Open access • Journal Article • DOI:10.1021/JA0763605

Synthesis and Use of Mechanism-Based Protein-Profiling Probes for Retaining β-D-Glucosaminidases Facilitate Identification of Pseudomonas aeruginosa NagZ

Keith A. Stubbs, Adrian Scaffidi, Aleksandra W. Debowski, Brian L. Mark ...+2 more authors

Institutions: University of Western Australia

Published on: 09 Jan 2008 - Journal of the American Chemical Society (American Chemical Society)

Topics: Hypothetical protein

Final version published as: Stubbs, K. A., Scaffidi, A., Debowski, A. W., Mark, B. L., Stick, R. V., & Vocadlo, D. J. (2008). Synthesis and Use of Mechanism-Based Protein-Profiling Probes for Retaining β-d-Glucosaminidases Facilitate Identification of Pseudomonas aeruginosa NagZ. Journal of the American Chemical Society, 130(1), 327–335. https://doi.org/10.1021/ja0763605.

Synthesis and Use of Mechanism-Based Protein-Profiling Probes for Retaining β-D-Glucosaminidases Facilitate Identification of *Pseudomonas aeruginosa* NagZ

Keith A. Stubbs,[†] Adrian Scaffidi,[‡] Aleksandra W. Debowski,[†] Brian L. Mark,[§] Robert V. Stick,[‡] and David J. Vocadlo*,[†]

Contribution from the Department of Chemistry, Simon Fraser University, 8888 University Drive, Burnaby, B.C. Canada, V5A 1S6, School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, Australia, 6009, and Department of Microbiology, University of Manitoba, Rm 418 Buller Building, Winnipeg, Manitoba, Canada, R3T 2N2

E-mail: dvocadlo@sfu.ca

Abstract: The NagZ class of retaining *exo*-glucosaminidases play a critical role in peptidoglycan recycling in Gram-negative bacteria and the induction of resistance to β-lactams. Here we describe the concise synthesis of 2-azidoacetyl-2-deoxy-5-fluoro-β-D-glucopyranosyl fluoride as an activity-based proteomics probe for profiling these *exo*-glycosidases. This active-site directed reagent covalently inactivates this class of retaining *N*-acetylglucosaminidases with exquisite selectivity by stabilizing the glycosyl-enzyme intermediate. Inactivated *Vibrio cholerae* NagZ can be elaborated with biotin or a FLAG-peptide epitope using the Staudinger ligation or the Sharpless−Meldal click reaction and detected at nanogram levels. This ABPP enabled the profiling of the *Pseudomonas aeruginosa* proteome and identification at endogenous levels of a tagged protein with properties consistent with those of PA3005. Cloning of the gene encoding this hypothetical protein and biochemical characterization enabled unambiguous assignment of this hypothetical protein as a NagZ. The identification and cloning of this NagZ may facilitate the development of strategies to circumvent resistance to β-lactams in this human pathogen. As well, this general strategy, involving such 5-fluoro inactivators, may prove to be of general use for profiling proteomes and identifying glycoside hydrolases of medical importance or having desirable properties for biotechnology.

Introduction

Glycoside hydrolases are an extremely large class of enzymes that act to cleave carbohydrate motifs. They are found throughout nature in organisms ranging from bacteria to humans and have been classified into over 100 families on the basis of structural similarity.[1] In light of their biological significance, the rapid detection of proteins involved in processing glycoconjugates is of considerable interest.[2] However, compared to activity-based methods for profiling protease and esterase activities,[3−7] which have met with considerable success, the methods used to profile the activities of glycan-processing enzymes within proteomes and the discovery of new families of these enzymes is lagging.[2]

Several activity-based proteomics probes (ABPPs) directed toward glycosidases have been developed[8−13] but only those incorporating a 2-deoxy-2-fluorosugar have proven useful for probing complex proteomes such as cell lysates. The first of these 2-deoxy-2-fluorosugar probes, directed against *exo*-glycosidases, inactivates the enzyme quite slowly owing to lost enzyme−carbohydrate interactions.[12] The second, directed against one class of *endo*-glycosidases, is not suitable for labeling *exo*-glycosidases owing to the structure of the ABPP.[13,14]

Indeed, a major complexity in the development of probes directed against glycoconjugate-degrading enzymes is that, unlike proteases, which commonly have a cleft-like active site, most carbohydrate processing enzymes are *exo*-acting and cleave only the terminal ends of glycoconjugates. These enzymes

[†] Simon Fraser University.
[‡] University of Western Australia.
[§] University of Manitoba.

(1) Henrissat, B.; Davies, G. *Curr. Opin. Struct. Biol.* **1997**, *7*, 637−644.
(2) Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. *Mol. Cell. Proteomics* **2002**, *1*, 781−790.
(3) Phillips, C. I.; Bogyo, M. *Cell. Microbiol.* **2005**, *7*, 1061−1076.
(4) Speers, A. E.; Cravatt, B. F. *Chem. Biol.* **2004**, *11*, 535−546.
(5) Ovaa, H.; van Swieten, P. F.; Kessler, B. M.; Leeuwenburgh, M. A.; Fiebiger, E.; van den Nieuwendijk, A. M.; Galardy, P. J.; van der Marel, G. A.; Ploegh, H. L.; Overkleeft, H. S. *Angew. Chem., Int. Ed.* **2003**, *42*, 3626−3629.
(6) Speers, A. E.; Cravatt, B. F. *ChemBioChem* **2004**, *5*, 41−47.
(7) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686−4687.
(8) Tsai, C. S.; Li, Y. K.; Lo, L. C. *Org. Lett.* **2002**, *4*, 3607−3610.
(9) Kurogochi, M.; Nishimura, S.; Lee, Y. C. *J. Biol. Chem.* **2004**, *279*, 44704−44712.
(10) Hinou, H.; Kurogochi, M.; Shimizu, H.; Nishimura, S. *Biochemistry* **2005**, *44*, 11669−11675.
(11) Romaniouk, A. V.; Silva, A.; Feng, J.; Vijay, I. K. *Glycobiology* **2004**, *14*, 301−310.
(12) Vocadlo, D. J.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2004**, *43*, 5338−5342.
(13) Williams, S. J.; Hekmat, O.; Withers, S. G. *ChemBioChem* **2006**, *7*, 116−124.
(14) Hekmat, O.; Kim, Y. W.; Williams, S. J.; He, S.; Withers, S. G. *J. Biol. Chem.* **2005**, *280*, 35126−35135.

commonly have pocket-shaped active site architectures that hinder the development of useful probes since there is little space to accommodate pendent biochemical reporter groups such as biotin. An exception are the *endo*-glycosidases that have a canyon-like active site that can tolerate bulky reporter groups attached to the probe.[13,14]

One distinct advantage in common with the serine and cysteine proteases, however, is that most retaining *exo*-glycosidases use a catalytic mechanism involving the transient formation of a covalent intermediate. These glycosyl-enzyme intermediates can be stabilized so that they persist by using either deoxy sugars[15] or, more commonly, deoxy-fluorosugars as mechanism-based inactivators or probes of enzyme mechanism.[16−18] The inclusion of a fluorine atom at C2 or C5 of the pyranose ring has the effect of destabilizing the two transition states that flank the covalent glycosyl-enzyme intermediate, thereby slowing both enzyme-catalyzed steps. A key functional requirement is that a very good leaving group be incorporated at the anomeric center. In this way, the intermediate is kinetically accessible since the leaving group compensates for the destabilizing effect of the fluorine, yet the breakdown of the intermediate is very slow since the excellent leaving group is no longer present. The net effect is that these fluorosugars act as both a recognition element and reactive group, since a stable covalent bond is formed between the enzymic nucleophile and fluorosugar.[16,17,19,20]

A key challenge for developing ABPPs for glycoside hydrolases is the point of attachment of the reporter group. As described earlier, *exo*-glycosidases have a pocket-shaped active site in which extensive contacts are formed between the protein and all substrate hydroxyl groups, making it impractical to incorporate a bulky biochemical reporter group such as biotin. One solution to this problem has been demonstrated for several families of retaining β-galactosidases by using a 6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranosyl fluoride ABPP where the pendant azide group could later be ligated to a reporter group.[12]

Several chemoselective ligations have been developed that make use of the small unobtrusive azide functionality as one reactant.[21−24] The reactive partner for the azide group varies for these different reactions; for the Staudinger ligation it is a phosphine, for the Sharpless−Meldal click reaction it is an alkyne, and for the strained Huisgen ligation it is a cyclooctyne derivative.[21−24] These chemoselective ligations can be carried out under physiological conditions and, owing to the entirely bioorthogonal nature of both partners, are so exquisitely selective that the large concentrations of biologically relevant functional groups do not interfere. Any of these reactive functional group pairs can be appended to various affinity probes or ligands as desired.

