Bisubstrate UDP–peptide conjugates as human O-GlcNAc transferase inhibitors

Vladimir S. BORODKIN*, Marianne SCHIMPL*, Mehmet GUNDOGDU*, Karim RAFIE*, Helge C. DORFMUELLER*†, David A. ROBINSON‡ and Daan M. F. van AALTEN*†[1]

*MRC Protein Phosphorylation und Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.
†Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.
‡Drug Discovery Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.

Inhibitors of OGT (O-GlcNAc transferase) are valuable tools to study the cell biology of protein O-GlcNAcylation. We report OGT bisubstrate-linked inhibitors (goblins) in which the acceptor serine in the peptide VTPVSTA is covalently linked to UDP, eliminating the GlcNAc pyranoside ring. Goblin1 co-crystallizes with OGT, revealing an ordered C_3 linker and retained substrate-binding modes, and binds the enzyme with micromolar affinity, inhibiting glycosyltransfer on to protein and peptide substrates.

Key words: bisubstrate analogue inhibitor, glycosyltransferase, O-GlcNAc, rational drug design.

INTRODUCTION

Reversible post-translational modification of nuclear and cytoplasmic proteins with β-linked O-GlcNAc in metazoa is involved in numerous signal transduction cascades that regulate almost every cellular process [1–3]. O-GlcNAc cycling is governed by a pair of antagonistic enzymes existing as single-copy genes in two to three splice variants, namely OGT (O-GlcNAc:polypeptidyl transferase) and OGA (O-GlcNAc hydrolase). An ogt gene knockout in mice has been shown to be lethal at the embryonic level [4]. Aberrant O-GlcNAc profiles on certain proteins are associated with the onset and progression of neurodegenerative disease [5]. Existing inhibitors of OGA have been used to induce cellular hyper-O-GlcNAcylation both *in vitro* and *in vivo* [6]. Conversely, in-depth elucidation of the biological implications of cellular hypo-O-GlcNAcylation is hampered by the dearth of suitable effectors of OGT. To date, only a limited number of OGT inhibitors have been reported, all targeting the UDP-GlcNAc-binding site. The compound BZX {4-methoxyphenyl 6-acetyl-2-oxobenzo[d]oxazole-3(2H)-carboxylate}, proposed to be a neutral pyrophosphate mimic, was identified as a cell-permeant irreversible inhibitor of hOGT (where h denotes human) [7]. The mechanism of hOGT inhibition with BZX involves cross-linking of the active-site residues Lys[842] and Cys[917] with an S-thiocarbamate link [8]. A unique approach to hOGT inhibition was reported previously [9], employing cell-penetrant per-acetylated 4Ac-5S-GlcNAc (2-acetamido-2-deoxy-5-thio-D-glucopyranose). Once inside the cell, this compound is deacetylated by non-specific esterases producing the free thiosugar 5S-GlcNAc, which is then a substrate for the UDP-GlcNAc biosynthetic pathway, leading to incorporation of the thiosugar into the OGT donor substrate analogue UDP-5S-GlcNAc. The latter inhibits hOGT *in vitro* ($K_i = 8 \mu$M) and *in vivo*, although the basis for

selectivity over other GlcNAc transferases remains to be fully explained [9]. A set of non-hydrolysable substrate analogues including the glycosyl thiophosphate (UDP-S-GlcNAc) and C-glycosylphosphonate (UDP-C-GlcNAc) have also been reported [10]. Although these compounds moderately inhibited hOGT, they are not expected to be selective probes. In common with many other glycosyltransferases, OGT is subject to product inhibition, and, accordingly, the most potent inhibitor of hOGT reported to date is the reaction product UDP (K_d 0.5 μM) [11]. However, this product inhibition is difficult to exploit for inhibitor design. Not only would the charged nature of the compound render it cell-impermeant, but, in addition, UDP is a central metabolite involved in nucleic acid (RNA) biosynthesis, as well as in the activation of monosaccharides as glycosyl donors for glycoconjugate biosynthesis (six out of the nine mammalian nucleotide sugars are UDP-sugars). UDP analogues consequently have the potential to interact with diverse classes of enzymes such as oxidoreductases, transferases, hydrolases, lyases and isomerases. Bisubstrate inhibitors would offer a means of engineering selectivity by capitalizing on the specific combination of substrates.

Recent structural snapshots of ternary hOGT complexes with substrate/product analogues have uncovered invaluable insights into the hOGT catalytic mechanism [11,12]. Upon binding of the acceptor substrate, hitherto solvent-exposed parts of the bound UDP-GlcNAc engage directly with the peptide backbone, aligning the incoming nucleophile and the anomeric carbon in a glycosyltransfer-competent conformation. This conformation also brings together the acceptor serine and the pyrophosphate moiety, an interaction that has been proposed to be essential for catalysis [11]. In the present study, we exploit these data for the rational design of new OGT bisubstrate inhibitors that combine elements of both substrates; an approach expected to result in selective inhibition of the enzyme compared with inhibitors limited to targeting the donor-binding site alone.

MATERIALS AND METHODS

Chemical synthesis

Inhibitors were synthesized as outlined in schemes 1–3; for a full description, see the Supplementary Online Data at http://www. biochemj.org/bj/457/bj4570497add.htm. Characterization by NMR and MS, **14a**: ^{31}P NMR (202 MHz, ^{2}H$_2$O) δ -11.08 (d, J_{P_α,P_β} 26.6 Hz), -11.56 (d, J_{P_α,P_β} 26.6 Hz); HRMS (high-resolution MS) (m/z) $[M-H]^- = 1157.4171$. **14b**: ^{31}P NMR (202 MHz, ^{2}H$_2$O) δ -10.97 (d, J_{P_α,P_β} 27.2 Hz), -11.53 (d, J_{P_α,P_β} 27.2 Hz); HRMS (m/z) $[M-H]^- = 1171.4343$.

Protein crystallography

Hanging-drop crystallization experiments with drops containing 2 μl of reservoir solution [1.45 M K$_2$HPO$_4$, 10 mM EDTA and 1% (w/v) xylitol] and 2 μl of 100 μM hOGT-(312–1031) (purified as described previously [11]) and 1 mM goblin1 (**14a**) in 20 mM Tris/HCl (pH 8.5) and 0.5 mM THP [tris-(3-hydroxypropyl)phosphine] gave hexagonal rod-shaped crystals within 3–4 days at 22°C. Crystals were cryoprotected by 2 s of immersion in a saturated Li$_2$SO$_4$ solution before flash-freezing in liquid nitrogen. Diffraction data were collected at Diamond Light Source beamline I03. Data were processed with Xia2 and scaled to 3.15 Å (1 Å = 0.1 nm) using SCALA [13]. The structure was solved by molecular replacement with PDB code 4AY5 and refined with REFMAC [14]. Model building was performed with Coot [15]. Ligand topology was provided by PRODRG [16].

In vitro glycosylation of hTAB1

Reaction mixtures containing 1 μM TAB1-(7–402) protein [TAB1 is TGF (transforming growth factor)-β-activated kinase-binding protein 1] (purified as described previously [17]), 0.125 μM hOGT-(312–1031) and 10 μM UDP-GlcNAc in a buffer of 50 mM Tris/HCl (pH 7.5) and 1 mM DTT were incubated at 37°C for 90 min, separated by SDS/PAGE (10% gels) and transferred on to nitrocellulose membranes. Membranes were probed with a TAB1-gSer^{395} (O-GlcNAcylated Ser^{395}) O-GlcNAc site-specific antibody [18], followed by an IR800-labelled secondary antibody and analysed using a LI-COR Odyssey scanner and associated quantification software. Data were fitted to a four-parameter equation for dose-dependent inhibition using GraphPad Prism 5.0.

Steady-state kinetics

Reactions contained 50 nM hOGT-(312–1031) in 50 mM Tris/HCl (pH 7.5), 0.1 mg/ml BSA, 10 μM sodium dithionate and 10 μM peptide (KKENSPAVTPVSTA) and various amounts of inhibitors in a total volume of 100 μl. Reaction mixtures were pre-incubated for 15 min and started by addition of UDP-GlcNAc to a final concentration of 3.2 μM. After 30 min of incubation at 22°C, assays were stopped by adding 200 μl of 25 mM Hepes (pH 7.4), 10 mM NaCl, 50% (v/v) methanol and 15 μM fluorophore, a UDP-sensitive xanthene-based Zn(II) complex prepared as described in [19,20]. Product formation was detected fluorimetrically on a Gemini EM fluorescence microplate reader (Molecular Devices) at excitation and emission wavelengths of 485 nm and 530 nm respectively. A non-linear regression curve fit was performed with Prism.

Biolayer interferometry

Measurements were made on a ForteBio Octet RED384 instrument at 25°C. Biotinylated hOGT-(312–1031) was prepared at 25 μg/ml in TBS (25 mM Tris/HCl, pH 7.5, and 150 mM NaCl) buffer containing 1 mM DTT and immobilized on superstreptavidin biosensors. Free streptavidin sites were blocked by incubation with biocytin. A parallel set of superstreptavidin biosensors were prepared with biotinylated streptavidin to act as a control. The assay was carried out in 384-well plates with a sample volume of 100 μl. Inhibitor solutions were prepared from solid stocks and dissolved in assay buffer and a concentration series of 3-fold dilutions from a top concentration of 100 μM was created. Cycles for analysis involved obtaining a 30 s baseline followed by a 60 s association step and a 120 s dissociation step. The assay was repeated with the reference biosensors to correct for non-specific interactions and the entire assay was repeated in triplicate for both compounds. Data were processed and kinetic parameters were calculated using ForteBio software.

RESULTS AND DISCUSSION

Inspection of the ternary hOGT–UDP-5S-GlcNAc–TAB1 peptide complex [11] shows that the anomeric carbon of the sugar is positioned at a distance of 3.4 Å from the modified peptide side chain (Figure 1A). Biophysical determination of the binding affinity for product and substrate revealed a 30-fold higher K_d for UDP-GlcNAc than for UDP [11], suggesting that, in the absence of an acceptor, the sugar moiety does not positively contribute to the binding affinity.

We consequently envisaged a set of bisubstrate OGT inhibitors in the form of UDP–peptide conjugates in which UDP is coupled to a variable peptide subunit by a suitable linker. The weakly binding GlcNAc moiety was omitted from the inhibitor structure and replaced with a linear three- or four-carbon tether to retain the spatial arrangement of UDP and peptide as observed in the Michaelis complex. The resulting compounds were named goblin1 (OGT bisubstrate-linked inhibitor 1) and goblin2 respectively.

