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 covariantly linked to UDP, eliminating the GlcNAc pyranoside ring. Goblin1 co-crystallizes with OGT, revealing an ordered C3 linker and retained substrate-binding modes, and binds the enzyme with micromolar affinity, inhibiting glycosyltransferase 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 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-oxobenzol[1]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^{422} and Cys^{477} 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 ($K_i 0.5 \mu M$) [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 glycol 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 anomic 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: 31P NMR (202 MHz, 2H2O) δ = −11.08 (d, J 2p 2p 26.6 Hz), −11.56 (d, J 2p 2p 26.6 Hz); HRMS (high-resolution MS) (m/z) [M − H]− = 1157.4171. 14b: 31P NMR (202 MHz, 2H2O) δ = −10.97 (d, J 2p 2p 27.2 Hz), −11.53 (d, J 2p 2p 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 K2HPO4, 10 mM EDTA and 1% (v/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 Li2SO4 solution before flash-freezing in liquid nitrogen. Diffraction data were collected at Diamond Light Source beamline I03. Data were processed with Xia2 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-gSer395 (O-GlcNAcylated Ser395)-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 dithionite 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 involving 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 for both 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 Kd 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 (OGTbisubstrate-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 polyphosphates 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 alklylation [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-diisopropylethylamine) (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
Bisubstrate UDP–peptide conjugates as human O-GlcNAc transferase inhibitors

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 Fo\(^{-}\)Fc electron density map is shown, contoured at 3.5\(\sigma\). Full goblin1 Fo\(^{-}\)Fc 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\(\mu\)M). 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-methylbenzhydrylamine) resin and HCTU [O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethylluronium 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 CD1 (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) AlBr (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 hOGT 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
(Supplementary Figure S3 at http://www.biochemj.org/bj/457/bj4570497add.htm), revealed unambiguous density for the entire compound, including the ordered C1 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 SmNodC (Sinorhizobium meliloti NodC) (Supplementary Figure S4 at http://www.biochemj.org/bj/457/bj4570497add.htm).

**Scheme 3** Synthesis of the ‘linker-only’ UDP derivative

(a) 16, 4,5-dicyanoimidazole, acetonitrile, room temperature, 1 h, then (b) I2/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%.

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.

**AUTHOR CONTRIBUTION**

Vladimir Borodkin performed chemical syntheses, Mehmet Gundogdu and Marianne Schimpl performed crystallography and structure determination, Karin Rafe and Helge Dortmuller measured enzyme activity and inhibition, David Robinson conducted binding affinity measurements. Vladimir Borodkin and Daan van Aalten conceived the original idea. All authors were involved in planning experiments, analysing results and writing the paper.

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**REFERENCES**

SUPPLEMENTARY ONLINE DATA

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

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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 then 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 NaHCO3 solution and brine. The aqueous layers were back extracted with the ethyl acetate two times more. 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% of the target product as clear oil.

\[ \alpha_\text{D} = +4.7 (c 1.0 in chloroform) \]

\[ ^1H \text{NMR (500 MHz, } [\text{H}]\text{chloroform}) \delta 6.82 (s, 4H), 5.86 (d, J 12.2, 10.5, 5.7 Hz, 1H), 5.42 (d, J 8.9 Hz, 1H), 5.29 (dq, J 17.3, 1.6 Hz, 1H), 5.19 (dq, J 10.4, 1.3 Hz, 1H), 4.66–4.55 (m, 2H), 4.45 (dt, J 8.8, 3.2 Hz, 1H). 3.95 (td, J 6.2, 1.0 Hz, 2H), 3.91–3.87 (m, 1H), 3.76 (s, 3H), 3.68 (dd, J 9.5, 3.3 Hz, 1H), 3.61 (qt, J 9.5, 6.1 Hz, 2H), 1.98 (p, J 6.2 Hz, 2H), 1.45 (s, 9H); \]

\[ ^13C \text{NMR (126 MHz, } [\text{H}]\text{chloroform}) \delta 170.4, 155.5, 153.8, 153.131.67, 118.4, 115.4, 114.6, 79.9, 70.8, 68, 65.9, 65.1, 55.7, 54.1, 29.5, 28.3. \]

