Profiling substrate promiscuity of wild-type sugar kinases for multi-fluorinated monosaccharides

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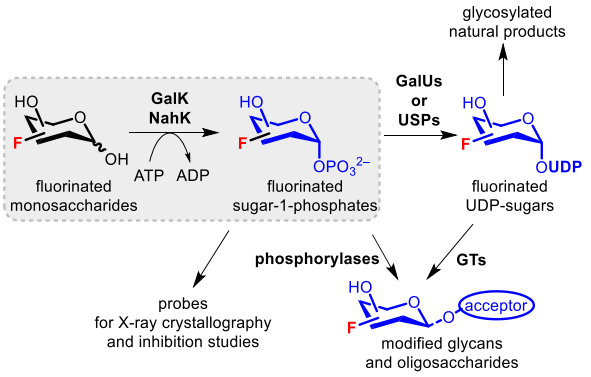
SUMMARY: Fluorinated sugar-1-phosphates are of emerging importance as intermediates in the chemical and biocatalytic synthesis of modified oligosaccharides, as well as probes for chemical biology. Here we present a systematic study of the activity of a wide range of anomeric sugar kinases (galacto- and N-acetylhexosamine kinases) against a panel of fluorinated monosaccharides, leading to the first examples of polyfluorinated substrates accepted by this class of enzymes. We have discovered four new N-acetylhexosamine kinases with a different substrate scope, thus expanding the number of homologs available in this subclass of kinases. Lastly, we have solved the crystal structure of a galactokinase in complex with 2-deoxy-2-fluoro galactose, giving insight into changes in the active site that may account for the specificity of the enzyme towards certain substrate analogues.

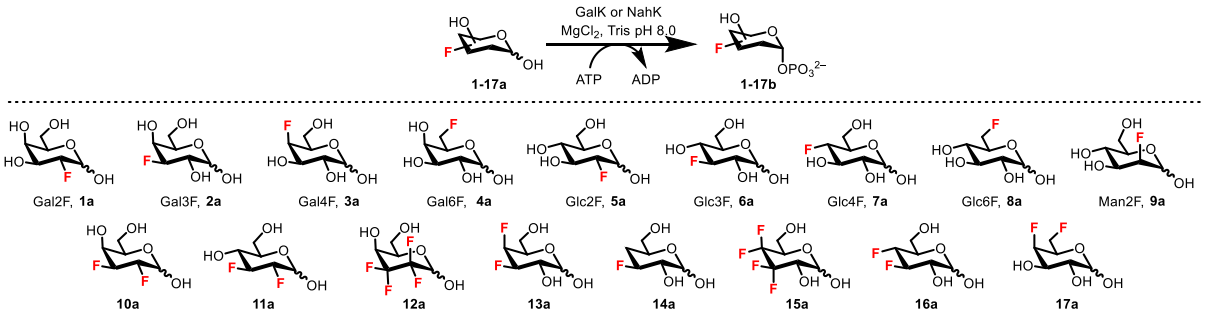
INTRODUCTION: Understanding the biological functions of complex oligosaccharides and the mechanisms that underpin carbohydrate recognition, synthesis and bioconversion, is an ever-expanding research area in glycobiology that has major implications in medicine and drug development. For these studies the supply of new substrates, probes and reference materials is often a major bottleneck, due to the high chemical complexity and cost of these target products. The chemical synthesis of functionalised oligosaccharides can be challenging, time-consuming and often inefficient.[1-4] In contrast, chemo-enzymatic strategies,[5] in which chemical syntheses are used to generate natural and non-natural substrate analogues, which are enzymatically functionalised to produce key intermediates that enable access to a range of modified glycans, have proven to be highly successful. A representative example of this strategy is the synthesis of modified sugar-1-phosphates. Sugar-1-phosphates have a central role in sugar metabolism and utilization,[6] often being the first intermediate biosynthesised from the reducing monosaccharide. Modified sugar-1-phosphates are commonly employed as basic building blocks towards the synthesis of diverse oligosaccharides, glycoproteins and glycosylated natural products.[7,8] Fluorinated sugar-1-phosphates in particular, are important (Scheme 1) as it is well known that the presence of fluorine can influence the stereo-electronic properties of a molecule.[9-11] Fluorinated sugar-1-phosphates are widely employed in the synthesis of fluorinated glycans, either directly via phosphorylases[12-14] or by conversion to the corresponding UDP-sugars by the action of uridyltransferases (GalUs) or UDP-sugar pyrophosphorylases (USPs)[15], followed by coupling via glycosyltransferases (GTs).[16] Similarly, fluorinated UDP-sugars have also been used as donors for the synthesis of a range of modified glycosylated natural products.[17] Furthermore, the peculiar stereo-electronic changes induced by the substitution of sugar ring hydroxyls for fluorine affects how these molecules interact with enzymes,[18] making them extremely useful probes for structural and biochemical investigations, as previously reported for GTs,[19,20] glycosidases,[21] mutases,[22-24] synthases,[25] lectins, [26] anti-carbohydrate antibodies,[27] and in metabolic studies.[28]