Synthesis of the target compounds entailed the preparation of phosphorylated 'stretched serine' derivatives (Scheme 1) suitable for Fmoc solid-phase peptide synthesis and peptide assembly followed by reaction of the phosphorylated peptides with activated nucleoside monophosphate (Scheme 2). Synthesis of analogous peptide–nucleotide polyphosphate conjugates as protein kinase bisubstrate inhibitors using an on-resin phosphorylation–pyrophosphorylation protocol has been reported previously [21]. In the first instance, we opted for off-resin pyrophosphorylation for the reason of operational and analytical convenience. We established a robust, albeit moderately yielding, two-step procedure for the preparation of the key 'stretched serine' allyl esters **3a/b** by alkylation [22] of the dianion generated from the commercially available N-Boc (N-t-butoxycarbonyl) serine **1** with MP (p-methoxyphenyl)-protected 3-bromopropan-1-ol or 4-bromobutan-1-ol **2a/2b** [NaH, DMF (dimethylformamide)] followed by esterification with allyl bromide/DIPEA (N,N-di-isopropylethylamine) (Scheme 1). Attempted alkylation of **1** under phase-transfer conditions [23] was unsuccessful, whereas more elaborate synthetic schemes proved to be inefficient. Ensuing N-protective group remodelling as well as removal of the terminal hydroxy group protection (**3a/3b→4a/4b→5a/5b**) was ensured by the perfect orthogonality of the MP protecting group, resulting in nearly quantitative yield for three steps. Finally, installation of the phosphate group with acid-labile MBn (p-methylbenzyl) protection and deblocking of the C-terminus yielded the required phosphorylated 'stretched serine' building blocks **8a/8b** with very good overall efficiency. Careful choice of the phosphate protecting group was essential, as the traditional
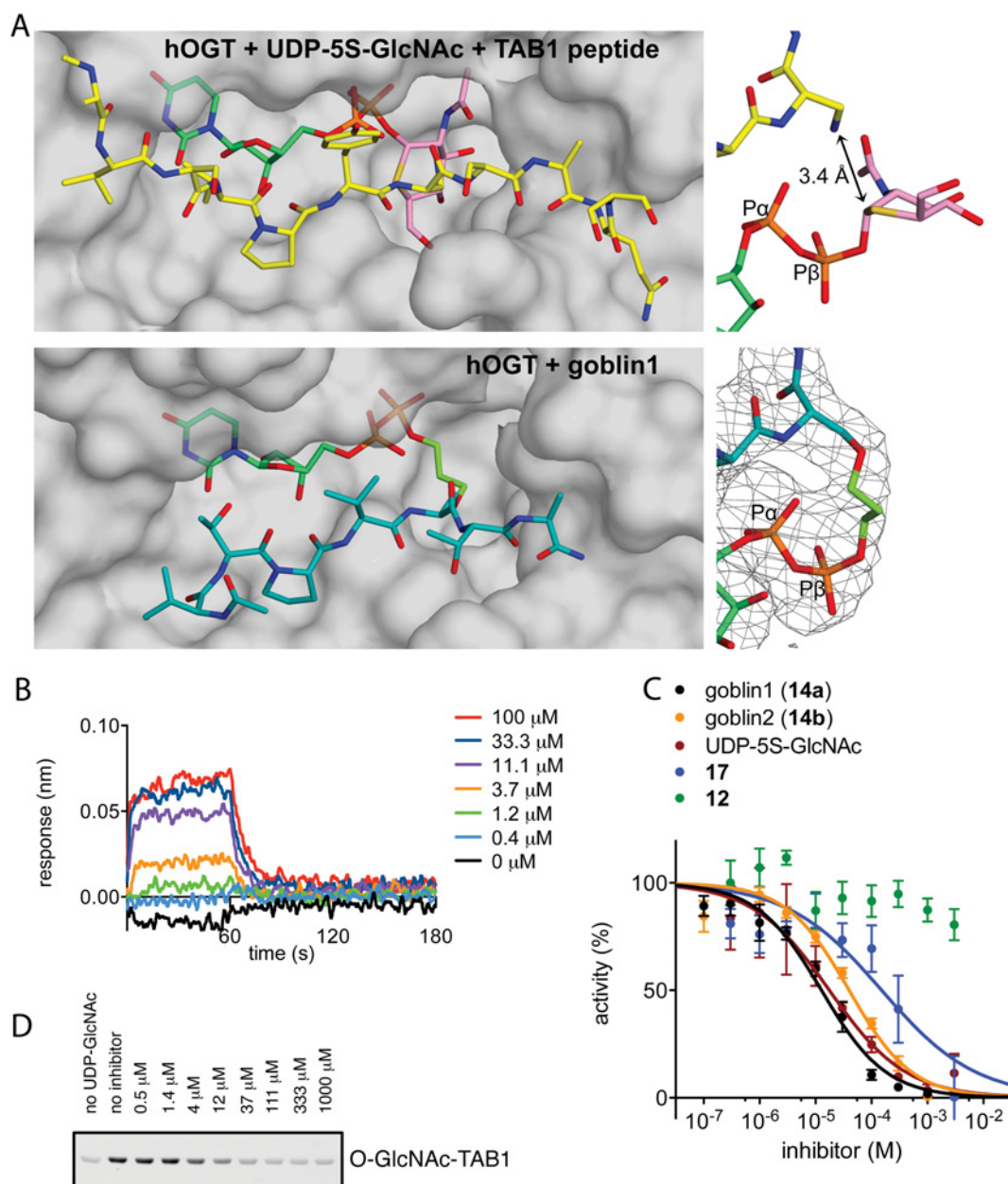
Figure 1   Analysis of goblin1 binding and inhibition

(**A**) Structure of hOGT Michaelis complex [11] (PDB code 4AY6) and the hOGT–goblin1 complex. hOGT is shown as a molecular surface. The donor substrate UDP-5S-GlcNAc (pink/turquoise carbon atoms), the acceptor peptide (yellow carbon atoms) and goblin1 (green carbon atoms) are shown as stick models. On the right, a close-up view of the compounds is shown, centred on the linker incorporated in goblin1. For the hOGT–goblin1 complex, an unbiased (i.e. before incorporating of any ligand) four-fold NCS (non-crystallographic symmetry) averaged $F_o - F_c$ electron density map is shown, contoured at 3.5σ. Full goblin1 $F_o - F_c$ maps are shown in Supplementary Figure S3 at http://www.biochemj.org/bj/457/bj4570497add.htm. (**B**) Biolayer interferometry was used to measure binding affinity of hOGT for goblin1 (K_d 7.9 μM). A representative binding profile is shown, obtained from a concentration series of goblin1 interacting with hOGT. Further data and curve fits are shown in Supplementary Figure S1 at http://www.biochemj.org/bj/457/bj4570497add.htm. (**C**) IC_{50} determination of goblin1 and goblin2 in comparison with the inhibitor UDP-5S-GlcNAc and fragments of the bisubstrate inhibitor, compounds **12** and **17**. Data points are means $\pm$ S.E.M. for three experiments. (**D**) Goblin1 inhibition of glycosylation of substrate protein TAB1, as detected by anti-O-GlcNAc Western blot. Densitometric analysis and IC_{50} determination are shown in Supplementary Figure S2 at http://www.biochemj.org/bj/457/bj4570497add.htm.

dibenzyl phosphate protection failed to be cleanly removed under the peptide cleavage conditions. Full details of the synthesis are given in the Supplementary Online Data.

Peptide synthesis was performed on a microwave-assisted CEM Liberty instrument using RinkAmide MBHA (4-methyl-benzhydrylamine) resin and HCTU [O-(1H-6-chlorobenzo-triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] as the coupling agent (for details, see the Supplementary Online Data). The synthesis of the UDP–peptide conjugates

14a/b is illustrated in Scheme 2. Crude phosphopeptides **11a/b** were reacted with a 10-fold excess of 2′,3′-O-diacetyl-UMP imidazolide prepared from triethylammonium salt **13** and CDI (1,1′-carbonylbisimidazole) overnight, deacetylated with methanol/triethylamine/water and finally purified by reverse-phase HPLC to furnish the targeted UDP–peptide conjugates **14a** (goblin1) and **14b** (goblin2) in fair yield. The identity of the newly synthesized compounds was confirmed by TOF–ESI–MS and NMR spectroscopy.
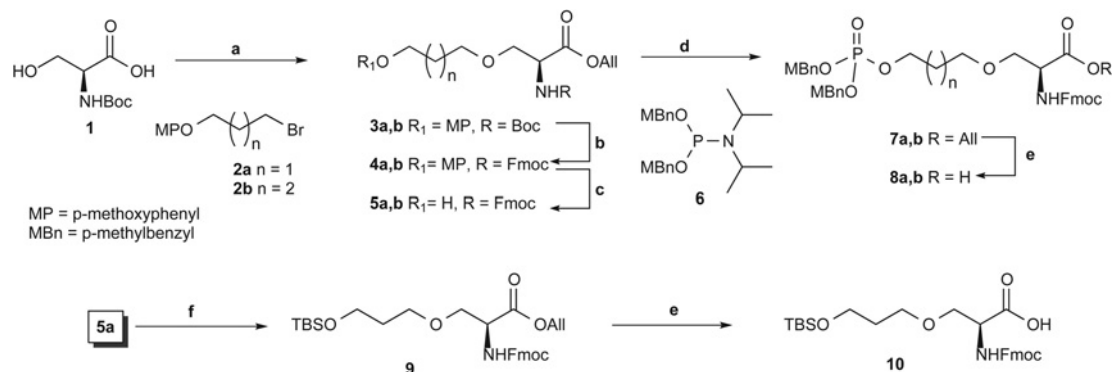
Scheme 1   Synthesis of the 'stretched serine' building blocks

(a) (i) NaH, Bu4NI (tetra-n-butylammonium iodide), **2a** or **2b**, DMF, 0°C then room temperature, 16 h, (ii) AllBr (allyl bromide), DIPEA, DMF, room temperature, 16 h, 30% for two steps; (b) (i) 95% TFA (trifluoroacetic acid), water, DCM (dichloromethane), room temperature, 1 h, (ii) FmocCl (Fmoc chloride), DIPEA, DCM, room temperature, 16 h, 90% for two steps; (c) CAN [ammonium cerium(IV) nitrate], acetonitrile, THF (tetrahydrofuran), water, room temperature, 1 h, 92%; (d) **6**, 4,5-dicyanoimidazole, acetonitrile, room temperature, 1 h then mCPBA (m-chloroperoxybenzoic acid), 0°C, 1 h, 85%; (e) Pd[(PPh₃)]₄ [tetrakis(triphenylphosphine)palladium(0)], morpholine, THF then Dowex 50WX8-100 H⁺, 100%; (f) TBSCl (t-butyl dimethylchlorosilane), DIPEA, DMAP (4-dimethylaminopyridine), DCM, room temperature,16 h, 92%. Boc, t-butoxycarbonyl.
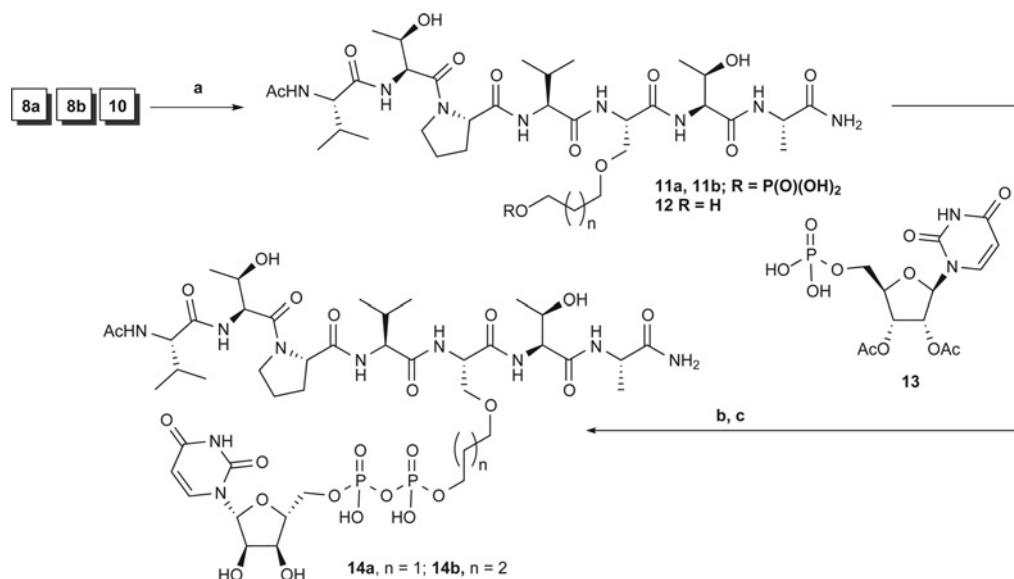
**Scheme 2   Synthesis of UDP–peptide conjugates and 'linker-only' peptide**

(a) Microwave-assisted Fmoc SPPS (solid-phase peptide synthesis); (b) (i) **13**, CDI (1,1'-carbonylbisimidazole), DMF (dimethylformamide), room temperature, 20 h then methanol then **8a/8b**, room temperature 16 h; (c) methanol, triethylamine, water, room temperature, 16 h then HPLC.