HRMS (m/z), [M + H]+ calculated for C22H35NO7, 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 NaHCO3 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; \[ \alpha_\text{D} = +11.4 (c 1.00 in chloroform); \]

\[ ^1H \text{NMR (500 MHz, } [\text{H}]\text{chloroform}) \delta 7.67 (d, J 7.5 Hz, 2H), 7.52 (t, J 6.6 Hz, 2H), 7.31 (t, J 7.5 Hz, 2H), 7.22 (td, J 7.5, 1.2 Hz, 2H), 6.78–6.68 (m, 4H), 5.78 (ddt, J 16.2, 10.9, 5.7 Hz, 1H), 5.62 (d, J 8.7 Hz, 1H), 5.21 (dq, J 17.1, 1.6 Hz, 1H), 5.11 (dq, J 10.4, 1.3 Hz, 1H), 4.54 (dt, J 5.7, 1.5 Hz, 2H), 4.49–4.42 (m, 2H), 4.34 (dd, J 10.6, 7.2 Hz, 1H), 4.26 (dd, J 10.6, 7.3 Hz, 1H), 4.15 (t, J 7.3 Hz, 1H), 3.87 (td, J 6.2, 1.8 Hz, 2H), 3.84 (dd, J 9.5, 3.2 Hz, 1H), 3.64 (s, 3H), 3.55 (qt, J 9.6, 6.2 Hz, 2H), 1.91 (p, J 6.1 Hz, 2H); \]

\[ ^13C \text{NMR (126 MHz, } [\text{H}]\text{chloroform}) \delta 170.1, 156, 153.8, 153, 143.9, 143.8, 141.3, 131.6, 127.7, 127.1, 125.22, 120, 118.6, 115.4, 114.7, 76.9, 70.7, 68.2, 67.2, 65.1, 55.7, 54.6, 47.2, 29.5. \]

HRMS (m/z), [M + H]+ calculated for C33H51NO7, 532.2335; found 532.2331.

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Co-ordinates and structure factors for human O-GlcNAc transferase complexed to the Gobin1 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

\[ \alpha = +13.5^\circ \] (c 1.00 in chloroform); ¹H NMR (500 MHz, [²H]chloroform) δ 7.80 (dd, J 7.4, 1.1 Hz, 2H), 7.70–7.60 (m, 2H), 7.43 (t, J 7.4, 0.9 Hz, 2H), 7.34 (td, 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; \[ \alpha = +11.3^\circ \] (c 1.00 in chloroform); ¹H NMR (500 MHz, [²H]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), 5.14 (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); ¹³C NMR (126 MHz, [²H]chloroform) δ 170.1,
Bisubstrate UDP–peptide conjugates as human O-GlcNAc transferase inhibitors

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 Ser395 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 ± S.E.M.

Compound 5b

$[\alpha]_D = +12.4^\circ$ (c 1.00 in chloroform); $^1$H NMR (500 MHz, [H]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 (ddd, J 16.5, 10.9, 5.7 Hz, 2H), 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 (ddd, J 10.7, 3.2 Hz, 1H), 4.40 (ddd, J 10.7, 3.2 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, [H]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$_{24}$H$_{28}$NO$_6$, 426.1917; found 426.1922.

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;
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 adsorbed on to silica gel and purified by flash chromatography
combined organic layer was dried and concentrated. The residue
was partitioned between DCM and 1 M phosphate (pH 7)
was suspended in methyl t-butyl ether (20 ml) and centrifuged
again. The supernatants were pooled and concentrated. 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-isopropylphosphoramide 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 to room temperature and reaction was stirred for 1 h. 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

**Compound 7b**

To a solution of 5b (0.22 g, 0.297 mmol) in THF (3 ml)
morpholine (0.05 ml, 0.53 mmol) and Pd(PPh 3)4 [tetrakis
(triphenylphosphine)palladium(0)] (0.009 g, 0.0078 mmol) were
added sequentially. The reaction was stirred for 1.5 h, quenched
by the 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 S3 Stereo image of hOGT with bisubstrate inhibitor showing unbiased F_{c} – F_{e} electron density after 4-fold averaging (3.5σ)**

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4-parameter IC50 equation in the GraphPad Prism program (http://www.graphpad.com). NodC
the elongation of 4-methylumbelliferyl-N-acetyl-
fluorigenic in vitro assay was performed in the presence of 1 mM–0.46
inhibitor, or by the goblin1 fragments containing either UDP and linker (Figure S4: The bacterial GlcNAc transferase NodC is not inhibited by goblin1)
the ODN of SmNodC, 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 IC50 equation in the GraphPad Prism program (http://www.graphpad.com). NodC
inhibits product inhibition by UDP (IC50 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