Scheme 1. Kinase-mediated synthesis of fluorosugar-1-phosphates and their applications.

Two main classes of enzymes are responsible for the *in vivo* synthesis of sugar-1-phosphates from non-activated monosaccharides: galactokinases (GalKs) which catalyse the ATP dependant phosphorylation of galactose (Gal) and *N*-acetylhexosamine kinases (NahKs, also known as HexNAcKs) which phosphorylate both *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc). Many well characterised examples of GalKs have been reported,[29-39] while only a few NahKs have been previously investigated.[40,41] Both classes are highly promiscuous, exhibiting a broad tolerance to natural and derivatised monosaccharides.

Table 1. Screening of fluorinated monosaccharides against GalKs and NahKs[a].



	1a	2a	3a	4a	5a	7a	9a	10a	12a	13a	14a
EcGalK	>95	<5 (6)	<5	<5	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
PfGalK	>95	<5 (48)	<5 (59)	<5 (10)	n.c.	n.c.	n.c.	<5 (14)	n.c.	n.c.	n.c.
SpGalK	>95	>95	>95	>95	n.c.	n.c.	5	>95	12 (55)	n.c.	n.c.
LlGalK	>95	83	72	74	n.c.	n.c.	n.c.	43	n.c.	n.c.	n.c.
LgGalK	>95	74	91	67	n.c.	n.c.	n.c.	45 (57)	n.c.	n.c.	n.c.
BiGalK	>95	>95	73	57 (85)	n.d.	n.d.	n.d.	81	<5 (11)	n.c.	n.c. (<5)
ScGalK	>95	27 (77)	54	<5	n.d.	n.d.	n.d.	<5	n.c.	n.c.	n.c.
BliNahK	25	n.c.	7	n.c.	64	23 (43)	>95	n.c.	n.c.	55 (97)	11 (77)
BllNahK	<5	n.c.	29	n.c.	26 (52)	<5 (12)	>95	n.c.	n.c.	14 (79)	<5 (57)
ApNahK	20	n.c.	>95	n.c.	30	37 (39)	>95	n.c.	n.c.	38 (56)	21 (25)
CxNahK	<5	n.c.	49	n.c.	n.c.	<5	>95	n.c.	n.c.	<5	<5 (6)
EtNahK	n.c.	n.c.	11	n.c.	n.c.	n.c.	93	n.c.	n.c.	n.c.	n.c.
TbNahK	5	n.c.	72	n.c.	7	<5	>95	n.c.	n.c.	<5	<5

Conv. [%][b]: 0, 20, 40, 60, 80

[a] Experimental conditions: 8 mM substrate, 10 mM ATP, 5 mM $MgCl_2$, 0.12 mg mL^{-1} NahK, 100 mM Tris pH 8.0, 37°C, 24 h. [b] Determined by ^{19}F NMR (n.c. = no conversion, n.d. = not determined). Optimised conversions shown in brackets. Limit of quantitation estimated at 5% conversion. Compounds 6a, 8a, 11a, 15a, 16a and 17a were not substrates for any kinases tested.

