Final version published as: Cecioni, S., & Vocadlo, D. J. (2017). Carbohydrate Bis-acetal-Based Substrates as Tunable Fluorescence-Quenched Probes for Monitoring exo-Glycosidase Activity. Journal of the American Chemical Society, 139(25), 8392–8395. https://doi.org/10.1021/jacs.7b01948

Carbohydrate Bis-acetal-Based Substrates as Tunable Fluorescence-Quenched Probes for Monitoring *exo*-Glycosidase Activity

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ABSTRACT: Tunable Förster resonance energy transfer (FRET)-quenched substrates are useful for monitoring the activity of various enzymes within their relevant physiological environments. Development of FRET-quenched substrates for *exo*-glycosidases, however, has been hindered by their constrained pocket-shaped active sites. Here we report the design of a new class of substrate that overcomes this problem. These Bis-Acetal-Based Substrates (BABS) bear a hemiacetal aglycon leaving group that tethers fluorochromes in close proximity, also positioning them distant from the active site pocket. Following cleavage of the glycosidic bond, the liberated hemiacetal spontaneously breaks down, leading to separation of the fluorophore and quencher. We detail the synthesis and characterization of GlcNAc-BABS, revealing a striking 99.9% quenching efficiency. These substrates are efficiently turned over by the human *exo*-glycosidase O-GlcNAcase (OGA). We find the hemiacetal leaving group rapidly breaks down, enabling quantitative monitoring of OGA activity. We expect this strategy to be broadly useful for the development of substrate probes for monitoring *exo*-glycosidases, as well as a range of other enzymes having constrained pocket-shaped active sites.

The creation of fluorescence-quenched fluorescent substrates has delivered valuable probes with tunable fluorescent properties. Various enzyme classes including, most notably, proteases have had such substrates deployed for a range of applications both in cells and *in vivo*.[1−4] Successful targeting of proteases likely stems from their cleft-like active site architecture, which accommodates positioning the requisite pair of fluorochromes at the N- and C-terminus of peptide-based substrates. Similarly, FRET-based substrates for *endo*-glycosidases, which cleave in the middle of glycan chains, have been generated.[5,6] More problematic has been the creation of such substrate probes for *exo*-acting enzymes, which have pocket-shaped active sites that are sterically demanding.[4]

We were attracted to this problem by the nearly 100 glycoside hydrolases encoded by the human genome.[7] Most are *exo*-glycosidases, and they are receiving increasing interest owing to their emerging roles in human health. Deficiencies in the activities of several *exo*-glycosidases found in lysosomes are well-known to be involved in the set of lysosomal storage diseases.[8] *Exo*-glycosidases have also emerged as therapeutic targets for diseases such as Parkinson's,[9] Alzheimer's,[10] and cancers.[11]

Efforts to create substrate probes that circumvent the steric constraints of the active site of *exo*-glycosidases have been made by using fluorogenic phenolate-based glycosides,[12,13] reactive quinone methide-based probes,[14−16] or spirocyclic rhodol and rhodamine-based glycosides.[17,18] While important tools, these probes may exhibit limitations in certain applications, such as the study of the regulation of enzyme activity in different cellular compartments. For example, the pH-dependent fluorescence of spirocyclic fluorochromes and phenolate-based substrates may be problematic in cellular compartments of different pH. Also, the potential inactivation of enzymes by reactive electrophiles may present challenges when aiming to quantitate enzyme activity. Tuning the photophysical properties of fluorogenic phenolate-based glycosides can also be challenging. Recently, a different approach has been pursued for quantitating human glucocerebrosidase activity in cells.[19] This "dark-to-bright" fluorescence-quenched substrate relied on modifying the primary hydroxyl group of the pyranose. While effective for glucocerebrosidase, this approach has not proven generally applicable since hydroxyl groups are often critical recognition elements for glycoside hydrolases.[20,21]

We were therefore interested in overcoming the steric limitations of *exo*-processing enzymes while preserving the carbohydrate recognition motif. We felt this could be accomplished by positioning both fluorochromes outside of the active site pocket, where they would not interfere with substrate recognition. We were inspired by the cyanogenic β-glucosidase,[22] which cleaves cyanogenic glycosides to form a cyanohydrin, which subsequently breaks down to liberate cyanide. We envisioned that a bis-acetal glycoside would position the two fluorochromes in close proximity and that *exo*-glycosidase catalyzed cleavage of the substrate would yield a hemiacetal that would in turn spontaneously breakdown to liberate two fragments (Figure 1). If each fragment could bear one fluorochrome, the net consequence would be that catalytic activity would lead to separation of these FRET partners and result in a convenient "dark-to-bright" change in fluorescence. One concern, however, is the known lability of acetals under acidic conditions that has been exploited in pH-sensitive prodrugs or linkers for drug delivery.[23,24] We recognized that carbohydrate anomeric acetals are chemically distinct from regular acetals. Due to stabilization by the endocyclic oxygen, we expected these bis-acetals would show stability across a range of physiological pH values.