In previous work, the small pendant azide group attached to the 2-fluoro sugar probe diminished, but did not prevent, binding of the saccharide moiety by *exo*-galactosidases.[12] In that study, the target enzyme could be covalently inactivated by the affinity probe bearing the azide group and then elaborated with a reporter group by using a ligation reaction.[12] As alluded to, however, this 2-fluorosugar probe, while highly selective, was slow to inactivate the target enzymes thereby limiting its use with unstable enzymes or proteomes rich in proteases.

Here we present a significantly improved strategy using an azide-derivatized 5-fluorosugar that quickly inactivates the target *exo*-glycosidases and forms a stable species (Figure 1A). A biochemical reporter group can be appended to this inactivated enzyme by using the Staudinger ligation or Sharpless−Meldal click reaction, enabling subsequent proteomic profiling and purification. Recent studies in our laboratory have focused on the enzyme NagZ,[25] which is involved in the murein recycling pathway in Gram-negative bacteria. This enzyme has been shown to regulate the inducible expression of AmpC β-lactamase in Gram-negative bacteria.[26,27] NagZ and other sequence related glucosaminidases are members of CAZy family 3[28,29] of glycoside hydrolases that also contains glycosidases active on a variety of other saccharides. NagZ is an *exo*-glycosidase that uses a catalytic mechanism involving the formation and subsequent breakdown of a covalent glycosyl−enzyme intermediate.[20] Based on previous mechanistic studies of *Vibrio furnisii* β-glucosaminidase using fluorinated saccharides[20] and structural studies of NagZ from *Vibrio cholerae*,[25] we envisioned that 2-azidoacetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl fluoride **1** (2AA5FGF) could be an efficient ABPP that would be useful for functional proteomic analysis of organisms that produce mechanistically related glucosaminidases.

Results and Discussion

A high-resolution crystal structure of *V. cholerae* NagZ (VCNagZ) in complex with an inhibitor has recently been reported and revealed the presence of a large pocket surrounding the 2-acetamido group.[25] This observation suggested to us that substrates and inhibitors bearing extensions at the 2-acetamido group should be well tolerated by the enzyme. This site therefore seemed suitable, and synthetically accessible, for the installation of an azide functionality.

Before embarking on a lengthy synthesis we decided to investigate whether an azide group attached to the 2-acetamido moiety of an *N*-acetyl-D-glucosaminyl substrate would be tolerated by the enzyme. We therefore prepared the known chromogenic substrate 4-nitrophenyl 2-azidoacetamido-2-deoxy-β-D-glucopyranoside (pNP-GlcNAz).[30] We found this compound to be a good substrate for the β-D-glucosaminidase VCNagZ, an enzyme that we had previously cloned.[25] Compared to the parent 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (pNP-GlcNAc; $k_{cat}/K_M = 8.6 \pm 0.66$ μmol mM^{-1} min^{-1} mg^{-1},

(15) Roeser, K. R.; Legler, G. *Biochim. Biophys. Acta* **1981**, *657*, 321−333.
(16) Wicki, J.; Rose, D. R.; Withers, S. G. *Methods Enzymol.* **2002**, *354*, 84−105.
(17) Withers, S. G.; Rupitz, K.; Street, I. P. *J. Biol. Chem.* **1988**, *263*, 7929−7932.
(18) Hartman, M. C. T.; Coward, J. K. *J. Am. Chem. Soc.* **2002**, *124*, 10036−10053.
(19) Vocadlo, D. J.; Davies, G. J.; Laine, R.; Withers, S. G. *Nature* **2001**, *412*, 835−838.
(20) Vocadlo, D. J.; Mayer, C.; He, S.; Withers, S. G. *Biochemistry* **2000**, *39*, 117−126.
(21) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010.
(22) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599.
(23) Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057−3064.
(24) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046−15047.
(25) Stubbs, K. A.; Balcewich, M.; Mark, B. L.; Vocadlo, D. J. *J. Biol. Chem.* **2007**, *282*, 21382−21391.
(26) Votsch, W.; Templin, M. F. *J. Biol. Chem.* **2000**, *275*, 39032−39038.
(27) Cheng, Q.; Li, H.; Merdek, K.; Park, J. T. *J. Bacteriol.* **2000**, *182*, 4836−4840.
(28) Henrissat, B.; Bairoch, A. *Biochem. J.* **1996**, *316*, 695−696.
(29) Henrissat, B.; Bairoch, A. *Biochem. J.* **1993**, *293*, 781−788.
(30) Vocadlo, D. J.; Hang, H. C.; Kim, E. J.; Hanover, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9116−9121.

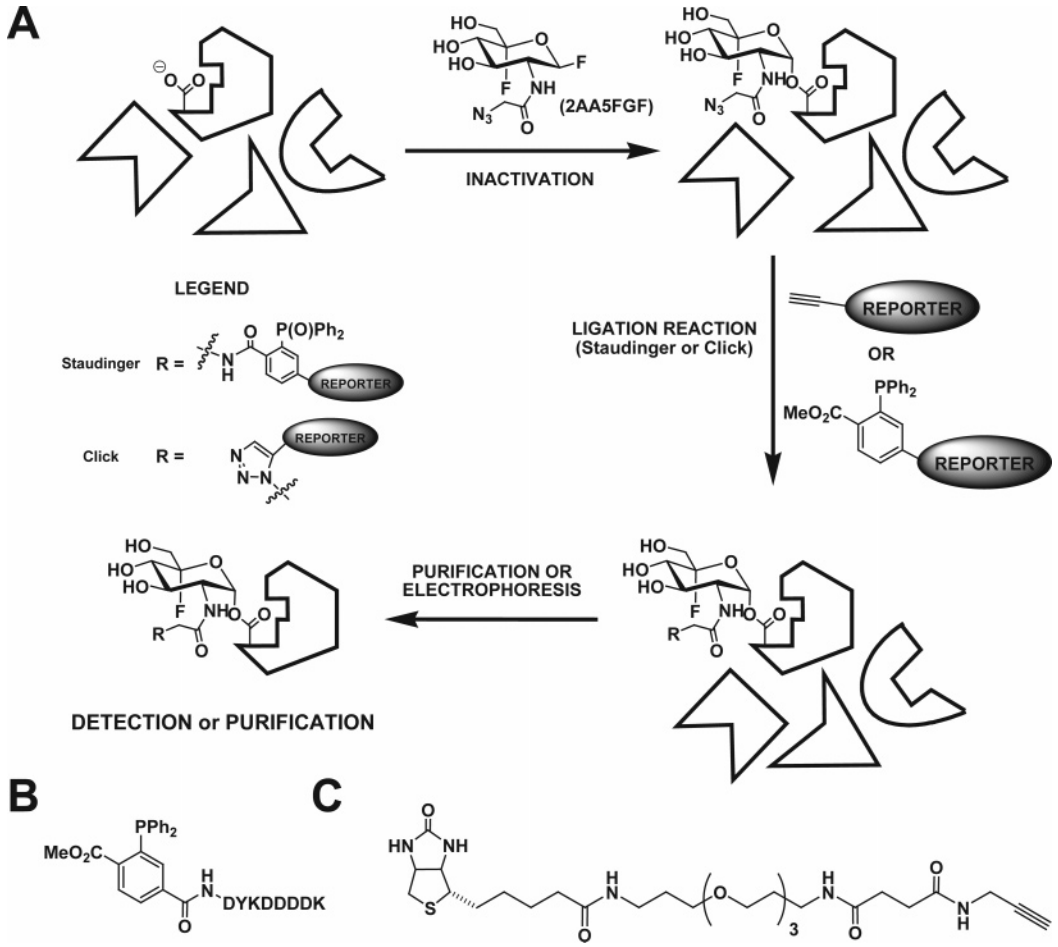


Figure 1. (A) The activity-based detection and purification strategy using 2-azidoacetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl fluoride (2AA5FGF). Incubation of the proteome with 2AA5FGF results in rapid inactivation and covalent labeling of the catalytic nucleophile of the β-glucosaminidase. Denaturation under acidic conditions and conjugation using either the Staudinger ligation or by the Sharpless-Meldal click reaction facilitates either profiling in lysates or purification and identification using an immobilized streptavidin stationary phase. (B and C) Reporter groups used in this study. (B) Phosphine-FLAG and (C) Alkyne-biotin.

