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Reverse thiophosphorylase activity of a glycoside phosphorylase in the synthesis of an unnatural Man β 1,4GlcNAc library†

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β -Mannosides are ubiquitous in nature, with diverse roles in many biological processes. Notably, Man β 1,4GlcNAc a constituent of the core *N*-glycan in eukaryotes was recently identified as an immune activator, highlighting its potential for use in immunotherapy. Despite their biological significance, the synthesis of β -mannosidic linkages remains one of the major challenges in glycoscience. Here we present a chemoenzymatic strategy that affords a series of novel unnatural Man β 1,4GlcNAc analogues using the β -1,4-D-mannosyl-*N*-acetyl-D-glucosamine phosphorylase, BT1033. We show that the presence of fluorine in the GlcNAc acceptor facilitates the formation of longer β -mannan-like glycans. We also pioneer a “reverse thiophosphorylase” enzymatic activity, favouring the synthesis of longer glycans by catalysing the formation of a phosphorolysis-stable thioglycoside linkage, an approach that may be generally applicable to other phosphorylases.

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Glycoside phosphorylases (GPs) naturally catalyse the breakdown of glycosidic bonds between glycans (phosphorolysis).¹ However, these useful biocatalysts can also be harnessed in a synthetic “reverse phosphorolysis” direction (Fig. 1A) requiring only simple sugar-1-phosphate donors for the synthesis of diverse glycosides.^{2,3} Yet the inherent reversibility of GPs can limit their utility in the synthetic direction. Herein we explore the use of unnatural substrates to favour “reverse phosphorolysis” using a GP active on β -mannosides and in the process pioneer “reverse thiophosphorylase” enzymatic activity, wherein formation of a phosphorolysis-stable thioglycoside linkage (Fig. 1B) facilitates the synthesis of longer glycans.

β -Mannosides are highly prevalent in nature,⁴ with diverse roles in biological processes including energy storage⁵ and cell wall biosynthesis.⁶ Notably the ubiquitous ManGlcNAc₂ motif within eukaryotic *N*-glycans⁷ contains a Man β 1,4GlcNAc disaccharide, which was recently identified as a novel immune modulator in autoimmune disease.^{8,9} Man β 1,4GlcNAc has shown potential as a new activator of STING (stimulator of interferon genes pathway) triggering a broad immune response in macrophages.⁸ STING is a component of the innate immune system and a key mediator of inflammation.¹⁰ Therefore small molecule activators are emerging as a promising strategy in cancer immunotherapy.¹¹ Despite its striking biological significance and recent advances in the chemical synthesis of such linkages,¹² the efficient assembly of β -mannosides still remains one of the major challenges in glycoscience.

Herein we utilize a GP-mediated chemoenzymatic approach¹³ for the synthesis of β -mannosides in the form of an unnatural library of Man β 1,4GlcNAc-based glycans, including a number of extended glycans. We incorporate unnatural functionality into the enzymatic building blocks through chemical synthesis and show that when a 4-SH nucleophile or 6F group are present in the GlcNAc acceptor, this facilitates the extension of Man β 1,4GlcNAc producing longer β -mannan like glycans. This approach not only affords access to a series of novel, unnatural Man β 1,4-GlcNAcs which may have potential in immunotherapy, but also represents a benchmark for the utility of GPs for thioglycoside synthesis. With more than 190 GPs that

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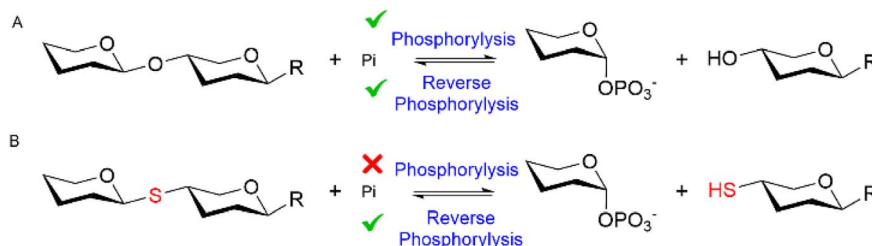


Fig. 1 The reversible GP catalyzed reaction (A). Proposed irreversible "reverse thiophosphorylase" activity with a 4SH-thiol acceptor (B).

have been characterized to date, if this "reverse thiophosphorylase" activity was observed more broadly in other GPs, it could provide straightforward access to a wide range of thioglycosides.¹⁴