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[α]D = +24.3° (c 1.00 in chloroform); δ 7.79–7.77 (m, 2H), 6.75 (dd, J 11.0, 7.5 Hz, 2H), 7.41 (td, J 7.4, 1.0 Hz, 2H), 7.73 (tt, J 7.4, 1.0 Hz, 2H), 7.27–7.20 (m, 4H), 16.1 (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), 4.04 (dq, J 9.5, 3.5 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); 1H NMR (500 MHz, [1H]chloroform) δ 172.1, 156.2, 144.3, 143.9, 141.3, 138.5, 132.6, 132.2, 131.0, 129.3, 128.2, 128.7, 127.7, 127.1, 125.3, 119.9, 69.7 (d, Jcsp 5.5 Hz), 69.6 (d, Jcsp 5.5 Hz), 69.6, 67.2, 66.8, 64.8 (d, Jcsp 5.1 Hz), 54.6, 47.1, 30.2, 30.1, 21.2; 13P NMR (202 MHz, [1P]chloroform) δ = –0.84. HRMS (m/z), [M – H]− calculated for C36H44NO9P, 672.2362; found 672.2357.

Compound 8b

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, 0.35 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 NaHCO3 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.

[α]D = +7.4° (c 1.23 in chloroform); δ 7.79–7.77 (m, 2H), 6.75–7.50 (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 (ddd, J 5.7, 4.2, 1.5 Hz, 2H), 4.48 (dq, 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, 9.7, 4.3 Hz, 3H), 3.56–3.54 (m, 2H), 1.71 (p, J 6.2 Hz, 2H), 0.85 (s, 9H), 0.00 (s, 6H); 1H NMR (126 MHz, [1H]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 C36H44NO9P, 689.2754; found 689.2781.

Compound 9

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(PPh3)4 (0.012 g, 0.024 mmol)
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) 6.41–6.56 (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 (ddd, 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 177.5, 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, Jc,P 8.9 Hz, 69.7, 69, 67.9, 67, 65, Jc,P 5.4 Hz, 63.6 (Jc,P 6.2 Hz, 60.3), 59.6, 59.4, 59, 57.3, 53.7, 49.5, 48.5, 30.1, 30, 29.8 (Jc,P 7.1 Hz), 29.5, 24.7, 21.6, 18.2, 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 = 26.6 Hz), –11.56 (d, J = 26.6 Hz). HRMS (m/z), [M – H]$^-$ calculated for C$_{12}$H$_{25}$N$_{2}$O$_{3}$P$_{2}$ 1157.4169; found 1157.4171.

Compound 14b

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 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 7.2 Hz, 3H), 0.94 (d, J 6.7 Hz, 3H), 0.93 (d, J 6.8 Hz, 3H).
Bisubstrate UDP–peptide conjugates as human O-GlcNAc transferase inhibitors

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/H2O/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 x 100 cm; flow rate 0.4 ml/min; elution with 0.25 M NH4HCO3). 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.

1H NMR (500 MHz, 2H2O) 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, CH3OCH2CH2CH2OP(O)], 3.42 [t, J 6.5 Hz, 1H, 2H, CH3OCH2CH2CH2OP(O)], 3.21 (s, 3H, CH3O), 1.77 [dq, J 6.4 Hz, 2H, CH3OCH2CH2CH2OP(O)]. 13C NMR (126 MHz, 2H2O) δ 170.37, 154.80, 141.15 (C-6), 102.77 (C-5), 88.56 (C-1'), 82.82 (d, J 6.9 Hz, C-4'), 73.66 (C-2'/C-3'), 69.55 (C-2'/C-3'), 68.93 [CH3OCH2CH2CH2OP(O)], 64.90 (d, J 6.5 Hz, C-5'), 63.50 [d, J 6.9 Hz, CH3OCH2CH2CH2OP(O)], 57.75 (CH3O), 29.58 [d, J 7.4 Hz, CH3OCH2CH2CH2OP(O)]. 31P NMR (202 MHz, 2H2O) δ -11.14 (d, J 19.7 Hz), -11.66 (d, J 19.8 Hz). HRMS (m/z), [M – H]− calculated for C44H73N10O23P2 1171.4325; found 1171.4343.

REFERENCES