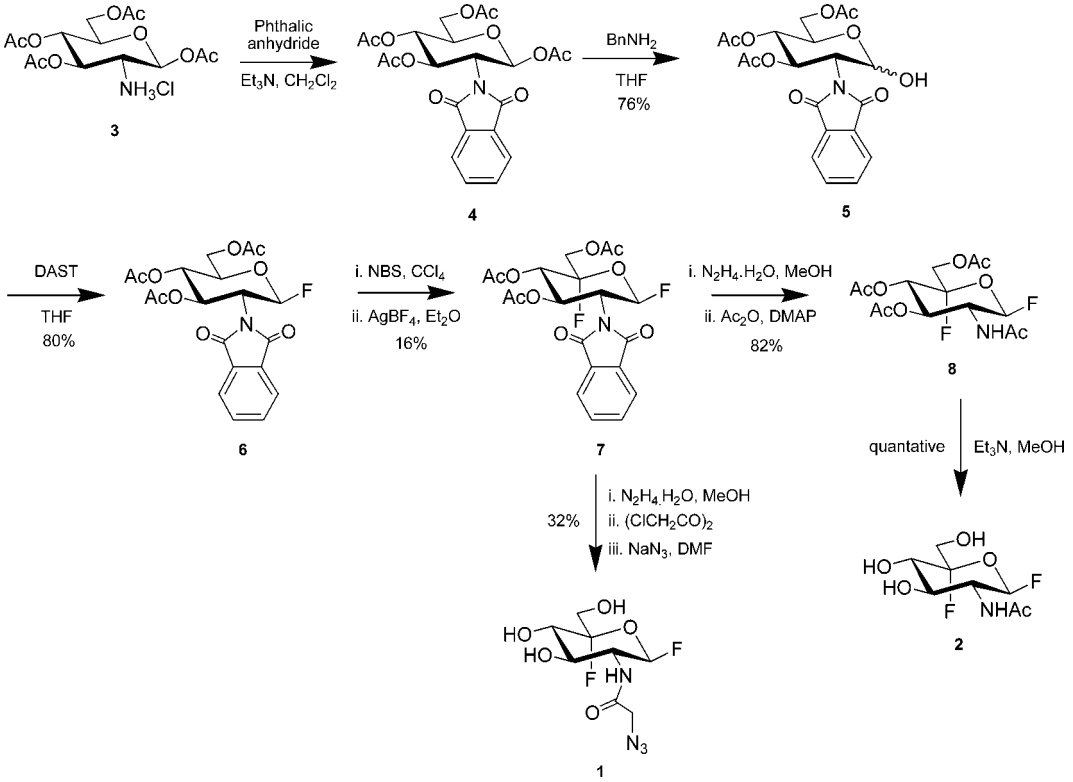
$k_{cat} = 4.4 \pm 0.08$ μmol min^{-1} mg^{-1}, $K_M = 0.51 \pm 0.03$ mM), the value of the second-order rate constant for the VCNagZ-catalyzed hydrolysis of pNP-GlcNAz is only 2.8 fold higher (pNP-GlcNAz; $k_{cat}/K_M = 24 \pm 5.7$ μmol mM^{-1} min^{-1} mg^{-1}, $k_{cat} = 0.48 \pm 0.02$ μmol min^{-1} mg^{-1}, $K_M = 0.02 \pm 0.004$ mM), indicating that the azide moiety only modestly affects enzyme activity (see Supporting Information).

Having established that the azide group is well tolerated by this representative member of the family 3 *exo*-β-D-glucosaminidases, we synthesized both 2-acetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl fluoride **2** (2A5FGF) and the azide analogue 2AA5FGF **1** as potential inactivators (Scheme 1). Using the hydrochloride **3**[31] as a starting point, the liberated amine was protected using a phthalimido group to give **4**. Selective removal of the anomeric acetyl group was effected using benzylamine, and the resulting hemiacetal **5**[32] was treated with (diethylamino)sulfur trifluoride (DAST) to yield the β-fluoride **6** in excellent yield. Photobromination of **6** gave the presumed 5-bromo intermediate that was immediately treated with silver tetrafluoroborate in diethyl ether to furnish exclusively the 5-fluoro D-*gluco* derivative **7**. Surprisingly, removal of the phthalimido group could be effected by hydrazine without harming either anomeric fluorine. Subsequent acetylation of the intermediate amine afforded the amide **8**, after which deprotection yielded the desired 2-acetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl fluoride **2**. The 2AA5FGF ABPP **1** was prepared similarly starting from the common intermediate **7**. This time, however, a one-pot procedure was used in which removal of the phthalimido group was followed by chloroacetylation. Installation of the azide moiety was effected by displacement of the α-chloride to yield the probe **1**, in good yield. It is worth noting that previous efforts to generate such 2-acetamido 1,5-difluoro compounds failed to yield the D-*gluco* epimer but rather afforded the L-*ido* analogue in very modest yield.[20] Furthermore, unlike the previous study, the synthesis presented here enables facile installation of an azide group, which is a prerequisite to develop this ABPP. Although the yield of the two-step photobromination/fluorination proceedure is modest, the overall synthesis is facile and readily furnishes more than adequate quantities of ABPP **1** since very small quantities of material are required for proteomic profiling.

With the putative mechanism-based inhibitors **1** and **2** in hand, we turned our attention to evaluating their ability to inactivate VCNagZ. Incubation of VCNagZ with 2A5FGF **2** results in rapid time-dependent inactivation of the enzyme relative to a control (Figure 2A). This result differs somewhat from a report

(31) Cunha, A. C.; Pereira, L. O. R.; de Souza, M. C. B. V.; Ferreira, V. F. *J. Chem. Ed.* **1999**, *76*, 79−80.
(32) Pozsgay, V.; Jennings, H. J. *J. Carbohydr. Chem.* **1990**, *9*, 333−343.

Scheme 1. Synthesis of the 2-Acetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl Fluoride **2** (2A5FGF) and the Azide Analogue 2AA5FGF **1**



by Vocadlo *et al*. who saw incomplete inactivation of a family 3 β-*N*-acetylglucosaminidase from *V. furnissi* when using the C-5 epimer, the L-*ido* inactivator.[20] The more natural D-*gluco* inactivator studied here may therefore offer a more appropriate scaffold for generation of ABPPs for β-*N*-acetylglucosaminidases. We carried out inactivation kinetics at several inactivator concentrations, and the kinetic parameters governing inactivation (k_{inact} and K_i) were determined by plotting k_{obs} versus [I] (Figure 2B). Inactivation of VCNagZ by 2A5FGF **2** revealed a K_i value of 86 $\pm$ 15 μM and a k_{inact} value of 0.74 $\pm$ 0.09 s^{-1}. In all cases the inactivation data fitted well to a single exponential decay equation.

Gratifyingly, 2AA5FGF **1** acts as an even more efficient time-dependent inactivator of VCNagZ; in only 7 minutes complete inactivation of VCNagZ is observed at concentrations as low as 40 μM (Figures 2C and 2D). The value of the second-order rate constant ($k_{inact}/K_i = 0.011 \pm 0.002\ \mu M^{-1}\ s^{-1}$) governing inactivation compares favorably with that of the parent inactivator 2A5FGF **2** ($k_{inact}/K_i = 0.009 \pm 0.003\ \mu M^{-1}\ s^{-1}$), consistent with the studies described above using the azide derivatized substrate. Consequently, the azide moiety has little effect on the inactivation process. 2AA5FGF **1** therefore inactivates family 3 β-D-glucosaminidases on a time scale appropriate for practical applications and suggests that this compound could be used to detect β-D-glucosaminidase activity within complex proteomes at endogenous levels of target protein.

The reporter group chosen for initial studies was the phosphine-FLAG ligand (Figure 1B).[21] After incubation of purified VCNagZ (0.84 mg mL^{-1}) with **1** (333 μM) for 10 minutes no residual enzyme activity could be detected. To stop the turnover of the presumed glycosyl-enzyme intermediate the protein was denatured under mild reducing conditions (pH 3.5 in the presence of urea). In this way both the labile acylal ester linkage and the azide moiety remained intact. Treatment of the sample with phosphine-FLAG[21] followed by SDS-PAGE and Western blot analysis revealed no signal if the acrylamide gel was subjected to electrophoresis for the normal time (90 V for 20 minutes and then 180 V for 65 minutes). This result was presumably owing to the acylal ester linkage of the glycosyl-enzyme intermediate breaking down under the basic conditions (pH 8.8) of the buffered gel. Decreasing the pH of the gel resulted in diffuse bands that were poorly resolved (data not shown).