For the synthesis of our Man β 1,4GlcNAc analogues we investigated the inverting β -1,4-D-mannosyl-N-acetyl-D-glucosamine phosphorylase from *Bacteroides thetaiotaomicron* (BT1033).¹³ BT1033 is a GH130 family phosphorylase, previously shown to catalyse the transfer of Man from α -D-mannose-1-phosphate (Man1-P) onto N-acetyl-D-glucosamine (GlcNAc) to produce Man β 1,4GlcNAc by reverse phosphorolysis. To investigate the substrate promiscuity of BT1033, we screened a series

of chemically synthesised Man1-P donors (3–10) and GlcNAc acceptors (11–14) (Fig. 2). The GlcNAc acceptors were designed with an azido-propyl handle to provide an accessible point for bioconjugation and this was exploited in our glycan detection methodology (Fig. 2A). Imidazolium-based ionic liquid tags (ITags) are highly sensitive mass spectrometry (MS) probes that enable low detection limits, due to their dominant ionizability by MS.¹⁶ To facilitate the semi-quantitative detection of the Man β 1,4GlcNAc products in our reactions, as well as any unreacted acceptor, the reaction products were labelled with an alkyne-functionalised ITag 1 using a copper-catalysed alkyne-azide cycloaddition (CuAAC) reaction and analysed by liquid-

