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A convenient synthesis of the C-1-phosphonate analogue of UDP-GlcNAc and its evaluation as an inhibitor of O-linked GlcNAc transferase (OGT)

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Abstract—The C-1-phosphonate analogue of UDP-GlcNAc has been synthesized using an α-configured C-1-aldehyde as a key intermediate. Addition of the anion of diethyl phosphate to the aldehyde produced the hydroxyphosphonate. The configuration of this key intermediate was determined by X-ray crystallography. Deoxygenation, coupling of the resulting phosphonic acid with UMP and deprotection gave the target molecule as a di-sodium salt. This analogue had no detectable activity as an inhibitor of (OGT).

Keywords: *O*-GlcNAc transferase; *N*-Acetylglucosamine; UDP-GlcNAc; Phosphate isostere; OGT inhibition

1. Introduction

O-GlcNAc transferase- (OGT)-mediated transfer of *N*-acetylglucosamine (GlcNAc) from α-linked UDP-GlcNAc to serine and threonine residues with the formation of β-glycosyl linkages represents an important post-translational modification of proteins. By generating UDP-GlcNAc, the hexose monophosphate biosynthetic pathway may be viewed as a nutrient-sensing signalling pathway.[1–3] Based on the targets modified by *O*-GlcNAc, the proposal has been made that the enzymes of *O*-GlcNAc metabolism modulate nuclear transport, transcription, cell growth and apoptosis in response to nutrient availability.[4,5] Development of new potent and selective inhibitors for OGT would facilitate delineation of the roles of this enzyme. We report herein one approach to such inhibitors.

In general, the important and diverse roles of the glycosidic bond in biological structure and function have prompted intense interest in the synthesis of structural analogues wherein the glycosidic oxygen is replaced with an isosteric mimic. Thus, our initial synthetic targets were analogues wherein the glycosidic oxygen of UDP-GlcNAc would be replaced with a methylene (compound **2**), fluoromethylene (compound **3**) or difluoromethylene (compound **4**) moiety. Although the simple phosphonate analogue **2** represents the more accessible synthetic target (the synthesis of the phosphono analogue **5** of *N*-acetyl-α-D-glucosamine-1-phosphate has been described[6] and a synthesis of **2** also has been reported recently),[7] fluorophosphonates and difluorophosphonates are closer mimics of the phosphate ester, both sterically and electronically. Inspired by early studies by Blackburn and co-workers,[8] fluorophosphonate analogues of several biologically important

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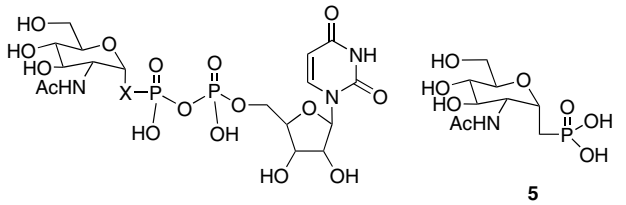
phosphates have been prepared and studied, including those of phosphoserine,[9] phosphotyrosine,[10] phosphoenol pyruvate[11] and others. Berkowitz et al. recently prepared the two CHF epimers of monofluorophosphonate isosters of glucose-6-phosphate as substrate mimics for glucose-6-phosphate dehydrogenase.[12] Particularly intriguing was their observation of discrimination by the enzyme between the two epimers. In another relevant example, Motherwell and co-workers have prepared a protected β-linked glucosyl difluoromethylenephosphonate by radical addition to a *gem*-difluoroenol ether precursor.[13]