Despite the interest in fluorinated sugar-1-phosphates and the wide availability of GalKs and NahKs, few examples of kinase mediated fluorosugar phosphorylation have been demonstrated. All four monodeoxy-monofluoro galactoses (2FGal **1a**, 3FGal **2a**, 4FGal **3a**, 6FGal **4a**) have long been recognised as substrates for yeast GalK,[42] and investigated subsequently as model substrates with EcGalK[15,17] and LgGalK.[35] Some groups have also reported the conversion of geminally fluorinated monosaccharide derivatives, such as 6,6-difluoro-D-fucose (6-deoxy-6,6-difluoro-D-galactose) [43,44] and N-trifluoroacetyl-D-glucosamine/-D-galactosamine.[41] However, the tolerance of anomeric kinases to vicinal dideoxy-difluorinated substrates, or monosaccharides with a higher degree of fluorination, is currently unexplored. This class of sugars is of interest in the study of protein-carbohydrate interactions.[18,22,24,45-48] Here, we describe a systematic approach in which fluorosugar reactivity of diverse anomeric kinases was explored, leading to examples of mono-, di- and tetrafluorinated substrates that are phosphorylated by these enzymes. We also describe the 3D-structure of BiGalK and showcase, through ligand complexes with 2FGal and Gal, subtle changes in the active site which can occur when a sugar ring-hydroxyl is replaced with fluorine.

RESULTS AND DISCUSSION: A panel of seven published GalKs (EcGalK[32], PfGalK,[38] SpGalK,[33] LlGalK,[39] LgGalK,[35] BiGalK[34] and ScGalK[37]) and two published NahKs (BliNahK[41] and BllNahK[41]) was chosen. Due to the lower diversity available in the latter family, four putative NahK homologs (49-54% sequence identity) from bacterial species (Supporting information) were also selected and included in the panel: ApNahK (WP_091282512), CxNahK (WP_045094128), EtNahK (ODM08532) and TbNahK (WP_062612735). The kinase panel was investigated for the conversion of a series of deoxyfluorinated galactoses, glucoses and a mannose derivative. While some examples of kinase mediated phosphorylation of monofluorinated galactoses have been reported previously[15,17,35,42], we rationalised that the inclusion of these compounds in our extensive screen could help to identify kinases with higher activity against these, as well as with a broader substrate scope. Kinase mediated conversion of the substrates to the sugar-1-phosphate was monitored by ^{19}F NMR (Table 1, Table S1). Phosphorylation at the anomeric position of the monosaccharides was evident, as shown by the formation of only a single new species in the ^{19}F NMR, in comparison to two species that were present in the reference spectrum of the monosaccharide, where the anomeric centre is in the hemiacetal form. Formation of the sugar-1-phosphate was further validated by HRMS analysis (Supporting information).

As expected, the GalKs demonstrated a strict preference for deoxyfluorinated galactoses. Deoxyfluorination at the 2-position was the most tolerated, as demonstrated by the near full conversion of 2FGal (**1a**) by all of the GalKs. All GalKs displayed some activity towards 3FGal (**2a**), 4FGal (**3a**) and 6FGal (**4a**), however there were clearly differences in the activity profiles towards these substrates, between the different enzymes. For example, BiGalK catalysed nearly full conversion of 3FGal but moderate conversions of 4FGal and 6FGal. In contrast, ScGalK catalysed moderate conversion of 4FGal but low-level conversions of 3FGal and 6FGal. Overall, SpGalK catalysed the highest conversions of all deoxyfluorinated galactoses (>95%), as well as the conversion of 2FMan (**9a**) at low levels, further indicating its promiscuity.