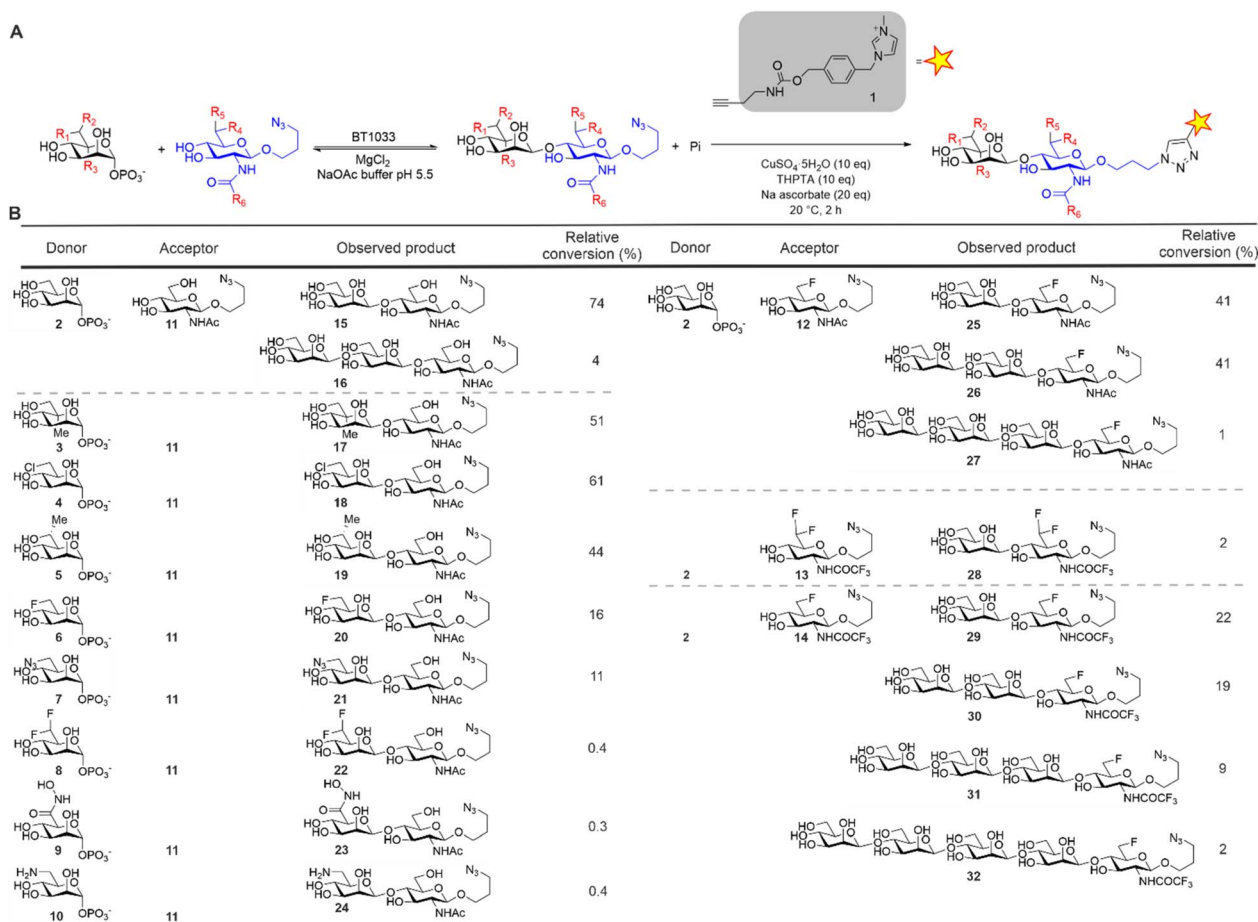


Fig. 2 (A) ITag screening methodology for BT1033 reactions. Reaction mechanism depicted in reverse phosphorolysis direction. (B) BT1033 activity towards unnatural donors and acceptors.^{3,15}

Next, we screened for activity towards fluorinated GlcNAc-N₃ acceptors **12–14**, with Man-1P **2** (Fig. 2B). Fluorination, whilst having little effect on the overall conformation of a glycan,¹⁷ is known to affect stereo-electronic properties and can therefore modulate biological function.¹⁸ 6F-GlcNAc-N₃ **12** and 6F-GlcNTFA-N₃ **14** were tolerated by the enzyme, producing 83% and 52% total conversion to product respectively. For both acceptors, not only were the anticipated disaccharide products observed at conversions of 41% (**12**) and 22% (**14**) respectively, but also masses consistent with the production of longer

Using **11**, we observed mostly acceptor (61%), some disaccharide (26%) and trisaccharide (11%), and low-level tetrasaccharide (1%). Comparatively, for **33** the majority of the product observed was disaccharide (61%), with some trisaccharide (25%) and tetrasaccharide (1%). These findings are consistent with the accumulation of phosphorolysis resistant Man β 1,4-S-GlcNAc-N $_3$ **34**. Although the specific activity of BT1033 towards GlcNAc-N $_3$ **11** (3.20 $\mu\text{mol min}^{-1} \text{mg}^{-1}$) was \sim 2-fold higher than towards SH-GlcNAc-N $_3$ **33** (1.59 $\mu\text{mol min}^{-1} \text{mg}^{-1}$), enzyme titration curves with the respective acceptors highlight the beneficial effect that the thiol has on the final conversion to product in the competing phosphorolysis reaction, with near full conversion of SH-GlcNAc-N $_3$ **33** achieved in

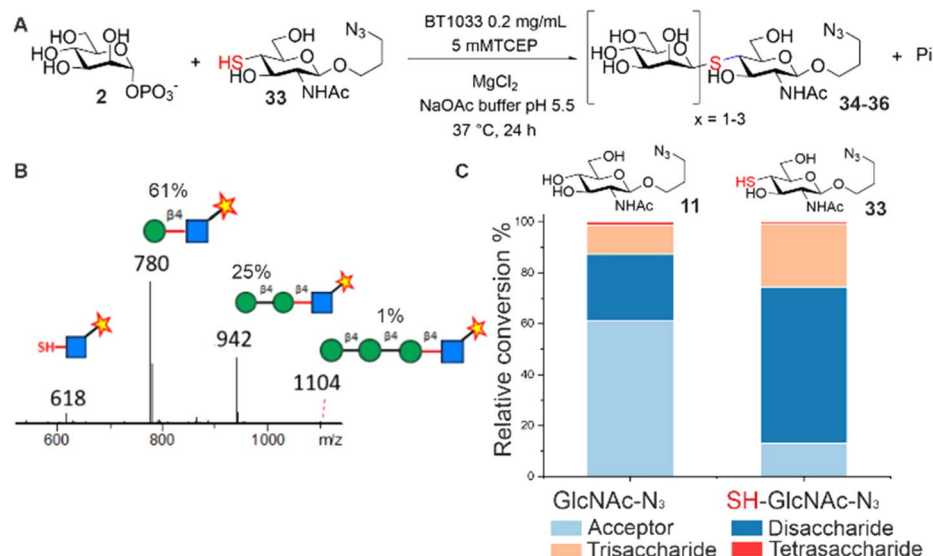


Fig. 3 (A) BT1033 turnover of thio-GlcNAc-N₃ **33** to produce the Manβ1,4-S-GlcNAc-N₃ **34** thioglycoside. Reaction mechanism depicted in the reverse phosphorolysis direction. (B) LC-MS analysis showing di-, tri- and tetrasaccharide thioglycoside formation. (C) Comparative product distribution in BT1033 reactions with **11** and **33**.

