# Chemoenzymatic synthesis of an unnatural Manβ1,4GlcNAc library using a glycoside phosphorylase with "reverse *thio*phosphorylase" activity

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**ABSTRACT:** β-Mannosides are ubiquitous in nature, with diverse roles in many biological processes. Notably, Manβ1,4GlcNAc a constituent of the core N-glycan in eukaryotes, was recently identified as a STING immune pathway 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 Manβ1,4GlcNAc 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 phosphorylysis-stable thioglycoside linkage, an approach that may be generally applicable to other phosphorylases.

β-Mannoside linkages are highly prevalent within the glycans[1] of plants, bacteria, protozoa and eukaryotes with diverse, essential roles in biological processes including energy storage[2] and cell wall biosynthesis.[3] Notably in eukaryotes the ubiquitous $ManGlcNAc_2$ motif within *N*-glycans[4] contains a Manβ1,4GlcNAc disaccharide, which was recently identified as a novel immune modulator in autoimmune disease.[5, 6] This Manβ1,4GlcNAc has shown potential as a new activator of STING (stimulator of interferon genes pathway), with the disaccharide triggering a broad immune response in macrophages.[5] STING is a component of the innate immune system and a key mediator of inflammation,[7] so small molecule activators are emerging as a promising strategy in cancer immunotherapy.[8]

Despite its striking biological significance and recent advances in the chemical synthesis of such linkages,[9] the efficient assembly of β-mannosides still remains one of the major challenges in glycoscience. Chemoenzymatic strategies have therefore emerged as a complementary approach, employing chemical synthesis to furnish glycosyl donors and acceptors before protecting group free enzyme-mediated glycosylation. Enzymatic β-mannoside bond formation is exemplified through the use of glycosyl transferases (GTs) such as Alg1, which has been used in the synthesis of $ManGlcNAc_2$.[10] However, despite exquisite stereo- and regioselectivity, the requirement for both a complex Leloir glycosyl donor (GDP-Man) and a complex acceptor in lipid-linked $GlcNAc_2$-PP-Dolichol, limits the GT's utility. Glycoside phosphorylases (GPs) which catalyse glycosidic bond breakdown (phosphorylysis), offer an attractive alternative approach for enzymatic β-mannosylation. This is because they can be harnessed in a "reverse phosphorylysis" synthetic direction (Figure 1A), requiring only simple sugar-1-phosphate donors, and have shown promiscuity with unnatural substrates.[11, 12]

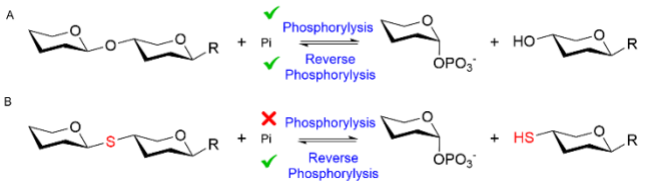


Figure 1. The reversible GP catalyzed reaction (A). Proposed irreversible "reverse thiophosphorylase" activity with a 4SH-thiol acceptor (B).

Herein we report the chemoenzymatic synthesis of a library of unnatural Manβ1,4GlcNAc analogues, using a GP-mediated strategy.[13] We incorporate unnatural functionality into the enzymatic building blocks through chemical synthesis and show that when fluorine is present in the GlcNAc acceptor, this facilitates further extension of Manβ1,4GlcNAc with Man producing longer β-mannan like glycans. Using the same GP, we pioneer "reverse thio- phosphorylase" enzymatic activity, favouring the synthesis of longer glycans by initially catalysing the formation of a phosphorylysis-stable thioglycoside linkage (Figure 1B). This approach represents a benchmark for the utility of GPs for thioglycoside synthesis, and here specifically affords access to a series of