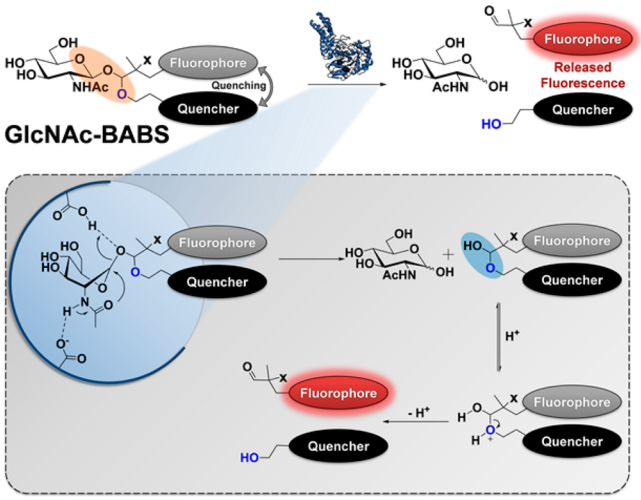
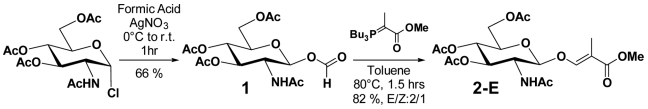


Figure 1. Enzymatic hydrolysis of fluorescence-quenched GlcNAc Bis-Acetal-Based Substrates (GlcNAc-BABS) releases a hemiacetal which in turn spontaneously decomposes, leading to separation of the fluorophore/quencher pair. Bis-acetal is shown in the orange oval, and hemiacetal motif in blue oval.

Here, we detail a generally applicable synthesis of carbohydrate bis-acetal-based substrates (BABS). This approach yields efficiently quenched substrates that enable "dark-to-bright" conversion upon enzymatic processing. We exemplify the approach using the *exo*-acting human *O*-GlcNAcase (hOGA), which cleaves *O*-linked *β*-*N*-acetylglucosamine (*O*-GlcNAc) units from serine and threonine residues of nuclear and cytoplasmic proteins.[25] We selected hOGA for study because it has emerged as an enzyme of high interest owing to its potential as a therapeutic target for tauopathies.[10] Furthermore, the complex biology of OGA creates a need for better probes that could help understand its regulation.

To test our approach, we set out to prepare GlcNAc bis-acetals. Our synthesis was motivated by Stoodley et al., who used a glucose pendant group as a chiral auxiliary to explore stereoselective functionalization of vinylogous carbonates.[26−28] We first generated *β*-formyl glycoside **1** (Scheme 1) using silver

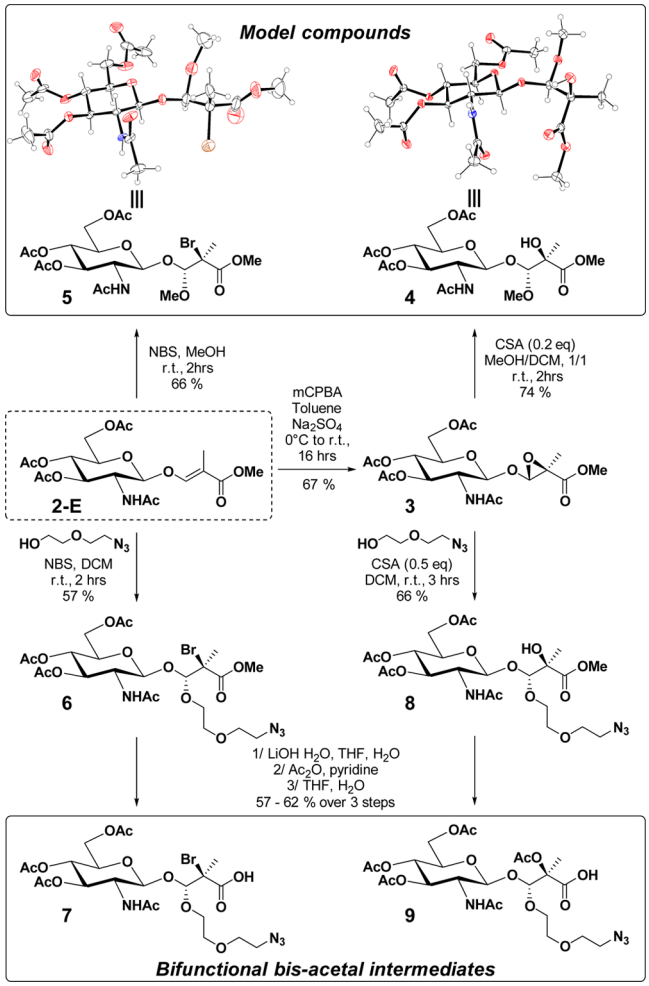
Scheme 1. Synthesis of GlcNAc Vinylogous Carbonate