30 min, with only a 3-fold excess of Man-1P donor relative to acceptor and 0.33 mg mL⁻¹ of enzyme (Fig. S35A†). In contrast, under the same conditions only ~20% conversion of GlcNAc-N₃ **11** was observed, while ~40% was observed with 0.04 mg mL⁻¹ of enzyme (Fig. S35B†).

To further showcase the utility of BT1033 for chemo-enzymatic β-mannosylation we assembled a library of unnatural azidopropyl-linked Manβ1,4-GlcNAc glycans on a semi-preparative scale, including thioglycoside di, tri and tetrasaccharides (**34–36**) and fluorinated di, tri, tetra and pentasaccharides (**25–27**, **37**), in isolated yields ranging from 5% to 68%, (Table 1, ESI Section 8†). The structures of synthesised glycans were validated by 1D and 2D NMR and HRMS (ESI Sections 8 and 9†), with β-glycosidic linkages confirmed using IPAP HSQC, which measures each anomeric carbons ¹J_{CH} coupling constant (Tables S3 and S4†).²² Although similar trends were observed the isolated product yields differed from the relative conversions measured by MS, which is likely a reflection of the change in scale and challenges associated with purification of longer oligosaccharides. To validate BT1033

was able to operate irreversibly as a “reverse thiophosphorylase” we investigated the stability of our purified glycan library to BT1033 catalysed phosphorolysis (Fig. 4). As expected Manβ1,4-GlcNAc **15** underwent rapid phosphorolysis, with ~50% breakdown to acceptor **11** observed after 2 min and ~65% after 24 h. Intriguingly, although Manβ1,4-6F-GlcNAc **25** showed a greater proportion of phosphorolysis over 24 h compared to **15** (78% vs. 70% breakdown to acceptor respectively), a lower amount of phosphorolysis was observed at 2 min (15% vs. 50% breakdown to acceptor respectively). This slower rate of phosphorolysis may therefore account for the observed formation of C6-fluorinated tri-, tetra-, and pentasaccharide by reverse phosphorolysis. The presence of the 6F-GlcNAc moiety appeared to have minimal effect on breakdown of fluorinated trisaccharide **26** to disaccharide **25**, when compared to trisaccharide **16**, which contains the natural GlcNAc moiety. Notably, 6Cl-Man β1,4-GlcNAc **18** also showed a much lower proportion (~20%) of phosphorolysis-mediated product after 24 h compared to **15** (~65%). Again, potentially accounting for the accumulation of **18** in the reverse phosphorolysis reaction when

Table 1 Manβ1,4-GlcNAc-N₃ analogues produced on scale

Donor	Acceptor	Product	Yield (%)	Amount (mg)
4	11	6Cl-Manβ1,4-GlcNAc 18	68	2.6
2	11	Manβ1,4-GlcNAc-N ₃ 15	56	6.5
		Manβ1,4-Manβ1,4-GlcNAc-N ₃ 16	15	2.4
2	12	Manβ1,4-6F-GlcNAc-N ₃ 25	12	1.4
		Manβ1,4-Manβ1,4-6F-GlcNAc-N ₃ 26	13	2.1
		Manβ1,4-Manβ1,4-Manβ1,4-6F-GlcNAc-N ₃ 27	7	1.4
		Manβ1,4-Manβ1,4-Manβ1,4-Manβ1,4-6F-GlcNAc-N ₃ 37	5	1.3
2	33	Manβ1,4-S-GlcNAc-N ₃ 34	20	3.8
		Manβ1,4-Manβ1,4-S-GlcNAc-N ₃ 35	23	5.9
		Manβ1,4-Manβ1,4-Manβ1,4-S-GlcNAc-N ₃ 36	11	3.4