The NahKs displayed a broader fluorinated substrate scope, demonstrating the conversion of some deoxyfluorinated galactoses, glucoses and the mannose derivative. These results also show the activity of the four putative NahKs, highlighting the importance of investigating different scaffolds with low sequence similarity in order to find new homologs with increased activity. Most NahKs displayed moderate activity towards 2- and 4- deoxyfluorinated analogues, while displaying little or no activity towards their 3- and 6- deoxyfluorinated counterparts. As expected, most of the NahKs phosphorylated both galacto- and gluco- configured substrates. This could be explained by a lack of hydrogen bonding interactions made between the enzyme active site residues and the 4-OH, as is shown in the crystal structure of BliNahK in complex with GlcNAc[49] (Figure S1). All NahKs catalysed the highest conversions of 2FMan (**9a**), consistent with previous observations that mannose was a better substrate for NahKs than galactose and glucose.[41]

The panel of kinases was then investigated for activity towards vicinal dideoxygenated monosaccharides having di- and tetrafluorinated motifs (Table 1, Table S2). Interestingly, most of the GalKs phosphorylated 2,3-dideoxy-2,3-difluoro galactose (**10a**) (Figure S4) but not 3,4-dideoxy-3,4-difluorogalactose (**13a**) or 4,6-dideoxy-4,6-difluoro galactose (**17a**), despite being able to convert monofluorinated monosaccharides 3FGal, 4FGal and 6FGal. Notably, SpGalK and BiGalK also converted 2,3-dideoxy-2,2,3,3-tetrafluoro-D-*threo*-hexopyranose (**12a**) but not 3,4-dideoxy-3,3,4,4-tetrafluoro-D-*erythro*-hexopyranose (**15a**). Although only trace amounts of product was observed under normal screening conditions, reaction optimisation by increasing the concentration of SpGalK and the reaction time, improved the conversion to 55% (Figure 1). The chemically synthesised sugar phosphate, 2,3-dideoxy-2,2,3,3-tetrafluoro-D-*threo*-hexopyranose phosphate (**12b**) was used as a ^{19}F NMR standard to validate the presence of the correct product. These findings further support observations made in the deoxyfluorinated monosaccharide screening, which highlighted the high activity of GalKs (particularly SpGalK and BiGalK) towards 2- and 3-deoxyfluorinated galactose derivatives. On the other hand, the GalKs did not show turnover of the gluco-configured substrates **11a** and **16a** or the vicinal 3,4-dideoxy-3-fluoro sugar (**14a**), further emphasising the importance of galacto-configuration at the 4-position in determining GalK substrate preference. In contrast to the GalKs, the NahKs were moderately active towards 3,4-dideoxy-3,4-difluorogalactose (**13a**) and the 3,4-dideoxy-3-fluoro sugar (**14a**) and conversions of up to 97% and 77% could be obtained for **13a** and **14a** respectively (Table 1), but displayed no activity towards 3,4-dideoxy-3,4-difluoroglucose (**16a**). Despite increasing the enzyme loading and reaction times, NahKs failed to reveal any activity towards analogues **10a, 11a, 12a, 15a** or **17a**. Remarkably, within this panel of 8 heavily fluorinated monosaccharides, 4 of them could be effectively phosphorylated (>50% conversion) by at least one of the kinases investigated.

The preference of GalKs for galacto-configured substrates fluorinated at the 2-position, emphasised by the preference of difluorinated analogue **10a** over analogues **13a** and **17a**, prompted us to investigate these interactions at the molecular level. Using BiGalK as a model example, the crystal structures of BiGalK in complex with 2FGal **1a** (PDB: 6TEQ), Gal (PDB: 6TER) and ADP (PDB: 6TEP) were solved (Table S3). Overall, the secondary structure architectures of BiGalK were nearly indistinguishable between the three ligand-bound complexes (Figure S2a). The position of the bound ADP was as expected (Figure S2b), when compared to the nucleotide binding sites in the structures of LlGalK[50] and PfGalK.[38] In the sugar-bound complexes 2FGal (**1a**) and Gal are present as a mixture of the alpha and beta forms. In the Gal-bound structure, the ligand is positioned through a network of hydrogen bonding interactions (Figure 2a).