nitrate and formic acid. Wittig-type olefination of the formyl glycoside using 2-tributylphosphorane-methylpropionate delivered vinylogous carbonates **2-E** and **2-Z**. Epoxidation of intermediate **2-E** using mCPBA provided epoxide **3** with complete stereoselectivity (Scheme 2). In model reactions using methanol, we tested both the acid-catalyzed epoxide opening of compound **3** using catalytic CSA and the bromoalkoxylation of compound **2-E** using NBS. Both approaches yielded GlcNAc bis-acetals with complete regioselectivity. The crystalline nature of these products allowed us to confirm the stereochemical outcome of these routes by X-ray diffraction of **4** and **5** (Table S1).

We next applied this methodology to the generation of bifunctional GlcNAc bis-acetals by bromoalkoxylation of **2-E** and epoxide opening of **3** using 2-(2-azidoethoxy)ethanol to afford

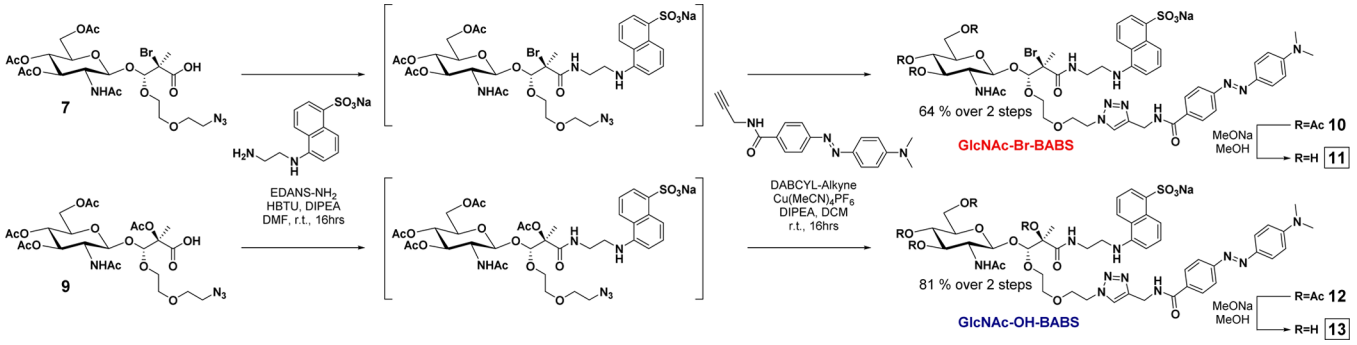
Scheme 2. Synthesis of Bifunctional Bis-acetal Intermediates



bis-acetals **6** and **8**, respectively (Scheme 2). We then developed a one-pot, three-step, protecting group manipulation that provided us with carboxylic acids **7** and **9**. GlcNAc bis-acetals **7** and **9** are flexible advanced intermediates for the late stage conjugation of fluorophores and quenchers. We decided to test our concept of fluorescence-quenched GlcNAc-BABS with the commonly used EDANS fluorophore and DACBYL quencher pair (Scheme 3). We therefore used a two-step coupling procedure first reacting EDANS-NH_2 with carboxylic acids **7** and **9** using HBTU and DIPEA in DMF. The second step consisted of copper-catalyzed azide−alkyne cycloaddition (CuAAC) with DABCYL-alkyne to yield the protected GlcNAc-BABS **10** and **12**. De-*O*-acetylation using potassium carbonate in methanol provided us with the target molecules GlcNAc-Br-BABS **11** and GlcNAc-OH-BABS **13**.