After some optimization a short electrophoresis protocol (90 V for 20 minutes and then 200 V for 8 minutes) was developed that when used in conjunction with Western blot analysis showed the enzyme was indeed labeled (Figure 3). To confirm that the phosphine-FLAG was reacting specifically with the azide of NagZ inactivated with **1** and not with the protein itself, various controls were performed (Figure 3A). The results clearly reveal that both the inactivation of NagZ using **1** and the subsequent Staudinger ligation are highly specific, and neither reagent on its own results in labeling of the enzyme.

To validate the selectivity of our approach toward those β-D-glucosaminidases that form a glycosyl-enzyme intermediate, we tested two enzymes from family 84 of glycoside hydrolases. These β-D-glucosaminidases differ in their catalytic mechanism in that they use substrate-assisted catalysis involving the 2-acetamido group as a nucleophile in place of an enzymatic nucleophile.[33−37] These enzymes therefore do not form a covalent glycosyl-enzyme intermediate, and as a result, they

(33) Tews, I.; Perrakis, A.; Oppenheim, A.; Dauter, Z.; Wilson, K. S.; Vorgias, C. E. *Nat. Struct. Biol.* **1996**, *3*, 638−648.
(34) Knapp, S.; Vocadlo, D.; Gao, Z. N.; Kirk, B.; Lou, J. P.; Withers, S. G. *J. Am. Chem. Soc.* **1996**, *118*, 6804−6805.
(35) Macauley, M. S.; Whitworth, G. E.; Debowski, A. W.; Chin, D.; Vocadlo, D. J. *J. Biol. Chem.* **2005**, *280*, 25313−25322.

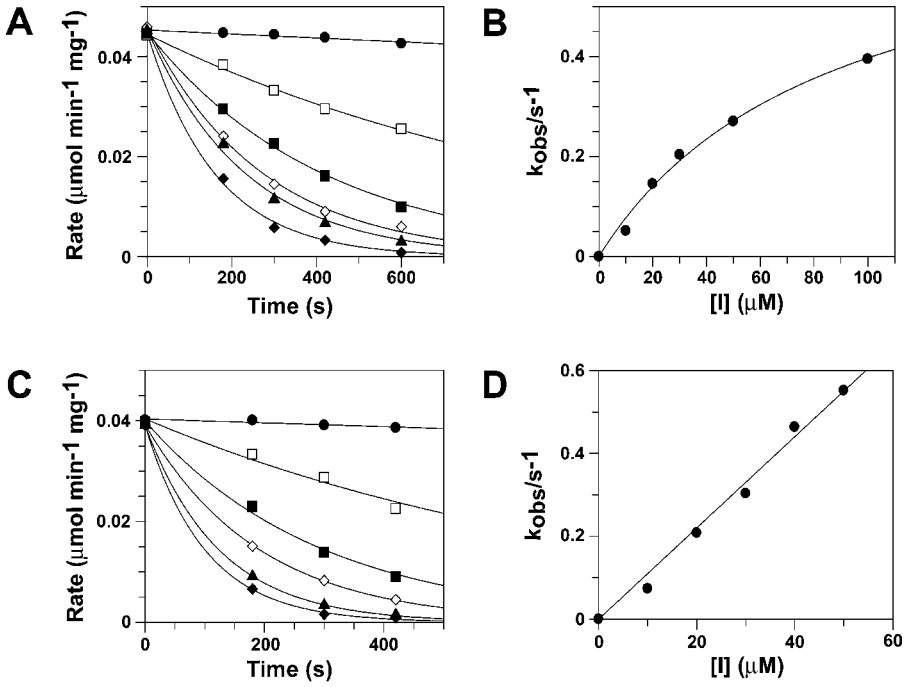


Figure 2. Time-dependent inactivation of VCNagZ. (A) Experimental data represent the VCNagZ activity of control samples (without **2**) over time. [**2**] used with VCNagZ: 0 (●), 10 (□), 20 (■), 30 (◇), 50 (▲), 100 (◆) μM. Curves are the nonlinear fits of data to a single-exponential decay equation. (B) Plot of the inactivation rate constants (k_{obs}) as a function of [**2**] for VCNagZ. (C) Time-dependent inactivation of VCNagZ by **1**: Experimental data represent the VCNagZ activity of control samples (without **1**) over time. [**1**] used with VCNagZ: 0 (●), 10 (□), 20 (■), 30 (◇), 40 (▲), 50 (◆) μM. Curves are the nonlinear fits of data to a single-exponential decay equation. (D) Plot of the inactivation rate constants (k_{obs}) as a function of [**1**] for VCNagZ.

should not be labeled using the approach described here. Repeating the experiment with VCNagZ along with the family 84 human *O*-GlcNAcase and a close bacterial homologue of *O*-GlcNAcase, *Bt*GH84, demonstrated the selectivity of the labeling strategy (Figure 3B). The family 3 β-D-glucosaminidase, VCNagZ, is labeled, but as predicted, the β-D-glucosaminidases from family 84 are not.

Having shown the probe is selective for family 3 β-D-glucosaminidases we determined the lower detection limit of our strategy. Incubating purified VCNagZ (0.03, 0.09, 0.3, and 0.84 mg mL^{-1}) with **1** revealed the lower limit for the detection of VCNagZ to be approximately 80 ng (Figure 4A). Even though it was obvious to us that we were losing signal the longer the gel was subjected to electrophoresis, the approach is still quite sensitive.

Having established that VCNagZ could be successfully labeled using **1**, our attention turned to extending this strategy to the analysis of β-D-glucosaminidase activity from cell lysates. The problem we faced, however, was that owing to the short electrophoresis protocol, a gel could not be obtained that would give sufficiently good resolution of cell lysates to resolve protein bands clearly. We therefore turned our attention to using a biotin-alkyne reporter group, which has the benefit of having a biotin moiety that can be used to immobilize labeled protein to avidin resin even under the acidic conditions required to stabilize the acylal ester linkage. The avidin resin should immobilize only those proteins bearing the glycosyl-enzyme intermediate conjugated to the reporter group. The washed resin, free of unlabeled proteins, can be mixed with SDS-PAGE loading buffer and boiled to cleave the acylal ester linkage, thereby releasing the enzyme. After electrophoresis of the supernatant, gels can be stained with SYPRO Orange to identify the affinity purified proteins. We decided to use the proven alkyne-linked biotin (Figure 1C).[13,38−41]

With this new reporter group in hand, we turned our attention to labeling VCNagZ. Samples of VCNagZ (0.027, 0.09, 0.16, and 0.3 mg mL^{-1}) were treated with **1** in the manner described above. Again, to prevent the turnover of the glycosyl-enzyme intermediate, the protein was denatured under mild reducing conditions (pH 3.5) by adding a solution of TCEP, the biotin tag in DMSO, and the triazole ligand[39] in a mixture of DMSO and *tert*-butanol. After incubation of the sample for five hours, followed by treatment with avidin resin, the sample was centrifuged. The pelleted beads were washed with PBS buffer, and SDS-PAGE loading buffer was then added and the sample boiled. After electrophoresis (10% acrylamide), proteins were visualized using SYPRO Orange. Gratifyingly, we observed a protein band corresponding to the molecular weight of VCNagZ (Figure 4B). These results indicate that both the Sharpless−Meldal click cycloaddition and the avidin binding assay are highly specific, but what is more encouraging was that this capture and release approach is highly sensitive and allows longer electrophoresis times. The lower limit for the detection of the VCNagZ after all sample handling is approximately 25

(36) Dennis, R. J.; Taylor, E. J.; Macauley, M. S.; Stubbs, K. A.; Turkenburg, J. P.; Hart, S. J.; Black, G. N.; Vocadlo, D. J.; Davies, G. J. *Nat. Struct. Mol. Biol.* **2006**, *13*, 365−371.
(37) Rao, F. V.; Dorfmueller, H. C.; Villa, F.; Allwood, M.; Eggleston, I. M.; van Aalten, D. M. *EMBO J.* **2006**, *25*, 1569−1578.
(38) Link, A. J.; Vink, M. K. S.; Agard, N. J.; Prescher, J. A.; Bertozzi, C. R.; Tirrell, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10180−10185.
(39) Link, A. J.; Tirrell, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 11164−11165.
(40) Wilbur, D. S.; Hamlin, D. K.; Vessella, R. L.; Stray, J. E.; Buhler, K. R.; Stayton, P. S.; Klumb, L. A.; Pathare, P. M.; Weerawarna, S. A. *Bioconjugate Chem.* **1996**, *7*, 689−702.
(41) Gayo, L. M.; Suto, M. J. *Tetrahedron Lett.* **1996**, *37*, 4915−4918.

