions with **4** (entry 2). The enhanced selectivity can be attributed to the phenyl protecting groups on **4**, which make the thiophosphate moiety more electron rich, driving it to the α position via the anomeric effect.

The *S*-linked isomer **7b** was isolated in an 8% yield following purification relative to the *O*-linked **7a** (16%) using the preferred DMAP/CH_2Cl_2 coupling conditions (combined yield = 24%) (entry 4). For the purification of **7a** and **7b**, triethylamine neutralization of the silica using a 95:5 hexane:triethylamine slurry was required. In the absence of triethylamine, the material was found to break down completely during purification and only the hydrolysis products were recovered. The *O*-linked species was found to breakdown in the presence of $CDCl_3$ to the free benzyl sugar and phenyl thiophosphoric acid within 24 hours, as was observed using TLC analyses, however, the compounds were found to be more stable in CD_2Cl_2. In CD_2Cl_2 an interesting isomerization (Scheme 4) with sugar-thiophosphate **7a** was observed, in which the exclusively α **7a** compound gradually interconverted to the *S*-linked species **7b** with a resulting α/β ratio of 4:1 within 48 hours, after isolation. This transformation was observed by the large up-field shift in the ^{31}P NMR spectrum when the phosphorous-sulfur bond shifts from a double bond, as in the case of the *O*-linked species, to a single bond as in the case of the *S*-linked species. The ^{31}P NMR spectra overlay of the various forms of the benzyl glucosyl thiophosphates (**6**–**7**) is shown in Figure 1. Compound **7b** was confirmed to be the *S*-linked isomer by ^{1}H NMR spectroscopy, which showed the H-P coupling of the α *O*-linked species ($^3J_{1,\,P}$ = 9.5 Hz, 6.20 ppm) had changed and shifted slightly up field ($^3J_{1,\,P}$ = 11.1 Hz, 6.30 ppm). An overlay of the H1 splitting patterns for compounds (**6**, **7a** and **7b**) is shown in Figure 2. The *S*-glycosyl thiophosphate appeared to be the more stable isomeric form and was not as vulnerable to glycosidic cleavage by $CDCl_3$ relative to its *O*-glycosyl counterpart.



Scheme 4 Proposed isomerization of glucopyranosyl-thiophosphate (**7a**)

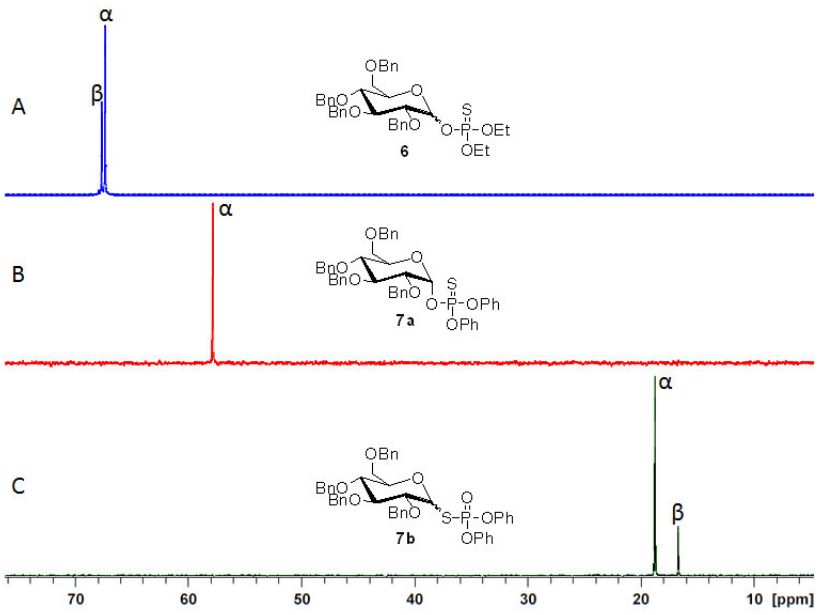


Figure 1 ^{31}P NMR spectra for glucosyl thiophosphates **6** (A) in $CDCl_3$, **7a** (B) and **7b** (C) in CD_2Cl_2.

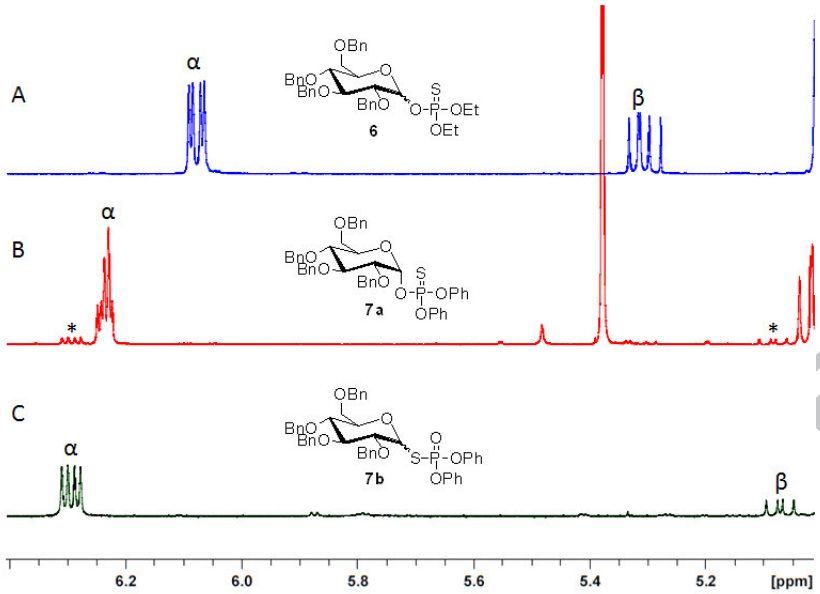


Figure 2 ^{1}H NMR spectra for glycosyl thiophosphates **6** (A) in $CDCl_3$; **7a** (B) and **7b** (C) in CD_2Cl_2 displaying α and β splitting patterns. The (*) indicates the start of isomerization from **7a** to **7b** ~6 hours after isolation.