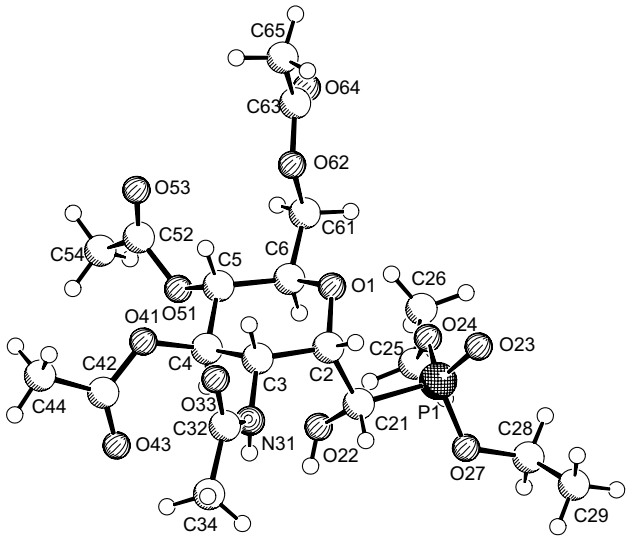


X= CH_2 (**2**), CHF (**3**), CF_2 (**4**) **5**

2. Chemistry

The strategy used by Nicotra and co-workers for the synthesis of **5** involved initial preparation of a phosphonate analogue of a glucose derivative.[6] The hydroxyl group in the 2-position was then converted to the amine functionality. While our own work was in progress, Finney and co-workers reported a synthesis of our target compound **2** that involved a new route to **5** that also used an oxygen to nitrogen transformation.[7] In our work, we chose to reinvestigate approaches based on glucosamine derivatives as precursors that we felt would be more direct. Horton and Cui noted that many problems associated with the functionalization of amino sugars are not manifested during free radical reactions.[14] We reasoned that the α-linked aldehyde **9** should serve as a convenient intermediate for the preparation of target compound **2**, and devised a strategy based on the recent preparation of an acetamido derivative **8** containing an α-linked propenyl group (Scheme 1). Cui and Horton have published the facile synthesis of the α-*C*-allyl precursor **7**.[14] Following their procedure, 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride was prepared by the chlorination of the peracetate of commercially available *N*-acetyl-D-glucosamine and was treated with allyltributyltin under free-radical conditions to give a single anomer of the C-1-allylation product **7**. As previously described by McGarvey et al. we converted **7** to the vinyl derivative **8** using transition metal-catalyzed double bond migration.[15] Ozonolysis of **8** afforded the desired aldehyde **9** as the α-anomer in very good yield. We have also explored oxidation with osmium tetroxide but this method[16] gave a very low yield of **9** (2%). We observed slow partial anomerization of aldehyde **9** to its β-anomer during attempted purification along with the formation of several unidentified decomposition products. For this reason we used aldehyde **9** directly in the next step immediately after its isolation.

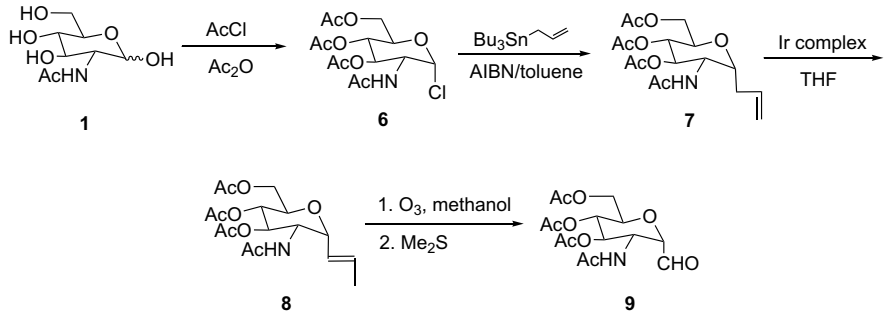
With a convenient route to **9** established, we next explored methods to form the carbon phosphorous bond. We were pleased to find that reaction of aldehyde **9** with the anion of diethyl phosphite cleanly produced the α-hydroxyphosphonate **10** as a mixture of two diastereoisomers (ratio 95/5). However, isomerization of **9** to the β-anomer needed to be ruled out due to the configurational lability of **9** noted above. For this reason, the structure of **10** was confirmed by X-ray analysis.