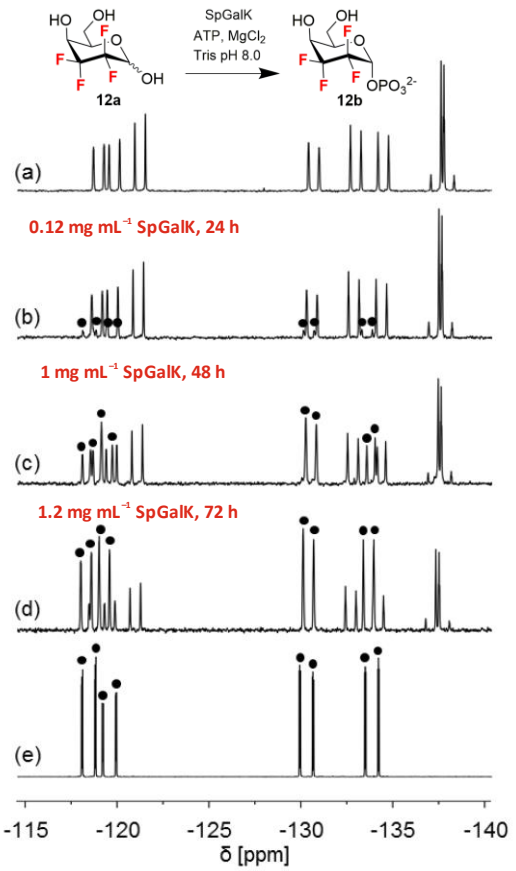


Figure 1. ^{19}F NMR analysis of SpGalK mediated phosphorylation of substrate **12a**. Peaks corresponding to product **12b** are marked (●). (a) Reference spectrum of **12a;** (b) Reaction under screening conditions; (c-d) Reactions with increased loading and reaction times; (e) reference spectrum of **12b.**[24]

The active site residues that interact directly with Gal (R40, H47, D49, G188, D191, Y248) are conserved in all bacterial GalKs investigated here (Figure S3). As expected for the alpha-configured sugar, the 1-OH is positioned within hydrogen bonding distance of R40 and close to D191 (within 3.2-3.6 Å). The corresponding residues in *L. lactis* GalK (R37, D186) were previously shown to have a role in catalysis by deprotonating the 1-OH of Gal.[50] The 2-OH forms hydrogen bonding interactions with the side chain of D191 and some water molecules, while the 3-OH hydrogen bonds to OD2 D49, N G188 and a water molecule. D49, which forms hydrogen bonds with both the 3-OH and 4-OH, appears to be important for substrate positioning. This potentially accounts for the absence of GalK activity towards **13a**, as fluorines at the 3- and 4-positions could not act as hydrogen bond donors. Whilst the sugar molecule is relatively accessible near the 2-OH where there is a network of hydrogen-bonded water molecules, the active site residues interact more closely around the 4-OH and 6-OH.

In the 2FGal-bound structure, the 2-fluorine can only serve as a hydrogen bond acceptor and therefore cannot form a hydrogen bond with the side chain of D191 (Figure 2b). There are one or two additional water molecules modelled in the region close to, and forming hydrogen bonds with the 2-fluorine in the 2FGal structure molecules, compared to those observed for the 2-OH group in the Gal structure. These extra water molecules may help to compensate for the loss of interactions with D191. The carbonyl group of G188 is flipped, allowing it to form a new hydrogen bond with the water molecule that interacts with the 3-OH. OG1 T187 appears to stabilise the water molecules which interact with the 2-fluorine and the 3-OH.

