Ketone Isosteres of 2-*N*-Acetamidosugars as Substrates for Metabolic Cell Surface Engineering

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Received August 9, 2000

Metabolic oligosaccharide engineering using unnatural substrates has provided an avenue for the introduction of novel chemical reactivity on cell surfaces.[1] This approach exploits the unnatural substrate tolerance of enzymes involved in carbohydrate biosynthesis (depicted schematically in Figure 1A). For example, derivatives of *N*-acetylmannosamine (ManNAc) which bear a selectively reactive chemical handle, such as a ketone[2] or azide,[3] on the *N*-acyl group are transformed into glycoconjugate-bound sialosides by human cells. The selective reactivity of ketones with aminooxy or hydrazide groups, and azides with modified phosphine reagents, permits exogenous chemical cell surface targeting.[4] If the substrate promiscuity exhibited by the enzymes and transporters of the sialic acid pathway is a general feature of other carbohydrate metabolic pathways, multiple avenues for metabolic engineering will be available. So far, few studies have addressed the unnatural substrate tolerance of other carbohydrate biosynthetic pathways.[1]

The ubiquitous presence of the 2-*N*-acetamidosugars,[5] *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), in glycoproteins, proteoglycans, and glycolipids makes them attractive targets for metabolic engineering. GlcNAc and GalNAc are converted within cells to their UDP-activated analogues via salvage pathways.[6] UDP-GlcNAc can be subsequently converted to ManNAc or UDP-GalNAc, or utilized by GlcNAc transferases that incorporate the sugar into various glycoconjugates. Likewise, GalNAc transferases utilize UDP-GalNAc as a substrate and deliver the sugar to numerous glycoconjugates. We considered the possibility that unnatural GlcNAc and GalNAc derivatives might gain access to the cell surface through their respective salvage pathways.

We designed "2-ketosugars", which are C_2-carbon isosteres of the 2-*N*-acetamidosugars (Figure 1B), as novel analogues that possess a ketone group for chemoselective reaction with aminooxy or hydrazide reagents. A concise synthesis of 2-ketosugars was developed from known 2-iodosugars, which are readily available by electrophilic iodination of commerically available glycals.[7]

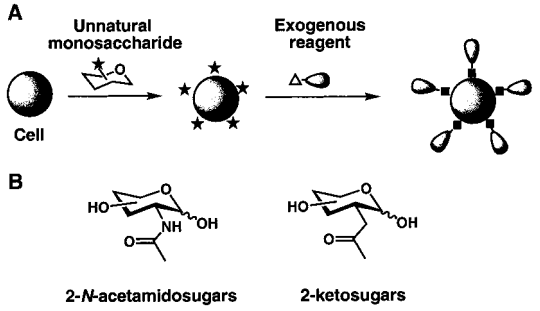
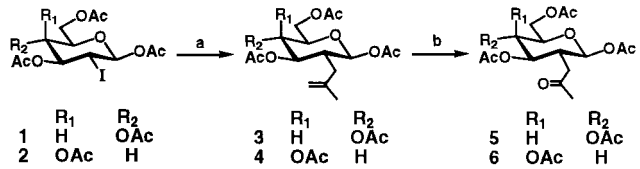


Figure 1. (A) Metabolic cell surface engineering using unnatural monosaccharide substrates. An electrophilic functional group (★) attached to a metabolic substrate is delivered to cell surface glycoconjugates. Reaction with a complementary nucleophile (△), delivered exogenously, produces a new covalent adduct (■) on the cell surface. (B) 2-Ketosugars as C_2-carbon isosteres of 2-*N*-acetamidosugars.

