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Synthesis and Biological Evaluation of New UDP-GalNAc Analogues for the Study of Polypeptide-α-GalNAc-transferases

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Abstract—A series of three *O*-methylated UDP-GalNAc analogues have been synthesised using a divergent strategy from a 3,6-di-*O*-pivaloyl GlcNAc derivative. The biological activity of these probes toward polypeptide-α-GalNAc-transferase T1 has been investigated. This study shows that this glycosyltransferase exhibits a very high substrate specificity.

It is becoming increasingly evident that glycoproteins, as markers in cell–cell communication events, are playing crucial regulatory functions in living organisms. Moreover, alteration of *N*- or *O*-linked oligosaccharides has been shown to be closely related to many diseases, including for instance the progression of cancer.[1]

The discovery that tunicamycin is a powerful inhibitor of the protein *N*-glycosylation pathway led to significant progress in understanding both biosynthesis and properties of *N*-glycosylated proteins.[2] In contrast, despite its biological importance, many aspects of the *O*-glycosylation process remain to be elucidated.[3,4]

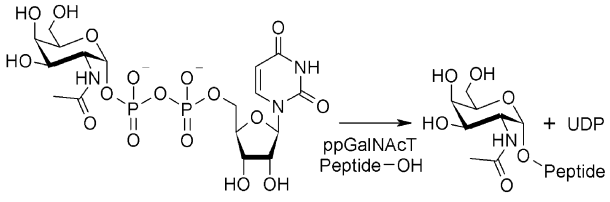
Polypeptide-α-GalNAc-transferases (ppGalNAcTs) catalyse the first glycosylation step in the biosynthesis of *O*-glycosylated proteins (Scheme 1) by transferring a GalNAc unit from UDP-GalNAc to the hydroxyl group of a serine or a threonine residue with retention of the anomeric configuration.[5]

Therefore, in order to further study the *O*-glycosylation process and to elucidate the importance of the O-glycans, there is a great interest in finding inhibitors of ppGalNAcTs. For that purpose, one of the best strategy in the field of glycosyltransferases is the design of bisubstrate-type inhibitors.[6] In order to determine where a linker arm could be attached to the sugar nucleotide substrate, we decided to investigate a series of mono-*O*-methylated UDP-GalNAc derivatives (**1**–**3**, Fig. 1). An analogous approach gave relevant results regarding the glycosyl transfer of β-1,4-GalT.[7]

The evaluation of these probes as substrates or inhibitors of GalNAc transferases would provide information about steric allowance as well as possible requirement of free OH groups for hydrogen bonding. To the best of our knowledge, this study is the first one dedicated to the donor substrate specificity of the ppGalNAcTs. In this communication, we describe the synthesis of UDP-GalNAc analogues **1**–**3** and their behaviour towards UDP-GalNAc transferase T1.

Synthesis of Probes

The syntheses of mono-*O*-substituted sugars are usually based on selective protection/deprotection strategies which are often time-consuming. Recently, an efficient three-step procedure for the preparation of GalNAc



Scheme 1. First step in the biosynthesis of *O*-glycosylated proteins.

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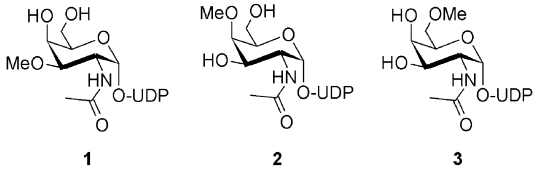


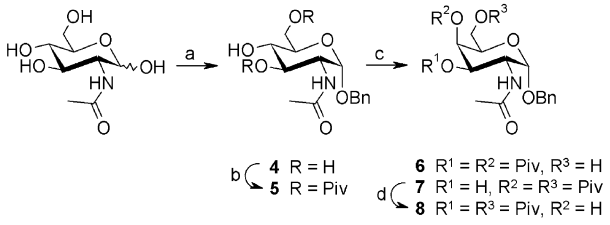
Figure 1. Mono-*O*-methylated analogues of UDP-GalNAc.

units starting from GlcNAc was reported, in which 3,4- and 4,6-di-*O*-pivaloyl-D-*galacto* derivatives **6** and **7** were the key intermediates.[8] Therefore, taking advantage of these results, the three precursors necessary for a regioselective methylation (**6**, **7** and **8**) were efficiently synthesised in four steps starting from GlcNAc (Scheme 2).