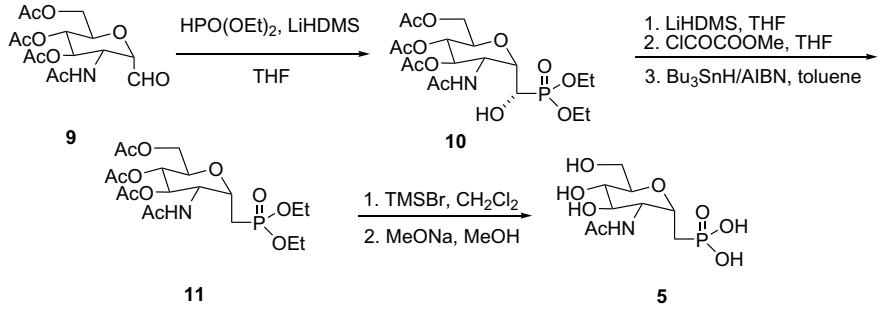
X-ray structure of **10**

The hydroxy phosphonate **9** seemed a promising intermediate not only for the synthesis of the phosphonate analogue of UDP-GlcNAc but also to fluorinated derivatives **3** and **4** since there are many procedures available for replacing a hydroxyl group with fluorine. However, all attempts to carry out this transformation with **10**, including fluorination with DAST or Deoxofluor, were unsuccessful. In each case we obtained complex mixtures of nonfluorinated products. There was evidence from mass spectroscopy that intramolecular reactions between the acetamido group and a presumed activated hydroxyl group were occurring more rapidly than the displacement with fluoride.

While pursuing the elusive fluorinated intermediate, we also continued work towards the preparation of nonfluorinated target **2**. Deoxygenation of **10** was carried out under the so-called Dolan–MacMillan conditions[17] to give phosphonate **11** in moderate yields. Deprotection of the phosphonate group of **11** by treatment with trimethylsilylbromide and subsequent deacetylation of the hydroxy groups of the sugar core led to the desired phosphono analogue of *N*-acetyl-α-D-glucosamine-1-phosphate **5** (Scheme 2). To obtain an elemental analysis



Scheme 1.



Scheme 2.

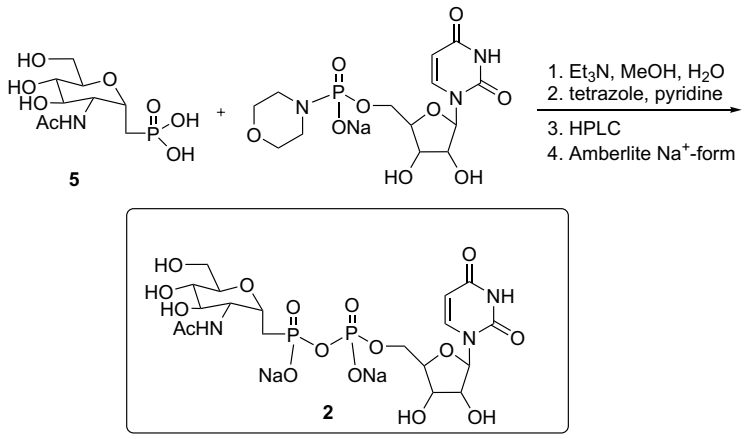
of **5** we converted the free acid form into its disodium salt which crystallized as the monohydrate.

Compound **5** shows an 11 Hz coupling constant between H-2 and H-3. This supports assignment of an axial orientation of H-2 and H-3. Similarly, the coupling constant of 2 Hz between H-1 and H-2 is indicative of the equatorial orientation of H-1. This coupling constant cannot be readily extracted from the complex multiplet of H-1. The optical rotation, carbon, proton and phosphorus NMR are consistent with those reported for **5**[6] (see Section 5). Confirmation of this structure validates this convenient alternative route to this GlcNAc analogue directly from the amino sugar.

With the key phosphonic acid **5** that possesses the requisite α-configuration in hand we considered methods to couple this with commercially available uridine monophosphomorpholidate. Several standard coupling methods are described.[18–20] The procedure described by Schmidt and co-workers[20] seemed most relevant to our own work so this was closely followed. Activation of phosphonate **5** with triethylamine and subsequent addition of activated uridine monophosphate in the presence of 1H-tetrazole as a catalyst[19,21,22] led to the desired coupling reaction (Scheme 3). After isolation by HPLC and ion-exchange chromatography to produce the sodium form, the target molecule **2** was obtained in modest yield, consistent with previous results using this procedure.[19,20] The structure was supported by high resolution mass spectrometry and ^{1}H, ^{13}C and ^{31}P NMR. The ^{1}H and ^{13}C NMR data were consistent with the data recently published by Finney and co-workers.[7]