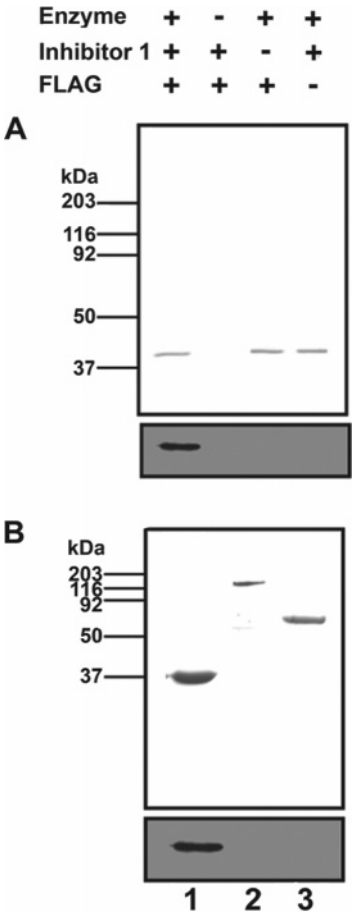


Figure 3. Visualization of **1**-labeled VCNagZ by denaturing SDS/PAGE-Western blot. After inactivation, the samples were labeled with phosphine-FLAG and analyzed by the Western blot technique using anti-FLAG-HRP (HRP, horseradish peroxidase): (A) SDS/PAGE-Western blot with the appropriate controls. Coomassie blue-stained 10% SDS-PAGE gel, electrophoresed fully, showing equal loading of lanes. The protein concentration used in each lane was approximately 1 μg. Western blot analysis shows all appropriate species must be present for a successful result. (B) Demonstrates the selectivity of the technique for those β-D-glucosaminidases that have a catalytic mechanism involving a glycosyl-enzyme intermediate. Lane (1) VCNagZ (2.8 μg), (2) family 84 human *O*-GlcNAcase (1.2 μg), (3) family 84 *Bt*GH84 (2 μg). The Coomassie blue-stained 10% SDS-PAGE gel, electrophoresed fully, shows the different molecular weights of the three proteins.

ng, which compares favorably with the approximate 80 ng detection limit obtained using the FLAG-phosphine method.

We next evaluated the proteome from *P. aeruginosa*, a Gram-negative bacterium that harbors a chromosomally inducible AmpC β-lactamase.[42] This opportunistic pathogen is problematic for patients suffering from cystic fibrosis, chronic obstructive pulmonary disease, and severe burns.[43−45] The genome sequence of *P. aeruginosa* is known, and one gene (PA3005) has been annotated as a family 3 β-D-glucosaminidase homologue[28,29] (For more information on the classification of glycoside hydrolases, see the CAZY Database available at afmb.cnrs-mrs.fr/CAZY/). By analogy to other inducible AmpC systems, this putative NagZ could be important in the induction of AmpC β-lactamase and could therefore be a target for the development

(42) Sanders, C. C.; Sanders, W. E., Jr. *Clin. Infect. Dis.* **1992**, *15*, 824−839.
(43) Govan, J. R.; Deretic, V. *Microbiol. Rev.* **1996**, *60*, 539−574.
(44) Lyczak, J. B.; Cannon, C. L.; Pier, G. B. *Clin. Microbiol. Rev.* **2002**, *15*, 194−222.
(45) Nagaki, M.; Shimura, S.; Tanno, Y.; Ishibashi, T.; Sasaki, H.; Takishima, T. *Chest* **1992**, *102*, 1464−1469.

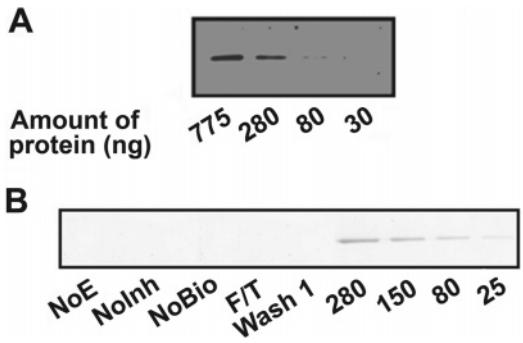


Figure 4. (A) Western blot showing the detection limit of **1**, used in conjunction with Staudinger ligation: amounts of VCNagZ were 775, 280, 80, 30 ng. (B) SYPRO-stained 10% SDS-PAGE showing the detection limit of **1**, used in conjunction with copper-catalyzed Sharpless-Meldal click reaction. Lanes shown (left to right); no enzyme, no inhibitor **1**, no biotin label, flow through, wash 1, 280 ng of VCNagZ, 150 ng of VCNagZ, 80 ng VCNagZ, 25 ng VCNagZ.

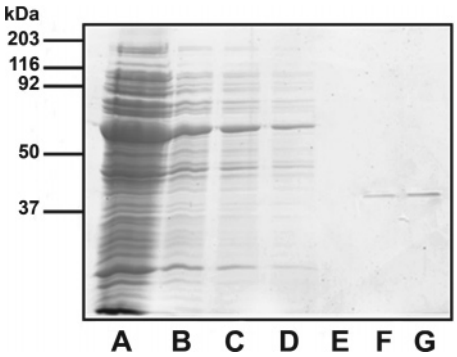


Figure 5. SYPRO-stained 10% SDS-PAGE analysis of a cell lysate (240 μg) from a culture of *P. aeruginosa* PAO1. The cell lysate was treated with **1** and then added to avidin resin. (A) Flow through, (B) wash 1, (C) wash 2, (D) wash 3, (E) wash 4, (F) elution of bound PANagZ, (G) standard of purified recombinant PANagZ (60 ng).

of strategies to combat this antibiotic resistance mechanism.[25] A small culture of *P. aeruginosa* was grown to exponential phase, harvested, and then lysed. The clarified lysate of *P. aeruginosa* was incubated with activity-based probe **1** (5.6 μg), treated with the biotin-alkyne reporter group (Figure 5), and incubated with streptavidin beads as described above for purified VCNagZ. Analysis of the beads by SDS-PAGE followed by SYPRO Orange staining revealed a band with a molecular weight consistent with that predicted for PA3005, a putative *P. aeruginosa* family 3 β-D-glucosaminidase homologue (predicted MW = 33 kDa). We therefore cloned this putative NagZ, PA3005, and after overexpression and purification we subjected it to the same protocol as that used with the cell lysate (Figure 5, Lane G). Gratifyingly, pure PA3005 protein product is labeled by **1** and migrates during electrophoresis to the same position as the labeled protein in the cell lysate. 2AA5FGF **1** therefore effectively labels this putative NagZ in a complex proteome with exquisite selectivity, allowing it to be both detected and purified. To verify the efficiency of this ABPP with this putative enzyme, termed PANagZ, we analyzed the purified enzyme as we did VCNagZ. Again, we find that selectivity is extremely high, and the detection limit using Western blot is similar (≈50 ng) (Figures 6A and B).

The protein product of PA3005 from *P. aeruginosa* bears 48% amino acid sequence identity with VCNagZ. To verify its activity as a β-*N*-acetylglucosmainidase we assayed the recombinant enzyme against a series of 4-nitrophenyl glycosides and

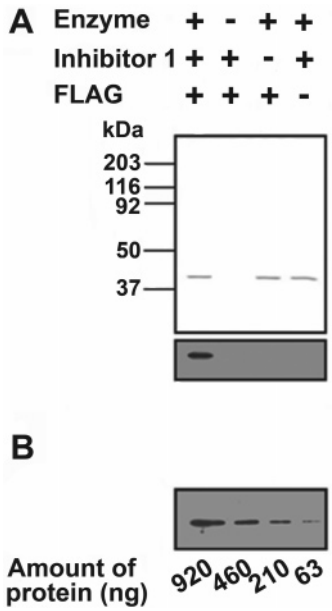


Figure 6. Characterization of PANagZ: (A) Visualization of **1**-labeled PANagZ by denaturing SDS/PAGE-Western blot with appropriate controls. After inactivation, the samples were treated as for VCNagZ SDS/PAGE-Western blot analysis. (B) Western blot showing the detection limit of **1**, used in conjunction with Staudinger ligation. For PANagZ amounts used were 920, 460, 210, and 63 ng.