To investigate the contribution of the separate parts of the UDP–peptide conjugate towards inhibition potency, we synthesized 'linker-only' peptide **12** using 'stretched serine' building block **10** (Scheme 1). We also prepared the matching 'linker-only' UDP analogue **17** starting from 3-methoxypropanol taking advantage of the one-pot/three-step pyrophosphorylation procedure mediated by di-(*p*-methoxybenzyl)-*N*,*N*-di-isopropylphosphoramidite **16** [24] (Scheme 3).

The affinity of goblin1 and goblin2 for hOGT was evaluated by biolayer interferometry (Figure 1B, and Supplementary Figure S1 at http://www.biochemj.org/bj/457/bj4570497add.htm) yielding K_d values of 7.9 and 4.9 μM respectively. *In vitro* glycosylation of a peptide substrate was inhibited in a dose-dependent manner with an IC$_{50}$ value of 18 μM for goblin1 and 40 μM for goblin2 (Figure 1C). Furthermore, the ability of goblin1 to inhibit O-GlcNAcylation of a well-characterized human substrate protein, TAB1 [18], was investigated by Western blotting employing

a TAB1 O-GlcNAc Ser[395] site-specific antibody (Figure 1D, and Supplementary Figure S2 at http://www.biochemj.org/bj/457/bj4570497add.htm). Dose-dependent inhibition of hOGT activity was observed, and densitometric quantification allowed the calculation of an IC$_{50}$ value of 8 μM (Supplementary Figure S2). In a set of control experiments with 'linker-only' compounds **12** and **17**, we observed only weak hOGT inhibition by **17** (IC$_{50}$ 300 μM) whereas 'linker-only' peptide **12** was proved to be neither a substrate nor an inhibitor (Figure 1C). Attempts to inhibit OGT in cells appeared unsuccessful, probably due to the size/negative charge of the compounds.

To confirm the binding mode of goblin1, we co-crystallized the compound with hOGT and determined the crystal structure of the complex (Supplementary Table S1 at http://www.biochemj.org/bj/457/bj4570497add.htm). Electron density difference maps at 3.15 Å, improved by 4-fold non-crystallographic averaging
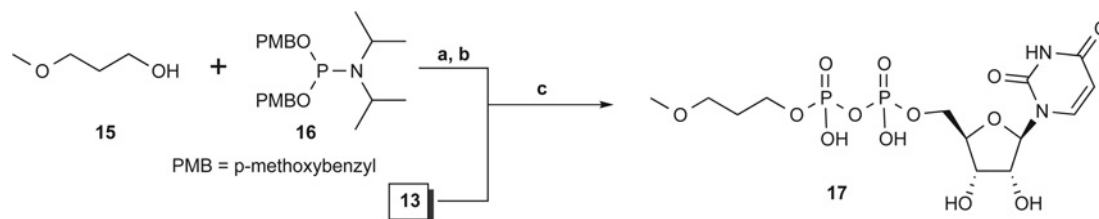
Scheme 3   Synthesis of the 'linker-only' UDP derivative

(a) **16**, 4,5-dicyanoimidazole, acetonitrile, room temperature, 1 h, then (b) I$_2$/Py (pyridine), 0°C, 1 h then **13**, room temperature, 16 h; (c) methanol, triethylamine, water, room temperature, 16 h, then size-exclusion chromatography 40 %.

(Supplementary Figure S3 at http://www.biochemj.org/bj/457/bj4570497add.htm), revealed unambiguous density for the entire compound, including the ordered C$_3$ linker (Figure 1A). As envisaged, UDP adopts the same conformation as observed in the hOGT Michaelis complex (Figure 1A; maximum atom shift, 0.6 Å) and the peptide occupies the -4 to $+2$ subsites with a similar backbone conformation (maximum backbone atom shift near O-GlcNAc site, 0.9 Å). The three-carbon linker connects the two components apparently without introducing any strain, allowing both the UDP moiety and the peptide part of the inhibitor to adopt the optimal position in the binding site, effectively mimicking the natural substrates.

One of the main objectives of combining components of donor and acceptor substrate into a bisubstrate inhibitor is the expected improved selectivity of such a construct when compared with inhibitors that compete with a single substrate only. Although accessing the selectivity of the novel OGT bisubstrate inhibitors over an exhaustive panel of GlcNAc transferases is beyond the scope of the present study, we were able to establish that goblin1 is not an inhibitor of the GlcNAc transferase *Sm*NodC (*Sinorhizobium meliloti* NodC) (Supplementary Figure S4 at http://www.biochemj.org/bj/457/bj4570497add.htm).

Concluding remarks

O-GlcNAc modification of proteins is abundant and essential, yet its precise cellular function remains to be uncovered. The development of small-molecule inhibitors of OGT is a prerequisite for the elucidation of the biological role of O-GlcNAc, and eventually the therapeutic intervention in diseases involving dysregulation of O-GlcNAc. With multisubstrate enzymes, such as OGT, specific inhibition can rarely be achieved with ligands that compete solely with one of the substrates. Peptide–nucleotide polyphosphate conjugates have been reported as bisubstrate inhibitors of protein kinases in the early 1990s; the discovery recently led to the identification of low-nanomolar cell-penetrant inhibitors with neutral linkers replacing the polyphosphate chain [25]. The pioneering work of Palcic et al. [26] introduced the concept of bisubstrate inhibition for glycosyltransferases, targeting fucosyltransferases [27].

In the present paper, we have reported the first examples of functional OGT bisubstrate inhibitors featuring a short linker replacing the GlcNAc moiety of the donor substrate and covalently connecting a uridyl diphosphate 'binding anchor' to an acceptor peptide. The resulting compounds, goblin1 and goblin2, exhibit low-micromolar affinity for OGT and are capable of inhibiting glycosylation of peptide as well as protein substrates *in vitro*. The crystal structure of the hOGT–goblin1 complex reveals the structural mimicry offered by the inhibitor, with both UDP and peptide moieties retaining the alignment observed in the

Michaelis complex. As expected for a bisubstrate inhibitor, both fragments add synergistically to the inhibition: the 'linker-only' derivative of UDP was shown to be an order of magnitude less potent an inhibitor, whereas the 'linker-only' acceptor peptide was neither a substrate for the OGT nor an inhibitor. The goblin scaffold allows for further increases in potency by optimization of linker length/type, peptide sequence and nucleotide modifications, whereas cell penetrance could be addressed by extending the peptide backbone with cell-penetrating peptide/peptoid motifs [28,29] potentially bearing intracellular localization signals, to provide tools for studying OGT function *in vivo*.

REFERENCES

1   Torres, C. R. and Hart, G. W. (1984) Topography and polypeptide distribution of terminal *N*-acetylglucosamine residues on the surfaces of intact lymphocytes: evidence for O-linked GlcNAc. J. Biol. Chem. **259**, 3308–3317

2   Zachara, N. E., Cheung, W. D. and Hart, G. W. (2004) Nucleocytoplasmic glycosylation, O-GlcNAc: identification and site mapping. Methods Mol. Biol. **284**, 175–194

3   Hart, G. W., Housley, M. P. and Slawson, C. (2007) Cycling of O-linked *β-N*-acetylglucosamine on nucleocytoplasmic proteins. Nature **446**, 1017–1022

4   Shafi, R., Lyer, S. P. N., Ellies, L. G., O'Donnell, N., Marek, K. W., Chui, D., Hart, G. W. and Marth, J. D. (2000) The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. Proc. Natl. Acad. Sci. U.S.A. **97**, 5735–5739

5   Dias, W. B. and Hart, G. W. (2007) O-GlcNAc modification in diabetes and Alzheimer's disease. Mol. Biosyst. **3**, 766–772

6   Macauley, M. S. and Vocadlo, D. J. (2010) Increasing O-GlcNAc levels: an overview of small-molecule inhibitors of O-GlcNAcase. Biochim. Biophys. Acta **1800**, 107–121

7   Gross, B., Kraybill, B. and Walker, S. (2005) Discovery of O-GlcNAc transferase inhibitors. J. Am. Chem. Soc. **127**, 14588–14589

8   Jiang, J., Lazarus, M. B., Pasquina, L., Sliz, P. and Walker, S. (2012) A neutral diphosphate mimic crosslinks the active site of human O-GlcNAc transferase. Nat. Chem. Biol. **8**, 72–77

9   Gloster, T., Zandberg, W., Heinonen, J., Shen, D., Deng, L. and Vocadlo, D. (2011) Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. Nat. Chem. Biol. **7**, 174–181

10 Dorfmueller, H. C., Borodkin, V. S., Blair, D. E., Pathak, S., Navratilova, I. and van Aalten, D. M. F. (2011) Substrate and product analogues as human O-GlcNAc transferase inhibitors. Amino Acids **40**, 781–792

11 Schimpl, M., Zheng, X., Borodkin, V. S., Blair, D. E., Ferenbach, A. T., Schuettelkopf, A. W., Navratilova, I., Aristotelous, T., Albarbarawi, O., Robinson, D. A. et al. (2012) O-GlcNAc transferase invokes nucleotide sugar pyrophosphate participation in catalysis. Nat. Chem. Biol. **8**, 969–974

12 Lazarus, M. B., Jiang, J., Gloster, T. M., Zandberg, W. F., Whitworth, G. E., Vocadlo, D. J. and Walker, S. (2012) Structural snapshots of the reaction coordinate for O-GlcNAc transferase. Nat. Chem. Biol. **8**, 966–968

13 Collaborative Computational Project, Number 4 (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. **50**, 760–763

14 Murshudov, G. N., Vagin, A. A. and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. **53**, 240–255

15 Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. **60**, 2126–2132

16 Schüttelkopf, A. W. and van Aalten, D. M. F. (2004) PRODRG: a tool for high-throughput crystallography of protein–ligand complexes. Acta Crystallogr. D Biol. Crystallogr. **60**, 1355–1363

17 Conner, S. H., Kular, G., Peggie, M., Shepherd, S., Schüttelkopf, A. W., Cohen, P. and van Aalten, D. M. F. (2006) TAK1-binding protein 1 is a pseudophosphatase. Biochem. J. **399**, 427–434

18 Pathak, S., Borodkin, V. S., Albarbarawi, O., Campbell, D. G., Ibrahim, A. and van Aalten, D. M. F. (2012) O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release. EMBO J. **31**, 1394–1404

19 Lee, H. S. and Thorson, J. S. (2011) Development of a universal glycosyltransferase assay amenable to high-throughput formats. Anal. Biochem. **418**, 85–88

20 Ojida, A., Takashima, I., Kohira, T., Nonaka, H. and Hamachi, I. (2008) Turn-on fluorescence sensing of nucleoside polyphosphates using a xanthene-based Zn(II) complex chemosensor. J. Am. Chem. Soc. **130**, 12095–12101

21 Medzihradszky, D., Chen, S. L., Kenyon, G. L. and Gibson, B. W. (1994) Solid-phase synthesis of adenosine phosphopeptides as potential bisubstrate inhibitors of protein-kinases. J. Am. Chem. Soc. **116**, 9413–9419

22 Liu, D., Dong, J., Yin, Y., Ma, R., Shi, Y., Wu, H., Chen, S. and Li, G. (2011) A concise synthesis of the HCV protease inhibitor BILN 2061 and its P3 modified analogs. Chin. J. Chem. **29**, 1489–1502

23 Palmer, M. J., Danilewicz, J. C. and Vuong, H. (1994) Phase-transfer mediated serine O-alkylation. Synlett **3**, 171–172