The significantly higher yields of the diethylthiophosphates relative to the diphenyl thiophosphate prompted an attempt at separating the α and β diastereomers of **6**. Many possible separation conditions were explored using various elutants (EtOAc/hexane, Water/MeOH), as well as normal phase silica, silver impregnated silica, reversed-phased C18 silica, alumina and basic alumina media conditions. Under all circumstances the product was either recovered as a diastereotopic mixture of anomers or hydrolyzed during the separation.

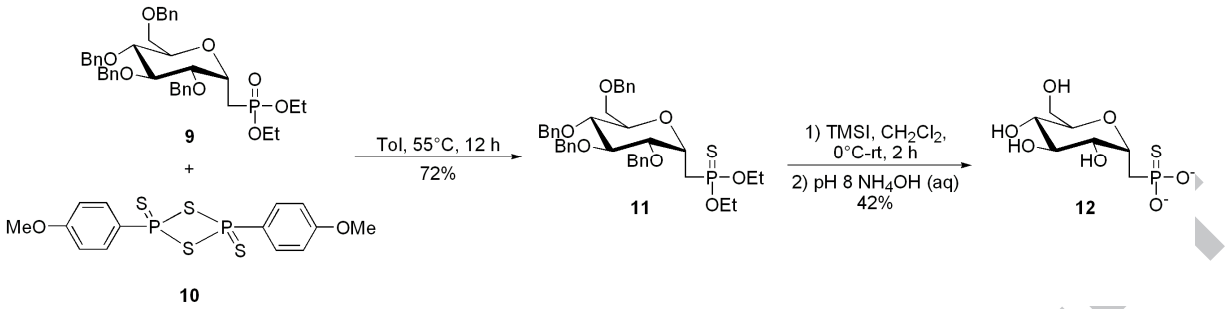
Despite unsuccessful separation attempts, the α/β mixtures of glycosyl thiophosphates may not be of crucial concern. Given the stereospecificity of nucleotidylyltransferases and glycosyltransferases, they can potentially select and drive the α/β equilibrium toward the preferred form (α or β) of the glycosyl thiophosphate. After attempted separation of the anomers, deprotection of the glycosyl thiophosphates was explored. The attempted literature deprotection conditions employed with benzyl sugar product are summarized in Table 2. Basic LiOH[19] conditions were found to be unsuccessful and the acidic conditions of TMSI,[20] TMSBr[21] and BCl_3[22] were all found to be too harsh, resulting in anomeric cleavage of the product. Hydrogenolysis of the products was explored next in the hope that it would remove the benzyl protecting groups on the sugar moiety and/or the phenyl protecting groups on the thiophosphate

moiety. While compounds containing sulfur have been well known to poison hydrogenation catalysts,[23,24] the incorporation of ammonium formate into the reaction media has been shown to circumvent this problem in certain circumstances involving sulfate hydrogenolysis.[25] In our hands none of these hydrogenolysis conditions enabled deprotection of the glucosyl thiophosphates.

Table 2 Attempted deprotection conditions for α/β-D-glucopyranosyl diethylthiophosphates

Compound	Deprotection Conditions	Result
6	LiOH(sat), dioxane/water, 50°C	No reaction
6	TMSI, CH_2Cl_2, 0°C	Anomeric cleavage
6	TMSBr, CH_2Cl_2, 0°C	Anomeric cleavage
7a, 7b	BCl_3, CH_2Cl_2, -78°C	Anomeric cleavage
7a, 7b	H_2 Pd/C, MeOH/EtOAc	No reaction
7a, 7b	H_2 PtO_2, MeOH/EtOAc	No reaction
7a, 7b	H_2 Pd/C or H_2 PtO_2, NH_4CO_2 (1-15 eq), MeOH/EtOAc	No reaction

We next turned our synthetic efforts towards the preparation of a non-scissile, isosteric analogue of α-D-glucose 1-phosphate containing sulfur. The synthesis of glucosyl thiophosphonate (**12**) was accomplished from globally protected glucosyl phosphonate (**11**), which was prepared as described previously.[26] Lawesson's Reagent (**10**) was used in the transformation of **9** to **11**. The use of **10** for the replacement of the oxo group of phosphorous (P=O) with the thio (P=S) group is relatively common.[27] While many reports require refluxing toluene conditions to affect transformation (P=O to P=S),[27,28] the optimal conditions were found to be heating at 55°C for 12 hours, with dry toluene. Compound **11** was isolated in 72% yield after purification (Scheme 5). The robust C—P thiophosphonate anomeric linkage of **11**, compared to the protected glucosyl thiophosphates O—P linkage of **5**-**7**, facilitated easy global deprotection. This was accomplished using TMSI, followed by purification via cellulose chromatography to afford **12** in a 42% yield.