Notably, we found no detectable hydrolysis of the bis-acetal motif at pH values as low as 2 and as high as 10 (Figure S1). This remarkable stability likely stems from the participation of the endocyclic oxygen in the bis-acetal motif. We also evaluated the quenching efficiency of GlcNAc-BABS, and we found 99.93% and 99.89% quenching efficiencies for **11** and **13** respectively (Figure S2). This remarkable 1000-fold fluorescence quenching highlights that this design positions the fluorophore and quencher in close proximity, potentially enabling contact quenching. Interestingly, at higher concentrations of GlcNAc-BABS, we observed an inner-filter effect (IFE) (Figure S3) that is presumably due to the fluorescence signal being screened

Scheme 3. Conjugation of Fluorophore and Quencher Enables Generation of GlcNAc-BABS



intermolecularly by nearby GlcNAc-BABS. This effect is commonly observed and corrected for when using fluorescence-quenched substrates.[29]

We next set out to test whether hOGA could hydrolyze these GlcNAc-BABS and whether the intermediate hemiacetal would quickly breakdown to produce measurable fluorescence on a suitable time scale. Incubation of GlcNAc-BABS **11** and **13** with hOGA showed increasing rates with increasing GlcNAc-BABS concentrations (Figure 2a). We measured these steady-state rates for various concentrations of GlcNAc-BABS and, after correction for IFE, determined the second-order rate constants for each substrate probe (Figure 2b, Figure S4). hOGA catalyzed hydrolysis of GlcNAc-Br-BABS **11**, and GlcNAc-OH-BABS **13** revealed k_{cat}/K_m values of 263 and 519 M^{-1} s^{-1}, respectively. Interestingly, these values are comparable to the second-order rate constant measured for methyl β-D-*N*-acetylglucopyranoside[30] (440 M^{-1} s^{-1}) and about 50-fold higher than the rate constants measured for *O*-GlcNAc-modified protein substrates.[31] We also confirmed that the observed rates were linearly dependent on hOGA concentrations (Figure 2c). Accordingly, both GlcNAc-BABS **11** and **13** are competent substrates that are processed at comparable rates as the natural substrates of hOGA.

During these assays, we noticed a short lag phase (~1 min) in our initial rate experiments. The initial velocities gradually reached linear rates (Figure 2a). We speculated that this lag stemmed from an increase in the concentration of a hemiacetal (HA) intermediate to reach a steady-state concentration. To test this idea and determine the first-order rate constant for decomposition of these hemiacetals (HA), we developed an assay in which we can rapidly halt enzyme activity (Figure 2d). The assay was initiated by addition of hOGA, allowed to proceed for 1 min, and then the enzymatic reaction was rapidly stopped by addition of a high concentration of the tight binding hOGA inhibitor Thiamet-G (100 μM; 50 000 × K_i; K_i = 2 nM).[32] We could then monitor the decomposition of any accumulated hemiacetal (HA) (Figure 2e–f). Interestingly, we found that the hemiacetal (HA) generated from GlcNAc-OH-BABS **13** broke down 4.5 times faster than the one generated from GlcNAc-Br-BABS **11**, with an observed first-order rate constant (k_3) of 4.4 × 10^{-2} and 1 × 10^{-2} s^{-1} respectively. These results are comparable to reported rate constants for the hydrolysis of acetaldehyde methoxyethyl hemiacetal (k_0 = ~5 × 10^{-2} s^{-1}, pH = 7.5).[33] In addition, the stabilizing effect of the electron-withdrawing bromine atom is in good agreement with the electronic effects of substituents on hemiacetal breakdown.[34] Notably, these rate constants are also consistent with the more rapid approach to steady state upon hOGA-catalyzed hydrolysis of GlcNAc-BABS