The starting material was heated at 90 °C in a large excess of benzyl alcohol in the presence of $BF_3{\cdot}Et_2O$ to afford, after recrystallisation, the benzyl glycoside (74%) **4**.[9] Following the previously mentioned procedure, treatment of **4** with 2.2 equivalents PivCl at 0 °C yielded the 3,6-di-*O*-pivaloyl derivative **5** (70%).

Inversion of configuration at C-4 was achieved by formation of the 4-*O*-triflate followed by intramolecular displacement by either of the adjacent pivaloyl groups, leading to a mixture of 3,4- and 4,6-di-*O*-protected GalNAc derivatives **6** and **7** (overall yield 90%). These regioisomers were easily separated by flash chromatography, affording **6** and **7** in 32 and 58% yield, respectively. Finally, the third required precursor **8** was obtained through a 4→3 migration induced by treating **7** with DBU.[10] However, in spite of a thermodynamically favourable switch from axial to equatorial position, the yield of this transformation never exceeded 50%. Since the starting material can be easily recovered after chromatography, the overall sequence, starting from 3 g GlcNAc, provided nearly 1 g of each precursor.

Methylation of **7** using standard Kuhn procedure (MeI, Ag_2O, DMF) furnished an inseparable mixture of *O*-methylated derivatives because, like their acetyl counterparts, pivaloates undergo migration under these conditions.[11] This result prompted us to envisage a two-step strategy which consists in forming a methylthiomethyl (MTM) derivative followed by its hydrogenolysis with Raney Ni, thus delivering the expected methyl ether (Scheme 3).[12]



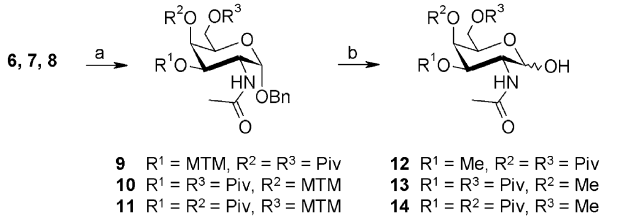
Scheme 2. (a) BF_3/Et_2O, BnOH, 90 °C, 5 h, 74%; (b) PivCl (2.2 equiv), pyridine, dichloromethane, 0 °C, 2 h, 70%; (c) (i) Tf_2O (1.3 equiv), pyridine, dichloroethane, −15 °C, 2 h; (ii) H_2O, reflux, 2 h, 90% (two steps); (iii) separation by flash chromatography, **6**: 32%, **7**: 58%; (d) (i) DBU (5 equiv), dichloromethane, rt, 2 h; (ii) separation by flash chromatography, **7**: 50%, **8**: 50%.

Among the various methodologies available to introduce the MTM group (mainly used as a protecting group), only one is performed under very mild conditions compatible with the ester functions.[13] Thus, compounds **6**, **7** and **8** were reacted with 4 equivalents Bz_2O_2 and 8 equivalents Me_2S in MeCN at 0 °C to provide their corresponding MTM derivatives **9**, **10** and **11** in good yields.

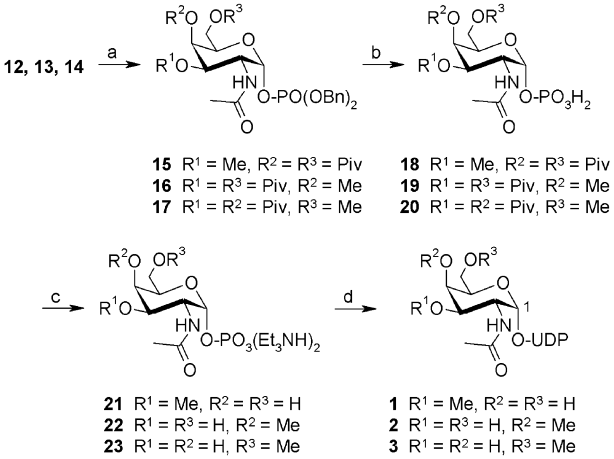
Hydrogenolysis with Raney Ni led to the expected C–S bond cleavage, as well as promoted the deblocking of the anomeric position, yielding quantitatively mono-*O*-methylated derivatives **12**, **13** and **14**.