Keck radical coupling[8] of methallyltributyltin with 2-iodo glucose analogue **1** or 2-iodo galactose analogue **2** afforded 2-methallylpyranosides (**3** and **4**) as the major products (6:1 and 7:1 equatorial:axial methallylation, respectively) (Scheme 1). The

Scheme 1[a]



[a] (a) Methallyltributyltin, AIBN, benzene, reflux, 65−70%; (b) (*i*) O_3, −78 °C, 10:1 CH_2Cl_2:MeOH; (*ii*) DMS, −78 °C to room temperature, 85−90%.

predominance of the equatorial over the axial isomer is in accordance with diastereoselectivities previously reported by Giese and co-workers.[9] Ozonolysis of compounds **3** and **4**, followed by reduction with dimethyl sulfide (DMS) and HPLC purification, gave the peracetylated 2-ketosugars **5** and **6**. These compounds were used directly in metabolic studies; the acetate esters improved uptake by passive diffusion across cellular membranes and were removed in situ by cytosolic esterases.[10]

The cellular metabolism of the 2-ketosugars **5** and **6** was investigated in a number of cell lines which exhibit varying patterns of *N*- and *O*-linked glycosylation. Jurkat cells (a human T-cell line) have a majority of *N*-linked glycoproteins, with few mature *O*-linked glycans, while HL-60 cells (human myeloid cell line) are rich in *O*-linked glycoproteins.[4a] HeLa cells (a human cervical epithelial tumor cell line) have a full complement of *N*- and *O*-linked glycoproteins. Chinese Hamster ovary (CHO) cells have a diverse array of cell surface glycoconjugates as well and are widely used for overexpression of recombinant glycoproteins.[11]

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10.1021/ja002962b CCC: $20.00
Published on Web 01/20/2001

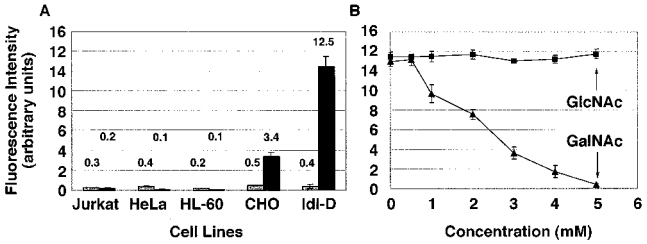

Figure 2. (A) Flow cytometry analysis of cell surface ketones produced by 2-ketosugars **5** (left bars) and **6** (right bars). (B) Effects of GalNAc (▲) and GlcNAc (■) on ketone expression produced by **6** (40 μM).

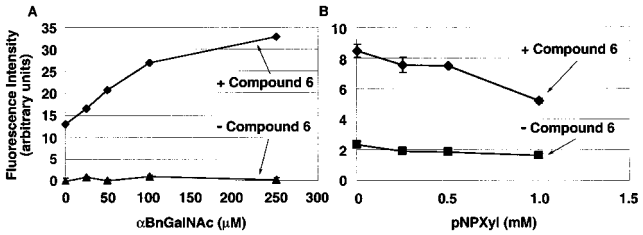

Figure 3. (A) αBnGalNAc increases ketone reactivity produced by **6** (40 μM) (◆), compared to control cells (▲). (B) pNP-Xyl decreases ketone expression produced by **6** (40 μM) (◆), compared to control cells (■).

In a typical experiment, cells were grown in the presence of **5** or **6** (40 μM) for 2−3 days and analyzed for the appearance of glycoconjugate-associated ketones using our previously reported assay.[4a] Briefly, the cells were reacted with biotin hydrazide, stained with fluorescein isothiocyanate-labeled avidin (FITC-avidin), and analyzed by flow cytometry to quantify ketones on cell surface glycoconjugates. As shown in Figure 2A, no ketones were detected on any cell line incubated with GlcNAc isostere **5**.[12] However, GalNAc isostere **6** produced a significant number of detectable ketones on CHO cells (approximately 20 000 ketones/cell were reacted with biotin hydrazide under these experimental conditions), while ketones were undetectable on the three human cell lines (Figure 2A).