24 van der Heden van Noort, G. J., Verhagen, C. P., van der Horst, M. G., Overkleeft, H. S., van der Movel, G. A. and Flippov, D. V. (2008) A versatile one-pot procedure to phosphate monoesters and pyrophosphates using di(*p*-methoxybenzyl)-*N*,*N*-diisopropylphosphoramidite. Org. Lett. **10**, 4461–4464

25 Lavogina, D., Enkvist, E. and Uri, A. (2010) Bisubstrate inhibitors of protein kinases: from principle to practical applications. ChemMedChem **5**, 23–34

26 Palcic, M. M., Heerze, L. D., Srivastava, O. P. and Hindsgaul, O. (1989) A bisubstrate analog inhibitor for $\alpha(1\rightarrow2)$-fucosyltransferase. J. Biol. Chem. **264**, 17174–17181

27 Izumi, M., Yuasa, H. and Hashimoto, H. (2009) Bisubstrate analogues as glycosyltransferase inhibitors. Curr. Top. Med. Chem. **9**, 87–105

28 Stewart, K. M., Horton, K. L. and Kelley, S. O. (2008) Cell-penetrating peptides as delivery vehicles for biology and medicine. Org. Biomol. Chem. **6**, 2242–2255

29 Koren, E. and Torchilin, V. P. (2012) Cell-penetrating peptides: breaking through to the other side. Trends Mol. Med. **18**, 385–393

SUPPLEMENTARY ONLINE DATA

Bisubstrate UDP–peptide conjugates as human O-GlcNAc transferase inhibitors

Vladimir S. BORODKIN*, Marianne SCHIMPL*, Mehmet GUNDOGDU*, Karim RAFIE*, Helge C. DORFMUELLER*†, David A. ROBINSON‡ and Daan M. F. VAN AALTEN*†[1]

*MRC Protein Phosphorylation und Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.
†Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.
‡Drug Discovery Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.

SYNTHESIS METHODS

Compound 3a

To a solution of the SerBocOH (N-t-butoxycarbonylserine) (2.25 g, 11 mmol) in DMF (40 ml) 60 % sodium hydride suspension in oil (0.88 g, 22 mmol) was added sequentially in two equal portions at 0 °C (ice-bath). When gas evolution ceased, the reaction was removed from the cooling bath and stirred at room temperature until gas evolution ceased (1 min). The reaction was returned to the ice-bath and tetra-n-butylammonium iodide (0.369 g, 1 mmol) and a solution of 1-(3-bromopropoxy)-4-methoxybenzene **2** (3.23 g, 13.2 mmol) in DMF (15 ml) were added in a succession. The reaction was removed from the ice-bath and stirred at room temperature for 16 h. The reaction was quenched by careful addition of a few drops of 10 % citric acid solution, diluted with ethyl acetate and washed successively with 10 % citric acid and water. The aqueous layers were back-extracted with the ethyl acetate two times more. The combined organic layer was dried and concentrated.

The residue was dissolved in DMF (25 ml) and treated with allyl bromide (1.3 ml, 15 mmol) in the presence of DIPEA (2.6 ml, 15 mmol) at room temperature for 16 h. The reaction was diluted with methanol and concentrated. The residue was partitioned between ethyl acetate and 1 M HCl, and the layers were separated. The organic layer was successively washed with water and a mixture of saturated $NaHCO_3$ solution and brine. The aqueous layers were back-extracted with ethyl acetate. The combined organic layer was dried and concentrated. The residue was adsorbed on to silica and purified by flash chromatography in petroleum ether/ethyl acetate 5–10–20 % to give 1.37 g (3.36 mmol, 30 %) of the target product as clear oil.

$[\alpha]_D = +4.7°$ (c 1.0 in chloroform). ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 6.82 (s, 4H), 5.86 (ddt, J 17.2, 10.5, 5.7 Hz, 1H), 5.42 (d, J 8.9 Hz, 1H), 5.29 (dq, J 17.3, 1.6 Hz, 1H), 5.19 (dq, J 10.4, 1.3 Hz, 1H), 4.66–4.55 (m, 2H), 4.45 (dt, J 8.8, 3.2 Hz, 1H), 3.95 (td, J 6.2, 1.0 Hz, 2H), 3.91–3.87 (m, 1H), 3.76 (s, 3H), 3.68 (dd, J 9.5, 3.3 Hz, 1H), 3.61 (qt, J 9.5, 6.1 Hz, 2H), 1.98 (p, J 6.2 Hz, 2H), 1.45 (s, 9H); ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 170.4, 155.5, 153.8, 153, 131.67, 118.4, 115.4, 114.6, 79.9, 70.8, 68, 65.9, 65.1, 55.7, 54.1, 29.5, 28.3. HRMS (m/z), $[M + H]^+$ calculated for $C_{21}H_{32}NO_7$, 410.2179; found 410.2183.

Compound 3b

$[\alpha]_D = +2.2°$ (c 1.0 in chloroform); ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 6.84 (s, 4H), 5.99–5.85 (m, 1H), 5.38 (d, 1H), 5.37–5.32 (m, 1H), 5.27–5.23 (m, 1H), 4.75–4.59 (m, 2H), 4.47 (dt, J 8.6, 3.4 Hz, 1H), 3.92 (t, J 6.2 Hz, 2H), 3.88 (dd, J 9.4, 3.3 Hz, 1H), 3.79 (s, 3H), 3.68 (dd, J 9.4, 3.4 Hz, 1H), 3.57–3.45 (m, 2H), 1.85–1.76 (m, 2H), 1.77–1.69 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 170.5, 155.5, 153.8, 153.2, 131.7, 118.4, 115.4, 114.5, 80, 71.1, 70.7, 68.4, 65.9, 55.7, 54.2, 28.3, 26.1. HRMS (m/z), $[M + H]^+$ calculated for $C_{22}H_{35}NO_7$, 425.2414; found 425.2422.
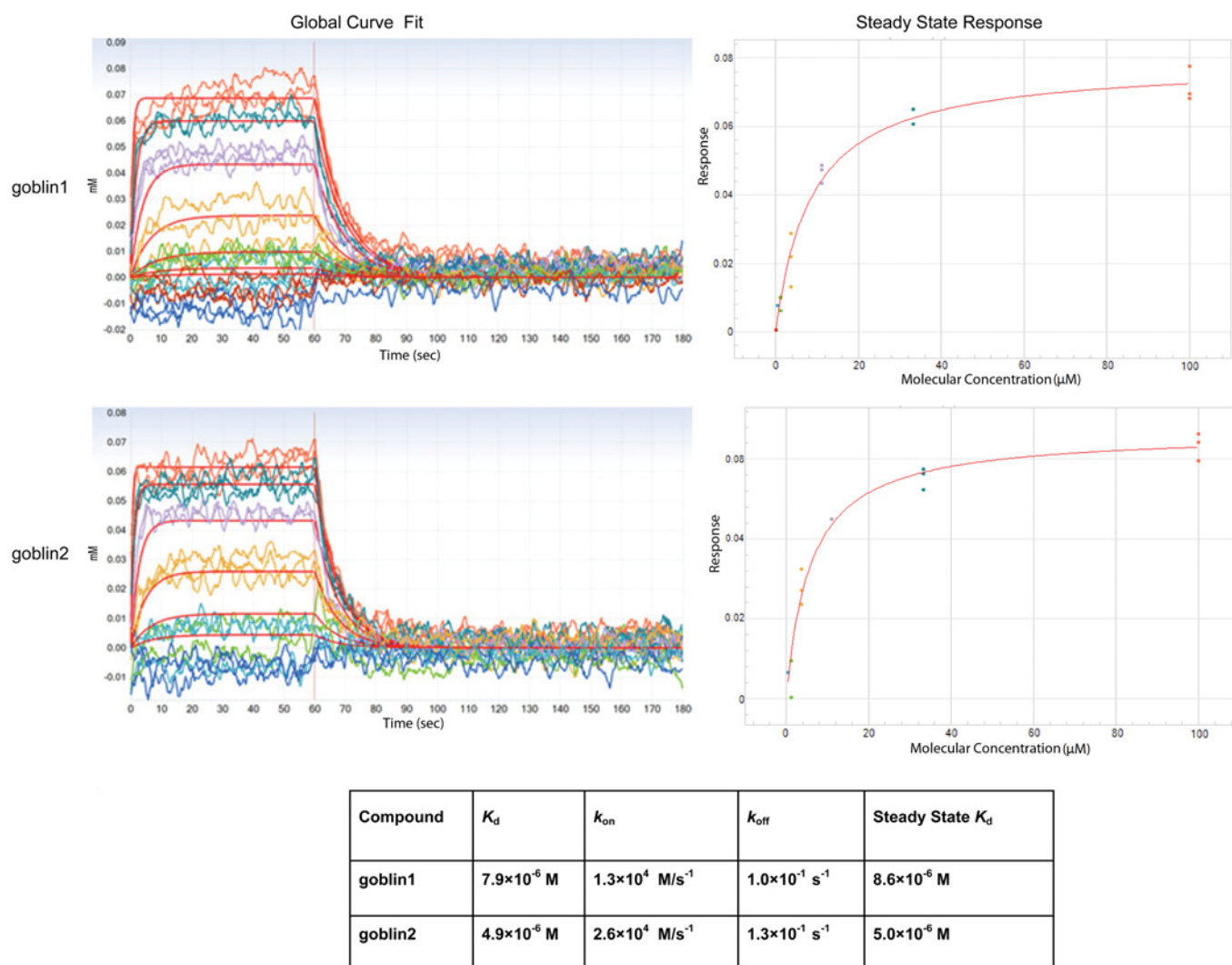
Compound 4a

To a cold (ice-bath) stirred solution of **3a** (1.18 g, 2.9 mmol) in DCM (dichloromethane) (15 ml) was added 95 % aqueous TFA (trifluoroacetic acid) (1.5 ml). The reaction was kept for 2 h at room temperature, diluted with toluene (10 ml) and concentrated. The residue was dissolved in a mixture of chloroform and toluene (1:3, v/v) (10 ml) and concentrated. This procedure was repeated once more. The residue was dissolved in DCM (20 ml) and treated with an excess of DIPEA (2 ml) and FmocCl (Fmoc chloride) (0.905 g, 3.5 mmol) at 0 °C (ice-bath). The reaction was kept at room temperature for 16 h, quenched with methanol (0.1 ml), stirred for 30 min and concentrated. The residue was dissolved in DCM and successively washed with 1 M HCl, water and a mixture of $NaHCO_3$ solution and brine. Aqueous layers were back-extracted with DCM. The combined organic layer was dried and concentrated. The residue was adsorbed on to silica gel and purified by flash chromatography in petroleum ether/ethyl acetate 5–10–20 % to give 1.42 g (2.67 mmol, 92 %) of the target product as crystalline solid. The analytical sample was crystallized from toluene/petroleum ether.

Melting point 103 °C; $[\alpha]_D = +11.4°$ (c 1.00 in chloroform); ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.67 (d, J 7.5 Hz, 2H), 7.52 (t, J 6.6 Hz, 2H), 7.31 (t, J 7.5 Hz, 2H), 7.22 (td, J 7.4, 1.2 Hz, 2H), 6.78–6.68 (m, 4H), 5.78 (ddt, J 16.2, 10.9, 5.7 Hz, 1H), 5.62 (d, J 8.7 Hz, 1H), 5.21 (dq, J 17.1, 1.6 Hz, 1H), 5.11 (dq, J 10.4, 1.3 Hz, 1H), 4.54 (dt, J 5.7, 1.5 Hz, 2H), 4.49–4.42 (m, 2H), 4.34 (dd, J 10.6, 7.2 Hz, 1H), 4.26 (dd, J 10.6, 7.3 Hz, 1H), 4.15 (t, J 7.3 Hz, 1H), 3.87 (td, J 6.2, 1.8 Hz, 2H), 3.84 (dd, J 9.5, 3.2 Hz, 1H), 3.64 (s, 3H), 3.55 (qt, J 9.6, 6.2 Hz, 2H), 1.91 (p, J 6.1 Hz, 2H); ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 170.1, 156, 153.8, 153, 143.9, 143.8, 141.3, 131.6, 127.7, 127.1, 125.22, 120, 118.6, 115.4, 114.7, 76.9, 70.7, 68.2, 67.2, 65.1, 55.7, 54.6, 47.2, 29.5. HRMS (m/z), $[M + H]^+$ calculated for $C_{31}H_{34}NO_7$, 532.2335; found 532.2331.