Further elaboration to UDP-sugars was achieved following the usual strategy, as outlined in Scheme 4. First attempts of phosphorylation at O-1 with BuLi and $ClPO(OBn)_2$[14] or DMAP and $ClPO(OPh)_2$[15] were unsuccessful in our hands. However, the one-pot treatment with $(BnO)_2PNEt_2$, followed by oxidation with hydrogen peroxide at −78 °C provided the glycosylphosphotriesters **15**, **16** and **17** in fairly good yields.

Conventional hydrogenolysis of the benzyl phosphates over Pd/C furnished phosphates **18**, **19**, and **20** in quantitative yields. After removal of pivaloyl groups using Zemplen conditions, the resulting sugar-mono-



Scheme 3. (a) Bz_2O_2 (4 equiv), Me_2S (8 equiv), MeCN, 0 °C to rt, 4 h, **9**: 91%, **10**: 76%, **11**: 68%; (b) Raney Ni, EtOH, rt, overnight, **12**, **13**, **14**: 99%.



Scheme 4. (a) (i) $(BnO)_2PNEt_2$ (1.5 equiv), 1*H*-tetrazole (1.5 equiv), THF, rt, 3 h; (ii) H_2O_2 (3 equiv), −78 °C, 1 h, **15**: 74%, **16**: 62%, **17**: 60% (two steps); (b) H_2, Pd/C (10% wt), EtOH, rt, 1 h, **18**, **19**, **20**: 99%; (c) (i) MeONa (2 equiv), MeOH, rt, overnight; (ii) Amberlite® IR-120 (Et_3NH^+ form), **21**: 95%, **22**: 95%, **23**: 96% (two steps); (d) UMP-morpholidate (2 equiv), 1*H*-tetrazole (3 equiv), pyridine, rt, 4 days **1**: 81%, **2**: 36%, **3**: 28%.

phosphates were converted into their triethylammonium salts **21**, **22** and **23** with a cationic exchange resin. Finally, coupling with UMP was carried out using Moffatt and Khorana procedure, with UMP-morpholidate as the activated UMP source and 1*H*-tetrazole as the catalyst.[16] After 4 days of reaction, UDP-sugars **1**, **2** and **3** were obtained in a yield range of 28–81%.[17] For the purification of those UDP-hexoses, we developed a new HPLC method on a Hypercarb column which allowed a convenient separation from the dimeric side-product UPPU.[18]

Biological Evaluation

The methylated sugar nucleotides were no donor substrates for the ppGalNAc transferase T1, as revealed by the mass spectrometric analysis of model acceptor peptides exposed to glycosylation conditions.[19] The peptides incubated with UDP-GalNAc as substrate were completely converted into glycopeptides whereas those incubated with the UDP(OMe)GalNAc analogues **1**–**3** remained completely unsubstituted. In addition, the methylated sugar nucleotides proved to be only weak competitive inhibitors of the enzyme. At an UDP-GalNAc concentration of 20 μM (about 2 times K_m) the 3-OMe derivative **1** showed an inhibition comparable to UDP-GlcNAc which likewise is not recognised as a donor substrate (Table 1). The concentrations of UDP-GlcNAc and UDP(3-OMe)GalNAc necessary for 50% inhibition (IC_{50}) were very similar at around 300 μM. The UDP(4-OMe)GalNAc and the UDP(6-OMe)-GalNAc analogues were weaker inhibitors with IC_{50}'s around 700 μM. These results lend further support to the observations that the sugar nucleotide binding site of the ppGalNAc transferase T1 has a very high affinity for UDP-GalNAc and a rather low affinity for other UDP-sugars or UDP and its derivatives. Affinity chromatography experiments[21] showed that the enzyme does not bind to UDP-hexanolamine agarose but binds efficiently to mercuri-UDP-GalNAc thiopropyl agarose where the sugar nucleotide is immobilised through the Hg on the uracil, thereby making the GalNAc freely accessible. Furthermore, the same study showed that the enzyme did not bind to mercuri-UDP-GlcNAc thiopropyl agarose suggesting a strong selectivity of the sugar nucleotide binding site. The different *O*-methyl groups may either prevent the sugar nucleotide from entering the binding pocket on the protein or free OH groups may be indispensable for substrate binding via hydrogen or water bridges. In particular, the OH group at C-4 and the *N*-acetyl group may be involved in substrate recognition since neither UDP-GlcNAc nor UDP-Gal are substrates for the ppGalNAc transferase T1 although another member of the ppGalNAc transferase family has been shown to use UDP-Gal as a substrate albeit very poorly.[22] Molecular modeling of the UDP-GalNAc binding site based on structural homologies with other glycosyltransferases of known three-dimensional structures and tested through site directed mutagenesis experiments[23] indicates that at least two of the OH groups are involved in the substrate binding and that the sugar nucleotide fits very tightly into the deep binding pocket mostly through the GalNAc part whereas the uridine part remains exposed to the medium at the surface of the protein. These observations may explain the weak inhibitory activity of the UDP(OMe)GalNAc derivatives. In the absence of crystallographic data, further studies with the deoxy analogues of UDP-GalNAc will be useful to determine which of the OH groups may be interacting with amino acids at the transferase active site. These results will be reported in due course.