Scheme 5 Synthesis of α-D-glucose 1C-thiophosphonate

α-D-glucose 1C-thiphosphonate (**12**) was evaluated as a substrate for thymidylyltransferase Cps2L, and compared to α-D-glucose 1-phosphate (**14**) and its phosphonate analogue α-D-glucose 1C-phosphonate (**13**) (Scheme 2). Compounds **12-14** were coupled with deoxythymidine triphosphate (**15**, dTTP) to produce sugar nucleotides **16-18**. Conversions were monitored at 30 minutes and 24 hours by HPLC analysis (Table 3). At varying concentrations of Cps2L (2 EU, 7 EU, 12 EU), the maximum conversion of glucosyl thiophosphonate **12** to sugar nucleotide **16** was 15% (entry 1). The HPLC analysis did not differentiate between whether oxygen or sulfur had acted as the nucleophile, giving rise to two potential products (X, Y = O, Z = S) or (X, Z = O, Y = S). Analysis of the ESI-MS/MS fragmentation pattern for the $[M-H]^-$ product peak injected from an aliquot of the enzyme assay revealed that oxygen operated as the nucleophile, rather than the sulfur atom, confirming the structure of the sugar nucleotide analogue as compound **16** (Figure 3). If the sulfur atom were acting as the nucleophile, an $[NMP]^-$ fragment of 377, as opposed to 321, would have been observed. It is conceivable that the β-thiophosphonate in **16** may exist as two diastereomers at the β-phosphorus. Our HPLC and mass spectrometric analyses were unable to differentiate between these two forms, however, the observation of only one peak by HPLC suggests tentatively that the compound exists as only one diastereomer at the βP position. The GlmU catalyzed synthesis of UDP(βS)-GlcNAc did not report whether two diastereomers were formed, nevertheless, inspection of their ^{31}P NMR suggests a single diastereomeric product.[16] If this discovery is corroborated, it demonstrates that both GlmU and Cps2L, and potentially this class of enzyme, are able to distinguish between the *pro*-R and *pro*-S thiophosphonate oxygen atoms. We have recently demonstrated that Cps2L has approximately a 10-fold difference in reactivity between the monofluoromethylene analogues of glucose ketosephosphonates,[17] demonstrating that the Cps2L active site is able to discriminate between isosteric non-physiological substrates

possessing chirality where no chirality is present in the physiological substrate. Glucosyl phosphonate **13** showed high turnover to its sugar nucleotide product **17**, as has been previously reported (entry 2),[26] and was a more active substrate than compound **12**.

Table 3 Comparison of conversions to sugar nucleotides for α-D-glucose 1-phosphate analogues

Entry	Compound	Product	Conversion 30 min	Conversion 24 h
1	**12**	**16**	2%	15%
2	**13**	**17**	95%	96%
3	**14**	**18**	95%	100%

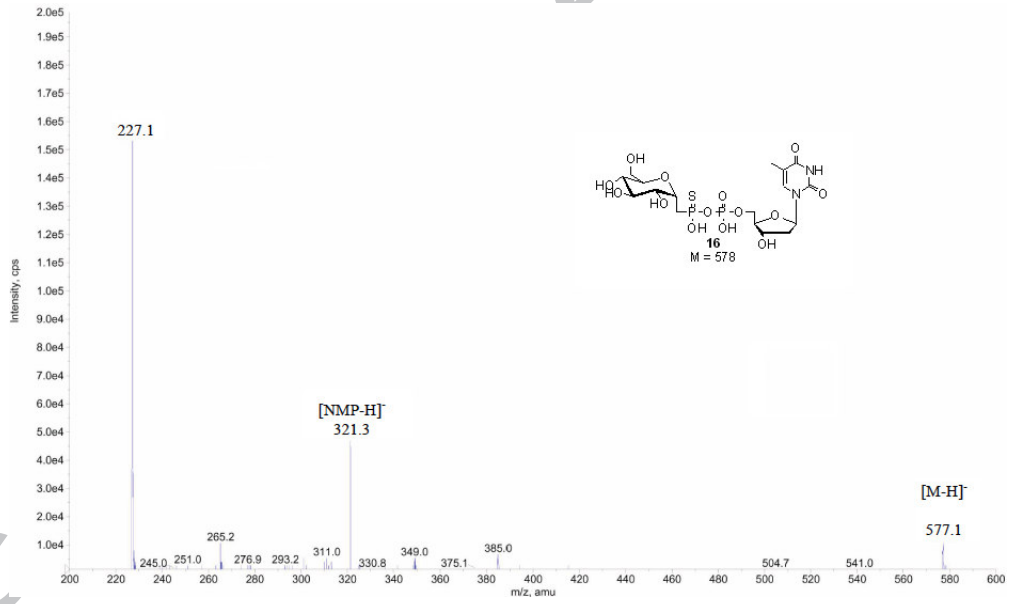


Figure 3 Tandem mass spectrometry (ESI-MS/MS EPI) scan of Cps2L product **16**.

3. Conclusion

Several methods for the synthesis of glucosyl-thiophosphates were explored and evaluated. It was found that the coupling method as well the protecting groups of the carbohydrates and thiophosphates had a significant impact on both the stereoselectivity and yield of the reactions. The diastereoselectivity did not appear to be effected by varying the temperature conditions. Separation of α/β diastereomers was not achieved for the protected thiophosphates. The first example of isomerization from an *O*-linked glucosyl thiophosphate to an *S*-linked species was reported. Deprotection of the various glucosyl thiophosphates was unsuccessful due to the fragility of the glycosidic bond or poisoning of the hydrogenolysis catalyst by sulfur. This highlights the need for alternative deprotection strategies or modified thiophosphorylating reagents. By contrast, α-D-Glucose 1-thiophosphonate was successfully synthesized and converted to a thiophosphonate-containing sugar nucleotide analogue utilizing the thymidylyltransferase Cps2L. The first example of a glycosyl thiophosphonate containing sugar nucleotide analogue was reported. The enzymatic formation of compounds which incorporate both phosphonate and thio- functionalities into their scaffold introduces a greater diversity of non-scissile, isosteric, sugar nucleotide analogues. These types of compounds may have biological applications as probes for glycosyltransferases or other related enzymes.