---

[1] To whom correspondence should be addressed (email dmfvanaalten@dundee.ac.uk).

Co-ordinates and structure factors for human O-GlcNAc transferase complexed to the Goblin1 inhibitor have been deposited in the PDB under code 4CDR.

Figure S1   Biolayer interferometry sensograms

Binding profiles obtained and kinetic parameters calculated for interactions between hOGT and goblin1 and goblin2. Concentration series of each compound (3-fold serial dilution, top concentration 100 μM) were prepared in triplicate. Left-hand panels: binding profiles and global curve fitting from which the kinetic parameters were calculated. Right-hand panels: steady-state binding response from which the steady-state K_d was calculated.

Compound 4b

$[\alpha]_D = +13.5°$ (c 1.00 in chloroform); ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.80 (dd, J 7.4, 1.1 Hz, 2H), 7.70–7.60 (m, 2H), 7.43 (tt, J 7.4, 0.9 Hz, 2H), 7.34 (td, J 7.5, 1.2 Hz, 2H), 6.85 (s, 4H), 5.94 (ddt, J 16.4, 10.9, 5.7 Hz, 1H), 5.70 (d, J 8.7 Hz, 1H), 5.37 (dq, J 17.2, 1.7 Hz, 1H), 5.32–5.22 (m, 1H), 4.79–4.65 (m, 2H), 4.58 (dt, J 8.7, 3.3 Hz, 1H), 4.46 (dd, J 10.6, 7.2 Hz, 1H), 4.40 (dd, J 10.6, 7.3 Hz, 1H), 4.28 (t, J 7.3 Hz, 1H), 3.94 (t, J 6.0 Hz, 3H), 3.79 (s, 3H), 3.62–3.48 (m, 2H), 1.80 (dq, J 25.9, 7.3 Hz, 4H). HRMS (m/z), $[M + H]^+$ calculated for $C_{32}H_{37}NO_7$, 547.2570; found 547.2558.

Compound 5a

To a stirred solution of **4a** (1.39 g, 2.61 mmol) in a mixture of THF (tetrahydrofuran)/acetonitrile (1:1, v/v) (15 ml) a solution of ammonium cerium(IV) nitrate (3 g, 5.47 mmol) in water (7.5 ml) was added at room temperature. The reaction was stirred for 20 min, diluted with 10% EDTA solution and ethyl acetate

and the layers were separated. The organic layer was washed successively with 10% aqueous $Na_2S_2O_5$ solution, water and a mixture of saturated $NaHCO_3$ solution and brine. The aqueous layers were back-extracted with ethyl acetate. The combined organic layer was dried and concentrated. The residue was adsorbed on to silica gel and purified by flash chromatography in (petroleum ether/DCM, 4:1, v/v)/ethyl acetate 5→45% to give 1.1 g (2.58 mmol, ~100%) of the target product as tan-coloured crystals. The analytical sample was crystallized from chloroform/petroleum ether.

Melting point 73°C; $[\alpha]_D = +11.3°$ (c 1.00 in chloroform); ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.76–7.62 (m, 2H), 7.54 (t, J 6.9 Hz, 2H), 7.32 (tt, J 7.6, 1.4 Hz, 3H), 7.24 (tt, J 7.3, 1.3 Hz, 2H), 5.84 (ddt, J 16.4, 10.4, 5.7 Hz, 1H), 5.72 (d, J 8.6 Hz, 1H), 5.26 (dq, J 17.2, 1.5 Hz, 1H), 5.18 (dt, J 10.4, 1.3 Hz, 1H), 4.65–4.55 (m, 2H), 4.48 (dt, J 8.6, 3.4 Hz, 1H), 4.35 (dd, J 10.6, 7.2 Hz, 1H), 4.29 (dd, J 10.6, 7.2 Hz, 1H), 4.16 (t, J 7.2 Hz, 1H), 3.82 (dd, J 9.7, 3.5 Hz, 1H), 3.66–3.61 (m, 3H), 3.57 (ddd, J 9.4, 6.5, 5.1 Hz, 1H), 3.51 (ddd, J 9.4, 6.7, 5.1 Hz, 1H), 1.80–1.62 (m, 2H); ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 170.1,
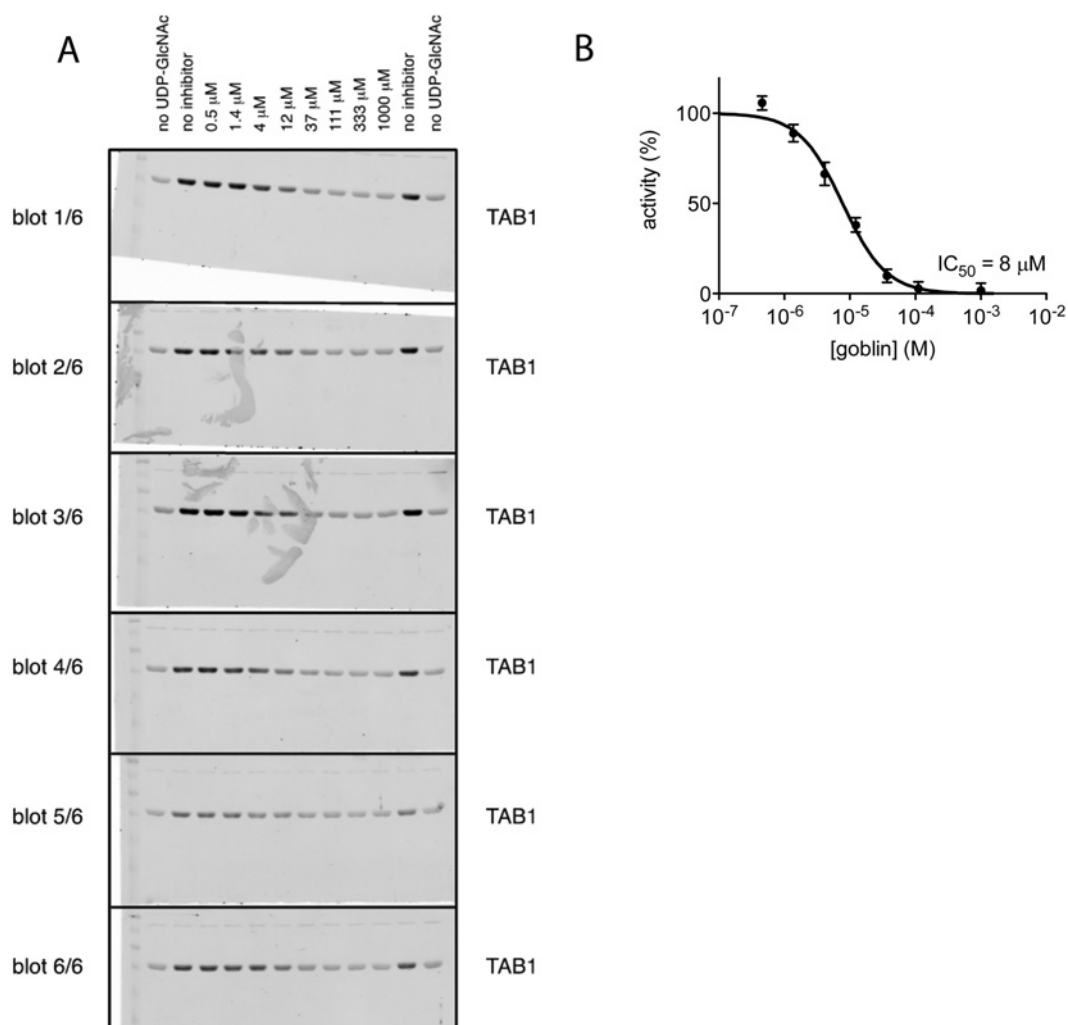
Figure S2   hOGT inhibition by goblin1

In vitro O-GlcNAcylation of TAB1-(7–402) protein was performed in the presence of 10 μM UDP-GlcNAc and 1 mM–0.46 μM goblin1. O-GlcNAc was detected by immunoblotting with a TAB1 O-GlcNAc Ser[395] site-specific antibody (**A**) [2]. The experiment was performed in six replicates and quantified by densitometric analysis, normalized and fitted to a four-parameter equation for dose-dependent inhibition in GraphPad Prism (http://www.graphpad.com) (**B**). Results are means $\pm$ S.E.M.

156.1, 143.9, 143.8, 141.3, 131.5, 127.7, 127.1, 125.2, 125.1, 120, 119, 71, 69.9, 67.2, 66.3, 60.8, 54.5, 47.1, 32.0. HRMS (*m/z*), $[M + H]^+$ calculated for $C_{24}H_{28}NO_6$, 426.1917; found 4261922.

Compound 5b

$[\alpha]_D = +12.4°$ (*c* 1.00 in chloroform); ^{1}H NMR (500 MHz, [2H]chloroform) δ 7.79 (d, *J* 7.5 Hz, 2H), 7.65 (t, *J* 7.1 Hz, 2H), 7.43 (t, *J* 7.5 Hz, 2H), 7.35 (t, *J* 7.4 Hz, 2H), 5.94 (ddt, *J* 16.5, 10.9, 5.7 Hz, 1H), 5.78 (d, *J* 8.7 Hz, 1H), 5.37 (d, *J* 17.1 Hz, 1H), 5.28 (d, *J* 10.4 Hz, 1H), 4.81–4.65 (m, 2H), 4.62–4.53 (m, 1H), 4.45 (dd, 1H), 4.40 (dd, *J* 10.7, 7.2 Hz, 1H), 4.28 (t, *J* 7.2 Hz, 1H), 3.93 (dd, *J* 9.8, 3.3 Hz, 1H), 3.73 (dd, *J* 9.6, 3.2 Hz, 1H), 3.66 (t, *J* 6.0 Hz, 2H), 3.58–3.46 (m, 2H), 1.78 (s, 2H), 1.66 (dq, *J* 18.0, 6.7 Hz, 4H); ^{13}C NMR (126 MHz, [2H]chloroform) δ 170.1, 156.1, 143.9, 143.8, 141.3, 131.6, 127.7, 127.1, 125.2, 125.1, 120, 118.7, 71.5, 70.6, 67.2, 62.5, 54.6, 47.2, 29.6, 26. HRMS (*m/z*), $[M + H]^+$ calculated for $C_{25}H_{31}NO_6$, 441.2151; found 441.2160.