The observation of ketones on CHO cells incubated with **6** suggested that this 2-ketosugar gains access to the salvage pathway. If so, then a reduction in the concentration of endogenous intermediates should increase the metabolic flux of unnatural intermediates. Endogenous UDP-GalNAc, produced primarily from UDP-GlcNAc inside the cells, is expected to compete with the UDP analogue of **6** in reactions mediated by GalNAc transferases. We therefore investigated the metabolism of **6** in a mutant CHO cell line termed ldl-D that is unable to synthesize endogenous UDP-GalNAc by virtue of a deficient UDP-GlcNAc-C_4-epimerase activity.[13] Ldl-D CHO cells treated with compound **6** displayed cell surface ketones at a level 3-fold higher than wild-type CHO cells (Figure 2A), supporting the notion that 2-ketosugar **6** is delivered to cell surface glycoconjugates via the GalNAc salvage pathway. As further confirmation, we incubated ldl-D CHO cells with compound **6** (40 μM) together with various concentrations of GalNAc or GlcNAc in the media. As shown in Figure 2B, exogenous GalNAc inhibited ketone expression where as GlcNAc had no effect.

In CHO cell glycoproteins, GalNAc residues occupy core positions of *O*-linked glycans α-linked to the hydroxyl group of threonine or serine, whereas relatively few reside at distal sites of glycans. To determine if 2-ketosugar **6** had replaced GalNAc in these core positions, we explored the effects of benzyl 2-*N*-acetamido-2-deoxy-α-D-galactopyranoside (αBnGalNAc), a known competitve inhibitor of *O*-glycan extension from peptide-proximal core GalNAc residues,[14] on the number of detectable cell surface ketones. αBnGalNAc increases the accessibility of core GalNAc residues by preventing further oligosaccharide elaboration,[15] and likewise the 2-ketosugar analogue should be more accessible for chemical reactions. By contrast, αBnGalNAc should prevent the expression of 2-ketosugars occupying distal positions on *O*-linked glycans. Simultaneous treatment of ldl-D CHO cells with compound **6** (40 μM) and αBnGalNAc increased the number of ketones detected on the cells in a dose-dependent fashion (Figure 3A). These data suggest that the 2-keto GalNAc isostere substitutes for GalNAc at the core positions of *O*-linked glycoproteins and is further elaborated by glycosyltransferases in the absence of αBnGalNAc.

In addition to *O*-linked glycoproteins, GalNAc residues reside in chondroitin sulfate proteoglycans. The incorporation of **6** into chrondroitin sulfate was determined using *p*-nitrophenyl-*β*-D-xyloside (pNP-Xyl), a known metabolic inhibitor of glycosaminoglycan elongation.[16] The simultaneous treatment of cells with **6** (40 μM) and pNP-Xyl reduced the cell surface ketone signal in dose-dependent fashion, down to 40% of control cells at 1 mM pNP-Xyl (Figure 3B). This suggests that the metabolic product of **6** is incorporated into chondroitin sulfate proteoglycans as well as core positions of *O*-linked glycoproteins.

In conclusion, we have demonstrated that a 2-keto isostere of GalNAc is a novel substrate for metabolic glycoprotein engineering in wild-type and ldl-D CHO cells. The corresponding GlcNAc analogue could not be detected on cells of any type, perhaps due to competition with endogenous GlcNAc and its downstream intermediates in the salvage pathway which are present at notoriously high intracellular concentrations.[17] The 2-keto GalNAc analogue should be incorporated into secreted glycoproteins as well as cell surface molecules, since both are similarly posttranslationally modified. This technique might be exploited to introduce unique chemical reactivity into secreted glycoproteins produced by large-scale recombinant expression, allowing further selective modification.

Acknowledgment. This research was supported by grants to C.R.B. from the National Institutes of Health (GM58867-01) and the Director, Office of Science, Office of Basic Energy Sciences, Division of Materials Sciences and Engineering and the Office of Energy Biosciences of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

Supporting Information Available: Synthetic procedures for all new compounds and experimental details of biological assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA002962B

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