4. Experimental

4.1. General Methods

All reagents and solvents were purchased and used without further purification. Synthetic reactions were performed under N_2 atmosphere unless otherwise indicated. Reaction progress was generally monitored by thin-layer chromatography using silica gel 60 F_{254} plates (Silicycle). Plates were visualized using a an ethanol dipping solution containing *p*-anisaldehyde (3.4%), sulfuric acid (2.2%) and acetic acid (1.1%) followed by charring or ultraviolet light (λ = 254 nm). Dry ice/acetone and dry ice/acetonitrile slurries were used to generate reaction temperature conditions of -78 and -40°C respectively. For attempted diastereomeric separation, the silver impregnated silica was generated by stirring normal silica in a 1 M solution of $AgNO_3$, followed by decantation of the solvent. The silica was then dried in the oven over night at 60°C and column loaded in a 10/90 EtOAc/hexane slurry. Hydrogenation reactions were generally

performed on 10–30 mg of glycosyl-thiophosphate with platinium(IV) oxide (80 mol%) or palladium on carbon (20 mol%) in 10 mL of 1:1 EtOAc:EtOH or 1:1 EtOAc:MeOH respectively. The mixtures were shaken under H_2 in a Parr apparatus at 54 PSI for 5–18 h. The mixtures were then filtered and TLC analysis, ^{31}P NMR spectroscopy or Low Resolution Mass Spectrometry was used as an indication of reaction progress. Bruker AV-300 or AV-500 MHz NMR spectrometers were utilized for all NMR spectra via the Nuclear Magnetic Resonance Research Resource (NMR^3). For $CDCl_3$ and CD_2Cl_2, ^{1}H NMR chemical shifts are reported as *d* in units of parts per million (ppm) downfield from tetramethylsilane (δ 0.0). ^{31}P NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to 85% H_3PO_4. Low resolution mass spectra were obtained with an Applied Biosystems hybrid triple quadrupole linear ion trap ionization (ESI) source. The mass spectrometer was coupled with an Agilent 1100 HPLC instrument with a Phenomenex Kinetex 2.6 u (150 x 2.10 mm) column. Samples were run using a flowrate of 120 µL/min and an isocratic 70/30 CH_3CN/2 mM aqueous ammonium acetate (pH 5.5) buffer. The capillary voltage was set to ±4500 kV, with a declustering potential of ±60V and a curtain gas of 20 (arbitrary units) for positive and negative mode, respectively. Analyst version 1.4.1 (Applied Biosystem) software was used for analysis. HPLC analysis for all enzymatic assays was performed as previously described[29] using a Hewlett Packard Series 1050 instrument with an Agilent Zorbax 5 µm Rx-C18 column (150 x 4.6 mm). Compounds containing a nucleotide base chromophore were monitored at 254 nm absorbance. HPLC runs were performed for 15 min at a flowrate of 1 mL/min. A linear gradient from 90/10 A/B to 40/60 A/B over 8 min followed by a 40/60 A/B plateau from 8-10 min and a linear decline to 90/10 A/B from 10-11 min followed by isocratic 90/10 A/B was used for all assays. Buffer A was aqueous 12 mM Bu_4NBr, 10 mM KH_2PO_4 AND 5% HPLC grade CH_3CN. Buffer B was HPLC grade CH_3CN.

4.1 HPLC enzyme assay conditions:

Cps2L was over-expressed, purified and assayed as previously described.[29] The concentration of Cps2L was measured spectrophotometrically at 280 nm using calculated extinction coefficient 29.8 $mM^{-1}cm^{-1}$. Enzyme reactions containing dTTP (1.0 mM) α-D-glucose 1-phosphate analogues **12-14** (2.0 mM), $MgCl_2$ (2.2 mM) and inorganic pyrophosphatase (0.5 EU) were initiated by addition of Cps2L (2 EU) in Tris-HCl buffer (20 mM final concentration, 50 µL final volume). The reactions were incubated for 30 minutes or 24 hours at 37°C, quenching with

methanol (50 µL), and centrifuged (5 min at 12,000 x *g*) to precipitate the denatured enzymes prior to HPLC analysis. Percent conversions were calculated as the ratio of sugar nucleotide product (**16-18**) formed relative to remaining dTTP. In the absence of Cps2L, no turnover to sugar nucleotide product was observed. Retention times can be found in the supporting information.

4.2. Synthesis

4.2.1. Diphenylchlorothiophosphate (4)

Diphenylchlorothiophosphate was synthesized using a previously established literature procedure.[30] Phenol (1.88 g, 20.0 mmol) was dissolved in CH_2Cl_2 (5.2 mL) and triethylamine (2.8 mL (20.0 mmol). Half of the solution was added dropwise over 15 minutes to a solution of $PSCl_3$ (1 mL, 10.0 mmol) in CH_2Cl_2 (3 mL) at 0°C. After 2.5 hours, TLC showed full consumption of phenol so the remaining half of the solution was added to the reaction mixture dropwise and the solution was allowed to stir overnight. TLC analysis after 18 hours showed one major product by TLC (Rf = 0.63 10/90 EtOAc/hexane). The material was isolated directly using normal phase chromatography (100% hexane) and collected as a white crystalline solid (1.400 g, 50%). δ_P ($CDCl_3$) 58.8 (s, 1P, P1).