Compound 6

The reaction was carried out in a 50 ml Falcon centrifuge tube. A solution of di-isopropylamine (3.5 ml, 25 mmol) in hexane (10 ml) was added dropwise to a stirred solution of phosphorus trichloride (1.1 ml, 12.6 mmol) in hexane (30 ml) at $-50°$C. The reaction was removed from the cooling bath, warmed to room temperature, and further vigorously stirred for 1 h and centrifuged at 3700 *g* for 15 min at 4°C. Approximately 30 ml of the supernatant was transferred into a recovery flask; the residue was resuspended in methyl t-butyl ether (15 ml) and centrifuged as before. The supernatant was added to the recovery flask. The solution was concentrated to give 1.93 g (9.55 mmol, 76%) of the crude target product (δ 169.9 p.p.m.) as a slightly yellowish liquid. A solution of the above residue (0.97 g, 4.8 mmol) in THF (5 ml) was added to a solution of *p*-methylbenzyl alcohol 1.17 g, 9.6 mmol) and di-isopropylethylamine (2.5 ml, 14.4 mmol) in THF (20 ml) at 0°C (ice-bath) (reaction was carried out in a 50 ml Falcon centrifuge tube). The reaction was removed from the cooling bath and stirred further for 2 h at room temperature;
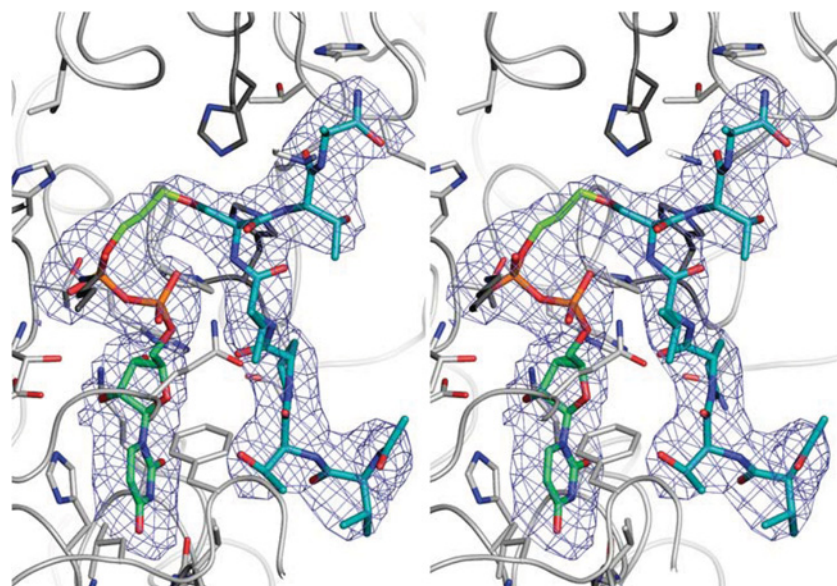
Figure S3 Stereo image of hOGT with bisubstrate inhibitor showing unbiased $F_o - F_c$ electron density after 4-fold averaging (3.5σ)

the precipitate started to fall in 10–15 min. The reaction was diluted with methyl t-butyl ether (total volume 40 ml) and centrifuged as before. The supernatant was collected; the residue was suspended in methyl t-butyl ether (20 ml) and centrifuged again. The supernatants were pooled and concentrated. The residue was partitioned between DCM and 1 M phosphate (pH 7) buffer (pH of the aqueous phase 7 after shaking) and the layers were separated. The organic layer was washed with brine. The aqueous layers were successively extracted with the same portion of DCM. The organic layers were dried, concentrated and dried under vacuum overnight to give 1.7 g of the crude product as a yellowish freely running oil, which was shown to be sufficiently pure by NMR (δ 146 p.p.m.).

Compound 7a

To a solution of **5a** (0.85 g, 2 mmol) and dimethylbenzyl *N*,*N*-di-isopropylphosphoramidite **6** (0.97 g, 2.6 mmol) in acetonitrile (10 ml), 4,5-dicyanoimidazole (0.307 g, 2.6 mmol) was added at room temperature and reaction was stirred for 1 h. The reaction mixture was cooled down to 0 °C (ice-bath) and 3-chloroperbenzoic acid (0.64 g, 2.6 mmol) was added in one portion. The reaction was stirred further for 1 h and quenched by the addition of a 20 % solution of $Na_2S_2O_5$, stirred for 30 min, removed from the cooling bath and diluted with DCM. The layers were separated; the organic layer was washed successively with water and a mixture of concentrated $NaHCO_3$ solution and brine. The aqueous layers were back-extracted with DCM. The combined organic layer was dried and concentrated. The residue was adsorbed on to silica gel and purified by flash chromatography in (petroleum ether/DCM, 4:1, v/v)/ethyl acetate 10–40 % to give 1.21 g (1.7 mmol, 85 %) of the target product as a clear oil.

$[\alpha]_D = +8.3°$(*c* 1.00 in chloroform);^{1}H NMR (500 MHz, [²H]chloroform) δ 7.79 (d, *J* 7.5 Hz, 2H), 7.70 (dd, *J* 11.5, 7.5 Hz, 2H), 7.45–7.39 (m, 3H), 7.34 (td, *J* 7.5, 1.0 Hz, 2H), 7.29–7.24 (m, 5H), 7.18 (d, *J* 7.6 Hz, 5H), 6.18 (d, *J* 8.6 Hz, 1H), 5.98–5.87 (m, 1H), 5.36 (dq, *J* 17.3, 1.6 Hz, 1H), 5.27 (dq, *J* 10.5, 1.3 Hz, 1H), 5.09–4.97 (m, 5H), 4.75–4.65 (m, 2H), 4.57 (dt, *J* 8.5, 3.3 Hz, 1H), 4.47 (dd, *J* 10.5, 7.2 Hz, 1H), 4.38 (dd, *J* 10.6, 7.4 Hz,

1H), 4.27 (t, *J* 7.3 Hz, 1H), 4.21–4.12 (m, 1H), 4.10 (m,1H), 3.93 (dd, *J* 9.5, 3.5 Hz, 1H), 3.70 (dd, *J* 9.5, 3.1 Hz, 1H), 3.52 (ddt, *J* 26.3, 9.5, 6.1 Hz, 2H), 2.37 (s, 6H), 1.88 (p, *J* 6.0 Hz, 2H); ¹³C NMR (126 MHz, [²H]chloroform) δ 170, 156.3, 144, 143.9, 141.3, 138.4, 132.93 (d, $J_{C,P}$ 6.4 Hz), 131.7, 129.3, 128.2, 128.2, 127.7, 127, 125.4, 125.3, 112, 118.6, 70.66, 69.27 (dd, $J_{C,P}$ 4.5 Hz), 67.2, 67, 66.1, 64.4 (d, $J_{C,P}$ 5.6 Hz), 54.7, 47.2, 30.24 (d, $J_{C,P}$ 6.1 Hz), 21.3; ³¹P NMR (202 MHz, [²H]chloroform) δ −0.55. HRMS (*m/z*), $[M + H]^+$ calculated for $C_{40}H_{45}NO_9P$ 714.2832; found 714.2826.

Compound 7b

$[\alpha]_D = +5.2°$ (*c* 2.5 in chloroform); ¹H NMR (500 MHz, [²H]chloroform) δ 7.79 (d, *J* 7.5 Hz, 2H), 7.70 (dd, *J* 11.5, 7.5 Hz, 2H), 7.45–7.39 (m, 3H), 7.34 (td, *J* 7.5, 1.0 Hz, 2H), 7.29–7.24 (m, 5H), 7.18 (d, *J* 7.6 Hz, 5H), 6.18 (d, *J* 8.6 Hz, 1H), 5.98–5.87 (m, 1H), 5.36 (dq, *J* 17.3, 1.6 Hz, 1H), 5.27 (dq, *J* 10.5, 1.3 Hz, 1H), 5.09–4.97 (m, 5H), 4.75–4.65 (m, 2H), 4.57 (dt, *J* 8.5, 3.3 Hz, 1H), 4.47 (dd, *J* 10.5, 7.2 Hz, 1H), 4.38 (dd, *J* 10.6, 7.4 Hz, 1H), 4.27 (t, *J* 7.3 Hz, 1H), 4.21–4.12 (m, 1H), 4.07 (q, 2H), 3.9 (dd, *J* 9.3, 3 Hz, 1H), 3.70 (dd, *J* 9.3, 3 Hz, 1H), 3.45 (m, 2H), 2.37 (s, 6H), 1.68 (m, 2H), 1.63 (m, 2H); ¹³C NMR (126 MHz, [²H]chloroform) δ 170, 156.3, 144, 143.9, 141.3, 138.4, 132.93 (d, $J_{C,P}$ 6.4 Hz), 131.7, 129.3, 128.2, 128.2, 127.7, 127, 125.4, 125.3, 112, 118.6, 70.66, 69.27 (dd, $J_{C,P}$ 4.5 Hz), 67.2, 67, 66.1, 64.4 (d, $J_{C,P}$ 5.6 Hz), 54.7, 47.2, 30.24 (d, $J_{C,P}$ 6.1 Hz), 21.3; ³¹P NMR (202 MHz, [²H]chloroform) δ −0.9. HRMS (*m/z*), $[M + H]^+$ calculated for $C_{41}H_{48}NO_9P$ 729.3067; found 729.306.

Compound 8a

To a solution of **7a** (0.22 g, 0.297 mmol) in THF (3 ml) morpholine (0.05 ml, 0.53 mmol) and Pd(PPh₃)₄ [tetrakis (triphenylphosphine)palladium(0)] (0.009 g, 0.0078 mmol) were added sequentially. The reaction was stirred for 1.5 h, quenched by addition of a suspension of Dowex 50WX8-100 H⁺ resin in methanol and stirred for 30 min. The resin was filtered off, and the filtrate was concentrated to give 0.195 g (0.29 mmol, 93 %) of the target product as a clear syrup.
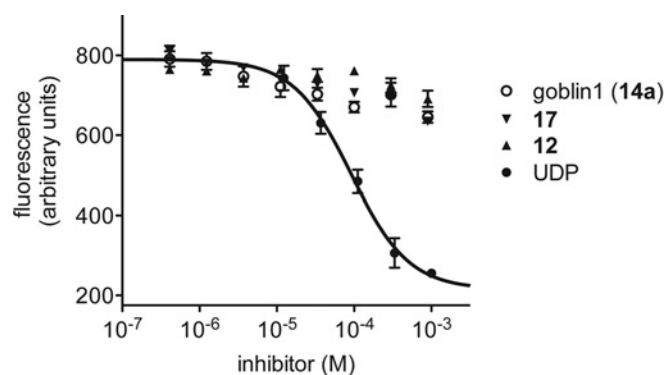
Figure S4   The bacterial GlcNAc transferase NodC is not inhibited by goblin1

*Sm*NodC, a GlcNAc transferase from *Sinorhizobium meliloti* that utilizes UDP-GlcNAc to synthesize chito-oligosaccharides, was assayed to investigate the selectivity of goblin1. A coupled fluorigenic *in vitro* assay was performed in the presence of 1 mM–0.46 μM of goblin1, **12**, **17** and UDP. The assay (details available from D. M. F. v. A. on request) relies on the elongation of 4-methylumbelliferyl-*N*-acetyl-β1,4-glucosamine by the GlcNAc transferase *Sm*NodC, and subsequent hydrolysis of the reaction products by *Aspergillus fumigatus* chitinase B; the release of 4-methylumbelliferone is detected fluorimetrically. Experiments were performed in triplicate, with results being means ± S.E.M. Dose–response curves were obtained using the 4-parameter IC$_{50}$ equation in the GraphPad Prism program (http://www.graphpad.com). NodC exhibits product inhibition by UDP (IC$_{50}$ 90 μM), but is not inhibited by either the goblin1 inhibitor, or by the goblin1 fragments containing either UDP and linker (**17**), or peptide and linker (**12**).