3. Biological testing

The in vitro inhibitory activity of isosteric *C*-glycosyl analogue of UDP-GlcNAc (**2**) towards OGT was evaluated by using Nup62, a nuclear pore protein known to be an excellent OGT substrate. The OGT activity was measured by quantitating UDP-[^{14}C]-GlcNAc incorporation into the recombinant protein substrate, Nup62, in the presence and absence of **2**. Somewhat unexpectedly, analogue **2** was found to be a very poor inhibitor with an IC_{50} of more than 5 mM for ncOGT. This suggests that the presence of a C(1)–O–P linkage is important to enzyme activity. A requirement for structural fidelity has been observed in other studies. For example, it has been shown[23] that a non-isosteric *C*-glycosyl ethylphosphonophosphate analogue of UDP-Gal with an extra methylene between the sugar and UDP are effective inhibitors of bovine milk β-1,4-galactosyltransferase ($IC_{50} = 40$ μM). In contrast, the inhibitory activity of the corresponding non-isosteric analogue of UDP-*C*-GlcNAc for *Neisseria meningitides* Gal (β1→4)-Glcβ1,3-*N*-acetyl-glucosaminyltransferase is very weak ($IC_{50} = 3.5$ mM). Given the fact that both GTs are inverting enzymes and require divalent manganese ions



Scheme 3.

for activity, this discrepant inhibitory activity exhibited by their natural sugar-nucleotide mimics may result from the different spatial tolerance in the enzyme active site. The structure of the catalytic region of OGT has not been solved yet, however as previously mentioned, mutational analysis of the catalytic domain of OGT has demonstrated that the geometry of the OGT enzyme active site tolerates little modification. When compared with the reported K_m value[24] of mOGT with respect to the natural UDP-GlcNAc substrate ($K_m = 0.5$ μM when Nup62 was used as an acceptor protein), the observed less than 50% inhibition of OGT by UDP-*C*-GlcNAc at even exaggerated concentration (5 mM) suggests that spatial arrangement of the amino acid residues involved in the enzyme site makes it extremely sensitive to the nucleotide-sugar substrate. Indeed, the OGT protein is highly conserved between species and it has a higher affinity for UDP-GlcNAc compared to the affinities of other GTs for their corresponding UDP-sugars.

As noted above, it has been postulated that OGT plays a key role in nutrient sensing[1] by responding to physiologic changes in levels of cytoplasmic UDP-GlcNAc, the end product of the hexosamine biosynthetic pathway (HBP). Having a recognition site with high affinity and high selectivity would facilitate this role through rapid and selective reaction of the enzyme with cytoplasmic pools of UDP-GlcNAc.[25]

4. Conclusion

Although a precise understanding of sugar nucleotide recognition must await a crystal structure, the result of the poor inhibitory activity of isosteric C-linked UDP-GlcNAc towards OGT shown here confirms the previous findings[25] that the active site of OGT has a comparatively rigid structure. This apparently allows OGT to detect even a subtle change of geometry generated by a single replacement of an *O*-glycosidic bond (bent geometry) with a *C*-glycosidic bond (tetrahedral geometry) of UDP-GlcNAc. In addition, the replacement of the oxygen with a methylene group will perturb the pKa of the neighbouring phosphate group.

5. Experimental

5.1. General procedures and chemicals

Solvents were purified according to standard procedures. *N*-Acetyl-α-D-glucosamine **1** was purchased from Sigma–Aldrich. Ozonolysis was performed with a Welsbach T-408 ozone generator. NMR spectra were recorded at 22 °C on a Varian Mercury-300 or Bruker Avance-600. Tetramethylsilane (TMS) or the residual resonance of the deuterated solvent was used as the internal standard; solvent: $CDCl_3$, $\delta = 7.24$; D_2O, $\delta = 4.63$; CD_3OD, $\delta = 1.09$, 3.49 ppm. For ^{31}P NMR, phosphoric acid was used as the external standard and spectra were broadband ^1H-decoupled. HRMS spectra were recorded on a Waters LCT Premier TOF mass spectrometer. Flash chromatography was performed on Biotage SP4. Preparative HPLC separations were performed on an Agilent 1100 Series. The column used was a YMC-Pack ODS-AM, 250 × 20 mm, 5 μm. Mixtures of acetonitrile and 0.5 M ammonium formate were used as the mobile phase. Optical rotations were measured at 25 °C with a Perkin Elmer 341 polarimeter.