4.2.2. 2,3,4,6-Tetra-*O*-acetyl-α/β-D-glucopyranosyl diethylthiophosphate (5)

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose (**1**) (0.35 mmol) was dissolved in anhydrous THF (2 mL) and cooled to -78°C using a dry ice/acetone slurry. *n*-Butyllithium (0.17 mL, 0.43 mmol) was added and the solution was stirred for one hour before diethylchlorothiophosphate (**3**) (0.063 mL, 0.4 mmol) was added dropwise. The solution turned a red-brown color upon warming to room temperature. The reaction was stirred overnight before being diluted with ice cold water (10 mL) and extracted with dichloromethane (10 mL). The organic extract was washed with ice cold aqueous HCl (0.5 M, 15 mL) and saturated sodium bicarbonate solution (15 mL). After drying with anhydrous sodium sulfate, the mixture was concentrated to a brown liquid. The material was purified using normal phase chromatography (isocratic 30/70 EtOAc/hexane) to afford the pure product **5** as a clear liquid, 1:3 α/β diastereotopic mixture (0.204g, 57% yield), R_F = 0.58 (40/60 EtOAc/hexane). δ_H ($CDCl_3$) 5.96 (dd, 0.25H, $^3J_{1,2}$ = 3.4 Hz, $J_{1,P}$ = 9.8 Hz, αH1), 5.48 (t, 0.25H, $^3J_{3,4} = J_{4,5}$ = 9.8 Hz, αH4), 5.34 (dd, 0.75H, $^3J_{1,2}$ = 7.9 Hz, $J_{1,P}$ = 10.6 Hz, βH1), 5.25 (t, 0.75H, $^3J_{3,4} = {}^3J_{4,5}$ = 9.8 Hz, βH4) 5.06-5.15 (m, 2H, H2, H3), 5.00 (m, 0.25H, αH5)

4.00-4.30 (m, 6H, $2OCH_2$, H6a, H6b), 3.82 (m, 0.75H, βH5), 2.07, 2.04, 2.03, 2.01 (4 x s, 4 x 3H, 4 x OAc), 1.28-1.39 (m, 6H, 2 x CH_3), δ_C 169.3-171.0 (4 x C, $\underline{C}(O)OCH_3$), 96.5 (d, CH, βC1, $^2J_{C,\,P}$ = 3.6 Hz), 94.0 (d, CH, $^2J_{C,\,P}$ = 4.1 Hz, αC1), 60.3-72.7 (4 x CH, C2-C5, 3 x CH_2, C6, $O\underline{C}H_2CH_3$), 14.3-32.0 (2 x CH_3 , $OCH_2\underline{C}H_3$, 4 x CH_3, $C(O)O\underline{C}H_3$), δ_P 67.09 (s, 0.25P αP=S), 66.94 (s, 0.75P, βP=S), HRMS (ESI^+): found $[M+Na]^+$ 523.0999. $C_{18}H_{29}Na_1O_{12}P_1S_1$ requires $[M+Na]^+$ 523.1010.

4.2.3. 2,3,4,6-Tetra-*O*-benzyl-α/β-D-glucopyranosyl diethylthiophosphate (6)

2,3,4,6-Tetra-*O*-benzyl-α/β-D-glucopyranose (**2**) (0.270 g, 0.50 mmol) was dissolved in anhydrous THF (3 mL) under nitrogen and cooled to -78°C using a dry ice/acetone slurry. *n*-Butyllithium (0.22 mL, 0.55 mmol) was added and the solution was stirred for one hour before diethylchlorothiophosphate (**3**) (0.1 mL, 0.64 mmol) was added dropwise. The reaction was allowed to come to room temperature overnight. The solution was poured into a suspension of diethyl ether (10 mL) and water (10 mL) and extracted with ethylacetate (7 mL) before being washed with saturated sodium bicarbonate (2 x 10 mL) and dried with anhydrous sodium sulfate. The mixture was concentrated *in vacuo* and collected as a clear liquid. The material was then purified using normal phase chromatography (isocratic 9/91 EtOAc/hexane) to afford the product (**6**) as a clear liquid, 2:1 α/β diastereotopic mixture (0.241 g, 67% yield), R_F = 0.32 (10/90 EtOAc/hexane). δ_H ($CDCl_3$) 7.20-7.50 (m, 20H, 4 x C_6H_5), 6.13 (dd, 0.67H, $^3J_{1,\,2}$ = 3.3 Hz, $^3J_{1,\,P}$ = 9.8 Hz, αH1), 5.4 (dd, 0.33H, $^3J_{1,\,2}$ = 7.9 Hz, $^3J_{1,\,P}$ = 10 Hz, βH1), 4.50-5.10 (m, 8H, 4 x $PhCH_2$), 4.20 (m, 4H, $2OCH_2$) 4.00-4.10 (m, 2H, H3, H4), 3.60-3.86 (m, H2, H5, H6a, H6b) 1.28-1.39 (m, 6H, 2 x CH_3), δ_C 137.7-138.7 (4 x C, C_6H_5), 127.7-128.6 (20 x CH, C_6H_5), 99.2 (d, CH, $^2J_{C,\,P}$ = 5.7 Hz, C1β), 95.8 (d, CH, $^2J_{C,\,P}$ = 5.7 Hz, C1α), 68.0-84.8 (5 x CH_2, CH_2Ph, C6, 4 x CH, C2-C5), 64.3-64.7 (m, $O\underline{C}H_2CH_3$), 15.8-16.0 (m, $OCH_2\underline{C}H_3$), δ_P 67.80 (s, 0.33P, βP=S), 67.38 (s, 0.67P, αP=S), HRMS (ESI^+): found $[M+Na]^+$ 715.2448. $C_{38}H_{45}Na_1O_8P_1S_1$ requires $[M+Na]^+$ 715.2465.