Table S1   Crystallographic data collection and structure refinement statistics

Values for highest-resolution shell are shown in parentheses.

	hOGT + goblin1
Data collection	
Beamline, wavelength (Å)	ID 23-1, 0.981
Space group	*P*321
Cell dimensions	
$a = b, c$ (Å)	273.7, 142.6
Resolution (Å)	30–3.15 (3.32–3.15)
R_{merge}	0.136 (0.505)
$I/\sigma I$	9.2 (3.1)
Completeness (%)	99.2 (99.7)
Redundancy	4.3 (4.5)
Refinement	
Resolution (Å)	30–3.15
Number of reflections	452654
Number of unique reflections	104870
R_{work}/R_{free}	0.197/0.213
Number of atoms	
Protein	22056
Ligand	312
B-factors	
Protein	54.5
Ligand	49.8
RMSDs	
Bond lengths (Å)	0.011
Bond angles (°)	1.34

$[\alpha]_D = +24.3°$ (*c* 1.00 in chloroform); ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.79–7.75 (m, 2H), 7.65 (dd, *J* 11.0, 7.5 Hz, 2H), 7.41 (td, *J* 7.4, 1.0 Hz, 2H), 7.33 (tt, *J* 7.4, 1.0 Hz, 2H), 7.27–7.20 (m, 4H), 7.16 (t, *J* 8.2 Hz, 4H), 6.31 (d, *J* 8.2 Hz, 1H), 5.11–4.95 (m, 4H), 4.61 (dt, *J* 8.2, 3.0 Hz, 1H), 4.41 (dd, *J* 10.5, 7.2 Hz, 1H), 4.31 (dd, *J* 10.5, 7.5 Hz, 1H), 4.24–4.14 (m, 2H), 4.10 (dq, *J* 11.2, 5.9 Hz, 1H), 3.92 (dd, *J* 9.5, 3.3 Hz, 1H), 3.79 (dd, *J* 9.5, 2.9 Hz, 1H), 3.58 (dt, *J* 10.4, 5.4 Hz, 1H), 3.47 (dt, *J* 9.8, 6.0 Hz, 1H), 2.37 (s, 3H), 2.35 (s, 3H), 1.87–1.80 (m, 2H); ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 172.1, 156.2, 144, 143.9, 141.3, 138.5, 132.6, 132.2, 130.1, 129.3, 128.27, 128.2, 127.7, 127.1, 125.35, 125.3, 119.9, 69.7 (d, $J_{C,P}$ 5.5 Hz), 69.6 (d, $J_{C,P}$ 5.5 Hz), 69.6, 67.2, 66.8, 64.8 (d, $J_{C,P}$ 5.1 Hz), 54.6, 47.1, 30.2, 30.1, 21.2; ^{31}P NMR (202 MHz, [^{2}H]chloroform) δ − 0.84. HRMS (*m/z*), $[M − H]^-$ calculated for C$_{37}$H$_{39}$NO$_9$P, 672.2362; found 672.2357.

Compound 8b

$[\alpha]_D = +19.2°$ (*c* 1.00 in chloroform); ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.79–7.75 (m, 2H), 7.65 (dd, *J* 11.0, 7.5 Hz, 2H), 7.41 (td, *J* 7.4, 1.0 Hz, 2H), 7.33 (tt, *J* 7.4, 1.0 Hz, 2H), 7.27–7.20 (m, 4H), 7.16 (t, *J* 8.2 Hz, 4H), 6.31 (d, *J* 8.2 Hz, 1H), 5.11–4.95 (m, 4H), 4.61 (dt, *J* 8.2, 3.0 Hz, 1H), 4.41 (dd, *J* 10.5, 7.2 Hz, 1H), 4.31 (dd, *J* 10.5, 7.5 Hz, 1H), 4.24–4.14 (m, 2H), 4.10 (dq, *J* 11.2, 5.9 Hz, 1H), 3.92 (dd, *J* 9.5, 3.3 Hz, 1H), 3.79 (dd, *J* 9.5, 2.9 Hz, 1H), 3.58 (dt, *J* 10.4, 5.4 Hz, 1H), 3.47 (dt, *J* 9.8, 6.0 Hz, 1H), 2.37 (s, 3H), 2.35 (s, 3H), 1.8 (m, 1H), 1.7 (m, 1H), 1.60 (m, 2H); ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 172.1, 156.2, 144, 143.9, 141.3, 138.5, 132.6, 132.2, 130.1, 129.3, 128.27, 128.2, 127.7, 127.1, 125.35, 125.3, 119.9, 69.7 (d, $J_{C,P}$ 5.5 Hz), 69.6 (d, $J_{C,P}$ 5.5 Hz), 69.6, 67.2, 66.8, 64.8 (d, $J_{C,P}$ 5.1 Hz), 54.6, 47.1, 30.2, 30.1, 21.2; ^{31}P NMR (202 MHz, [^{2}H]chloroform) δ − 0.94. HRMS (*m/z*), $[M + H]^+$ calculated for C$_{38}$H$_{44}$NO$_9$P, 689.2754; found 689.2781.

Compound 9

To a solution of **5a** (0.123 g; 0.29 mmol) in DCM (5 ml) and DIPEA (0.116 ml, 0.7 mmol), TBSCl (t-butyldimethylsilyl chloride) (0.075 g, 0.5 mmol) and DMAP (4-dimethylaminopyridine) (0.04 g, 0.035 mmol) were added sequentially. The reaction mixture was stirred for 16 h at room temperature. The reaction was quenched by the addition of methanol, stirred for 30 min and concentrated. The residue was partitioned between ethyl acetate and 10 % aqueous citric acid solution and the layers were separated. The organic layer was washed successively with water and a mixture of saturated NaHCO$_3$ solution and brine. The aqueous layer was additionally extracted with the same portion of ethyl acetate. The combined organic layer was dried and concentrated. The residue was purified by flash chromatography on silica; gradient elution petroleum ether/ether 10–30 % to give 0.14 g (0.26 mmol, 90 %) of the target product as a clear syrup.

$[\alpha]_D = +7.4°$ (*c* 1.23 in chloroform) ^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.79–7.67 (m, 2H), 7.63–7.50 (m, 2H), 7.40–7.28 (m, 2H), 7.34–7.23 (m, 2H), 5.93–5.81 (m, 1H), 5.62 (d, *J* 8.8 Hz, 1H), 5.29 (dq, *J* 17.2, 1.6 Hz, 1H), 5.20 (dq, *J* 10.6, 1.3 Hz, 1H), 4.63 (ddt, *J* 5.7, 4.2, 1.5 Hz, 2H), 4.48 (dt, *J* 8.8, 3.2 Hz, 1H), 4.39 (dd, *J* 10.6, 7.1 Hz, 1H), 4.31 (dd, *J* 10.6, 7.3 Hz, 1H), 4.20 (t, *J* 7.2 Hz, 1H), 3.86 (dd, *J* 9.5, 3.1 Hz, 1H), 3.62 (tdd, *J* 12.8, 9.9, 4.7 Hz, 3H), 3.56–3.43 (m, 2H), 1.71 (p, *J* 6.2 Hz, 2H), 0.85 (s, 9H), 0.00 (s, 6H). ^{13}C NMR (126 MHz, [^{2}H]chloroform) δ 170.12, 156.09, 143.95, 143.77, 141.29, 127.71, 127.08, 125.21, 125.15, 119.99, 118.54, 77.30, 77.26, 77.05, 76.79, 70.57, 68.28, 67.24, 66.10, 59.67, 54.57, 47.13, 32.63, 25.95, 18.34, − 5.33. HRMS (*m/z*), $[M + H]^+$ calculated for C$_{30}$H$_{42}$NO$_6$Si 540.2781; found 540.2831.

Compound 10

To a solution of **9** (0.13 g, 0.24 mmol) in THF (2.5 ml) morpholine (0.04 ml, 0.454 mmol) and Pd(PPh$_3$)$_4$ (0.012 g, 0.024 mmol)

were added sequentially. The reaction was stirred for 1.5 h; TLC (petroleum ether/DCM, 4:1, v/v)/ethyl acetate 40% and DCM/methanol 10% showed disappearance of the starting material and formation of a more polar new product. The reaction was quenched by addition of a suspension of Dowex 50WX8-100 H$^+$ resin in methanol and stirred for 10 min. The resin was filtered off, and the filtrate was concentrated to give 0.123 g (0.24 mmol, quant) of the target product as a yellow syrup.

^{1}H NMR (500 MHz, [^{2}H]chloroform) δ 7.70 (d, J 7.6 Hz, 2H), 7.57 (t, J 7.2 Hz, 2H), 7.34 (t, J 7.5 Hz, 2H), 7.26 (q, J 7.4, 6.4 Hz, 3H), 5.71 (d, J 8.5 Hz, 1H), 4.58–4.37 (m, 2H), 4.37–4.29 (m, 2H), 4.19 (t, J 7.2 Hz, 1H), 3.63 (p, J 10.8 Hz, 3H), 3.51 (dt, J 10.0, 4.6 Hz, 2H), 1.72 (dd, J 8.3, 4.0 Hz, 2H), 0.84 (s, 9H), 0.00 (s, 6H). HRMS (m/z), [$M+$H]$^+$ calculated for $C_{43}H_{71}N_{10}O_{23}P_2$ 500.2468; found 500.2461.

Compound 11a

Peptide synthesis was performed on RinkAmide MBHA (4-methylbenzhydrylamine) low-load resin (0.38 mmol/g, 0.13 g, 0.05 mmol) (Novabiochem). Standard couplings were performed using 5 equiv. of amino acid, 5 equiv. of HCTU [O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and 10 equiv. of DIPEA in a microwave-heated reactor (CEM Liberty microwave peptide synthesizer) for 5 min at 70 °C. Deprotections were performed with 20% piperidine in DMF initially for 0.5 min at 70 °C and then with a fresh portion of deprotection mixture for 3 min at 70 °C. Addition of the 'stretched serine' building block **8a** was performed using 2.5 equiv. of the amino acid for 20 min at 60 °C. After this point, deprotections were performed initially for 5 min at room temperature and then with a fresh portion of deprotection mixture for 15 min at room temperature. The peptide was cleaved from the resin with a mixture of TFA/water/TIPS (tri-isopropylsilane) 92.5:5:2.5, by vol. (2 ml) for 3 h. The cleavage mixture was filtered into the centrifuge tube containing cold (0 °C) diethyl ether (40 ml). The resin was additionally twice washed with 1 ml of TFA for 5 min each and the washings were added to the same tube. The tube was kept in the freezer (-18 °C) for 16 h. The precipitated peptide was centrifuged at 3700 **g** at 4 °C for 15 min; the supernatant was discarded. The pellet was suspended in diethyl ether and centrifuged again. The procedure was repeated once more. The pellet was blow-dried with argon, dissolved in 20% aqueous acetic acid and freeze-dried to give 0.025 g (0.03 mmol) of the crude peptide. HRMS (m/z), [$M-$H]$^-$ calculated for $C_{34}H_{60}N_8O_{15}P$, 851.3916; found 851.3907.

Compound 11b

HRMS (m/z), [$M-$H]$^-$ calculated for $C_{35}H_{62}N_8O_{15}P$, 865.4072; found 865.4100

Compound 12

The peptide was synthesized using 'stretched serine' building block **10** (2.5 equiv.) according to the general procedure.

^{1}H NMR (500 MHz, ^{2}H$_2$O) δ 4.61–4.56 (m, 2H), 4.45–4.41 (m, 1H), 4.31 (d, J 4.7 Hz, 1H), 4.30–4.25 (m, 1H), 4.19 (tt, J 6.5, 5.4 Hz, 1H), 4.13–4.03 (m, 3H), 3.91–3.83 (m, 1H), 3.76 (d, J 5.6 Hz, 2H), 3.69 (ddd, J 10.7, 8.6, 6.8 Hz, 1H), 3.58 (dtd, J 10.1, 8.5, 7.5, 2.6 Hz, 4H), 2.31–2.21 (m, 1H), 2.09–2.02 (m, 2H), 2.01 (s, 2H), 1.95 (dt, J 13.1, 7.3 Hz, 1H), 1.87 (dq, J 13.7, 6.9 Hz, 1H), 1.76 (td, J 6.4, 1.4 Hz, 2H), 1.37 (d, J 7.2 Hz, 3H), 1.22 (d,

J 6.4 Hz, 3H), 1.17 (d, J 6.4 Hz, 3H), 0.94 (d, J 6.7 Hz, 3H), 0.93 (d, J 6.8 Hz, 3H), 0.90 (d, J 6.5 Hz, 3H), 0.88 (d, J 6.8 Hz, 3H).