5.2. [2-(Acetylamino)-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl]methanal (9)

Ozone was bubbled into a stirred solution of **8**[15] (640 mg, 1.7 mmol) in 30 mL of dry MeOH at −78 °C for a period of 30 min (until the solution turned to a dark blue colour). The flow of ozone was stopped and the mixture was stirred another 30 min at −78 °C. The excess of ozone was removed by bubbling nitrogen

through the solution until the blue colour disappeared. Dimethylsulfide (3 mL) was then added and the stirred mixture was allowed to warm to room temperature and stored overnight. The MeOH was removed by rotary evaporation and the residue was dried under vacuum to constant weight to afford crude aldehyde **9** (540 mg, 87%) as a slightly yellow viscous oil. The purity of the crude product was 90% (LC) and due to low stability it was used in the next step without any further purification. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.82 (d, 1H, $^3J_{HH} = 1$, CHO), 6.24 (d, 1H, $^3J_{HH} = 8$, NH), 5.04–5.14 (m, 3H), 4.16–4.36 (m, 4H), 2.12 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 1.97 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 201.28 (s, CHO), 170.91 (s, CO), 170.05 (s, CO), 169.36 (s, CO), 169.19 (s, CO), 76.96 (s, CH), 73.71 (s, CH), 70.55 (s, CH), 68.11 (s, CH), 61.72 (s, CH_2), 49.12 (s, CH), 20.86 (s, CH_3), 20.73 (s, CH_3), 20.70 (s, CH_3), 20.61 (s, CH_3). HR-MS (ESI): $C_{15}H_{21}NO_9$ calculated 360.1295 (M+1); found: 360.1289 (M+1).

5.3. Diethyl [2-(acetylamino)-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl]-1-hydroxymethyl]phosphonate (10)

To a solution of diethyl phosphite (174 mg, 1.3 mmol) in 5 mL of THF was added lithium bis(trimethylsilyl)-amide (1.3 mL of 1 M solution in THF) at −78 °C. After 15 min a solution of aldehyde **9** (411 mg, 1.1 mmol) in 10 mL THF was added and the resulting mixture was stirred for 1 h. The reaction mixture was quenched by the addition of saturated solution of NH_4Cl (3 mL) and 30 mL of ether. The mixture was stirred for 15 min at −78 °C and then warmed to room temperature. Five millilitres of water was added and organic layer was separated. The aqueous layer was extracted in 2 × 10 mL of diethylether and the combined organic layers were dried over $MgSO_4$, filtered and concentrated. ^{1}H NMR of the crude product showed the presence of two diastereoisomers with ratio 95/5 (LC, ^{31}P NMR). Silica gel column chromatography (CH_2Cl_2/MeOH = 30:1) afforded white crystals of **10** (301 mg, 52.9%) as one diastereoisomer. $[\alpha]_D$ +63° ($c = 1$, acetone). Mp 170–172 °C (ethyl acetate/hexane). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.60 (d, 1H, $^3J_{HH} = 9$, NH), 5.72 (dd, 1H, $^3J_{HH} = 9$, $^3J_{HH} = 11$, CH), 5.48 (m, 1H), 5.16 (dd, 1H, $2 \times {}^3J_{HH} = 10$, CH), 4.54 (m, 1H, CH), 4.25–4.39 (m, 5H), 4.06–4.20 (m, 3H), 3.86 (dd, 1H, $^2J_{HP} = 50$, $^3J_{HH} = 13$, CH), 2.09 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.97 (s, 3H, CH_3), 1.39 (t, 3H, $^3J_{HH} = 7$, CH_3), 1.32 (t, 3H, $^3J_{HH} = 7$, CH_3). ^{13}C NMR ($CDCl_3$, 75.5 MHz): 171.77 (s, C=O), 171.11 (s, C=O), 170.83 (s, C=O), 169.56 (s, C=O), 73.64 (s, CH), 72.82 (s, CH), 72.32 (s, CH), 69.98 (d, $^1J_{CP} = 170$, CH), 68.62 (s, CH), 65.09 (d, $^2J_{CP} = 7$, CH_2), 63.31 (d, $^2J_{CP} = 8$, CH_2), 62.07 (s, CH_2), 51.16 (d, $^2J_{CP} = 11$, CH), 23.12 (s, CH_3), 21.04 (s, CH_3), 20.98 (s, CH_3), 20.88 (s, CH_3), 16.83 (d, $^3J_{CP} = 5$, CH_3), δ 16.53 (d, $^3J_{CP} = 6$, CH_3). ^{31}P NMR ($CDCl_3$, 121.4 MHz): δ 23.28 (s, 1P, ds_2), 22.54 (s, 1P, ds_1). HR-MS (ESI): $C_{19}H_{32}NO_{12}P$ calculated 498.1740 (M+1), found 498.1733 (M+1). Anal. Calcd for $C_{19}H_{32}NO_{12}P$: C, 45.88; H 6.48. Found: C, 45.60; H, 6.52.