found no detectable activity under the conditions of our assays (1 mM substrate, 25 μM enzyme) with pNP α-L-fucopyranoside, pNP β-D-glucopyranoside, pNP β-D-galactopyranoside, pNP β-D-glucuronide, pNP α-D-mannopyranoside, and pNP α-D-GlcNAc. A full kinetic analysis of the enzyme using pNP-GlcNAc and pNP-GlcNAz[30] revealed kinetic parameters (see Supporting Information; pNP-GlcNAc: $k_{cat}/K_M = 6.6 \pm 0.57$ μmol mM^{-1} min^{-1} mg^{-1}, $k_{cat} = 2.97 \pm 0.06$ μmol min^{-1} mg^{-1}, $K_M = 0.45 \pm 0.03$ mM; pNP-GlcNAz: $k_{cat}/K_M = 4.3 \pm 0.42$ μmol mM^{-1} min^{-1} mg^{-1}, $k_{cat} = 0.38 \pm 0.007$ μmol min^{-1} mg^{-1}, $K_M = 0.089 \pm 0.007$ mM) similar to those observed for VCNagZ (pNP-GlcNAc: $k_{cat}/K_M = 8.6 \pm 0.66$ μmol mM^{-1} min^{-1} mg^{-1}, $k_{cat} = 4.4 \pm 0.08$ μmol min^{-1} mg^{-1}, $K_M = 0.51 \pm 0.03$ mM; pNP-GlcNAz: $k_{cat}/K_M = 24 \pm 5.7$ μmol mM^{-1} min^{-1} mg^{-1}, $k_{cat} = 0.48 \pm 0.02$ μmol min^{-1} mg^{-1}, $K_M = 0.02 \pm 0.004$ mM) providing good support for assignment of this enzyme as a NagZ. These values also reveal that the azide group has a similarly small effect on catalysis for both of the enzymes, and the values obtained for pNP-GlcNAc are consistent with those measured for previously cloned family 3 *N*-acetyl-β-D-glucosaminidases from *V. furnisii* and *E. coli*.[46,47] The inactivation of PANagZ with 2A5FGF **2** and 2AA5FGF **1** also reveals the kinetic similarity of PANagZ and VCNagZ (see Supporting Information), consistent with our ability to inactivate and capture this enzyme. The second-order rate constants governing inactivation using both probes **2** ($k_{inact}/K_i = 0.013 \pm 0.004$ μM^{-1} s^{-1}, $K_i = 75 \pm 14$ μM, $k_{inact} = 1.0 \pm 0.1$ s^{-1}) and **1** ($k_{inact}/K_i = 0.010 \pm 0.003$ μM^{-1} s^{-1}) are also very similar (see Supporting Information) to those measured for VCNagZ. Altogether, this data strongly support the bioinformatic assignment of PA3005 as an *N*-acetyl-β-D-glucosaminidase and as an NagZ.

(46) Chitlaru, E.; Roseman, S. *J. Biol. Chem.* **1996**, *271*, 33433−33439.
(47) Tsujibo, H.; Hatano, N.; Mikami, T.; Hirasawa, A.; Miyamoto, K.; Inamori, Y. *Appl. Environ. Microbiol.* **1998**, *64*, 2920−2924.

Conclusion

In summary, this ABPP (2AA5FGF **1**) is the first highly efficient probe for analysis of retaining *exo*-glycosidases at endogenous levels from complex proteomes. This probe is useful for profiling and affinity-based purification of β-D-glucosaminidases that use a catalytic mechanism involving a covalent glycosyl-enzyme intermediate. The exquisite selectivity of both the fluorosugar inactivation and the Sharpless−Meldal click reaction also enables purification of this class of enzyme from complex mixtures containing the enzymes of interest at low native levels, as found within cell lysates. In this case we used ABPP 2AA5FGF **1** to validate a bioinformatics assignment of PA3005 as a β-D-glucosaminidase and verified this biochemically by cloning and characterization of *P. aeruginosa* NagZ as a potential target for inhibiting the chromosomally inducible AmpC β-lactamase pathway. We anticipate that this general strategy will find utility in analyses of glycoside hydrolases from various proteomes for both biotechnological and perhaps therapeutic purposes.

Experimental Section

General. ^{1}H and ^{13}C NMR spectra were obtained on a BrukerAV600 (600 MHz for ^{1}H and 150.8 MHz for ^{13}C) spectrometer. Unless stated otherwise, deuterated chloroform ($CDCl_3$) was used as the solvent with $CHCl_3$ (δ_H 7.26) or $CDCl_3$ (δ_C 77.0) being employed as internal standards. NMR spectra run in D_2O used internal CH_3OH (δ_H 3.34, δ_C 49.0) as the standard. Melting points were determined on a Reichert hot stage melting point apparatus. Optical rotations were performed with a Perkin-Elmer 141 Polarimeter in a microcell (1 mL, 10 cm path length) in $CHCl_3$ at room temperature, unless stated otherwise. Mass spectra were recorded with a VG-Autospec spectrometer using the fast atom bombardment (FAB) technique, with 3-nitrobenzyl alcohol as a matrix, unless otherwise stated. Elemental analyses of all synthesized compounds used in enzyme assays were performed at the Simon Fraser University. Flash chromatography was performed on BDH silica gel or Geduran silica gel 60 with the specified solvents. Thin-layer chromatography (TLC) was effected on Merck silica gel 60 F_{254} aluminum-backed plates that were stained by heating (≥200 °C) with 5% sulfuric acid in EtOH. Percentage yields for chemical reactions as described are quoted only for those compounds that were purified by recrystallization or by column chromatography, and the purity was assessed by TLC or ^{1}H NMR spectroscopy. All solvents except DMF and MeCN were distilled before use and dried according to the methods of Burfield and Smithers.[48] "Usual workup" refers to dilution with water, repeated extraction into an organic solvent, sequential washing of the combined extracts with hydrochloric acid (1 M, where appropriate), saturated aqueous sodium bicarbonate and brine solutions, followed by drying over anhydrous magnesium sulfate, filtration, and evaporation of the solvent by means of a rotary evaporator at reduced pressure. *V. cholerae* NagZ was expressed as previously described.[25]

3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucose (5). To 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucose **4** (5.5 g, 12 mmol) dissolved in THF (30 mL) was added benzylamine (3.0 mL, 28 mmol), and the mixture kept (1 h). Concentration of the reaction mixture followed by flash chromatography (EtOAc/petrol, 1:1) furnished **5** as a colorless powder (3.8 g, 76%). The spectral data were consistent with those previously reported.[32]

3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucosyl Fluoride (6). (Diethylamino)sulfur trifluoride (0.80 mL, 6.0 mmol) was added to **5** (1.5 g, 3.7 mmol) in CH_2Cl_2 (25 mL) at 0 °C, and the solution kept (0.5 h). The reaction mixture was quenched with aqueous $NaHCO_3$

(48) Burfield, D. R.; Smithers, R. H. *J. Org. Chem.* **1983**, *48*, 2420−2422.

followed by usual workup and removal of the solvent. Crystallization of the residue (Et_2O) yielded **6** as a colorless powder (1.2 g, 80%). The spectral data were consistent with those previously reported.[49]

3,4,6-Tri-*O*-acetyl-2-deoxy-5-fluoro-2-phthalimido-β-D-glucosyl Fluoride (7). *N*-Bromosuccinimide (370 mg, 2.10 mmol) was added to **6** (300 mg, 0.70 mmol) dissolved in CCl_4 (10 mL), and the mixture was irradiated using a 200 W bulb (3 h). Concentration of the reaction mixture followed by flash chromatography (EtOAc/toluene, 1:19) gave the presumed bromide as a gum (150 mg) that was immediately treated with $AgBF_4$ (64 mg, 0.33 mmol) in dry Et_2O (1 mL). The mixture was stirred (0.3 h), and upon consumption of the bromide the mixture was cooled and quenched with Et_3N (0.2 mL). The mixture was then filtered through silica and concentrated. Flash chromatography (EtOAc/toluene, 1:9) followed by crystallization (MeOH) furnished **7** as fine needles (50 mg, 16%), mp 145−148 °C, $[\alpha]_D^{20}$ +21.8°. δ_H (600 MHz) 7.90−7.75 (4H, m, Ar), 6.44 (dd, 1H, $J_{1,2}$ = 8.1 Hz, $J_{1,F1}$ = 53.3 Hz, H1), 6.04 (dd, 1H, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = 9.6 Hz, H3), 5.43 (dd, 1H, $J_{4,F5}$ = 23.2 Hz, H4), 4.58 (ddd, 1H, $J_{2,F1}$ = 14.0 Hz, H2), 4.42 (dd, 1H, $J_{6,F5}$ = 7.6 Hz, $J_{6,6}$ = 11.9 Hz, H6), 4.15 (dd, 1H, $J_{6,F5}$ = 4.2 Hz, H6), 2.87, 2.15, 2.10 (9H, 3s, CH_3). δ_C (150.9 MHz) 169.65, 169.52, 169.20, 167.11 (C=O), 134.54, 131.11, 123.79 (Ar), 109.48 (dd $J_{5,F1}$ = 9.2 Hz, $J_{5,F5}$ = 230.7 Hz, C5), 102.12 (dd, $J_{1,F1}$ = 219.1 Hz, $J_{1,F5}$ = 5.1 Hz, C1), 68.27 (d, $J_{4,F5}$ = 24.0 Hz, C4), 66.61 (d, $J_{3,F1}$ = 9.9 Hz, C3), 61.48 (d, $J_{6,F5}$ = 36.9 Hz, C6), 53.89 (d, $J_{2,F1}$ = 22.5 Hz, C2), 20.46, 20.31, 20.16 (3C, CO*C*H_3). HR-MS *m*/*z* (ES) 494.0668; $[M + K]^+$ requires 494.0665.