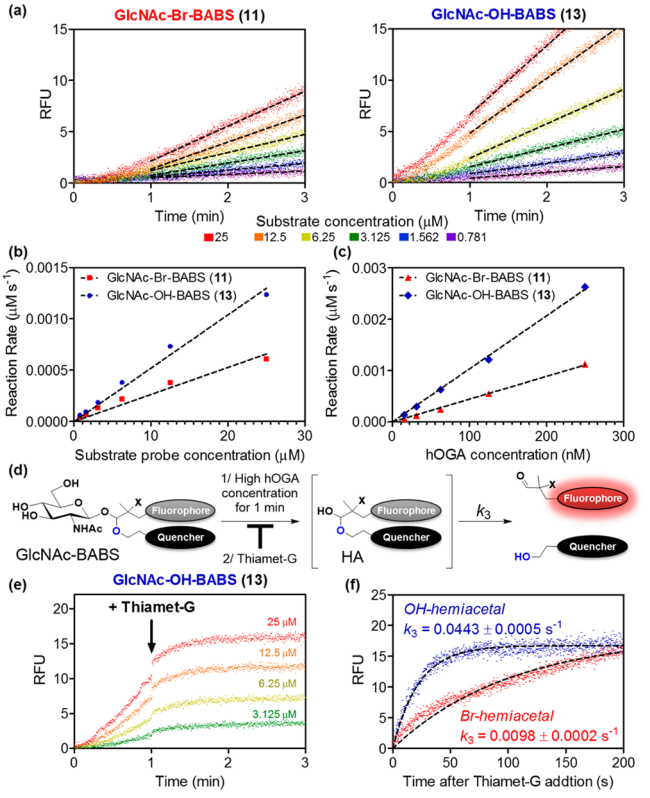


Figure 2. GlcNAc-BABS probes are turned over by hOGA. (a) Evolution of fluorescence (RFU) for different concentrations of GlcNAc-BABS in the presence of hOGA. Dotted lines represent the linear rates reached at steady state. (b) Rates of hOGA catalyzed hydrolysis of different concentrations of GlcNAc-BABS. (c) Rates of hydrolysis of GlcNAc-BABS depend on the concentrations of hOGA. (d) Hemiacetal breakdown assay design containing high hOGA concentration. Stopped after 1 min by addition of Thiamet-G. (e) Evolution of fluorescence for the hemiacetal breakdown assay with different concentrations of GlcNAc-OH-BABS. (f) Measurement of first-order rate constants for the breakdown of hemiacetal after addition of Thiamet-G.

13 as compared to **11** (Figure 2a). To evaluate the compatibility of the BABS design with complex cellular extracts, we also measured the turnover of GlcNAc-Br-BABS in SK-N-SH cell lysate in the presence of increasing concentrations of hOGA (Figure S5). This substrate is efficiently processed within lysates, at rates comparable to that observed in buffer. Taken together, these results support that the steady-state initial rates reliably report on the kinetics of the enzymatic cleavage of these GlcNAc-BABS probes.

Collectively these data validate the proof of concept for the Glyco-BABS design as efficient fluorescence-quenched substrates and suggest that Glyco-BABS probes may prove to be valuable for monitoring glycosidase activities in cells. Cell active fluorescence-quenched Glyco-BABS could provide useful information regarding target engagement by inhibitors directly in live cells as well as permit understanding of endogenous factors that regulate enzyme activity. Accordingly, these substrate probes should prove complementary to covalent activity-based probes (ABP)[1,4] for *exo*-glycosidases,[14,35] which report on the levels of active enzyme in cells. The modular approach used here will enable late-stage incorporation of bright fluorophores, including cell-compatible red-shifted fluorophores to help move toward quantitative monitoring of hOGA activity in cells. Such cell active Glyco-BABS should complement other substrate probes[12] and could be particularly valuable in cases where these other substrate designs show limitations. For example, Glyco-BABS derivatives targeting lysosomal and endolysosomal enzymes may prove useful, especially because pH-insensitive fluorochromes can be installed. Indeed, one of the main advantages of this design is its versatility, as developing a probe for another member of the large class of *exo*-glycosidases may simply require another core carbohydrate residue. Finally, we also expect this acetal and bis-acetal concept could be applied to other classes of hydrolases including, for example, sulfatases and phosphatases which have similarly constrained active sites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b01948.

Methods, compound characterization (PDF)
Crystallographic data (CIF)

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Notes

The authors declare the following competing financial interest(s): The authors note they have filed a provisional patent application pertaining to this research.

■ ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (RGPIN/-2015-05426) and the Canadian Institutes of Health Research (MOP-102756). D.J.V. thanks the Canada Research Chairs Program for support as a Tier I CRC in Chemical Biology. S.C. thanks the CIHR for a postdoctoral fellowship. We thank Dr. John R. Thompson for assistance with X-ray data collection and analysis.

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