X-ray crystal structure analysis for **10**: formula $C_{19}H_{32}NO_{12}P$, $M = 497.43$, colourless crystal 0.60 × 0.25 × 0.10 mm, $a = 9.4518(1)$, $b = 9.4016(1)$, $c = 14.8704(1)$ Å, $\beta = 107.655(1)°$, $V = 1259.18(2)$ Å^3, $\rho_{calc} = 1.312$ g cm^{-3}, $\mu = 1.498$ mm^{-1}, empirical absorption correction ($0.467 \leqslant T \leqslant 0.865$), $Z = 2$, monoclinic, space group $P2_1$ (No. 4), $\lambda = 1.54178$ Å, $T = 223$ K, ω and φ scans, 9484 reflections collected ($\pm h$, $\pm k$, $\pm l$), $[(\sin\theta)/\lambda] = 0.60$ Å^{-1}, 3047 independent ($R_{int} = 0.045$) and 3031 observed reflections [$I \geqslant 2\sigma(I)$], 329 refined parameters, $R = 0.036$, $wR^2 = 0.097$, Flack parameter 0.07(2), max. residual electron density 0.28 (−0.21) e Å^{-3}, ethyl group at O27 refined with split positions, hydrogen atom at N31 from difference Fourier maps, others calculated and refined as riding atoms.

The data set was collected with a Nonius KappaCCD diffractometer. Programs used: data collection COLLECT (Nonius B.V., 1998), data reduction Denzo-SMN,[26] absorption correction Denzo,[27] structure solution SHELXS-97,[28] structure refinement SHELXL-97 (G.M. Sheldrick, Universität Göttingen, 1997), graphics SCHAKAL (E. Keller, Universität Freiburg, 1997).

Crystollographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data centre as supplementary publication no. CCDC 649280. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (Fax: +44 (0)1223 336033 or email: deposit@ccdc.cam.ac.uk).

5.4. Diethyl [2-(acetylamino)-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl]methylphosphonate (11)

To a solution of hydroxymethylphosphonate **10** (247 mg, 0.5 mmol) in anhydrous THF (10 mL) was added lithium bis(trimethylsilyl)amide (1 M in THF, 0.6 mL, 0.6 mmol) at −78 °C. After 15 min methyl oxalylchloride (79 mg, 0.6 mmol) was added and the resulting mixture was stirred and warmed to 0 °C over a period of 2 h. The reaction was quenched with a 10% aqueous solution of sodium bicarbonate (5 mL), extracted with ethyl acetate (2 × 15 mL), dried over $MgSO_4$ and concentrated. The residue was dried over vacuum overnight. This was dissolved in anhydrous toluene and AIBN (6 mg, 0.04 mmol) and n-Bu_3Sn (188 mg, 0.6 mmol) were added under an argon atmosphere. The resulting mixture was stirred for 3 h under reflux. Toluene was evaporated and the residue was

purified by silica gel chromatography (CH_2Cl_2/MeOH = 30: 1) to afford 158 mg of product **11** (66.2%) as a viscous oil. $[\alpha]_D$ +30° ($c = 1$, acetone) ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.49 (d, 1H, $^3J_{HH} = 9$, NH), 5.14 (m, 2H, CH_2), 4.60–4.69 (m, 1H, CH), 4.40–4.47 (m, 1H, CH), 4.37 (dd, 2H, $^2J_{HP} = 21$, $^3J_{HH} = 5$), 4.24 (m, 4H, CH_2), 4.06 (m, 1H, CH), 2.20 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 1.44 (t, 6 H, CH_3, $^3J_{HH} = 7$). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 170.66 (s, C=O), 170.57 (s, C=O), 170.03 (s, C=O), 168.91 (s, C=O), 70.72 (s, CH), 69.70 (s, CH), 67.89 (d, $^3J_{CP} = 3$, CH), 67.69 (s, CH), 62.21 (d, $^2J_{CP} = 7$, CH_2), 61.90 (d, $^2J_{CP} = 7$, CH_2), 61.39 (s, CH_2), 50.33 (d, $^2J_{CP} = 11$, CH), 25.69 (d, $^2J_{CP} = 144$, CH_2), 22.97 (s, CH_3), 20.66 (s, CH_3), 20.61 (bs, $2 \times CH_3$), 16.34 (s, CH_3), 16.28 (s, CH_3). ^{31}P NMR ($CDCl_3$, 121.4 MHz): δ 27.63 (m, 1P). HR-MS (ESI): $C_{19}H_{32}NO_{11}P$ calculated 482.1791 (M+1), found 482.1799 (M+1). Anal. Calcd for $C_{19}H_{32}NO_{11}P$: C, 47.40; H, 6.70. Found: C, 47.35; H, 6.79.