^{13}C NMR (126 MHz, ^{2}H$_2$O) δ 177.46, 174.38, 174.09, 173.92, 173.64, 171.69, 171.21, 170.02, 69.12, 68.22, 67.05, 67.00, 60.37, 59.59, 59.49, 58.87, 58.69, 57.06, 53.59, 49.50, 48.50, 31.22, 30.16, 30.05, 29.50, 24.69, 21.65, 18.74, 18.66, 18.45, 18.38, 17.80, 17.60, 16.74. HRMS MS–TOF (positive): m/z 773.4434, calculated for $C_{34}H_{61}N_8O_{12}$ 773.4409, [$M+$H]$^+$

Compound 14a

2′,3′-O-diacetyl-UMP triethylammonium salt **13** (0.17 g, 0.3 mmol) was rendered dry by co-evaporation with DMF (3 ml), dissolved in DMF (3 ml) and treated with 1,1′-carbonylbisimidazole (0.096 g, 0.59 mmol). The reaction was kept for 16 h at room temperature, quenched by addition of methanol (0.032 ml, 0.77 mmol), kept for 30 min at room temperature and concentrated. ^{31}P NMR showed the presence of a single principal product [^{31}P NMR (202 MHz, ^{2}H$_2$O) δ -10.4]; assumed to be phosphoimidazolide. A solution of the crude peptide **11a** (0.025 g, 0.03 mmol) in DMF (2 ml) was treated with triethylamine (0.03 ml) and concentrated. To the residue was added a solution of the crude phosphoimidazolide in DMF (2 ml) and the reaction was kept for 20 h at room temperature. The reaction products were concentrated, dissolved in a mixture of methanol (2 ml), water (0.5 ml) and triethylamine (0.25 ml), and kept for 16 h at room temperature. The reaction was concentrated, dissolved in acetonitrile/water 95:5 (v/v) in 0.1% TFA (10 ml), filtered through a 0.45 μm syringe filter and purified by HPLC (Waters 19 × 100 Peptide separation technology column, gradient 5–95% acetonitrile in 0.1% TFA, detection at 214/254 nm). The fractions containing product were pooled and freeze-dried. The powder was dissolved in 0.25 M NH$_4$HCO$_3$ buffer and freeze-dried to give 0.015 g (0.013 mmol) of the target product as a fluffy powder.

^{1}H NMR (500 MHz, ^{2}H$_2$O) δ 7.89 (d, J 8.1 Hz, 1H), 5.94–5.91 (m, 1H), 5.90 (d, J 8.1 Hz, 1H), 4.54 (td, J 6.0, 5.6, 2.4 Hz, 2H), 4.41 (dd, J 8.3, 6.1 Hz, 1H), 4.32–4.28 (m, 2H), 4.28–4.23 (m, 2H), 4.22 (d, J 2.8 Hz, 1H), 4.19–4.16 (m, 1H), 4.16–4.13 (m, 1H), 3.95 (q, J 6.6 Hz, 2H), 3.87–3.81 (m, 1H), 3.74 (d, J 5.5 Hz, 2H), 3.66 (dt, J 10.2, 7.2 Hz, 1H), 3.58 (q, J 6.3 Hz, 2H), 2.30–2.20 (m, 1H), 2.07–1.89 (m, 2H), 1.98 (s, 2H), 1.85 (q, J 7.3, 6.3 Hz, 3H), 1.34 (d, J 7.2 Hz, 3H), 1.19 (d, J 6.3 Hz, 2H), 1.15 (d, J 6.5 Hz, 3H), 0.91 (dd, 6H), 0.87 (dd, 6H); ^{13}C NMR (126 MHz, ^{2}H$_2$O) δ 177.5, 174.4, 174.1, 173.9, 173.7, 171.8, 171.3, 170, 166.1, 151.8, 141.7, 102.6, 88.3, 83.2 (d, $J_{C,P}$ 8.9 Hz), 69.7, 69, 67.9, 67, 65 (d, $J_{C,P}$ 5.4 Hz), 63.6 (d, $J_{C,P}$ 6.2 Hz), 60.3, 59.6, 59.4, 59, 57, 53.8, 53.7, 49.5, 48.5, 30.1, 30, 29.8 (d, $J_{C,P}$ 7.1 Hz), 29.5, 24.7, 21.6, 18.72, 18.7, 18.44, 18.4, 17.7, 17.6, 16.7; ^{31}P NMR (202 MHz, ^{2}H$_2$O) δ -11.08 (d, $J_{P\alpha,P\beta}$ 26.6 Hz), -11.56 (d, $J_{P\alpha,P\beta}$ 26.6 Hz). HRMS (m/z), [$M-$H]$^-$ calculated for $C_{43}H_{71}N_{10}O_{23}P_2$ 1157.4169; found 1157.4171.

Compound 14b

^{1}H NMR (500 MHz, ^{2}H$_2$O) δ 7.91 (d, J 8.1 Hz, 1H), 5.93 (d, J 4.6 Hz, 1H), 5.91 (d, J 8.4 Hz, 1H), 4.58–4.52 (m, 1H), 4.41 (dd, J 8.2, 6.1 Hz, 1H), 4.33–4.29 (m, 2H), 4.28 (d, J 4.5 Hz, 1H), 4.26 (d, J 7.3 Hz, 1H), 4.24–4.20 (m, 1H), 4.20–4.15 (m, 1H), 4.13 (dd, J 5.6, 3.0 Hz, 1H), 4.09–4.03 (m, 1H), 4.08 (dd, J 9.7, 7.4 Hz, 2H), 3.86 (dq, J 15.9, 6.8, 6.0 Hz, 3H), 3.73 (d, J 5.6 Hz, 2H), 3.67 (dt, J 10.2, 7.1 Hz, 1H), 3.54–3.46 (m, 2H), 2.29–2.21 (m, 1H), 2.08–2.00 (m, 2H), 1.99 (s, 3H), 1.97–1.90 (m, 1H), 1.82–1.89 (m, 1H), 1.57 (m, 4H), 1.35 (d, J 7.2 Hz, 3H), 1.20 (d,

J 6.4 Hz, 3H), 1.15 (d, J 6.4 Hz, 3H), 0.91 (dd, 6H), 0.87 (dd, 6H); ^{13}C NMR (126 MHz, ^{2}H$_2$O) δ 177.5, 174.4, 174.1, 173.9, 173.6, 171.7, 171.2, 167, 166.1, 151.8, 141.7, 102.7, 88.4, 83.3 (d, J 9.0 Hz), 73.8, 71, 69.7, 68.9, 67 (d, $J_{C,P}$ 7.2 Hz), 66.3 (d, $J_{C,P}$ 5.6 Hz), 64.9 (d, $J_{C,P}$ 5.0 Hz), 60.3, 59.6, 59.5, 59, 57, 53.6, 49.5, 48.5, 30.1, 30, 29.5, 26.4 (d, $J_{C,P}$ 7.2 Hz), 25.1, 24.7, 21.6, 18.73, 18.66, 18.43, 18.4, 17.8, 17.6, 16.7; ^{31}P NMR (202 MHz, ^{2}H$_2$O) δ -10.97 (d, $J_{P\alpha,P\beta}$ 27.2 Hz), -11.53 (d, $J_{P\alpha,P\beta}$ 27.2 Hz). HRMS (m/z), $[M - H]^-$ calculated for $C_{44}H_{73}N_{10}O_{23}P_2$, 1171.4325; found 1171.4343.

Compound 17

To a cold (0°C) solution of 3-methoxypropanol **15** (0.018 g, 0.25 mmol) in acetonitrile (5 ml) and PMBPA [di-(p-methoxybenzyl)-N,N-diisopropylphosphoramidite] **16** [1] (0.26 g, 0.5 mmol) was added DCI (0.059 g, 0.5 mmol). After 30 min, a 0.5 M solution of iodine in pyridine (1.1 ml, 0.55 mmol) was added to the reaction mixture, to produce a tan-coloured solution, which was stirred further for 1 h. Then, 2′,3′-O-diacetyl-UMP triethylammonium salt **13** (0.14 g, 0.25 mmol) in 2.5 ml of acetonitrile was added and stirred for 16 h at room temperature. The reaction was concentrated and dissolved in a 5:2:1 (by vol.) methanol/H$_2$O/triethylamine mixture (8 ml) and stirred for 16 h at room temperature. The reaction mixture was concentrated, diluted with water and extracted with chloroform. The mixture was centrifuged at 3700 g for 15 min at 4°C. The clear layers were separated; the organic layer was extracted with water and centrifuged once more. The combined aqueous layer was extracted with chloroform, centrifuged and concentrated with the addition of n-butanol to give an oily residue. This was purified by size-exclusion chromatography (Bio-Gel P2 fine; column 2.6 cm × 100 cm; flow rate 0.4 ml/min; elution with 0.25 M NH$_4$HCO$_3$). The fractions containing the product were pooled and freeze-dried to give 0.05 g (0.1 mmol, 40%) of the target product as a white fluffy solid.

^{1}H NMR (500 MHz, ^{2}H$_2$O) δ 7.76 (d, J 8.1, 1H, H-6), 5.86 (d, 1H, H-1′), 5.80 (d, J 8.1, 1H, H-5), 4.28–4.18 (m, 2H, H-2′, H-3′), 4.15–4.08 (m, 2H, H-4′, H-5a′), 4.05 (ddd, J 11.6, 5.4, 2.8 Hz, 1H, H-5b′), 3.86 (q, J 6.6 Hz, 2H, CH$_3$OCH$_2$CH$_2$C$\underline{H_2}$OP(O)], 3.42 [t, J 6.5 Hz, 1H, 2H, CH$_3$OC$\underline{H_2}$CH$_2$CH$_2$OP(O)], 3.21 (s, 3H, C$\underline{H_3}$O), 1.77 [dq, J 6.4 Hz, 2H, CH$_3$OCH$_2$C$\underline{H_2}$CH$_2$OP(O)]. ^{13}C NMR (126 MHz, ^{2}H$_2$O) δ 170.37, 154.80, 141.15 (C-6), 102.77 (C-5), 88.56 (C-1′), 82.82 (d, $J_{C,P}$ 9 Hz, C-4′), 73.66 (C-2′/C-3), 69.55 (C-2′/C-3), 68.93 [CH$_3$O$\underline{C}$H$_2$CH$_2$CH$_2$OP(O)], 64.90 (d, $J_{C,P}$ 5.5 Hz, C-5′), 63.50 [d, $J_{C,P}$ 5.5 Hz, CH$_3$OCH$_2$CH$_2$$\underline{C}H_2$OP(O)], 57.75 ($\underline{C}H_3$O), 29.58 [d, $J_{C,P}$ 7.4 Hz, CH$_3$OCH$_2$$\underline{C}H_2CH_2$OP(O)]. ^{31}P NMR (202 MHz, ^{2}H$_2$O) δ -11.14 (d, J 19.7 Hz), -11.66 (d, J 19.8 Hz). HRMS (m/z), $[M - H]^-$ calculated for $C_{13}H_{21}N_2O_{13}P_2$ 475.0519; found 475.0531.

REFERENCES

1 van der Heden van Noort, G. J., Verhagen, C. P., van der Horst, M. G., Overkleeft, H. S., van der Marel, G. A. and Filippov, D. V. (2008) A versatile one-pot procedure to phosphate monoesters and pyrophosphates using di(p-methoxybenzyl)-N,N-diisopropylphosphoramidite. Org. Lett. **10**, 4461–4464

2 Pathak, S., Borodkin, V. S., Albarbarawi, O., Campbell, D. G., Ibrahim, A. and van Aalten, D. M. (2012) O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release. EMBO J. **31**, 1394–1404