4.2.4. 2,3,4,6-Tetra-*O*-benzyl-α/β-D-glucopyranosyl diphenylthiophosphate (7)

2,3,4,6-Tetra-*O*-benzyl-α/β-D-glucopyranose (**2**) (0.270 g, 0.5 mmol) and 4-dimethylaminopyridine (1.293 g, 1.23 mmol) were dissolved in dichloromethane (10 mL) and stirred under N_2 at 0°C for 15 minutes. A solution of diphenylchlorothiophosphate (**4**) (0.321 g, 2.28 mmol) in CH_2Cl_2 (5 mL) was added dropwise and stirred under N_2 at 0°C for 3 hours. The

reaction was quenched with distilled water (15 mL), extracted with dichloromethane (2 x 15 mL) and dried with anhydrous sodium sulfate. The mixture was concentrated *in vacuo* and collected as a clear viscous liquid. The material was purified using normal phase chromatography (isocratic 20/80 EtOAc/hexane) using basic silica (5% triethylamine, 95% hexanes) to afford two products (0.095 g, combined yield 24%). The oxygen linked thiophosphate **7a** $R_F = 0.74$, and the sulfur linked thiophosphate **7b** $R_F = 0.52$ (25% EtOAc/75% hexane).

1-*O*-Diphenylthiophosphate-2,3,4,6-tetra-*O*-benzyl-α/β-D-glucopyranose (7a)

δ_H (CD_2Cl_2) 7.15-7.45 (m, 30H, 6 x C_6H_5), 6.20 (dd, 1H, $^3J_{1,\,2}$ = 3.3 Hz, $^3J_{1,\,P}$ = 9.5 Hz, H1), 4.48-5.03 (m, 8H, 4 x $PhCH_2$), 3.97-4.16 (2H, H3, H4), 3.52-3.83 (m, 4H, H2, H5, H6a, H6b), δ_C 137.5-138.7 (6 x C, C_6H_5), 121.0-129.0 (30 x CH, C_6H_5), 93.7 (CH, C1), 67.9-81.6 (5 x CH_2, CH_2Ph, C6, 4 x CH, C2-C5), δ_P 57.86 (s, 1P, P1), HRMS (ESI$^+$): found $[M+Na]^+$ 811.2451 $C_{46}H_{45}Na_1O_8P_1S_1$ requires $[M+Na]^+$ 811.2465.

1-*S*-Diphenylthiophosphate-2,3,4,6-tetra-*O*-benzyl-α/β-D-glucopyranose (7b)

δ_H (CD_2Cl_2) 7.10-7.40 (m, 30H, 6 x C_6H_5), 6.30 (dd, 0.80 H, $^3J_{1,\,2}$ = 5.0 Hz, $^3J_{1,\,P}$ = 11.1 Hz, αH1), 5.07 (dd, 0.20 H, $^3J_{1,\,2}$ = 9.7 Hz, $^3J_{1,\,P}$ = 14.2 Hz, βH1), 4.96-4.39 (m, 8H, 4 x $PhCH_2$), 3.51-3.91(m, 5H, H2, H3, H4, H5, H6a), 3.20 (dd, 1H, $^2J_{6b,\,6a}$ = 1.76, $^3J_{6b,\,5}$ = 10.91, H6b), δ_C 135.4-139.0 (6 x C, C_6H_5), 120.4-130.4 (30 x CH, C_6H_5), 67.9-82.7 (5 x CH_2, CH_2Ph, C6, 4 x CH, C2-C5), δ_P 18.77 (s, 0.80P, αP=O), 16.69 (s, 0.20P, βP=O), HRMS (ESI$^+$): found $[M+Na]^+$ 811.2462 $C_{46}H_{45}Na_1O_8P_1S_1$ requires $[M+Na]^+$ 811.2465.

Diethyl (tetra-*O*-benzyl-α-D-heptulopyranosyl)-1-thiophosphonate (**11**)

To a suspension of **9** (0.099 g, 0.146 mmol) in toluene (8 mL) was added Lawesson's reagent (**10**) (0.059 g, 0.146 mmol) in one portion. The reaction was warmed to 55°C for 12 h and then cooled to room temperature. The yellow reaction mixture was filtered, concentrated *in vacuo* and purified using normal phase chromatography (isocratic 15/85 EtOAc/hexane) to afford the product (**9**) as a white solid (0.072 g, 71% yield), $R_F = 0.61$ (80/20 EtOAc/hexane). δ_H ($CDCl_3$) 7.17-7.39 (m, 20H, 4 x C_6H_5), 4.45-4.87 (m, 9H, 4 x $PhCH_2$, H2), 4.16 (q, 2H, $^3J_{H,\,H}$ = 7.0 Hz, $OC\underline{H}_2CH_3$), 4.12 (q, 2H, $^3J_{H,\,H}$ = 7.0 Hz, $OC\underline{H}_2CH_3$), 3.66-3.82 (m, 6H, H3, H4, H5, H6, H7a,

H7b), 2.47 (m, 2H, H1a, H1b), 1.33 (t, 3H, $^{3}J_{H,\,H}$ = 7.0 Hz, $OCH_2C\underline{H_3}$), 1.28 (t, 3H, $^{3}J_{H,\,H}$ = 7.0 Hz, $OCH_2C\underline{H_3}$), δ_C 127.7-138.8 (4 x C, C_6H_5, 20 x CH, C_6H_5), 72.1-82.2 (4 x CH_2, CH_2Ph, 3 x CH, C4-C6), 79.3 (d, CH, $^{3}J_{3,\,P}$ = 14.1 Hz, C3), 70.2 (d, CH, $^{2}J_{2,\,P}$ = 3.2 Hz, C2), 68.7 (CH_2, C7), 62.7 (d, CH_2, $^{2}J_{C,\,P}$ = 6.9 Hz, $O\underline{C}H_2CH_3$), 62.6 (d, CH_2, $^{2}J_{C,\,P}$ = 6.9 Hz, $O\underline{C}H_2CH_3$,), 30.5 (d, CH_2, $^{1}J_{1,\,P}$ = 116.0 Hz, C1), 16.4 (m, $OCH_2\underline{C}H_3$), δ_P 96.81 (s, 1P, P1), HRMS (ESI^+): found $[M+Na]^+$ 713.2494 $C_{39}H_{47}Na_1O_7P_1S_1$ requires $[M+Na]^+$ 713.2672.