5.5. *C*-(2-Acetylamine-2-deoxy-α-D-glucopyranosyl)methylphosphonic acid (5)

To a stirred mixture of compound **11** (101 mg, 0.021 mmol) in 10 mL of dichloromethane was added trimethylsilylbromide (0.12 mL, 0.084 mmol) dropwise at 0 °C. The resulting mixture was stirred under argon at room temperature. The reaction was monitored by ^{1}H NMR. After 3 days the signals of the ethyl groups disappeared and dichloromethane and the excess of trimethylsilylbromide were removed under a stream of nitrogen. The residue was dried under vacuum overnight. The crude product was dissolved in dry MeOH and to the resulting solution was added dropwise at 0 °C freshly prepared sodium methanolate (4 mg of sodium, 0.017 mmol) in 5 mL of MeOH. The reaction was monitored by TLC. After 1 h the reaction was almost finished and the mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched by the addition of DOWEX-H^+ and the pH of the solution was adjusted to 5. The mixture was filtered, evaporated and dried under vacuum. To the residue diethylether (10 mL) and water (5 mL) were added and the mixture was stirred vigorously for 30 min. The water phase was separated and lyophilized to afford 51 mg (0.017 mmol, 81.2%) of compound **5** as a white highly hygroscopic solid. About 20 mg of **5** was stirred with Amberlite IR-120 Na^+ for 10 min and the resulting disodium salt was used for elemental analysis. $[\alpha]_D$ +56° ($c = 1$, H_2O). ^{1}H-COSY NMR (D_2O, 600 MHz): δ 3.80 (ddd, 1H, $^3J_{HH} = 11$, $^3J_{HH} = 6$, $^3J_{HH} = 2$, CH), 4.27 (m, 1H), 3.61 (m, 2H, CH_2), 3.51 (dd, 1H, $^3J_{HH} = 11$, $^3J_{HH} = 9$, CH), 3.43 (ddd, 1H, $^3J_{HH} = 10$, $^3J_{HH} = 4$, $^4J_{HH} = 3$, CH), 3.30 (dd, 1H, $2 \times {}^3J_{HH} = 10$, CH), 2.11 (ddd, 1H, $^2J_{HP} = 16$, $^2J_{HH} = 16$, $^3J_{HH} = 12$, CH_2P), 1.85 (s, 3H, CH_3), 1.77 (ddd, 1H, $^2J_{HP} = 21$, $^2J_{HH} = 16$, $^3J_{HH} = 3$, CH_2P). ^{13}C NMR (CD_3OD, 75.5 MHz): δ 175.17 (s, C=O), 73.58 (s, CH), 70.97 (s, CH), 70.90 (s, CH), 70.33 (d, $^4J_{CP} = 5$, CH), 61.16 (s, CH_2), 54.02 (d, $^3J_{CP} = 13$, CH), 24.39 (d, $^2J_{CP} = 139$, CH_2P), 22.50 (s, CH_3). ^{31}P NMR (D_2O, 121.4 MHz): δ 26.03 (s, 1P). HR-MS (ESI): $C_9H_{18}NO_8P$ calculated 298.0692 (M−1), found 298.0690 (M−1). Anal. Calcd for monohydrate of disodium salt of **5**: $C_9H_{18}Na_2O_9P$: C, 29.93; H, 5.02. Found: C, 29.76; H, 5.18.