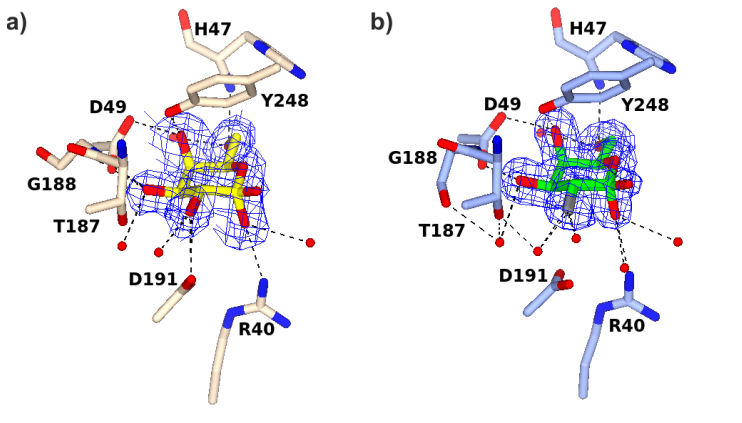


Figure 2. BiGalK active site residues interacting with Gal (a) and 2FGal (b). Electron density maps are REFMAC maximum-likelihood/σA weighted 2Fo−Fc maps contoured at an r.m.s.d. level of 1.0 σ for both (0.31 and 0.36 electrons/Å^3 for (a) and (b), respectively). Alpha and beta anomers are modelled at 0.9/0.1 occupancy in (a) and 0.6/0.4 occupancy in (b), respectively.

These subtle variations in the ligand-water hydrogen bonding network may explain why fluorination at the 2- and 3-positions is well tolerated by the enzyme. One could envisage that the restricted space around the 4-OH and 6-OH prohibits access for water molecules which could form compensatory hydrogen bonds when these groups are substituted with fluorine, as in **17a**.

CONCLUSIONS: In summary, we have demonstrated that wild-type anomeric sugar kinases can be exploited to access fluorinated monosaccharide-1-phosphates. We have systematically profiled kinase activity towards fluorinated analogues, demonstrating the first examples of polyfluorinated substrates accepted by this enzyme class. Our screen consisted of a large and diverse panel of kinases, highlighting that the selection of homologs with low sequence similarity can facilitate the identification of novel substrate specificities. We have shown that GalKs show a strong preference for galacto-configuration and a 4-OH, while NahKs accepted both galacto- and gluco-configured substrates. We have demonstrated the effective phosphorylation of 11 different fluorinated monosaccharides and through optimised reaction conditions, obtained conversions of >90% for 7 of these, setting the scene for further optimisation by rational design and/or random mutagenesis. Through the structural characterisation of a GalK in complex with 2FGal, we have gained valuable insight into subtle changes in the active site which can occur when a sugar ring-hydroxyl is replaced with fluorine. These results pave the way to the one-step synthesis of useful intermediates for enzymatic glycosylations and probes for chemical biology.

ASSOCIATED CONTENT

Supporting information

Experimental details and supporting data (PDF)

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AUTHOR CONTRIBUTIONS

F.P., T.K., K.H. and A.M. performed the enzymatic transformations; J.M., C.Q.F. and J.B.V. performed chemical synthesis and characterisation; T.K. and W.O. performed crystallography; P.B., C.Y. and T.K. performed sequence identification, cloning and protein production; A.H. developed ^{19}F NMR methodology; M.A.F., S.L.F., B.L., G.J.D., S.J.C. supervised the project; M.A.F., S.L.F. and B.L. designed the study; T.K. and F.P. wrote the paper, and all authors commented on the paper.

NOTES

The authors declare no competing financial interests: Prozomix is a commercial enzyme producer.

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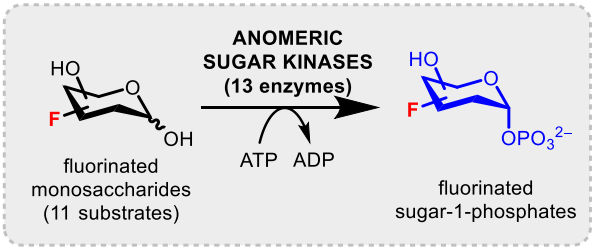
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