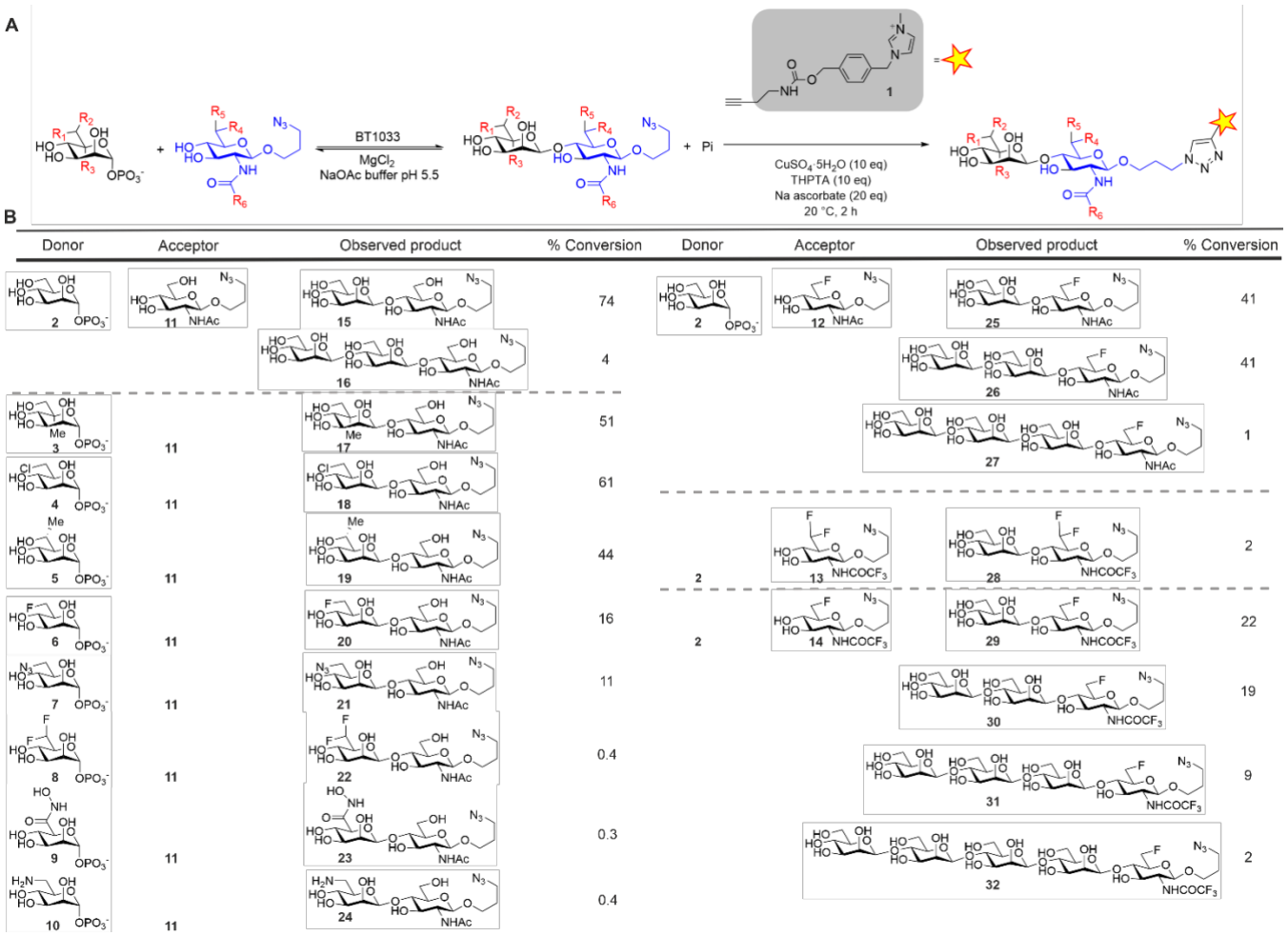


Figure 2A. ITag screening methodology for BT1033 reactions. Reaction mechanism depicted in reverse phosphorylysis direction. B. BT1033 activity towards unnatural donors and acceptors.[12, 14]

novel, unnatural Manβ1,4-GlcNAcs with direct potential as STING-based activators for immunotherapy.

For the synthesis of our Manβ1,4GlcNAc 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 Manβ1,4GlcNAc by reverse phosphorylysis. To investigate the substrate promiscuity of BT1033, we screened a series of chemically synthesised Man1-P donors (**3-10**) and GlcNAc acceptors (**11-14**) (Figure 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 (Figure 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.[15] To facilitate the detection of the Manβ1,4GlcNAc 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-chromatography coupled to mass spectrometry (LC-MS). The conversion of starting material to product was determined by comparing the relative ionisation intensities of the unreacted azido-propyl linked GlcNAc (GlcNAc-$N_3$) acceptor to the azido-propyl linked Manβ1,4GlcNAc products (Figure 1B, Supporting information section 5).