Table 1. Inhibitory activity of **1**–**3** and of UDP-GlcNAc; relative inhibition of ppGalNAc transferase T1 activity at various inhibitor concentrations (%)

Concn (μM)	**1**	**2**	**3**	UDP-GlcNAc
20	0	0	0	0
200	30	7	17	39
1000	74	72	76	79

Conclusion

The initial plans to build bisubstrate analogues by linking acceptor surrogates to one of the free OH group of the donor UDP-GalNAc appear to be thwarted by the high substrate specificity of the transferase. Nevertheless, the work reported here provides new and useful information on structure–activity relationships in an important enzymatic system for which studies with substrate analogues are just starting to be undertaken.[24]

Acknowledgements

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References and Notes

1. (a) Seitz, O. *Chembiochem*. **2000**, *1*, 214. (b) Toyokuni, T.; Singhal, A. K. *Chem. Soc. Rev*. **1995**, 231. (c) Barchi, J. J. *Curr. Pharm. Des*. **2000**, *6*, 485. (d) Dennis, J. W.; Granovsky, M.; Warren, C. E. *Biochim. Biophys. Acta* **1999**, *1473*, 21. (e) Brockhausen, I. *Biochem. Soc. Trans*. **1997**, *25*, 871. (f) Kim, Y. J.; Varki, A. *Glycoconjugate J*. **1997**, *14*, 569. (g) Brockhausen, I. *Biochim. Biophys. Acta* **1999**, *1473*, 67. (h) Rutishauser, U. *Curr. Opin. Cell. Biol*. **1996**, *8*, 679. (i) Hakomori, S.-I. In *Glycoproteins and Disease*; Montreuil, J., Vliegenthart, J., Schachter, H., Eds.; Elsevier: New York, 1996; p 243. (j) Hakomori, S. *Adv. Cancer Res*. **1989**, *52*, 257. (k) Hakomori, S.; Zhang, Y. *Chem. Biol*. **1997**, *4*, 97.
2. (a) Elbein, A. *Ann. Rev. Biochem*. **1987**, *56*, 497. (b) Elbein, A. *Methods Enzymol*. **1987**, *138*, 661. (c) Tkacz, J. S.; Lampen, J. O. *Biochem. Biophys. Res. Commun*. **1975**, *65*, 218. (d) Kornfeld, R.; Kornfeld, S. *Ann. Rev. Biochem*. **1985**, *54*, 631.

3. Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683.
4. Clausen, H.; Bennett, E. P. *Glycobiology* **1996**, *6*, 635.
5. (a) Joziasse, D. H. *Glycobiology* **1993**, *3*, 97. (b) Bennett, E. P.; Hassan, H.; Mandel, U.; Mirgorodskaya, E.; Roepstorff, P.; Burchell, J.; Taylor-Papadimitriou, J.; Hollingsworth, M. A.; Merkx, G.; van Kessel, A. G.; Eiberg, H.; Steffensen, R.; Clausen, H. *J. Biol. Chem.* **1998**, *273*, 30472. (c) Bennett, E. P.; Hassan, H.; Mandel, U.; Hollingsworth, M. A.; Akisawa, N.; Ikematsu, Y.; Merkx, G.; van Kessel, A. G.; Olofson, S.; Clausen, H. *J. Biol. Chem.* **1999**, *274*, 25362. (d) Ten Hagen, K. G.; Hagen, F. K.; Balys, M. M.; Beres, T. M.; Van Wuyckhuyse, B.; Tabak, L. A. *J. Biol. Chem.* **1998**, *273*, 27749. (e) Ten Hagen, K. G.; Tetaert, D.; Hagen, F. K.; Richet, C.; Beres, T. M.; Gagnon, J.; Balys, M. M.; Van Wuyckhuyse, B.; Bedi, G. S.; Degand, P.; Tabak, L. A. *J. Biol. Chem.* **1999**, *274*, 27867.
6. (a) Waldscheck, B.; Streiff, M.; Notz, W.; Kinzy, W.; Schmidt, R. R. *Angew. Chem. Int. Ed.* **2001**, *40*, 4007. (b) Khan, S. H.; Matta, K. L. *Glycoconjugates* **1992**, 361. (c) Palcic, M. M.; Heerze, L. D.; Srivastava, O. P.; Hindsgaul, O. *J. Biol. Chem.* **1989**, *264*, 17174. (d) Hashimoto, H.; Endo, T.; Kajihara, Y. *J. Org. Chem.* **1997**, *62*, 1914. (e) Compain, P.; Martin, O. R. *Bioorg. Med. Chem.* **2001**, *9*, 3077, and refs cited therein.
7. Endo, T.; Kajihara, Y.; Kodama, H.; Hashimoto, H. *Bioorg. Med. Chem.* **1996**, *4*, 1939.
8. Rochepeau-Jobron, L.; Jacquinet, J.-C. *Carbohydr. Res.* **1998**, *305*, 181.
9. Shulman, M. L.; Khorlin, A. Y. *Carbohydr. Res.* **1973**, *27*, 141.
10. (a) Becker, B.; Thiem, J. *Carbohydr. Res.* **1998**, *308*, 77. (b) Becker, B.; Thiem, J. *Tetrahedron: Asymmetry* **1994**, *5*, 2339.
11. (a) Angyal, S. J.; Melrose, G. J. H. *J. Chem. Soc.* **1965**, 6501. (b) Bonner, W. A. *J. Org. Chem.* **1959**, *24*, 1388.
12. Projer, P. M.; Angyal, S. J. *Austr. J. Chem.* **1978**, *31*, 1031.
13. Medina, J. C.; Salomon, M.; Kyler, K. S. *Tetrahedron Lett.* **1988**, *29*, 3773.
14. Inage, M.; Chaki, H.; Kusumoto, S.; Shiba, T. *Chem. Lett.* **1982**, 1281.
15. Sabesan, S.; Neira, S. *Carbohydr. Res.* **1992**, *223*, 169.
16. (a) Roseman, S.; Distler, J. J.; Moffatt, J. G.; Khorana, H. G. *J. Am. Chem. Soc.* **1961**, *83*, 659. (b) Wittmann, V.; Wong, C.-H. *J. Org. Chem.* **1997**, *62*, 2144.
17. NMR data: (**1**): ^{1}H (D_2O) δ 5.59 (dd, $J_{1,2}=3.5$ Hz, $J_{1,P}=7.0$ Hz, H-1); ^{13}C (D_2O): δ 175.0, 171.3, 166.5, 152.7, 142.7, 102.9, 94.8 ($J_{C,P}=6.1$ Hz, C-1), 89.6, 83.7 ($J_{C,P}=9.3$ Hz), 76.7, 74.2, 72.2, 70.1, 69.9, 64.4 ($J_{C,P}=5.1$ Hz), 61.0, 56.3 (OCH_3), 49.1 ($J_{C,P}=8.3$ Hz), 22.8; ^{31}P (D_2O): δ −10.64 (d, $J_{P,P}=20.5$ Hz, Pα), −12.24 (d, Pβ). (**2**): δ5.56 (dd, $J_{1,2}=3.5$, $J_{1,P}=6.5$ Hz, H-1); ^{31}P (D_2O): δ −10.70 (d, $J_{P,P}=20.4$ Hz, Pα), −12.33 (d, Pβ). (**3**); ^{1}H (D_2O) δ 5.56 (dd, $J_{1,2}=3.5$, $J_{1,P}=7.5$ Hz, H-1); ^{31}P (D_2O): δ −10.63 (d, $J_{P,P}=20.8$ Hz, Pα), −12.26 (d, Pβ).
18. Column: Hypercarb® 250×10 mm; elution: 45 mM aq ammonium formate and gradient MeCN. More details on the separation procedure will be provided in a separate publication.