3,4,6-Tri-*O*-acetyl-2-acetamido-2-deoxy-5-fluoro-β-D-glucosyl Fluoride (8). Hydrazine hydrate (0.5 mL) was added to **7** (30 mg, 0.07 mmol) in MeOH (2 mL), and the mixture kept (5 h). The mixture was concentrated, and the residue dissolved in pyridine (3 mL), then Ac_2O (0.5 mL, 5.3 mmol) and DMAP (2 mg) were added, and the mixture was again kept (2 h). The mixture was quenched with MeOH and subjected to a usual workup followed by flash chromatography (EtOAc/petrol, 3:2) to yield **8** as a colorless powder (20 mg, 82%), mp 221−223 °C, $[\alpha]_D^{20}$ −328°. δ_H (600 MHz) 5.70 (dd, 1H, $J_{1,2}$ = 7.5 Hz, $J_{1,F1}$ = 53.3 Hz, H1), 5.45−5.35 (m, 2H, H3,H4), 4.40 (dd, 1H, $J_{6,F5}$ = 8.8 Hz, H6), 4.25−4.17 (m, 1H, H2), 4.11 (dd, 1H, $J_{6,F5}$ = 5.2 Hz, $J_{6,6}$ = 12.0 Hz, H6), 1.94, 2.00, 2.05, 2.08 (4s, 12H, $COCH_3$). δ_C (150.9 MHz) 170.91, 171.28, 171.42, 173.64 (4C, *C*OCH_3), 111.14 (dd, $J_{5,F1}$ = 8.1 Hz, $J_{5,F5}$ = 228.0 Hz, C5), 106.29 (dd, $J_{1,F5}$ = 4.2 Hz, $J_{1,F1}$ = 219.1 Hz, C1), 69.68 (d, $J_{3,F1}$ = 9.6 Hz, C3), 69.54 (d, $J_{4,F5}$ = 23.9 Hz, C4), 62.76 (d, $J_{6,F5}$ = 36.7 Hz, C6), 54.78 (d, $J_{2,F1}$ = 23.4 Hz, C2), 20.34, 20.38, 22.59 (CO*C*H_3). HRMS *m*/*z* (ES) 406.0722; $[M + K]^+$ requires 406.0716.

2-Acetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl Fluoride (2). Triethylamine (0.5 mL) was added to **8** (15 mg) in MeOH (2 mL), and the mixture was stirred at 30 °C (3 h). Concentration of the reaction mixture gave **2** as a colorless powder (10 mg), mp 153−155 °C. δ_H (600 MHz, CD_3OD) 5.46 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{1,F1}$ = 53.9 Hz, H1), 3.93−3.87 (m, 1H, H2), 3.83−3.77 (m, 2H, H3,H6), 3.73 (dd, 1H, $J_{3,4}$ = 9.4 Hz, $J_{4,F5}$ = 23.9 Hz, H4), 3.66 (dd, 1H, $J_{6,F5}$ = 4.4 Hz, $J_{6,6}$ = 11.9 Hz, H6), 2.00 (s, 3H, $COCH_3$). δ_C (150.9 MHz, CD_3OD) 173.89 (*C*OCH_3), 113.99 (dd, $J_{5,F1}$ = 8.0 Hz, $J_{5,F5}$ = 226.8 Hz, C5), 107.15 (dd, $J_{1,F5}$ = 3.9 Hz, $J_{1,F1}$ = 215.5 Hz, C1), 71.52 (d, $J_{4,F5}$ = 24.8 Hz, C4), 70.47 (d, $J_{3,F1}$ = 9.8 Hz, C3), 62.64 (d, $J_{6,F5}$ = 33.9 Hz, C6), 56.89 (d, $J_{2,F1}$ = 20.0 Hz, C2), 22.84 (CO*C*H_3). HR-MS *m*/*z* (ES) 505.1427; $[M_2 + Na]^+$ requires 505.1421. Anal. calcd for $C_8H_{13}F_2NO_5$: C, 39.84; H, 5.43. Found: C, 39.67; H, 5.61%.

2-[(Azidoacetyl)amino]-2-deoxy-5-fluoro-β-D-glucopyranosyl Fluoride (1). Hydrazine hydrate (1.0 mL) was added to **7** (70 mg, 0.20 mmol) in MeOH (5 mL), and the mixture kept (5 h). The mixture was concentrated to yield a gum that was dissolved in MeOH (5 mL) and then treated with Et_3N (1 mL) and $(ClCH_2CO)_2O$ (~20 mol equiv) until the reaction was judged complete (TLC). Concentration of the mixture followed by flash chromatography (MeOH/$CHCl_3$, 3:17) gave a colorless solid that was dissolved in DMF (2 mL) and treated with NaN_3 (20 mg, 0.31 mmol), and the resulting mixture was stirred at 80 °C (2 h). Concentration of the mixture followed by flash chromatography (MeOH/$CHCl_3$, 3:17) furnished **1** as colorless flakes (15 mg, 32%), mp 161−163 °C, υ_{max}(film)/cm^{-1} 2070 (N_3). δ_H (600 MHz, CD_3OD) 5.53 (dd, $J_{1,F1}$ = 54.0 Hz, H1), 3.98−3.93 (m, 3H, H2, CH_2N_3), 3.86 (dd, 1H, $J_{2,3}$ = 10.2 Hz, H3), 3.82 (dd, 1H, $J_{6,F5}$ = 9.3 Hz, H6), 3.73 (dd, 1H, $J_{3,4}$ = 9.4 Hz, $J_{4,F5}$ = 24.1 Hz, H4), 3.68 (dd, 1H, $J_{6,F5}$ = 4.4 Hz, $J_{6,6}$ = 11.9 Hz, H6). δ_C (150.9 MHz, CD_3OD) 170.85 (*C*OCH_2N_3), 113.99 (dd, $J_{5,F1}$ = 8.1 Hz, $J_{5,F5}$ = 227.1 Hz, C5), 106.91 (dd, $J_{1,F5}$ = 3.9 Hz, $J_{1,F1}$ = 215.3 Hz, C1), 71.53 (d, $J_{4,F5}$ = 24.8 Hz, C4), 70.47 (d, $J_{3,F1}$ = 9.8 Hz, C3), 62.62 (d, $J_{6,F5}$ = 33.8 Hz, C6), 56.94 (d, $J_{2,F1}$ = 19.9 Hz, C2), 53.03 (CH_2N_3). HR-MS *m*/*z* (FAB) 283.0849; $[M + H]^+$ requires 283.0854. Anal. calcd for $C_8H_{12}F_2N_4O_5$: C, 34.05; H, 4.29. Found: C, 34.31; H, 4.23%.

Inactivation Kinetics. The time-dependent inactivation of each enzyme by **1** or **2** was monitored by measuring the residual enzyme activity over time. This was accomplished by incubation of the enzyme (0.053 mg mL^{-1} for VCNagZ and 0.082 mg mL^{-1} for PANagZ) in 500 μL of PBS buffer solutions containing either inactivator **1** or **2**. For VCNagZ and PANagZ with **2**, five inactivation concentrations (0, 10, 20, 30, 50, and 100 μM) were used. For the inactivation of VCNagZ with **1**, different concentrations (0, 10, 20, 30, 40, and 50 μM) were used. The control mixture contained the same concentration of the appropriate enzyme but no inactivator. Both inactivation and control samples were incubated at room temperature (25 °C), and at various time intervals an aliquot (5 μL) of each inactivation mixture was added to a solution of the substrate, 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in PBS (50 mM NaP_i, 100 mM NaCl, 0.1% BSA, pH 7.4), so that the final assay contained VCNagZ (active + inactive) at a concentration of 3.3 μg mL^{-1} and 0.5 mM substrate in PBS buffer; the total volume was 80 μL (for PANagZ the final concentration of enzyme was 5.1 μg mL^{-1}). The initial rates were then measured under steady-state conditions by spectrophotometric monitoring of the release of 4-nitrophenolate ion at 400 nm. Pseudo-first-order rate constants, $k_{obs} = k_i[I]/(K_i + [I])$, for the decay of activity at each inactivator concentration were determined from fitting the decay curve to a single-exponential decay equation using the nonlinear regression analysis package of the program Grafit.