First, we assessed the suitability of GlcNAc-$N_3$, **11** as an acceptor mimic for BT1033, with Man1-P **2** as a donor. LC-MS analysis showed an ion consistent with the mass of the Manβ1,4GlcNAc-ITag disaccharide (*m/z* 764) as expected (Figure S11). Additionally, we observed an ion consistent with the mass of the Manβ1,4GlcNAc-ITag disaccharide + 162 Da (*m/z* 926). BT1033 was previously shown to have weak synthetic activity with D-mannose as an acceptor, when using Man-1P as a donor.[13] Additionally, the enzyme could use chitobiose as an acceptor, demonstrating that it is capable of producing longer-glycans. Therefore, we proposed that the product at *m/z* 926 was a $Man_2$β1,4GlcNAc-ITag trisaccharide. Overall, we observed 74% conversion to disaccharide **15** and 4% to trisaccharide **16** (Figure 2B). Next, we screened BT1033 for activity towards eight unnatural Man-1P analogues (**3-10**), with acceptor **11** (Figure 2B, Figure S3-S10). C6-Chloro Man-1P **4** was best tolerated by BT1033, with 61% conversion to disaccharide observed after 24 h (Figure S3). Moderate conversions of C5-methyl Man-1P **3** and C6-methyl Man-1P **5** to disaccharide were also observed at 51% and 44%, respectively (Figure S4-S5). Conversion of C6-fluoro Man-1P

**6** and C6-azido Man-1P **7** to disaccharide was significantly lower at 16% and 11% respectively (Figure S6, S8), suggesting that these were poor substrates for the enzyme. No conversion of C6-gem-difluoro Man-1P **8** was observed, which was not surprising considering the poor turnover of **6**. Additionally, no turnover of C6-hydroxamic acid Man-1P **9** or C6-amine Man-1P **10** were observed (Figure S7, S9-S10). There was no evidence of longer glycan chain formation when using any of the unnatural Man-1Ps. Overall, these findings suggest that BT1033 has little or no activity towards C6-modified analogues with groups larger than the native $CH_2OH$. While poor turnover of C6-azido **7** and C6-amine **10** Man-1Ps was observed, the chlorine in disaccharide **18** could allow for further derivatization at the C6-position to an azide or amine.

Next, we screened for activity towards fluorinated GlcNAc-$N_3$ acceptors **12-14**, with Man-1P **2** (Figure 2B). Fluorination, while having little effect on the overall conformation of a glycan,[16] is known to affect stereo-electronic properties and can therefore modulate biological function.[17] 6F-GlcNAc-$N_3$ **12** and 6F-GlcNTFA-$N_3$ **14** were well tolerated by the enzyme, producing 41% and 22% conversion to disaccharide respectively. Additionally, we observed masses consistent with the production of longer mannan-type glycans. For example, with **12** we observed products consistent with disaccharide (*m/z* 766, Manβ1,4-6F-GlcNAc-ITag, 41%), trisaccharide (*m/z* 928, $Man_2$β1,4-6F-GlcNAc-ITag, 41%) and tetrasaccharide (*m/z* 1090, $Man_3$β1,4-6F-GlcNAc-ITag, 1%) formation (Figure S12). With **14**, in addition to the expected disaccharide (*m/z* 820, Manβ1,4-6F-GlcNTFA-ITag, 22%) we observed trisaccharide (*m/z* 982, $Man_2$β1,4-6F-GlcNTFA-ITag, 19%), tetrasaccharide (*m/z* 1144, $Man_3$β1,4-6F-GlcNTFA-ITag, 9%) and pentasaccharide (*m/z* 1306, $Man_4$β1,4-6F-GlcNTFA-ITag, 2%, Figure S14). In contrast, only low-level conversion of 6,6-diF-GlcNTFA **13** to disaccharide (2%) was observed (Figure S13). Taken together, this data indicates BT1033 can tolerate acceptors with fluorination at C6 position and within the NAc substituent. Increasing the number of fluorines in the acceptor resulted in poorer turnover by BT1033, with such presence in carbohydrate substrates previously shown to reduce the catalytic efficiency of some enzymes. [18] However, the presence of fluorine in the acceptor interestingly appeared to facilitate the formation of longer glycans by BT1033, when compared to GlcNAc-$N_3$ **11**. We hypothesized that fluorination in the acceptors may reduce the rate of the competing phosphorylysis reaction, altering the reaction equilibrium and resulting in an accumulation of the reverse phosphorylysis disaccharide product, which could subsequently serve as an acceptor for further mannosylation using **2**. To investigate this further, we tested BT1033 for activity with a 4-SH-GlcNAc-$N_3$ analogue **33** and compared this to its activity towards **11** under the same conditions (Figure 3). We anticipated that the reaction would yield a Manβ1,4-*S*-GlcNAc-$N_3$ **34** thioglycoside (Figure 3A). Thioglycosides are carbohydrate mimetics that are often resistant to hydrolysis and have elicited significant interest in recent years as probes for structural and biological studies, and as enzyme inhibitors.[19, 20] We hypothesized that if BT1033 was able to use a thiol as an acceptor with **2** (in the synthetic direction) the reaction may become irreversible due to the stability of the resultant thioglycoside to phosphorylysis.