19. The conditions used were essentially those described earlier[20] with modifications. The enzyme source was the bovine polypeptide: α-*N*-acetyl-galactosaminyl transferase T1 (ppGalNAc transferase) (EC 2.4.1.41) expressed as a secreted, 6His-tagged protein in insect cells after infection with a recombinant baculovirus, isolated from the culture medium by affinity chromatography on Ni-trinitrilotetraacetic acid agarose (Qiagen, Hilden, Germany) to a specific activity of 0.5 μmol GalNAc transferred per min and mg protein (=0.5 U/mg). Enzyme assays were carried out in a total volume of 25 μL containing 17.5 nmol peptide substrate (STPSTPSTPSTPSTP, Sigma-Genosys, St. Quentin Fallavier, France), 0.5 nmol UDP[^{3}H]GalNAc (Amersham, Orsay, France) at a specific activity of 20,000 cpm/nmol and 50 ng enzyme in 50 mM morpholinoethanesulfonic acid buffer (pH 6.5) with 15 mM $MnCl_2$. After 15-min incubation at 37 °C, the reaction was stopped by the addition of 750 μL of ice-cold water and the solution applied to a SepPak C18 cartridge (Waters, St. Quentin en Yvelines, France), washed with 10 mL of water, the peptide and the labelled glycopeptide eluted with methanol and the radioactivity of the eluate determined by scintillation counting. For inhibition studies, the nucleotide sugar analogues were added at the concentrations indicated and the tests carried out as above.
In order to determine whether the selectively methylated sugar nucleotides could be used as donor substrates by the ppGalNAc transferase, larger scale transfer reactions were set up. The reaction mixture contained 70 nmol of peptide, 75 μU of ppGalNAc transferase, 2.5 U alkaline phosphatase from calf intestine (New England Biolabs, Beverlay, MA, USA), 100 nmol of nucleotide sugar in 50 mM MES pH 6.5 and 15 mM $MnCl_2$ in a total volume of 100 μL. After 16 h at 37 °C, a second aliquot of the enzymes and the nucleotide sugars were added and the incubation continued for 24 h. The reaction was terminated by the addition of 750 μL of 50% MeOH, the precipitated proteins removed by centrifugation and the mixture applied onto a column (0.5×2.5 cm) of Dowex 1X8 (Cl^-) (100–200 mesh) (Bio-Rad, Marne-la-Coquette, France). The flow-through and washings were combined, evaporated under reduced pressure, taken up in water and purified as above on a SepPak C-18 cartridge. After concentration in a Speed-Vac concentrator the samples were processed for electrospray mass spectrometry on a Quattro II mass spectrometer (Micromass, Manchester, UK).
20. Craig, A. G.; Park, M.; Fischer, W. H.; Kang, J.; Compain, P.; Piller, F. *Toxicon* **2001**, *39*, 809.
21. Wang, Y.; Abernethy, J. L.; Eckhardt, A. E.; Hill, R. L. *J. Biol. Chem.* **1992**, *267*, 12709.
22. Wandall, H. H.; Hassan, H.; Mirgorodskaya, E.; Kristensen, A. K.; Roepstorff, P.; Bennett, E. P.; Nielsen, P. A.; Hollingsworth, M. A.; Burchell, J.; Taylor-Papadimitriou, J.; Clausen, H. *J. Biol. Chem.* **1997**, *272*, 23503.
23. Duclos, S., Da Silva, P., Vovelle, F., Piller, F. and Piller, V. Manuscript in preparation.
24. Lazarevic, D.; Thiem, J. *Carbohydr. Res.* **2002**, *337*, 2187.