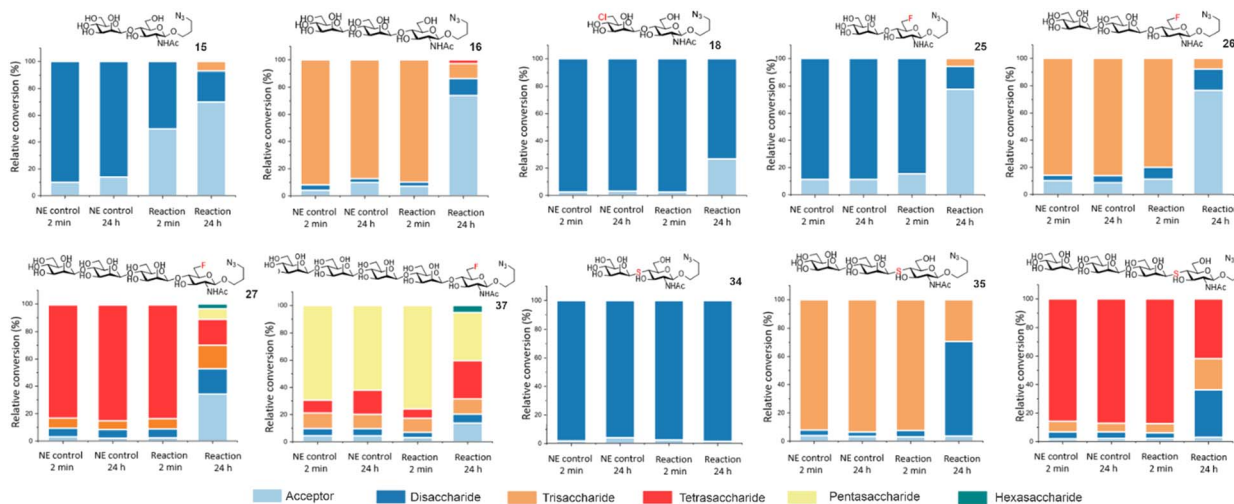


Fig. 4 Phosphorolysis of Man β 1,4-GlcNAc-N $_3$ analogues, with relative conversions determined by ITag LC-MS analysis. NE denotes: no enzyme control.

using **4**. As hypothesised Man β 1,4-*S*-GlcNAc **34** proved resistant to phosphorolysis, with no cleavage of the thioglycoside observed after 24 h indicating that the replacement of the alcohol nucleophile with a thiol in the acceptor does enable the phosphorolase to operate irreversibly in the synthetic direction. However, the presence of the Man β 1,4-*S*-GlcNAc thioglycoside linkage appears to have no effect on the extent of phosphorolysis of trisaccharide **35** to thioglycoside **34**, compared to the natural trisaccharide **16**, similar to observations for **26**. Tetrasaccharides containing the 6F functionality **27** and the thioglycoside linkage **36** respectively, were subjected to phosphorolysis and showed the expected breakdown to trisaccharide after 24 h. Whilst the phosphorolysis of **27** afforded a distribution of products (from acceptor to even longer glycans, indicating reverse phosphorolysis was occurring), the reaction with **36** halted as disaccharide accumulated due to the stability of the thioglycoside linkage. Finally, the 6F pentasaccharide **37**, similarly to **27**, afforded a distribution of products from acceptor to hexasaccharide, indicative of reverse phosphorolysis having occurred.

BT1033 belongs to the GH130 enzyme family, which includes β -mannoside phosphorylases MGP (4-*O*- β -D-mannosyl-D-glucose phosphorylase) and Uhgb_MP (β -1,4-mannosyl-*N*-glycan phosphorylase) from *Bacteroides* sp.^{13,23,24} Guided by structural studies of MGP and Uhgb_MP, GH130 catalysis is proposed to proceed through a “proton shuttle” mechanism (Fig. 5A). For the synthetic reaction, it is hypothesised that mannose in the -1 subsite, existing in an unstable B $_{2,5}$ boat conformation, is deprotonated by a catalytic Asp residue (Asp131 in MGP or Asp104 in Uhgb_MP) at the 3-OH which subsequently deprotonates the incoming GlcNAc acceptor *via* its 3-OH group.^{23,25} Amino acid sequence alignment of BT1033 and Uhgb_MP, identified Asp101 as the putative catalytic residue in BT1033 (Fig. S36†). Superimposition of a BT1033 alphafold model with the structure of Uhgb_MP in complex with β -D-mannose and phosphate, showed that BT1033 Asp101 overlaid with

Uhgb_MP Asp104, supporting this hypothesis (Fig. 5B). To reinforce this proposed role of Asp101 in BT1033 catalysis, we also produced a BT1033 D101A mutant and investigated the synthetic activity of the enzyme with both GlcNAc-N $_3$ **11** and SH-