**Table 1. Manβ1,4-GlcNAc-$N_3$ analogues produced on scale**

Following LC-MS analysis of reactions with **33** under disulfide reducing conditions, we observed masses consistent with the expected disaccharide (*m/z* 780, Manβ1,4-*S*-GlcNAc-ITag), as well as trisaccharide (*m/z* 942, $Man_2$β1,4-*S*-GlcNAc-ITag) and tetrasaccharide (*m/z* 1104, $Man_3$β1,4-*S*-GlcNAc-ITag) formation (Figure 3B). Overall, there was a greater proportion of reverse phosphorylysis product (i.e disaccharide) at the end of the reaction using **33**, compared with **11** (Figure 3C).

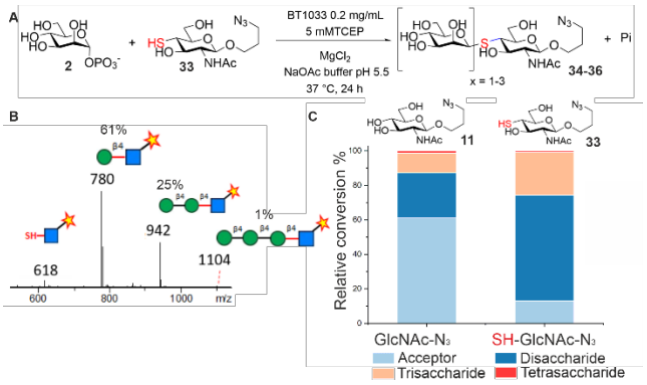


Figure 3A BT1033 turnover of thio-GlcNAc-$N_3$ **33** to produce the Manβ1,4-*S*-GlcNAc-$N_3$ **34** thioglycoside. Reaction mechanism depicted in the reverse phosphorylysis direction. B. LC-MS analysis showing di-, tri- and tetrasaccharide thioglycoside formation. C. Comparative product distribution in BT1033 reactions with **11** and **33**.

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 phosphorylysis resistant Manβ1,4-*S*-GlcNAc-$N_3$ **34**.

To showcase the utility of BT1033 for chemoenzymatic 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, Supporting information section 8). To validate BT1033 was able to operate irreversibly as a "reverse thiophosphorylase" we investigated the stability of our glycan library to BT1033 catalysed phosphorylysis (Figure 4). As expected Manβ1,4-GlcNAc **15** underwent rapid phosphorylysis, 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 phosphorylysis over 24 h, a significantly lower amount of phosphorylysis was observed at 2 min compared to **15**. This slower rate of phosphorylysis may therefore account for the observed formation of C6-fluorinated tri-, tetra-, and pentasaccharide by reverse phosphorylysis; particularly as 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** containing the natural GlcNAc moiety. Notably, 6Cl-Man β1,4-