Ammonium-(1-deoxy-α-D-heptulopyranosyl)thiophosphonate (12)

Under N_2 at 0ºC, a stirring solution of **11** (0.072 g, 0.105 mmol) in anhydrous CH_2Cl_2 (0.5 mL) was treated with iodotrimethylsilane (740 µL, 5.25 mmol) and allowed to warm to room temperature over 2 h. The reaction was monitored by TLC analysis using 6:3:1 n-propanol-NH_3-H_2O (Rf: 0.70), and quenched by methanol. The mixture was concentrated *in vacuo*, dissolved in H_2O (10 mL) and washed with diethyl ether (8 x 10 mL). The resulting acidic aqueous layer was immediately adjusted to pH 8 with aqueous NH_4OH (0.2 M), concentrated to 5 mL and purified by cellulose column (1.5 x 10 cm, 2.5 g) (n-propanol-NH_3-H_2O, 80/10/10). The resulting fraction was lyophilized to afford the target phosphonate **10** as a white film (0.013 g, 42%). δ_H (D_2O) 4.43 (m, 1H, H2), 3.68 (d, 1H, $^{2}J_{7a,\,7b}$ = 11.2 Hz, H7a), 3.44-3.56 (m, 4H, H3, H4, H6, H7b), 3.37 (m, 1H, H5), 2.41 (m, 2H, H1a, H1b), δ_C 71.4-73.5 (2 x s, 2 x CH, C4, C6), 72.1 (d, CH, $^{2}J_{2,\,P}$ = 4.1 Hz, C2), 70.3 (d, CH, $^{3}J_{3,\,P}$ = 13.7 Hz, C3), 69.5 (CH, C5), 60.4 (CH_2, C7), 28.7 (d, CH_2, $^{1}J_{1,\,P}$ = 112.0 Hz, C1), δ_P 87.90 (s, 1P, P1), HRMS (ESI^-): found $[M-1]^-$ 257.0463 $C_7H_{14}O_7P_1S_1$ requires $[M-1]^-$ 257.0245.

Supplementary Data

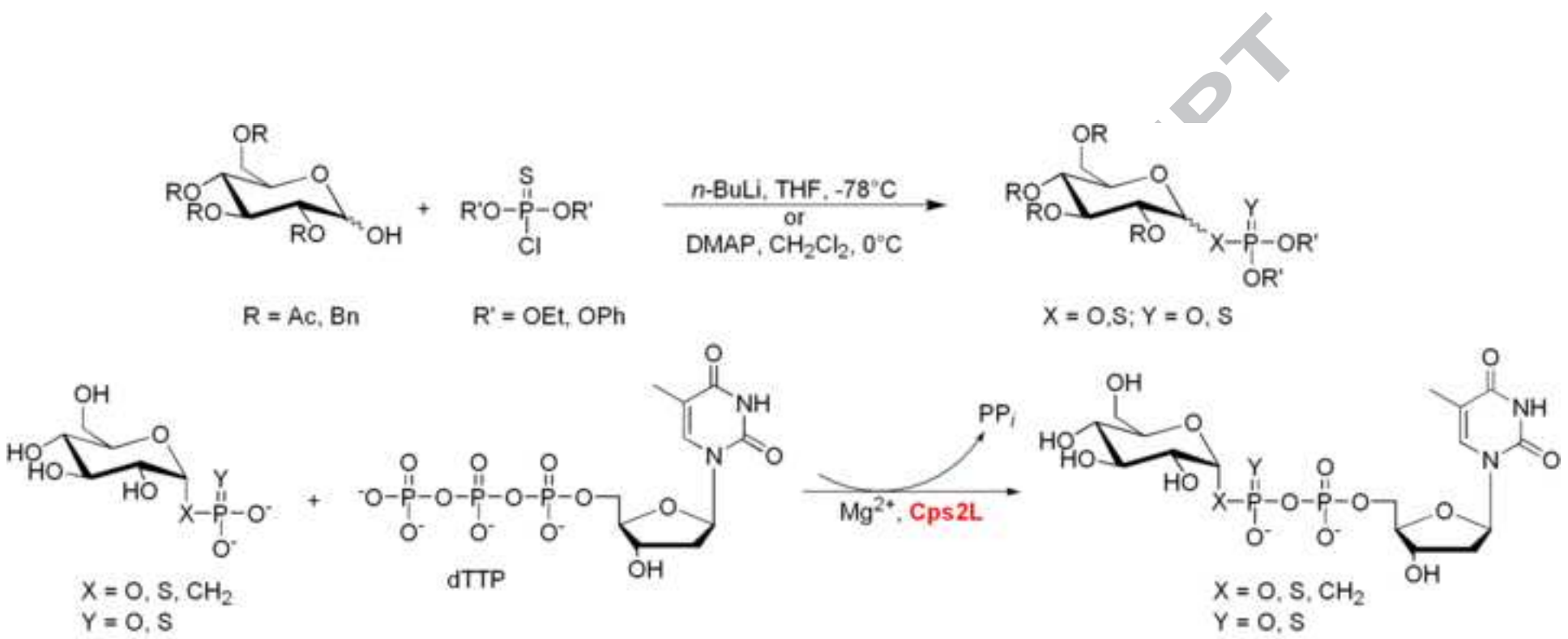
Supplementary data (NMR spectra for compounds **5**-**7**, **11**-**12**, and HPLC retention times for sugar nucleotides and related compounds).