Chemoselective Ligation Experiments Using Phosphine-FLAG. An aliquot of enzyme sample (50 μL) was treated with a solution (10 μL) of **1** (2.0 mM, final [**1**] = 333 μM) in PBS and incubated at room temperature for 10 min. An aliquot of this mixture (50 μL) was taken, and 1 M pH 2.0 sodium phosphate (20 μL), saturated urea (one-third of the total volume), and water (30 μL) were added, in that order, to yield a solution with pH ≈ 3.5. An aliquot of this mixture (25 μL) was then taken and added to an equivalent volume of a solution of FLAG-phosphine in water (500 μM, 250 μM final concentration), and the mixture was allowed to incubate at room temperature for 6 h. The sample was then mixed with SDS-PAGE loading buffer and without heating the sample (20 μL) was loaded onto precast 10% Tris-HCl polyacrylamide gels. After electrophoresis, the samples were electroblotted to nitrocellulose membrane (0.45 μm, Biorad). Transfer was verified by visual inspection of the transfer of prestained markers (Biorad, product 161−0318). The membrane was blocked using PBS containing 5% bovine serum albumin and 0.1% Tween-20 (blocking buffer) with rocking for 1 h at room temperature. The blocking solution was then decanted, and a solution of blocking buffer containing anti-FLAG-HRP mAb conjugate (1:3500, Sigma) was added. Membranes were incubated with rocking at room temperature for 1 h after which the blocking solution containing antibody was decanted and the membrane rinsed with PBS, containing 0.1% Tween-20 (wash buffer). Membranes were then washed for 2 × 5 min and 2 × 20 min with wash buffer. Detection of membrane-bound FLAG-HRP conjugates was

(49) Blomberg, L.; Norberg, T. E. *J. Carbohydr. Chem.* **1992**, *11*, 751−760.

accomplished by chemiluminescent detection using a SuperSignal West Pico Chemiluminescent Detection Kit (Pierce) and film (Pierce CL-XPosure).

Using Biotin−Avidin. The experiment involving recombinant VCNagZ was similar to that described above up to the point just after the addition of the inactivator **1**. An aliquot of the inactivation mixture (44 μL) was added to a solution (1 μL) of the biotin tag (120 × stock in DMSO), followed by 10 μL of a 5 mM solution of TCEP (60 × stock in water) and 5 μL of a 100 μM solution of ligand (15 × stock in DMSO:*t*-butanol 1:4) solution, giving a final *tert*-butanol concentration of 5%. Samples were gently vortexed, and 1 mM $CuSO_4$ (60 × stock in water, 1 μL) was added to each sample, making the total reaction volume 60 μL. Samples were gently vortexed again and allowed to react at room temperature for 5 h, at which time 50 μL of avidin resin (Promega) were added and allowed to stand for 4 h at 4 °C. The suspension was then centrifuged (Eppendorf 5417C) for 3 min at 5000 rpm. The supernatant was removed and a fresh 50 μL of PBS buffer was added, and after mixing, the suspension was kept for 1 h. The sample was washed in this way three more times, and then 2 × SDS-PAGE loading buffer (reducing) was added (50 μL). After boiling the sample, an aliquot (25 μL) was loaded onto a precast 10% Tris-HCl polyacrylamide gel. After electrophoresis (0.05% SDS running buffer), the gel was rinsed with water and treated with SYPRO-Orange stain in 7.5% aqueous acetic acid for 30 min. The liquid was decanted, and the gel was washed with 7.5% aqueous acetic acid for 20 min and then rinsed with water. The gel was then scanned with a TYPHOON imager using a 488 nm blue laser and 580 nm filter.

Probing *P. aeruginosa* Cell Lysates. Cultures (5 mL) of *P. aeruginosa* PAO1 were grown at 37 °C overnight to exponential phase, and the cells were harvested by centrifugation for 3 min at 13 000 rpm (3500 × g). The cells were washed with 250 μL of PBS buffer, pelleted, and then resuspended in fresh PBS buffer again. This suspension was then sonicated (4 × 15 s at 20% power, Fisher Scientific, model 500) after which the cell debris was removed by centrifugation at 15 000 rpm for 60 min. A sample of the supernatant (50 μL) was treated with a solution (10 μL) of **1** (2.0 mM, final[1] = 333 μM, 5.6 μg total) in PBS and incubated at room temperature for 10 min. An aliquot of this mixture (44 μL) was treated as described above for pure VcNagZ.

Cloning of *P. aeruginosa* NagZ. Genomic DNA from *P. aeruginosa* PAO1 was used as the template for cloning. The PCR was accomplished using the following primers 5′-GATATA**CATATG**CAAGGCTCTCT-GATGCTC-3′ (NdeI cut site shown in bold) and 5′-GATATA**G-GATCC**ATCAATCAGTTGCGCAGC-3′ (BamHI cut site shown in bold). The conditions used for the PCR were as follows: 28 cycles of denaturation at 95 °C for 30 s followed by annealing of primers for 30 s at 60 °C; extension for 5 min at 72 °C using *pfu* DNA polymerase. The reaction volume was 50 μL and contained 0.25 mM NTPs, 0.4 μM of each primer, 50 ng of template DNA, and 1 μL of *pfu* DNA polymerase. The PCR reaction mixture was subjected to electrophoresis through a 1.4% agarose gel for 45 min at 90 V, and the product was excised and purified using a gel extraction kit (Qiagen). Double digest reaction mixtures using both restriction endonucleases (NdeI and BamHI) were carried out on both the PCR product and empty pET28a vector. The reaction volumes were 50 μL and consisted of approximately 5 μg of DNA and 1.0 U of each restriction enzyme (Fermentas) and were allowed to proceed for 3−4 h at 37 °C. The reaction mixtures were then subjected to electrophoresis through a 1.4% agarose gel, and the desired DNA was gel purified as described above. 1 μL of the digested plasmid DNA was then combined with 5 μL of the digested PCR product and ligated together using T4 DNA Ligase (New England Biolabs) for 1 h at 18 °C. 2 μL of this ligation mixture were used to transform *E. coli* ultracompetent cells as described above except plates containing 50 μg/mL of kanamycin were used. A colony was picked and cultured, and the plasmid DNA was harvested the following day as described above. The entire insert was sequenced to ensure accuracy. DNA encoding NagZ in the pET28a expression vector was used to transform Tuner(λDE3) cells (Novagen). A colony was selected and cultured to exponential phase in LB media (5 mL) containing 50 μg/mL of kanamycin. This culture was used to inoculate a 750 mL culture that was grown to an $OD_{600} \approx 0.8$ at 37 °C. At this point protein expression was induced using 1 mM IPTG (Bioshop) for 2.5 h at 25 °C. Postinduction cells were harvested by centrifugation (Beckman J2-HS) for 10 min at 5000 rpm and resuspended in 20 mL of nickel-column binding buffer (50 mM Na_2PO_4, 500 mM NaCl, 5 mM imidazole; pH 7.4) for each liter of cell culture. The resuspended cells were incubated on ice for 30 min with 1 mg/mL of lysozyme (USB) and 1 mM PMSF followed by sonication (6 × 20 s at 60% power, Fisher Scientific, model 500). The cell debris was then removed by centrifugation at 14 000 rpm for 75 min, and the supernatant was loaded *via* syringe onto a 5 mL FF HisTrap column (Amersham Biosciences). The column was washed with 100 mL of wash buffer (same as binding buffer but containing 60 mM imidazole) and eluted with 30 mL of elution buffer (same as binding buffer but containing 250 mM imidazole). Purified enzyme was subsequently dialyzed overnight against 4 L of PBS (pH 7.4) containing 10% (w/v) glycerol, and the protein concentration of the resulting solution was quantified by a Bradford assay using BSA as a standard.

Acknowledgment. Ethan D. Goddard-Borger is thanked for technical assistance. D.J.V. is a scholar of the MSFHR and a Tier II Canada Research Chair in Chemical Glycobiology. We thank the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health research for funding.

Supporting Information Available: Details of kinetic analysis of VCNagZ and PANagZ with the substrates 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and pNP-GlcNAz as well as details of inactivation kinetic analysis of PANagZ with **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.