| 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 |

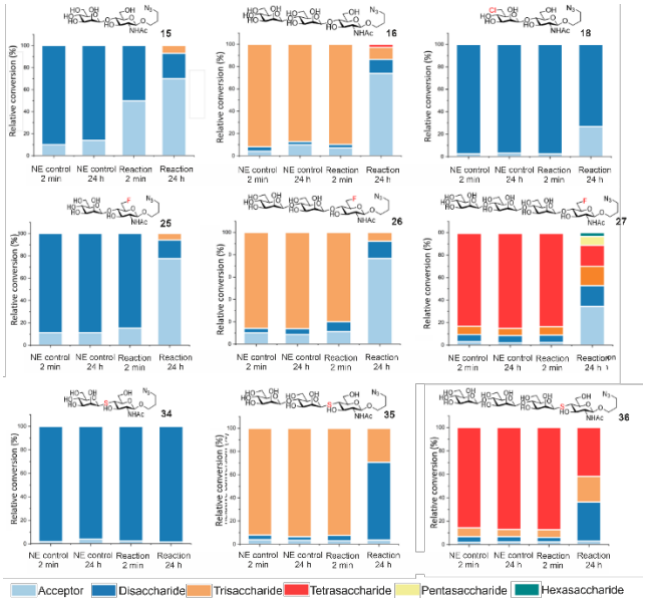


Figure 4. Phosphorylysis of Manβ1,4-GlcNAc-$N_3$ analogues, with conversions determined by ITag LC-MS analysis.

GlcNAc **18** also showed a much lower proportion (~20%) of phosphorylysis-mediated product after 24 h compared to **15** (~65%). Again, potentially accounting for the accumulation of **18** in the reverse phosphorylysis reaction when using **4**. As hypothesized, Manβ1,4-S-GlcNAc **34** proved resistant to phosphorylysis, 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 phosphorylase to operate irreversibly in the synthetic direction. However, the presence of the Manβ1,4-*S*-GlcNAc thioglycoside linkage appears to have no effect on the extent of phosphorylysis of trisaccharide **35** to thioglycoside **34**, compared to the natural trisaccharide **16**, similar to observations for **26**. Finally, tetrasaccharides containing the 6F functionality **27** and the thioglycoside linkage **36** respectively, were subjected to phosphorylysis and showed the expected breakdown to trisaccharide after 24 h. Whilst the phosphorylysis of **27** afforded a distribution of products (from acceptor to even longer glycans, indicating reverse phosphorylysis was occurring), the reaction with **36** halted as disaccharide accumulated due to the stability of the thioglycoside linkage.

In summary, we have demonstrated that BT1033 can be exploited to access diverse Manβ1,4-GlcNAc analogues, and longer β-mannan like glycans. We have incorporated unnatural functionality into the enzymatic building blocks through chemical synthesis, enabling a systematic approach in screening 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**. While fluorinated acceptors were also converted by the enzyme, the presence of the fluorine appears to facilitate further extension of Manβ1,4-GlcNAc with Man to produce longer β-mannan like glycans, likely through slowing the rate of phosphorylysis. Additionally, the novel demonstration of "reverse thiophosphorylase" activity is presented, also favouring the synthesis of longer glycans by initially catalysing the formation of a stable thioglycoside linkage. To date, the enzymatic synthesis of diverse thioglycosides using "thioglycoligases", glycosidase mutants with their catalytic acid/base residues mutated to an alanine or glycine, have been extensively explored by the Withers group[21] and others[19, 22]. However, to our knowledge the use of a wildtype GP to synthesise thioglycosides has not been explored. If this "reverse thiophosphorylase" activity were generally applicable to other GH130 phosphorylases, it could provide a very simple yet dynamic access to a diverse range of thioglycosides. Here specifically, we have produced a series of novel, unnatural Manβ1,4-GlcNAc containing glycans, with potential as non-hydrolysable STING-based activators for immunotherapy.

## ASSOCIATED CONTENT

**Supporting Information**. This material is available free of charge via the Internet at http://pubs.acs.org." Experimental procedures, chemical synthesis, chemoenzymatic synthesis, LC-MS and NMR data (PDF).

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### Author Contributions

‡TK and NH contributed equally. TK and NH screened donors and acceptors and performed chemoenzymatic synthesis. TK performed phosphorylysis 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 and MAF wrote the manuscript and designed the study. All authors analysed the data and commented on the manuscript.

### Notes

The authors declare no competing financial interest.

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