References

1. Pale, P.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 4547-4549.
2. Fujita, S.; Oka, N.; Matsumura, F.; Wada, T. *J. Org. Chem*. **2011**, *76*, 2648–2659.

3. Eckstein, F. *J. Amer. Chem. Soc.* **1970**, *92*, 4718-4723.
4. Goody, R. S.; Eckstein, F. *J. Amer. Chem. Soc.* **1971**, *93*, 6252-6257.
5. Cullis, P. M.; Maxwell, A., Weiner, D. P. *Biochemistry* **1997**, *36*, 6059-6068.
6. Baxter, N. J.; Olguin, L. F.; Golicnik, M.; Feng, G.; Hounslow, A. M.; Bermel, W.; Blackburn, G. M.; Hollfelder, F.; Waltho, J. P.; Williams, N. H. *Proc. Natl. Acad. Sci.* **2006***, 103*, 14732-14737.
7. Xu, Y.; Jiang, G.; Tsukahara, R.; Fujiwara, Y.; Tigyi, G.; Prestwich, G. D. *J. Med. Chem*. **2006**, *49*, 5309-5315.
8. Nakai, H.; Kitaoka, M.; Svensson, B.; Ohtsubo, K. *Curr. Opin. Chem. Biol.* **2013**, *17* 301-309.
9. Levander F.; Andersson, U.; Radstrom, P. *Appl. Environ. Microbiol.* **2001**, *67*, 4546–4553.
10. Sjoberg, A.; Hahn-Hagerdal, B.; *Appl. Environ. Microbiol.* **1989**, *55*, 1549–1554.
11. Alphey, M. S.; Pirrie, L.; Torrie, L. S.; Boulkeroua, W. A.; Gardiner, M.; Sarkar, A.; Maringer, M.; Oehlmann, W.; Brenk, R.; Sherman, M. S.; McNeil, M.; Rejzek, M.; Field, R. A.; Singh, H. M.; Gray, D.; Westwood, N. J.; Naismith, J. H. *ACS Chem. Biol.* **2013**, *8*, 387–396.
12. Qu, H.; Xin, Y.; Dong, X.; Ma, Y. *FEMS Microbiol. Lett.* **2007**, *275*, 2437-243.
13. Melo, A.; Glaser, L. *J. Biol. Chem.* **1965***, 240*, 398-405.
14. Zhang, G.; Yu, B.; Deng, S.; Hui, Y. *J. Carbohydr. Chem*. **1998**, *17*, 547-556.
15. Piekutowska, M.; Pakulski, Z. *Carbohydr. Res*. **2008**, *343*, 785-792.
16. Cai, L.; Ban, L.; Guan, W.; Mrksich, M.; Wang, P. W. *Carbohydr. Res*. **2011**, *346*, 1576-1580.
17. Forget, S. M.; Bhattasali, D.; Hart, V. C.; Cameron, T. S.; Syvitski, R. T.; Jakeman, D. L. *Chem. Sci.* **2012**, *3*, 1866-1878.
18. Huestis, M.; Aish, G.; Hui, J.; Soo, E.; Jakeman, D. L. *Org. Biomol. Chem*., **2009**, *6*, 477-484.
19. Coppola, C.; Saggiomo, V.; Di Fabio, G.; De Napoli, L.; Montesarchio, D. *J. Org. Chem*, **2007**, *72*, 9679-9689.

20. Heissigerova, H.; Kocalka, P.; Hlavackova, M.; Imberty, A.; Breton, C.; Chazalet, V.; Moravcova, J. *Collect. Czech. Chem. Commun*. **2006**, *71*, 1659-1672.
21. McKenna, C. E.; Schmidhauser, J. *J. Chem. Soc., Chem. Commun.*, **1979**, *0*, 739.
22. Elgibo-Gabas, M.; Canals, D.; Casas, J.; Llebaria, A.; Delgado, A. *ChemMedChem* **2007**, *2*, 992-994.
23. Albers, P.; Pietsch, J.; Parker, S. F. *J. Mol. Catal. A: Chem.* **2001**, *173*, 275-286.
24. Vassilakis, D.; Barbouth, N.; Oudar, J. *Catal. Lett.* **1990**, *5*, 321-330.
25. Ali, A. M.; Taylor, S. D. *J. Pept. Sci.* **2010**, *16*, 190-199.
26. Beaton, S. A.; Huestis, M. P.; Sadeghi-Khomami, A.; Thomas, N. R.; Jakeman, D. L. *Chem. Commun.* **2009**, 238-240.
27. Ozturk, T.; Ertas, E.; Mert, O. *Chem. Rev.* **2007**, *107*, 5210-5278.
28. Karp, G. M. *J. Org. Chem.* **1999**, *64*, 8156-8160.
29. Timmons, S. C.; Mosher, R. H.; Knowles, S. A.;; Jakeman, D. L. *Org. Lett.* **2007**, *9*, 857-860.
30. Hoque, M. E. U.; Shuchismita, D.; Guha, A. K.; Kim, C. K.; Lee, B. S.; Lee, H. W. *J. Org. Chem.* **2007**, *72*, 5493-5499.



Preparation of protected forms of glucose 1-thiophosphate
Isomerization from an *O*-linked to *S*-linked glucosyl thiophosphate
Preparation of glucose 1-thiophosphonate
Enzymatic conversion of glucose 1-thiophosphonate by thymidylyltransferase