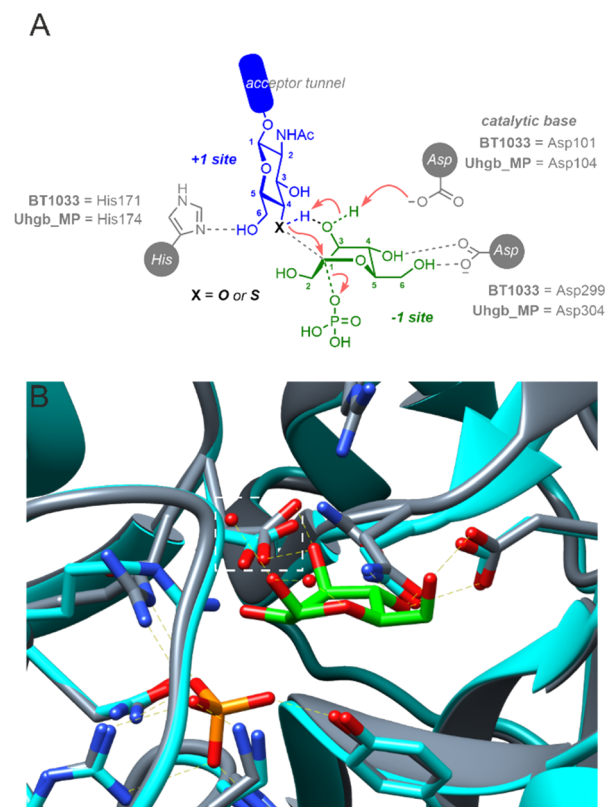


Fig. 5 (A) Proposed mechanism of BT1033. (B) Crystal structure of Uhgb_MP (4UDJ, grey) superimposed with a BT1033 alphafold model (turquoise), showing that BT1033 Asp101 overlays with Uhgb_MP Asp104 (orange box). Uhgb_MP structure shown in complex with β -D-mannose and phosphate.



GlcNAc-N₃ **33**, using the natural Man-1P **2** donor (Fig. S23†). As anticipated, BT1033 D101A displayed no activity towards GlcNAc-N₃ **11**, confirming that this residue is required for catalysis. Interestingly, no activity towards SH-GlcNAc-N₃ **33** was observed either implying that despite the lower pK_a of the thiol acceptor, deprotonation of the incoming nucleophile within the active site is still required. Previously the enzymatic synthesis of diverse thioglycosides using “thioglycoligases”, glycosidase mutants with their catalytic acid/base residues mutated to an alanine or glycine, have been achieved and extensively explored by the Withers group²⁶ and others.^{20,27} In contrast to thioglycoligases, we demonstrate here that the reverse thiophosphorylase activity of BT1033 is abolished in the absence of the catalytic base, thus suggesting that a deeper understanding of the proposed GH130 ‘proton shuttle’ mechanism may be required to aid the design of more efficient reverse thiophosphorylases.

In summary, we have demonstrated that BT1033 can be exploited to access diverse Manβ1,4-GlcNAc analogues, and longer β-mannan like glycans. We also establish novel reverse thiophosphorylase activity favouring the synthesis of longer glycans by initially catalysing the formation of a stable thioglycoside linkage. Following incorporation of unnatural functionality into the enzymatic building blocks through chemical synthesis, we systematically screened BT1033 for activity towards these unnatural donors and acceptors in a MS-based strategy using a “clickable” ITag to facilitate product ionisation and detection. BT1033 displayed activity towards C6-modified donors, most notably 6Cl-Man-1P **4**. Fluorinated acceptors were also turned over by the enzyme, and interestingly the presence of the fluorine appears to also facilitate extension of Manβ1,4-GlcNAc with Man to produce longer β-mannan like glycans, likely through slowing the rate of phosphorylation. Whilst enzymatic strategies for the synthesis of thioglycosides to date have focused on the exploitation of “thioglycoligases”,^{20,26,27} to our knowledge the use of a wildtype GP to synthesise thioglycosides has not been explored. If this “reverse thiophosphorylase” activity was generally applicable to other GH130 phosphorylases, it could provide simple yet dynamic access to a diverse range of thioglycosides. As Manβ1,4-GlcNAc has shown potential as an immune activator,¹⁰ the thioglycoside products of the reverse thiophosphorylase activity of BT1033 could have potential as non-hydrolysable β-mannose containing activators for immunotherapy. Furthermore, extension of the reverse thiophosphorylase approach to thiol substituted sugar-1P donors, could have potential utility in the construction of thiooligosaccharide homopolymers.

Data availability

The datasets supporting this article have been uploaded as part of the ESI.†

Author contributions

TK and NH screened donors and acceptors and performed chemoenzymatic synthesis. TK performed phosphorylation

experiments. TK, NH and JW performed protein production. JP, AW, SA, DW JBV and MG performed the chemical synthesis. MAF, GJM, CG and BL supervised the project. TK, NH, GM and MAF wrote the manuscript and designed the study. All authors analysed the data and commented on the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

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