5.6. [2-(Acetylamino)-2-deoxy-α-D-glucopyranosyl]methylphosphonoyl uridin-5′-yl phosphate disodium salt (2)

Compound **5** (42 mg, 14 μmol) was dissolved in MeOH (3 mL) and water (0.2 mL). The mixture was treated with Et_3N (0.1 mL), stirred at rt for 10 min and concentrated. The residue was dried on a vacuum pump overnight. It was then co-evaporated 5 times with dry pyridine. The resulting residue was dissolved in dry pyridine (2 mL) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine-5′-monophosphomorpholidate (29 mg, 42 μmol) was added. This was followed by the addition of a solution of 1*H*-tetrazole in MeCN (0.45 M, 0.94 mL, 0.42 mmol). The mixture was stirred for 3 days under argon at room temperature. After this period the reaction was quenched by adding water (2 mL) and diisopropylethylamine (0.1 mL). The mixture was stirred for 15 min and then concentrated under vacuum. The resulting residue was purified by HPLC (0.5 M ammonium formate buffer + 0.5% MeCN). Chromatography afforded the ammonium salt of **2**. This salt was transformed to the disodium salt by ion exchange chromatography (Amberlite IR-120 Na^+) to afford 9 mg of compound **2** (10%) as a white solid. ^{1}H NMR (D_2O, 300 MHz): δ 7.98 (d, 1H, $^3J_{HH} = 8$ Hz), 5.98 (m, 2H), 4.19–4.39 (m, 6H), 3.99 (m, 1H), 3.70–3.87 (m, 4H), 3.53 (m, 1H), 3.48 (m, 1H), 2.30 (m, 2H), 2.05 (s, 3H, CH_3). ^{13}C NMR (D_2O, 75.5 MHz): δ 175.57 (s, CO), 167.38 (s, CO), 152.88 (s, CO), 142.72 (s, =CHN), 103.67 (s, =CHCO), 89.54 (s), 81.19 (s), 74.85 (s), 73.82 (s), 71.68 (s), 71.33 (s), 70.60 (s), 66.96 (s), 65.70 (s), 61.72 (s), 54.57 (d, $^2J_{CP} = 14$, $POCH_2$), 25.22 (d, $^1J_{CP} = 151$, PCH_2), 23.04 (s, CH_3). ^{31}P NMR (D_2O, 121.4 MHz): δ 13.95 (d, 1P, $^2J_{PP} = 27$), −10.93 (d, 1P, $^2J_{PP} = 27$). HR-MS (ESI): $C_{18}H_{27}N_3Na_2O_{16}P_2$ calculated 626.0764 (M−Na^+), found 626.0764 (M−Na^+).

5.7. *O*-GlcNAc transferase inhibition assay[24]

Human nucleocytoplasmic O-linked *N*-acetylglucosaminyltransferase (hncOGT) was expressed in BL21 (DE3) cells (Novagen) using the pET 43.1(Ek/LIC) expression vector and bacterial extract containing hncOGT was prepared as previously described.[1] Bacterial extract

expressed with the empty vector was shown to have no OGT activity. Recombinant-purified Nup62 prepared as previously described[1] was used as a macromolecular substrate (GlcNAc acceptor) and bacterially overexpressed hncOGT was used with no further purification. Bacterial extract containing hncOGT was added to a 36 μL reaction mixture containing 18 μM of UDP-[^{14}C]-GlcNAc (American Radiolabeled Chemicals), 1.5 μg of recombinant purified Nup62 in 50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 12.5 mM $MgCl_2$ in the absence and presence of different concentrations of **2**. The 36 μL-reaction mixtures were incubated at 37 °C for 30 min with shaking. Reactions were stopped by adding 4× SDS-PAGE sample buffer (Invitrogen) and boiling samples for 5 min. SDS-PAGE was performed using precast 4-12% NuPage gel (Invitrogen), followed by staining with Simply Blue Safestain (Invitrogen) for 1 h and by destaining with distilled water for 1 h. Subsequently it was treated with En3Hance (Perkin Elmer, Wellesley) for 1 h and washed with 10% Polyethylene glycol (MW 7000–9000). Glycosylation of Nup62 with [^{14}C]-GlcNAc was visualized by exposure to a phosphoimage screen, which was then developed on the Fujifilm BAS-1500 phosphoimager. Densitometry of the phosphoimage data was performed with Image Gauge 3.0 software.

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